CHARACTERIZATION OF HUMAN PROSTATE TUMOUR-INITIATING CELLS

CHARACTERIZATION OF A POPULATION OF TUMOUR-INITIATING CELLS WITH STEM-LIKE PROPERTIES IN HUMAN PROSTATE CANCER

By:

ADRIAN P. RYBAK, B.Sc., M.Sc.

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2013) McMaster University
(Medical Sciences) Hamilton, Ontario, Canada

TITLE: Characterization of a population of tumour-initiating cells with stem-like properties in human prostate cancer

AUTHOR: Adrian P. Rybak, B.Sc. (McMaster University), M.Sc. (McMaster University)

SUPERVISOR: Damu Tang, Ph.D.

NUMBER OF PAGES: xx, 244

ABSTRACT

There is increasing evidence that prostate tumours are organized as a hierarchy with rare cancer stem cells (CSCs) implicated in initiating and maintaining the tumour. However, prospective prostate cancer stem cells (PCSCs) have not been thoroughly characterized from primary tissue specimens. Using the DU145 cell line, PCSCs have been propagated as non-adherent spheres *in vitro*. Approximately 1.25% of monolayer DU145 cells formed primary spheres while 26% of sphere cells formed subsequent spheres; a measure of PCSC self-renewal capacity. Spheres are enriched for cells expressing prostate basal and luminal cytokeratins and CSC markers (CD44, CD24, integrin $\alpha 2\beta 1$). PCSCs initiate xenograft tumours with enhanced capacity compared to monolayer cells. While epidermal growth factor (EGF) promoted PCSC propagation, basic fibroblast growth factor (bFGF) inhibited these events. Activation of EGF receptor (EGFR) signalling, following EGF treatment or expression of constitutively-active EGFR (EGFRvIII), increased sphere formation. Conversely, attenuation of EGFR signalling inhibited PCSC self-renewal. Consistent with the MEK-ERK pathway being a major target of EGFR signalling, the MEK-ERK pathway contributes to EGFR-facilitated PCSC propagation. Inhibition of ERK activation following MEK inhibitor treatment, expression of dominant-negative MEK1(K97M), or knockdown of ERK1 or ERK2 reduced PCSC propagation. Therefore, EGFR signalling promotes PCSC self-renewal by activating the MEK-ERK pathway.

SOX2 is an essential transcription factor for stem cells, however, its role in PCSCs remains unclear. SOX2 protein is upregulated in PCSCs propagated as spheres, and its

expression is regulated by EGFR signalling. EGFR activation, following EGF treatment or expression of constitutively-active EGFRvIII, increased SOX2 expression and PCSC self-renewal, while being attenuated by EGFR inhibitor treatment. Ectopic SOX2 expression enhanced EGF-induced PCSC self-renewal, while SOX2 knockdown renders PCSCs non-responsive to EGF-induced self-renewal and reduced their anchorage-independent growth. Furthermore, SOX2 expression is associated with the ability of PCSCs to form aggressive xenograft tumours. Collectively, SOX2 regulates EGFR-mediated PCSC self-renewal.

FOREWORD

This thesis is presented as a "sandwich" thesis, composed of three manuscripts that were prepared for publication during the author's Ph.D. candidacy. Two manuscripts have been published (Chapters 2 and 3) and one manuscript is submitted and under review for publication (Chapter 4) at the time this thesis was prepared. The manuscripts are presented as separate chapters that include prefaces detailing each author's contributions, as well as a description of the overall context wherein the manuscript was prepared. In addition, an introductory chapter that discusses previous research into prostate cancer stem cells and other topics, and outlines the overall basis for the thesis is included. A concluding chapter (Chapter 5) discusses the contributions of the work in Chapters 2, 3 and 4 to the field of study and proposes directions for future research.

References cited within each manuscript are entirely independent and are consistent with the style of each respective journal. Other citations used throughout the thesis appear in a separate References section following Chapter 5. Canadian spelling and grammar was carried throughout this dissertation, however, due to journal requirements, American spelling was used for manuscripts submitted for publication (Chapters 2, 3 and 4). Appendices are also included which provide unpublished data. This data is referred to as Appendix 1 and Appendix 2 in Chapter 5. In addition, the license agreement with Elsevier Limited is included in Appendix 3.

ACKNOWLEDGEMENTS

I would like to first thank my supervisor, Dr. Damu Tang, for providing me with the opportunity to work in his laboratory, and for giving me valuable insight, both personal and professional, throughout my Ph.D. studies. My appreciation also goes out to my co-supervisors, Dr. Laurie Doering and Dr. Richard C. Austin. Dr. Laurie Doering, thank you for providing me with excellent ideas and suggestions throughout my project. Dr. Richard Austin, you have been a great mentor to me. I will always value your faith in my scientific abilities, and you have taught me the value of scientific collaboration. I aspire to become a great mentor and collaborative researcher like you some day. I would also want to extend my gratitude to other faculty and researchers who have provided me tools and valuable insight during my Ph.D. studies: Dr. Khalid Al-Nedawi, Dr. Joan Krepinsky, Dr. Jean-Claude Cutz, Dr. Anil Kapoor, Dr. Alistair J. Ingram, Dr. Jane A. Foster, Dr. Judith West-Mays and Dr. Jeffrey G. Dickhout.

Many thanks also go out to current and past lab members at St. Joseph's hospital, particularly Anthony Bruce, Aubrey Gillis, Lizhi He, Nazihah Bakhtyar, Elizabeth Crane, Celeste Collins, Rachel Carlisle, Sana Basseri, and Ali Al-Hashimi. I can truly call them friends, having helped me in so many ways. Great appreciation goes out to Anthony Bruce, a great friend and an amazing researcher who taught me the value of objective science, who emphasized that a protein's function relates to its structural domains, and that elucidating a molecular mechanism requires "exposing the conditions". To work alongside someone who can think critically about science, ask amazing ('earth-shattering') questions, someone who was always willing to help me both personally and

professionally was an honour. You will be a great mentor and hopefully an amazing principal investigator some day!

A big thank you also goes out to John Dykyj, Adrian Woloszanskyj, Tomasz Niewadomski and Robert Cowan for their friendship, encouragement, advice and laughs throughout my Ph.D. studies.

Most importantly, I would like to thank my parents, Peter and Teresa Rybak, and my siblings, Anastazia and Taras Rybak (Ph.D. candidate), for their constant love, support and unwavering confidence in me, even when I did not think I could stay sane any longer in my Ph.D. studies. Lastly, I would like to thank my cousin, Larysa Baran, and my nieces, Ariana and Eliseya Snihura-Baran, who could always make me smile and laugh, and always brightened my day by taking my mind off of science.

I am truly fortunate to have the greatest, most supportive family in the world!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABC	avidin-biotin complex
ADT	androgen deprivation therapy
AEG	anophthalmia-esophageal-genital
AKT-P	AKT phosphorylation on Ser473
AML	acute myelogenous leukemia
AR	androgen receptor
bFGF	basic fibroblast growth factor
BPH	benign prostatic hyperplasia
BSA	bovine serum albumin
CARN	castration-resistant NKX3.1-expressing
CD	cluster of differentiation
CK	cytokeratin
CRPC	castration-resistant prostate cancer
CSC	cancer stem cell
ddH_2O	distilled/deionized water
DMEM	Dulbecco's modified essential medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGFR-P	EGFR phosphorylation on Tyr1068
EGFRvIII	EGFR variant III
EGTA	ethylene glycol tetraacetic acid
ERK	extracellular signal-regulated kinase
ERK1/2-P	ERK1 phosphorylation on Thr 202 and Tyr204; ERK2 phosphorylation on Thr185 and Tyr187
ESC	embryonic stem cell
FBS	fetal bovine serum
Fig	Figure
GFs	growth factors
Grb2	growth factor-receptor bound protein 2
h	hour
HMG	high mobility group
HMW	high molecular weight
HRPC	hormone refractory prostate cancer
hTERT	human telomerase reverse transcriptase
IF	immunofluorescence
IgG	immunoglobulin G
IHC	immunohistochemistry
iPS	induced pluripotent stem
kDa	kilodalton

lacZ	gene that encodes for the β-galactosidase enzyme
Lin	lineage
MEM	minimal essential medium
MAPK	mitogen-activating protein kinase
MEF	Myeloid Elf-1-like factor
MNL	monolayer
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NaCl	sodium chloride
NE	neuroendocrine
NLS	nuclear localization signal
NOD/SCID	non-obese, diabetic/severe-combined immunodeficient
p	passage
p42	protein 42 kDa (ERK2)
p44	protein 44 kDa (ERK1)
PBS	phosphate-buffered saline
PC	prostate cancer
PCR	polymerase chain reaction
PCSC	prostate cancer stem cell (or prostate cancer stem-like cell)
PDK1	phosphoinositide-dependent protein kinase 1
PI3K	phosphatidylinositol 3 kinase
PIP_2	phosphatidylinositol (4,5) diphosphate
PIP_3	phosphatidylinositol (3,4,5) triphosphate
PIN	prostatic intraepithelial neoplasia
PKB	protein kinase B (AKT)
PMSF	phenylmethylsulfonyl fluoride
PSA	prostate specific antigen
PSC	prostate stem cell
PTEN	phosphatase and tensin homolog on chromosome 10
RICTOR	rapamycin insensitive companinon of mTOR
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
RTKs	receptor tyrosine kinases
Sca-1	stem cell antigen-1
SCC	squamous cell carcinoma
SDACs	sphere-derived adherent cells
S.E.M. (or SE)	standard error of mean
Ser	serine
SF	serum-free
SFM	serum-free medium
SH2	Src-homology 2
Sos	Son of sevenless
SOX2	Sry-related HMG Box gene 2
STAT3	signal transducer and activator of transcription 3

STAT3-P	STAT3 phosphorylation on Tyr705
TBST	Tris-buffered saline containing 0.1% Tween-20
Thr	threonine
TGF-α	transforming growth factor-alpha
TIC	tumour-initiating cell
Tris	tris(hydroxymethyl)aminomethane
Tyr	tyrosine
UGSM	embryonic urogenital sinus mesenchyme
UV	ultraviolet
YFP	yellow fluorescent protein

DECLARATION OF ACADEMIC ACHIEVEMENT

This thesis is presented as a culmination of three manuscripts – two published papers and a manuscript in submission, as follows:

Adrian P. Rybak, Lizhi He, Anil Kapoor, Jean-Claude Cutz, and Damu Tang. 2011. Characterization of sphere-propagating cells with stem-like properties from DU145 prostate cancer cells. *Biochim Biophys Acta*. 1813(5): 683-94. doi: 10.1016/j.bbamcr.2011.01.018.

Adrian P. Rybak, Alistair J. Ingram, and Damu Tang. 2013. Propagation of Human Prostate Cancer Stem-Like Cells Occurs through EGFR-Mediated ERK Activation. *PLoS One*. 8(4):e61716. doi: 10.1371/journal.pone.0061716.

Adrian P. Rybak, and Damu Tang. 2013. SOX2 Plays a Critical Role in EGFR-mediated Self-Renewal of Human Prostate Cancer Stem-like Cells. *Mol Oncol* (submitted May 2013).

In addition, the author, Adrian P. Rybak, contributed to the following publications:

Yanyun Xie, Judy Yan, Jean-Claude Cutz, **Adrian P. Rybak**, Lizhi He, Fengxiang Wei, Anil Kapoor, Valentina A. Schmidt, Lijian Tao, and Damu Tang. 2012. IQGAP2, A candidate tumour suppressor of prostate tumorigenesis. *Biochim Biophys Acta*. 1822(6):875-884. doi: 10.1016/j.bbadis.2012.02.019.

Lizhi He, Cathy Fan, Anil Kapoor, Alistair J. Ingram, **Adrian P. Rybak**, Richard C. Austin, Jeffrey Dickhout, Jean-Claude Cutz, James Scholey, and Damu Tang. 2011. α-Mannosidase 2C1 attenuates PTEN function in prostate cancer cells. *Nat Commun*. 2:307. doi: 10.1038/ncomms1309.

Lizhi He, Alistair Ingram, **Adrian P. Rybak**, and Damu Tang. 2010. Shank-interacting protein-like 1 promotes tumorigenesis via PTEN inhibition in human tumor cells. *J Clin Invest*. 120(6):2094-2108. doi: 10.1172/JCI40778.

Cathy Fan, Lizhi He, Anil Kapoor, **Adrian P. Rybak**, Jason De Melo, Jean-Claude Cutz, and Damu Tang. 2009. PTEN inhibits BMI1 function independently of its phosphatase activity. *Mol Cancer*. 8:98. doi: 10.1186/1476-4598-8-98.

Cathy Fan, Lizhi He, Anil Kapoor, Aubrey Gillis, **Adrian P. Rybak**, Jean-Claude Cutz, and Damu Tang. 2008. Bmi1 promotes prostate tumorigenesis via inhibiting p16(INK4A) and p14(ARF) expression. *Biochim Biophys Acta*. 1782(11):642-648. doi: 10.1016/j.bbadis.2008.08.009.

CHAPTER 1

Introduction

1.1. Prostate epithelial cells and their susceptibility to tumorigenesis

1.1.1. The prostate gland - Anatomy, histology and its regenerative capacity

The prostate is a walnut-sized glandular tissue in males that surrounds the urethra at the base of the bladder. In adult males, the prostate gland contributes to production of secretory proteins which are present within the seminal fluid (Shen and Abate-Shen 2010). Although formation of the prostate occurs during embryogenesis through epithelial budding of cells in the urogenital sinus, these prostate epithelial buds undergo extensive ductal outgrowth and branching due to androgen stimulation during puberty [reviewed in (Abate-Shen and Shen 2000)]. This maturation process, which involves the interplay between cells of epithelial and mesenchymal origin within the urogenital sinus, leads to the development of the prostate epithelium which serves in its secretory function (Hayward, et al. 1997).

The prostate epithelium is comprised of four differentiated cell types: luminal secretory cells, basal cells, intermediate cells and neuroendocrine cells (Figure 1). Luminal cells are a differentiated androgen-dependent cell-type that produce and release prostatic secretory proteins into the lumen (Abate-Shen and Shen 2000) including prostate specific antigen (PSA) (Bonkhoff, et al. 1994), a serine protease responsible for preventing semen coagulation following ejaculation (Armbruster 1993). Furthermore, these cells are characterized by the expression of androgen receptor (AR) (De Marzo, et al. 1998), and cytokeratins 8 and 18 (Sherwood, et al. 1990). Basal cells are another

epithelial cell type located between the luminal cells and the basement membrane. Prostate basal cells are characterized by their expression of cluster of differentiation (CD) 44 (CD44) (Liu and True 2002), p63 (Yang, et al. 1998), as well as cytokeratins 5 and 14 (Sherwood et al. 1990). In addition, prostate basal cells display low AR expression (De Marzo et al. 1998) and lack PSA expression (Bonkhoff et al. 1994). Intermediate cells are luminally-located cells in budding acini during prostate development that express cytokeratins (CK) found in basal and luminal cells ($CK5^{\pm}CK14^{\pm}CK18^{+}$) (Xue, et al. 1998). Neuroendocrine cells, a rare AR-negative cell-type which is dispersed in the basal cell layer of the prostate epithelium, express chromogranin A and synaptophysin (Shen and Abate-Shen 2010).

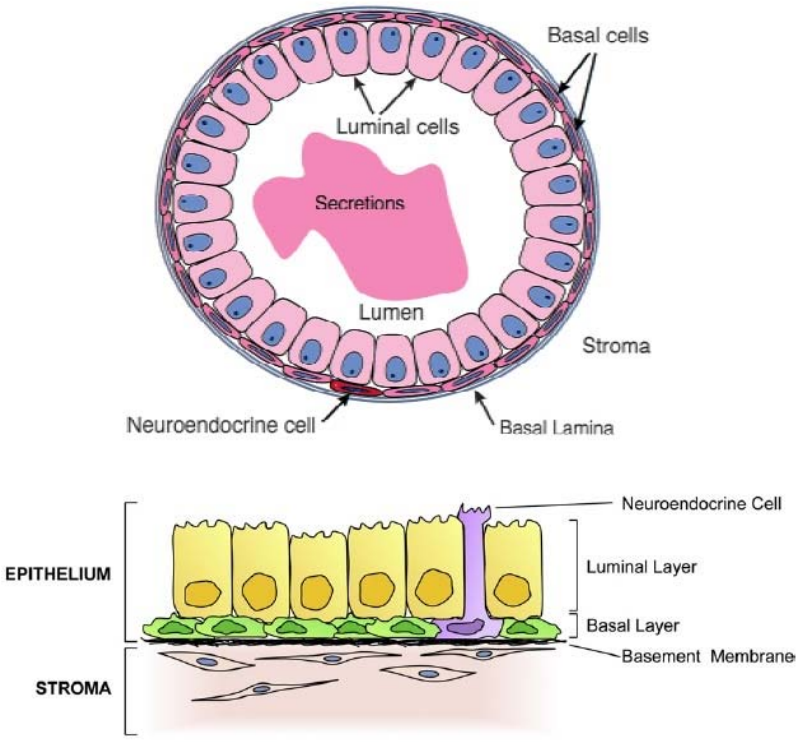


Figure 1. Schematic representation of the architecture of the human prostate epithelium. The prostate epithelium consists of an inner layer of secretory luminal cells. Basal cells form a continuous layer of cells around the luminal cells, and in contact with the basement membrane (top panel) [adapted from (Abate-Shen and Shen 2000)], which serves as a barrier between the epithelium and the stromal compartment (bottom panel) [adapted from (Oldridge, et al. 2012)]. In addition, intermediate cells displaying both basal and luminal markers and rare neuroendocrine cells are scattered throughout the prostate epithelium.

1.1.2. Prostate stem cells in mouse tissues

Tissue-specific stem cells are defined by their capacity for self-renewal and to produce mature progenies, which include non-renewing progenitors and terminally-differentiated effector cells (Wagers and Weissman 2004) that constitute distinct cell types of the tissue of interest. Self-renewal is defined as the ability of stem cells to maintain an undifferentiated state through cell division without losing their identity or functional potential (Chambers and Smith 2004; Sneddon, et al. 2012). The concept of a stem cell compartment in the prostate epithelium was first realized upon evaluating the regenerative capacity of the prostate gland following castration-induced atrophy in adult rats (English, et al. 1987; Sugimura, et al. 1986). Castration results in prostate regression in response to androgen deprivation, with a stable number of cells remaining in a regressed state. Upon re-administration of androgen, the prostate epithelium regenerates over a two-week period (English et al. 1987; Sugimura, et al. 1986), even after undergoing several rounds of regeneration and regression following androgen restoration and ablation (Wang, et al. 2013). This suggests that the prostate epithelium contains a long-term surviving population of stem cells that are resistant to castration.

In the mouse prostate, there is evidence for distinct stem cells with either a basal or luminal phenotype. In mouse studies, prostate cells expressing stem cell antigen-1 (Sca-1) were characterized as a subpopulation capable of reconstituting prostatic ducts lined with basal and luminal cells (Burger, et al. 2005). These reconstituted prostatic ducts, generated upon combining Sca-1^+ cells with embryonic urogenital sinus mesenchyme (UGSM) cells under the renal capsule, contained secretory material within the lumen of

prostate epithelia (Burger et al. 2005), further suggesting that these newly-derived prostatic ducts were functional. Using specific cell surface markers to further discriminate basal epithelial ($CD24^{-}CD49f^{+}$) $Sca\text{-}1^{+}$ cells from prostate stromal ($CD34^{+}$), luminal epithelial ($CD24^{+}CD49f^{-}$), haematopoietic ($CD45^{+}$, $Ter119^{+}$), and endothelial ($CD31^{+}$) lineages, $CD45^{-}CD31^{-}Ter119^{-}Sca\text{-}1^{+}CD49f^{+}$ cells were sorted from the mouse prostate and demonstrated to have self-renewal abilities *in vitro*. In addition, these cells were localized to the basal layer and formed *in vivo* prostatic ducts containing basal and luminal cells (Lawson, et al. 2007). Moreover, a single mouse prostate cell, defined by the $Lin^{-}Sca\text{-}1^{+}CD133^{+}CD44^{+}CD117^{+}$ surface profile, was capable of generating an intact prostate when transplanted with UGSM cells under the renal capsule (Leong, et al. 2008).

Although the functional prostate regeneration assay has demonstrated that basal cells in the murine prostate are bipotent, capable of generating both prostate basal and luminal cell lineages, these tissue reconstitution assays involve co-culturing the basal cells with UGSM cells (Burger et al. 2005; Lawson et al. 2007; Leong et al. 2008). The UGSM cells provide a strong inductive influence when combined with prostate epithelial tissue fragments and grafted under the kidney capsule of mice (Xin, et al. 2003). To avoid any unexpected plasticity that may manifest upon removing basal cells from their endogenous tissue microenvironment, genetic lineage-tracing experiments have explored the nature of basal or luminal-derived prostate epithelial cells towards prostate formation *in situ* following castration-driven prostate regression and androgen-mediated prostate regeneration studies [as reviewed by (Sharpe, et al. 2013)]. Mice expressing a tamoxifen-inducible Cre-recombinase under the control of the cytokeratin 5 (CK5) promoter have

been used to label prostate basal cells with the yellow fluorescent protein (YFP) and analyze their fate at single cell resolution *in situ*. This lineage-tracing experiment demonstrated that rare CK5-labelled basal cells within the prostate epithelium give rise to both basal and luminal cell progeny following androgen-mediated prostate regeneration (Wang, et al. 2013). This finding differs from the conclusions of a recent report that suggests that basal and luminal lineages are self-sustaining (unipotent), as CK14-labelled basal cells and CK18-labelled luminal cells produced only labelled basal and luminal cells, respectively, and did not undergo lineage conversion to generate other cell lineages (Choi, et al. 2012).

Further evidence in support of other prostate stem cells present within the prostate that are not of basal cell origin was obtained from tissue reconstitution assays carried out using prostatic grafts from p63-null mice. The p63-null prostate lacks basal cells, however, a prostate epithelium containing luminal cells formed upon grafting p63-null prostate epithelial cells along with UGSM cells under the renal capsule and supplementing with testosterone (Kurita, et al. 2004). Furthermore, a PSA-labelled luminal cell population is capable of surviving castration and reconstituting the luminal cell compartment following androgen treatment (Liu, et al. 2011). In addition, a population of castration-resistant NKX3.1-expressing (CARN) cells display a luminal phenotype in the regressed prostate which give rise to both basal and luminal cells following androgen-mediated regeneration, suggesting that the CARN cell population are bipotent in nature (Wang, et al. 2009). Therefore, regenerated luminal epithelial cells following castration appear to be derived from pre-existing luminal cells that survive

androgen deprivation (Choi et al. 2012; Liu et al. 2011; Wang et al. 2009). The reason for these discrepancies is unclear at present, and suggests that the prostate epithelial lineage hierarchy has not been clearly characterized, with distinct pools of epithelial cells with stem cell properties existing in the mouse prostate.

1.1.3. Prostate stem cells in human tissues

In the human prostate, evidence suggests the existence of a prostate tissue stem cell confined to the basal cell compartment. Human prostate cells with a basal phenotype ($CD44^+CD49f^+CK5^+p63^+CK8^-AR^-PSA^-$) have been shown to self-renew *in vitro* (Garraway, et al. 2010), with the capacity to reconstitute the prostate epithelium containing basal and luminal cells in a prostate regeneration assay (Garraway et al. 2010; Goldstein, et al. 2010). A number of cell surface markers have been used to identify prospective human prostate stem cells (PSCs). CD44, which serves as a receptor for hyaluronic acid, has been used to enrich for human PSCs in the basal cell layer which possess prostate regenerative activity (Garraway et al. 2010). Another putative marker of stem cells expressed by primary cultures of prostate epithelial cells includes integrin $\alpha_2\beta_1$ (Collins, et al. 2001). Integrin $\alpha_2\beta_1$ enriches for prostate basal epithelial cells based on their ability to bind to type I collagen (Collins et al. 2001). High integrin $\alpha_2\beta_1$ ($\alpha_2\beta_1^{hi}$) expression has been used to enrich for a population of epithelial cells that form large, slow-proliferating colonies (>21 days growth) and which demonstrate a basal cell phenotype ($CK5^+CK14^+PSA^-$). When these cells were combined with stromal cells and subcutaneously-injected into immuno-compromised male mice that are athymic and

lacking T cells, an epithelial structure formed (8/30 mice) consisting of both basal and secretory luminal (PSA^+AR^+) cells which was considered as evidence of prostate stem cell activity (Collins et al. 2001).

Another putative prostate stem cell marker is the cell surface marker CD133, a five-transmembrane-domain cell surface glycoprotein (Miraglia, et al. 1997). Several monoclonal antibodies have been developed against CD133, the most commonly used being AC133 (CD133/1) and AC141 (clone 293C3; CD133/2) which have been reported to recognize distinct epitopes (Green, et al. 2000). AC133 is a glycosylated epitope of CD133 (CD133/AC133) (Mak, et al. 2011) which has been used to enrich for human haematopoietic stem cells (Yin, et al. 1997), and it has been used as a marker to identify normal human neural stem cells (Singh, et al. 2003). Richardson et al. (2004) reported that CD133/$AC133^+$ human prostate epithelial cells are restricted to the integrin $\alpha_2\beta_1$-expressing subpopulation of basal cells. These CD133/$AC133^+\alpha_2\beta_1^{hi}$ cells had a greater colony-forming efficiency and proliferative potential *in vitro* than CD133/$AC133^-\alpha_2\beta_1^{hi}$ cells. However, CD133/$AC133^+\alpha_2\beta_1^{hi}$ colonies appeared 5 days later compared to CD133/$AC133^-\alpha_2\beta_1^{hi}$ colonies, suggesting that CD133/$AC133^+\alpha_2\beta_1^{hi}$ cells were in a more quiescent state (Richardson, et al. 2004). Immunofluorescent staining of CD133/$AC133^+$ cells identified them as being contained within the basal cell layer, accounting for approximately 0.75% of the total basal cell population, and demonstrating a basal phenotype by expressing CK5 and CK14 (Richardson et al. 2004). To demonstrate that CD133/$AC133^+\alpha_2\beta_1^{hi}$ cells display stem-like activity, CD133/$AC133^+\alpha_2\beta_1^{hi}$ and CD133/$AC133^-\alpha_2\beta_1^{hi}$ cells were subcutaneously implanted into nude male mice. Grafts

from CD133/AC133$^{+}\alpha_2\beta_1^{hi}$ cells were able to generate prostate acini *in vivo* which resemble prostate epithelia consisting of both basal and luminal cells (Richardson et al. 2004). Although evidence suggests that a stem cell population exists in the basal compartment of the human prostate epithelium, CD133/AC133^{+} cells from SV40-immortalized prostate cells did not generate tumours while CD133/AC133^{-} cells formed tumour grafts when recombined with either UGSM cells or cancer-induced fibroblasts under the renal capsule of immuno-compromised mice (Taylor, et al. 2012). Taken together, this suggests that the stem cell population and the cell-of-origin in prostate cancer may be distinct cell types.

1.1.4. Prostate cancer

Prostate cancer is the most common male malignancy and the second leading cause of cancer-related deaths in males in the developed world (Siegel, et al. 2012; Williams and Powell 2009). During the process of prostate tumorigenesis, oncogenic signalling pathways promote the progression of hormone-dependent carcinomas to hormone refractory prostate cancer (referred to as castration-resistant prostate cancer (CRPC)), the major contributing factor in prostate cancer fatalities (Moon, et al. 2008; Ross 2007). The primary strategy to treat localized prostate cancer is either radiation therapy or complete removal of the prostate, which is termed 'radical prostatectomy'. However, patients relapse in many cases and neoplastic growth of aberrant prostate cells resumes. Relapsed patients are often subjected to androgen deprivation therapy (ADT) in which either chemical or surgical castration is performed along with anti-androgen treatment (Abate-

Shen and Shen 2000). Tumour relapse, which is accompanied with an increase in PSA levels, frequently occurs post-surgery, and such patients are said to possess CRPC (Lee, et al. 2008).

Histological studies of prostate cancer tissues have identified a specific lesion of prostatic intraductal dysplasia (McNeal and Bostwick 1986), now commonly referred to as prostatic intraepithelial neoplasia (PIN). PIN is characterized by the appearance of luminal epithelial hyperplasia, enlarged nuclei and nucleoli, a reduction in the number of basal cells (Bostwick 1989) while maintaining an intact basement membrane (Bostwick, et al. 1993). PIN is generally regarded as a preneoplastic lesion in humans (Abate-Shen and Shen 2000). The majority of prostate cancers are pathologically classified as adenocarcinoma and display a luminal phenotype, with the absence of p63 and CK5 and CK14 staining for basal cells (Grisanzio and Signoretti 2008; Humphrey 2007) (Figure 2). Neuroendocrine-derived prostate cancer, which is generally classified as small cell carcinoma, represents a small fraction of prostate cancer cases (Grignon 2004). Furthermore, prostate cancer is multifocal in nature, as neoplastic lesions within a section of prostate cancer tissue can be genetically distinct from one another (Bostwick, et al. 1998; Macintosh, et al. 1998; Miller and Cygan 1994). Therefore, the presence of multiple lesions with unique genetic compositions in prostate cancer suggests that different cells within prostate epithelia may be vulnerable to tumorigenesis.

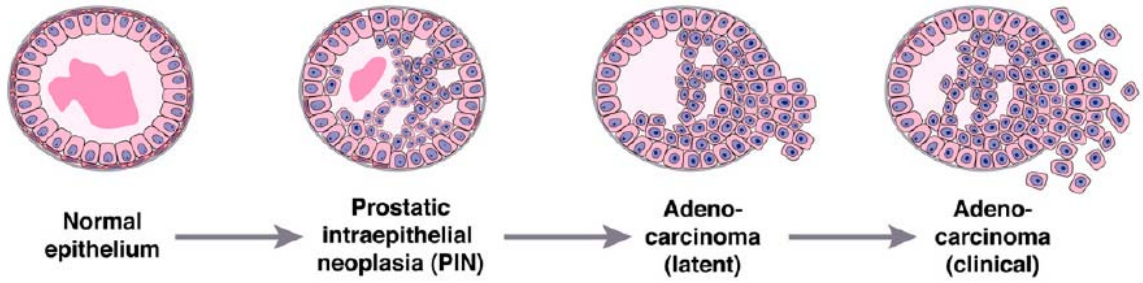


Figure 2. Stages of prostate cancer progression. Abnormalities in the normal prostate epithelium of prostatic glands result in prostatic intraepithelial neoplasia (PIN) featuring luminal epithelial hyperplasia and a reduction in the number of basal cells. PIN lesions progress to adenocarcinoma with loss of the basal layer and basement membrane, resulting in various grades of prostate cancer beginning with indolent (latent) to more aggressive (clinical) forms of the disease [adapted from (Shen and Abate-Shen 2010)].

1.1.5. Cell-of-origin in prostate cancer: basal versus luminal disease

Elucidation of the lineage relationships within the prostate epithelium is relevant for understanding the origins of prostate cancer. Given that prostate adenocarcinoma has a luminal phenotype, the cell of origin would be expected to correspond to a luminal cell that has undergone oncogenic transformation or a basal cell that differentiates and proliferates as luminal progeny following tumorigenesis [as reviewed by (Shen and Abate-Shen 2010)]. These possibilities have been examined in mouse models of prostate cancer. A basal cell of origin for prostate cancer has been suggested as human basal cells expressing androgen receptor (AR), AKT and ERG oncogenes can form carcinoma in

tissue reconstitution assays. These reconstituted prostate carcinomas, characterized by the loss of basal cells ($p63^-$) and expansion of luminal cells expressing PSA, were observed to be histologically similar to clinical human prostate cancer (Goldstein et al. 2010). Genetic lineage-tracing experiments have also explored the contribution of basal or luminally-derived prostate epithelial cells towards prostate transformation *in situ* using transgenic mouse models of prostate cancer [as reviewed by (Sharpe et al. 2013)]. Basal cells from the mouse prostate can undergo transformation (Choi et al. 2012; Lawson, et al. 2010; Wang et al. 2013). In contrast, deletion of the *PTEN* (phosphatase and tensin homolog on chromosome 10) tumour suppressor in castration-resistant Nkx3-1-expressing (CARN) luminal cells resulted in carcinoma formation after androgen treatment (Wang et al. 2009). While prostate carcinomas generated following *PTEN* deletion in basal (CK5- or CK14-targeted, respectively) cells or luminal cells (CARNs or CK8-targeted, respectively) were observed to be histologically similar (Choi et al. 2012; Wang et al. 2013), prostate basal cell-derived carcinomas arose more slowly (Wang et al. 2013). This may be due to the fact that loss of PTEN activity induces basal cells to differentiate into transformation-competent luminal cells (Choi et al. 2012). Although basal cells are capable of undergoing transformation, they are relatively resistant towards transformation induced by oncogenic stimuli *in situ* (Choi et al. 2012). While mouse tumours of basal and luminal origin are histologically similar (Choi et al. 2012; Wang et al. 2013), luminal cell-derived murine prostate cancer displays a molecular gene signature which is highly correlated with poor human patient prognosis (reduced biochemical-free survival) and significantly upregulated in high-risk patients (death within 12 months) (Wang et al.

2013). Taken together, cell-of-origin models propose that distinct cell types within the prostate lineage hierarchy are vulnerable towards tumorigenesis, which may give rise to prostate cancer subtypes with different prognoses and/or treatment responses. At least in a mouse model, luminal cell-derived tumours seem to recapitulate the more aggressive types of human prostate cancer (Wang et al. 2013). This implies a scenario in which human prostate tumours that are associated with different prognoses may originate from basal and/or luminal cells, respectively.

1.1.6. Cancer stem cells or tumour-initiating cells

Cancer is characterized by the excessive and uncontrolled growth of abnormal cells that can invade, propagate in and destroy foreign tissues (Hanahan and Weinberg 2000). However, within the bulk population of malignant cells in a given tumour, it has been recognized that phenotypic, proliferative and functional heterogeneity exists. Transplantation of primary human acute myelogenous leukemia (AML) cells into immuno-compromised mice led to the discovery that rare $CD34^{+}CD38^{-}$ cells, termed SCID leukemia-initiating cells, are capable of initiating leukemic growth and differentiate into leukemic blasts *in vivo* (Bonnet and Dick 1997). This was the first clear *in vivo* demonstration in support of the cancer stem cell (CSC) hypothesis, which suggests that human cancers are organized in a hierarchical manner with a phenotypically distinct subset of cancer cells, termed cancer stem cells or tumour-initiating cells, having the ability to form a new tumour and sustain tumorigenesis (Pardal, et al. 2003; Reya, et al. 2001; Visvader and Lindeman 2008).

The cancer stem cell or tumour-initiating model has also been demonstrated in solid tumours, including breast, brain and colon cancers. Al-Hajj and colleagues (2003) demonstrated that a subpopulation of human breast cancer cells with a $CD44^{+}CD24^{-}ESA^{+}$ phenotype display tumour-initiating potential when injected into female immuno-compromised mice. Furthermore, these breast tumour-initiating cells were capable of generating a phenotypically diverse population of cells following their engraftment into mouse mammary fat pads (Al-Hajj, et al. 2003). In brain cancers (medulloblastoma and glioblastoma multiforme), CD133 (AC133 epitope; CD133/AC133) has been used to purify a population of cells capable of generating tumours that resemble the original patient's tumour following intracranial injection in immuno-compromised mice, while $CD133/AC133^{-}$ cells were incapable of forming tumours (Singh, et al. 2004). More recently, a subset of colon cancer cells capable of tumour initiation have been identified in patient tumours upon isolating $CD133/AC133^{+}$ cells and injecting them subcutaneously (Ricci-Vitiani, et al. 2007) or in the renal capsule (O'Brien, et al. 2007) of immuno-compromised mice. Collectively, cancer cells have been isolated from various solid tumours via cell surface marker expression, which display tumour-initiating potential and are capable of re-establishing tumour heterogeneity *in vivo*.

1.1.7. In vitro propagation of cancer cells displaying stem-like properties

An *in vitro* approach towards isolating, propagating and evaluating the stem-like properties of putative CSCs derived from solid tumours is the non-adherent sphere formation assay (Visvader and Lindeman 2008) (Figure 3). The ability of stem-like cells

to be propagated as non-adherent spheres was first identified upon plating mouse embryonic neural cells in serum-free, epidermal growth factor (EGF)-supplemented conditions. The resulting sphere culture was enriched in cells (~20%) capable of self-renewal (ie. self-maintenance), forming secondary spheres upon re-plating dissociated sphere cells (Reynolds and Weiss 1996). This enriched embryonic neural precursor cell population could be subsequently differentiated into cells that display antigenic and morphologic characteristics of neurons, oligodendrocytes and astrocytes (Reynolds and Weiss 1996). Following this initial discovery, the sphere culture method has been used for the enrichment, propagation and evaluation of self-renewal capacity of neural stem cells (Jensen and Parmar 2006), and has been adapted to propagate human mammary epithelial stem cells (Dontu, et al. 2003).

A critical examination of the sphere formation assay has suggested that this assay evaluates the potential of a cell to exhibit both functional properties of stem cells (self-renewal capacity and differentiation potential) when removed from its *in vivo* niche (Pastrana, et al. 2011). Given that cells may form spheres in a cell autonomous fashion (independent of their *in vivo* microenvironment) or following exogenous mitogen treatment, *in vitro* sphere formation may not reflect the *in vivo* stem cell frequency. In addition, other experimental manipulations such as cell seeding density may affect the clonality of spheres, while sphere formation itself may influence cell surface-marker profiles (Pastrana et al. 2011). Furthermore, quiescent stem cells that may not be capable of forming spheres, or form spheres at a slower rate than the time frame of the sphere culture period, may not be propagated and reflected in the sphere culture (Pastrana et al.

2011). Finally, extended passaging of sphere-forming cells has been suggested to be necessary in order to interpret stem cell self-renewal activity by reducing the impact of progenitor-like (non-stem) cells that may be present in early passage (clonal) cultures and which display limited self-renewal potential (Reynolds and Rietze 2005). As the sphere assay has been used to evaluate the self-renewal capacity of a stem-like cell, their true clonality can only be guaranteed upon plating a single cell per well (Figure 3) (Pastrana et al. 2011).

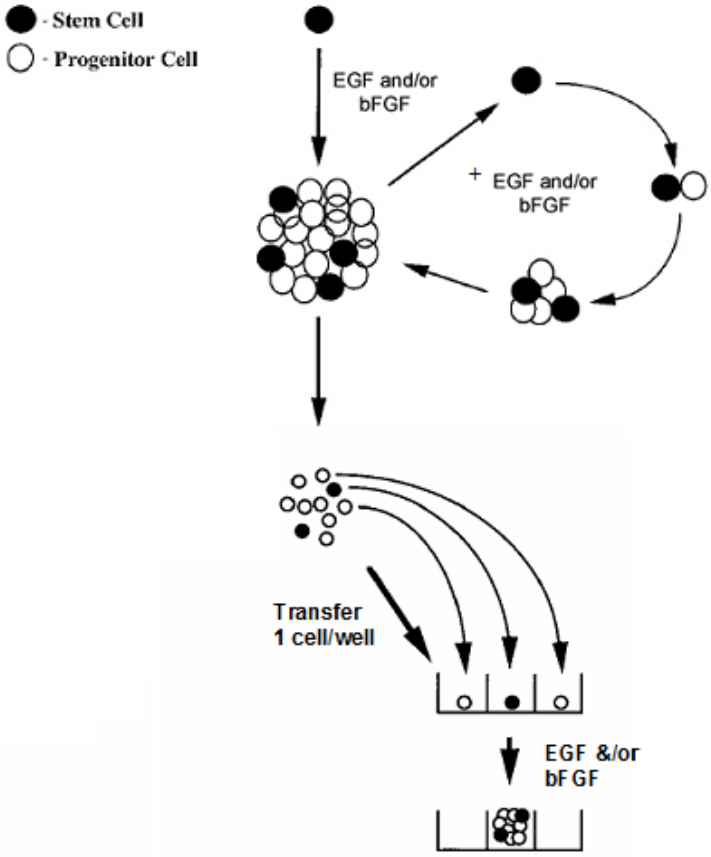


Figure 3. Schematic representation of the sphere formation assay and the experimental approach towards evaluating cancer stem cell (CSC) self-renewal activity. Under serum-free, growth factor-supplemented [epidermal growth factor (EGF) and/or basic fibroblast growth factor (bFGF)] conditions, CSCs from various solid tumours will form non-adherent spheres *in vitro*, composed of CSCs as well as progenitor-like cells that are incapable of reforming new spheres. To evaluate CSC self-renewal activity, sphere-derived cells are seeded in 96-well plates at a density of 1 cell/well. The proportion of new spheres formed is a measure of the self-renewal capacity [adapted from (Reynolds and Weiss 1996)].

Similar studies have shown that CSCs residing in solid tumours can be propagated as spheres *in vitro* and demonstrated to promote tumour initiation *in vivo*. A subpopulation of cells in brain cancers can form non-adherent spheres under serum-free media conditions, express CD133/AC133 on the cell surface of sphere cells, and subsequently express elevated levels of neuronal (β-tubulin III) and/or astrocyte (GFAP) markers upon culturing sphere cells under serum-supplemented (ie. differentiating) conditions (Hemmati, et al. 2003; Singh et al. 2003). Furthermore, prospective human brain CSCs displayed elevated *in vitro* self-renewal capacity and proliferative potential compared to human neural stem cells (Singh et al. 2003), and promoted *in vivo* tumour formation upon injecting enriched CD133/AC133^{+} cells into the frontal lobe of immuno-compromised mice (Singh et al. 2004). Human breast cells are capable of forming and propagating as spheres under serum-free conditions in the presence of EGF and basic fibroblast growth factor (bFGF) (Ponti, et al. 2005). These sphere-derived breast cancer cells have also been reported to differentiate into different mammary epithelial lineages under serum-supplemented conditions (Ponti et al. 2005). Furthermore, colon cancer cells that generate spheres remain as CD133/AC133^{+} cells that display increased *in vivo* tumorigenic potential compared to sphere cells propagated as adherent cells under serum-supplemented conditions (Ricci-Vitiani et al. 2007). Therefore, prospective CSCs derived from solid tumours have been shown to propagate as spheres *in vitro*, which are capable of recapitulating the original tumour *in vivo*.

1.1.8. Identification of human prostate tumour-initiating cells

Prostate cancer, like many other carcinomas, exhibits a functional hierarchy of cells with tumour-initiating cells positioned at the apex and having the ability to develop (or differentiate) into a spectrum of more mature progenies (Patrawala, et al. 2007; Patrawala, et al. 2006). The cancer stem cell hypothesis postulates that only a subset of cells within the tumour are capable of sustaining tumorigenesis and driving disease progression, while establishing the cellular heterogeneity that constitutes the primary tumour (Visvader and Lindeman 2012). Stem-like cancer cells, often referred to as cancer stem cells (CSCs) or tumour-initiating cells, have been experimentally defined by their ability to initiate tumours upon implanting in immuno-compromised mice (Pardal et al. 2003; Reya et al. 2001; Visvader and Lindeman 2008). These stem-like cancer cells generate non-tumorigenic progeny, resulting in intrinsically different populations of tumorigenic and non-tumorigenic cells within the tumour (Magee, et al. 2012). Therefore, the ability of an isolated, phenotypically distinct population of cancer cells to initiate tumour formation in immuno-compromised mice is the 'gold standard' for evaluating the presence of CSCs (Visvader and Lindeman 2008).

There are several pieces of evidence that suggest the existence of human PCSCs. A small population of primary prostate cells expressing the cell surface antigenic profile $CD44^{+}\alpha_2\beta_1{}^{hi}CD133/AC133^{+}$ was suggested to be the candidates of PCSCs (Collins, et al. 2005). This possibility is supported by several reports. Cancer stem cells are known to share similar surface antigens with their normal tissue stem cell counterparts (Zhao, et al. 2008). Consistent with this concept, human $\alpha_2\beta_1{}^{hi}CD133/AC133^{+}$ prostate epithelial cells

possess the properties of prostate stem cells (Richardson et al. 2004). Additionally, primary human prostate epithelial cells and malignant cells that ectopically express human telomerase reverse transcriptase (hTERT) displayed the $CD44^{+}\alpha_2\beta_1{}^{hi}CD133^{+}$ cell surface profile (Gu, et al. 2007; Li, et al. 2008; Miki, et al. 2007). Furthermore, hTERT-transformed primary prostate epithelial cells derived from a patient with advanced prostate cancer were able to generate prostate tumours that resembled the original tumour (Gu et al. 2007). These observations are consistent with a report that $CD44^{+}$ subpopulations isolated from several cultured human prostate cancer cell lines were more tumorigenic in immuno-compromised mice than the isogenic $CD44^{-}$ population of cells (Patrawala et al. 2007; Patrawala et al. 2006). In addition, DU145 prostate cancer cells expressing the prospective stem cell markers CD44 and CD133 (AC141 epitope; CD133/AC141) have been enriched following propagation under serum-free conditions (Dubrovska, et al. 2009). However, despite these observations supporting the candidacy of $CD44^{+}\alpha_2\beta_1{}^{hi}CD133^{+}$ cells as prospective PCSCs, it still remains to be demonstrated whether $CD44^{+}\alpha_2\beta_1{}^{hi}CD133^{+}$ cells isolated from primary prostate cancer tissues are capable of initiating tumour formation in immuno-compromised mice.

To date, bona-fide PCSCs have not been isolated from human primary prostate cancer tissues. This is largely due to the challenging nature of isolating prostate cancer cells since there is a limited amount of clinically available material, which itself is highly heterogeneous in nature. Due to the constraints in obtaining a sufficient amount of human prostate cancer material in order to subsequently isolate and characterize prospective PCSCs, established human prostate cancer cell lines and/or derived prostate tumour

xenograft models have become useful surrogate sources for investigating PCSCs. Enriched cells expressing the basal marker integrin α2β1 from the non-malignant, SV40-transformed BPH-1 human prostate cell line form xenografts with malignant characteristics when the epithelial cells were CD133/AC133-negative (Taylor et al. 2012). CD133/AC133^{+} BPH-1 basal cells did not form malignant xenografts, based on the author's criteria, upon co-injecting with cancer-associated fibroblasts in the renal capsule of immuno-compromised mice (Taylor et al. 2012), suggesting that proliferative CD133/AC133^{-} basal cells (Richardson et al. 2004) are more susceptible to tumorigenesis compared to slow-growing, prospective prostate basal stem-like (CD133/AC133^{+}) cells (Collins et al. 2005). Using the CWR22 tumour xenograft model, Rajasekhar and colleagues (2011) demonstrated that a small subset of cells demonstrated stem-like properties, forming non-adherent spheres in serum-free media (1 sphere/2500 total tumour cells) and displaying tumour-forming efficiencies that exceeded that of the bulk tumour cells (100% vs. 0% tumours, respectively) when transplanted subcutaneously or into the prostate of immuno-compromised male mice. Furthermore, these stem-like cells displayed the novel pluripotency marker TRA-1-60, as well as CD166 and CD151, with triple-marker positive CWR22 and DU145 cells displaying enhanced sphere forming efficiencies and tumour formation in immuno-compromised mice (Rajasekhar, et al. 2011). Given the limitations towards obtaining clinically-relevant primary prostate cancer tissue, the CWR22 tumour xenograft model offers an *in vivo* source of human prostate cancer cells, maintained in the mouse prostate over successive transplantations, which can be used to subsequently isolate for and study human PCSCs.

Although *in vitro* propagated prostate cancer cells with CSC properties have been studied, these cells have not been thoroughly investigated. Different methods have been used to culture PCSCs, including suspension or low adherence culture on a layer of agar (Li et al. 2008; Miki et al. 2007) or using low cell adherence plates (Dubrovska et al. 2009; Gu et al. 2007). In general, two types of media have been used to culture prostate cancer stem-like cells: media containing either fetal bovine serum (FBS) (Gu et al. 2007; Patrawala et al. 2007; Patrawala et al. 2006), or the combination of epidermal growth factor (EGF; 10 or 20 ng/ml) and basic fibroblast growth factor (bFGF; 10 or 20 ng/ml) (Dubrovska et al. 2009; Miki et al. 2007; Rajasekhar et al. 2011). DU145 prostate cancer cells can form primary spheres in growth factor-supplemented serum-free media, expressing the cell surface markers CD44 and CD133/AC141, compared to monolayer cells (12.2±0.2% versus 0.65±0.2%, respectively). Furthermore, $CD44^{+}CD133/AC141^{+}$ DU145 cells have been subsequently shown to promote tumour formation in immuno-compromised mice (Dubrovska et al. 2009). However, these sphere-generating cells derived from the DU145 prostate cancer cell line (Dubrovska et al. 2009) or from CWR22 xenografts (Rajasekhar et al. 2011) have not been cultured for an extended period of time in order to quantitatively evaluate stem-like properties such as self-renewal capacity. Therefore, whether these derived cells are progenitors or bona-fide PCSCs remains to be determined.

1.2. Cell signalling pathways utilized by solid tumours and their tumour-initiating cells

1.2.1. ErbB biology, EGFR signalling and cancer

The mammalian ErbB receptor tyrosine kinases, which include ErbB1 (also known as EGFR), ErbB2, ErbB3 and ErbB4, have been implicated in the progression of a variety of human cancers. In addition, EGFR is the first receptor tyrosine kinase to be directly associated with human tumours [as reviewed by (Hynes and Lane 2005)]. Protein kinases, which catalyze the transfer of a phosphoryl (PO_3^{2-}) group from adenosine 5'-triphosphate (ATP) to the hydroxyl group of serine, threonine and/or tyrosine residues on its protein substrate [as reviewed by (Roskoski 2012a)], are responsible for signal transduction in eukaryotic cells. Protein phosphorylation, which in part regulates protein-protein association, is responsible for propagating the signal [as reviewed by (Hunter 2000)]. The *EGFR* gene, which is located on chromosome 7, consists of 28 exons spanning a 200 kb region (Reiter, et al. 2001) and encodes a 1186 amino acid protein consisting of an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain that serves as a docking site for various adaptor proteins which mediate downstream signalling [as reviewed by (McCubrey, et al. 2006; Wheeler, et al. 2010)].

Initiation of EGFR transactivation and downstream signalling requires the binding of EGFR ligands to its ectodomain. The ligands that are specific for EGFR include EGF, transforming growth factor-alpha (TGF-α) and amphiregulin. These EGF-family of

growth factors are produced as transmembrane precursors that, once cleaved by cell surface proteases, results in the release of soluble ligands that can act in a paracrine and/or autocrine fashion [as reviewed by (Harris, et al. 2003)]. EGFR activation stimulates many complex intracellular signalling pathways that are tightly regulated by the presence and identity of the ligand, ErbB receptor dimer composition, and the availability of phosphotyrosine-binding proteins. The two primary signalling pathways activated by EGFR include the RAS/Raf/MEK/ERK (referred to as mitogen-activating protein kinase; MAPK) and the phosphatidylinositol 3 kinase (PI3K)-AKT signalling cascades, which result in the activation of cell proliferation and survival programs [as reviewed by (McCubrey et al. 2006; Wheeler et al. 2010)].

EGFR gene amplifications, which lead to enhanced EGFR activation, are found in human cancers, with these amplifications being commonly associated with EGFR mutations. One common *EGFR* mutation is a rearrangement that leads to genomic loss of exons 2 to 7 resulting in an in-frame deletion (267 amino acids) of the extracellular domain (Ekstrand, et al. 1994; Wong, et al. 1992). This mutation results in the loss of the ectodomain and produces a ligand-independent constitutively-active EGFR variant (referred to as EGFR variant III, or EGFRvIII) observed in lung (Garcia de Palazzo, et al. 1993), prostate (Olapade-Olaopa, et al. 2000), breast (Del Vecchio, et al. 2012), ovarian and brain cancers (Moscatello, et al. 1995).

1.2.2. EGFR signal activation in prostate cancer

Recent evidence suggests that the development of an androgen-independent (hormone-refractory) state in prostate cancer advancement may be due to the upregulation of growth factor-receptor signalling pathways, particularly through epidermal growth factor receptor (EGFR; ErbB1) activation (Shah, et al. 2006; Traish and Morgentaler 2009). In the prostate, immunohistochemical staining for EGFR in normal glands demonstrated that EGFR expression is restricted to the basal cell layer (Maygarden, et al. 1992). Di Lorenzo et al. (2002) investigated EGFR expression in prostate cancer tissue from 58 patients. Twenty-nine of these patients had primary tumours and were treated with radical prostatectomy as first-line therapy, with EGFR expression being positive in 41.4% of patients. In a second group of 29 patients with primary prostate tumours, the investigators first treated patients with ADT and with anti-androgens before radical prostatectomy; EGFR expression was found in 75.9% of patients. In a third cohort of 16 patients displaying metastatic disease and CRPC, all patients were found to be positive for EGFR expression. These data suggest that EGFR expression is associated with progression of prostate cancer towards an androgen-independent state (Di Lorenzo, et al. 2002). A number of other studies have also investigated the relationship between EGFR expression and prostate cancer progression, demonstrated a strong correlation between EGFR expression in prostate cancer tissues with androgen-independent status (Hernes, et al. 2004; Shah et al. 2006). The detection of the constitutively-active EGFRvIII in human prostate cancers, with higher levels of EGFRvIII protein expression being detected in androgen-resistant and metastatic prostate cancers, is indicative of an aggressive

phenotype of the disease (Olapade-Olaopa et al. 2000). In addition, a number of EGFR tyrosine kinase domain mutants (Cai, et al. 2008) that display activated EGFR signalling have been detected in human prostate cancer, further validating the significance of EGFR signal activation in prostate cancer progression.

Activation of EGFR signalling in prostate cancer has been shown to regulate the autonomous growth of prostate cancer cells *in vitro*, with EGF-mediated EGFR activation being associated with increases in downstream MAPK and PI3K-AKT signalling in DU145 and PC3 prostate cancer cells (Gan, et al. 2010). PC3 cells secrete factors, particularly the EGFR ligand known as tumour growth factor-alpha (TGF-α) into the conditioned medium. This conditioned media has been shown to be mitogenic for the less aggressive prostatic carcinoma cell lines, DU145 and LNCaP. Furthermore, PC3 cell proliferation was inhibited under serum-free conditions by antibodies directed against TGF-α and/or the EGF receptor (Hofer, et al. 1991). In addition, serum-free media conditioned by DU145 cells showed immunological TGF-α activity (MacDonald, et al. 1990), while treatment of DU145 cells with a monoclonal antibody towards the EGF receptor inhibited the growth of these cells in both FBS-supplemented and serum-free media (Connolly and Rose 1991). Taken together, these results suggest that prostate cancer cell growth is regulated by an EGF-mediated autocrine loop.

1.2.3. MAPK signalling in prostate cancer

The mammalian mitogen-activated protein kinases (MAPKs) are cytoplasmic protein serine/threonine kinases that transduce signals to the interior of the cell by activating

extracellular signal-regulated kinases (ERKs). ERK1 and ERK2 (ERK1/2) are related protein serine/threonine kinases (84% identical in sequence) that participate in the Ras-Raf-MEK-ERK signal transduction cascade, which is often referred to as the MAPK or ERK cascade (Roskoski 2012a). Ligand-induced activation of receptor protein-tyrosine kinases like EGFR, leading to receptor dimerization and phosphorylation of the intracellular C-terminal domains [as reviewed by (Lemmon and Schlessinger 2010)], allows Grb2 (growth factor-receptor bound protein 2) to bind to the activated receptor through its Src-homology 2 (SH2) domain (Lowenstein, et al. 1992) and subsequently recruits Sos (Son of sevenless), a guanine nucleotide exchange factor, to the activated receptor leading to Ras activation (Egan, et al. 1993; Li, et al. 1993; Lowenstein et al. 1992; Rozakis-Adcock, et al. 1993). As a result, GDP-bound Ras (H-Ras, K-Ras and N-Ras) is converted to active GTP-bound Ras [as reviewed by (Karnoub and Weinberg 2008)], which can subsequently bind to Raf-1 (c-Raf) (Moodie, et al. 1993; Warne, et al. 1993; Zhang, et al. 1993). Raf-1 mediated activation of MEK1 requires phosphorylation at two of its serine residues (S218 and S222); site-directed mutagenesis of either of these serine residues on MEK1 abolished ERK1 activity (Zheng and Guan 1994). MEK1 and MEK2 (MEK1/2) are dual-specificity protein kinases that mediate phosphorylation of ERK1 and ERK2 [as reviewed by (Roskoski 2012b)]. Site-directed mutagenesis of threonine and tyrosine sites in human ERK1 (Thr202/Tyr204) and rat ERK2 (Thr183/Tyr185, which are equivalent to Thr185/Tyr187 in human ERK2) inhibited their phosphorylation and kinase activities *in vitro* (Robbins, et al. 1993). ERK1 and ERK2, which share many functions, regulate as many as 160 documented cytoplasmic and

nuclear target proteins [as reviewed by (Yoon and Seger 2006)]. As a result, the MAPK signalling cascade participates in various cellular processes, including cell cycle progression, cell survival and proliferation, cell adhesion, differentiation, cell metabolism and transcriptional regulation [as reviewed by (Roskoski 2012a; Yoon and Seger 2006)].

Activation of the MAPK pathway has been shown to be associated with prostate cancer progression. In 60 primary and 18 metastatic human prostate cancer specimens, phosphorylation of ERK1 and ERK2 (ERK1/2) was examined using an antibody that specifically recognizes the dually phosphorylated MAP kinases (Gioeli, et al. 1999) at their conserved Thr-Xxx-Tyr motif implicated in their activity (Anderson, et al. 1990; Robbins et al. 1993). While non-neoplastic tissue displayed little to no staining for the phosphorylated forms of ERK1/2 (ERK1/2-P), intense nuclear with more diffuse cytoplasmic staining was detected infrequently in prostate basal cells rather than in secretory luminal cells. Of the 60 primary prostate cancer specimens evaluated, approximately 32% of the specimens had high levels (≥10% tumour cells positive) of ERK1/2-P. However, the levels of ERK1/2-P increased with increasing tumour stage; 66% of stage IV tumours demonstrated high levels of activated ERK1/2-P, while 17% of stage II tumours displayed high levels of ERK1/2-P. Furthermore, 44% of the 18 metastatic specimens examined (7 of 15 lymph node metastases and 1 of 3 bone metastases) demonstrated high levels of ERK1/2-P (Gioeli et al. 1999). Taken together, evidence suggests that activation of MAPK signalling increases as prostate cancer progresses to a more advanced disease.

Elevated MAPK signalling may also be selected for during androgen deprivation therapy (ADT). Although Gioeli and colleagues (1999) did not report a statistical correlation between MAPK signal activation and androgen-independent status following ADT in the specimens examined in their study, two case studies were reported in which the patients who had failed ADT displayed elevated ERK1/2 signal activation in their recurrent tumours. In a human prostate cancer tissue microarray analysis composed of 194 patients, ERK1/2-P staining levels were significantly increased in patients receiving neoadjuvant hormone therapy and in recurrent patients with CRPC as compared to benign prostatic hyperplasia specimens (Mulholland, et al. 2012). Further evidence in support of a relationship between MAPK signalling and progression of prostate cancer towards an androgen-independent state has been demonstrated *in vitro*. Ectopic expression of a Ras mutant (H-Ras(T35S)) in androgen-sensitive LNCaP cells, which preferentially activates Raf, maintained ERK activation (ERK1/2-P) under serum-free conditions and subsequently displayed increased *in vivo* tumorigenicity and *in vitro* anchorage-independent growth compared to parental LNCaP cells. In addition, 69% of these Ras-expressing LNCaP xenograft tumours were resistant to castration and displayed robust ERK activation post-castration (Bakin, et al. 2003b). Conversely, inducible expression of a dominant-negative Ras mutant (H-Ras(T17N), more commonly referred to as H-RasN17) in the androgen-independent, highly tumorigenic derivative LNCaP cell line, C4-2, inhibited ERK activation and restored androgen sensitivity to these cells by causing xenograft tumour regression in surgically-castrated mice (Bakin, et al. 2003a). Furthermore, MEK-ERK activation is necessary and sufficient to mediate Raf-induced

androgen receptor (AR) downregulation in prostate cancer cell lines irrespective of AR sensitivity (Hong, et al. 2011), further supporting the role of MAPK signalling in the advancement of prostate cancer.

1.2.4. PI3K-AKT signalling in prostate cancer

In addition to activating MAPK signalling, EGFR activation also initiates signalling events through the PI3K-AKT pathway (McCubrey et al. 2006). The PTEN (phosphatase and tensin homolog on chromosome 10) tumour suppressor is a lipid phosphatase that negatively regulates PI3K-AKT signalling by dephosphorylating phosphatidylinositol (3,4,5) triphosphate (PIP_3), a product of PI3K, to phospatidylinositol (4,5) diphosphate (PIP_2) (Stambolic, et al. 1998). Class IA PI3K is a heterodimer consisting of a regulatory (p85) and catalytic (p110) subunit which mediates PI3K activation from activated receptor tyrosine kinases (RTKs). Phosphorylated tyrosine residues on activated RTKs can interact with the SH2 domains of p85, which in turn recruits the p85-p110 heterodimer to the plasma membrane where PIP_2 resides [as reviewed by (Engelman 2009)]. Accumulation of PIP_3 results in the recruitment of pleckstrin-homology (PH) domain-containing proteins to the plasma membrane, like 3-phosphoinositide-dependent protein kinase 1 (PDK1) and AKT (also known as protein kinase B; PKB). The phosphorylation of AKT at Thr308 (which is in the activation loop of AKT) by PDK1 and at Ser473 (which is in a hydrophobic motif of AKT) by mammalian target of rapamycin (mTOR) and its associated protein RICTOR (rapamycin insensitive companion of mTOR) results in activation of this protein kinase (Sarbassov, et al. 2005).

As a result, activated AKT phosphorylates several cellular proteins to facilitate protein synthesis, cell growth and proliferation, cell metabolism and survival [as reviewed by (Engelman 2009)].

The relevance of PTEN in cancer arises from the discovery that the *PTEN* gene, located on chromosome 10, is highly mutated and lost in several cancers (Li and Sun 1997; Li, et al. 1997; Steck, et al. 1997). Subsequently, dysregulation of PI3K-AKT signalling, due to PTEN loss, has been largely associated with cancer. The frequency of loss of heterozygosity for *PTEN* in prostate cancer is approximately 35% (Engelman, et al. 2006). *PTEN* genomic deletions have been detected in 20.2% of prostate cancers, including 8.1% heterozygous and 12.1% homozygous deletions, and have been associated with advanced tumor stage, high Gleason grade, presence of lymph node metastasis, and androgen-independent disease (Krohn, et al. 2012). Integrative analysis of transcriptome (mRNA and microRNA), copy number analysis profiling and exon sequencing for somatic mutations conducted on a cohort of prostate cancer patients (24% of patients had a favorable long-term clinical outcome of >5 years of recurrence-free survival) revealed that the PI3K-AKT pathway was altered in 42% of primary cancers and 100% of metastases, while the Ras-Raf pathway was altered in 43% of primary specimens and 90% of metastases (Taylor, et al. 2010). Both PTEN loss and AKT activation, as measured by Ser473 phosphorylation (AKT-P), have been associated with biochemical recurrence (elevated PSA levels) following radical prostatectomy (Ayala, et al. 2004; Bedolla, et al. 2007). In addition, PTEN loss is associated with prostate tumour

progression and has also been shown to predict a shorter time for metastasis-free survival (Lotan, et al. 2011), implicating PI3K-AKT signalling in prostate cancer development.

The role of PI3K-AKT signalling in prostate cancer has also been examined in mouse models. *PTEN* heterozygous (+/-) mice were first reported to display hyperplastic lesions in the prostate, with benign PIN lesions observed in some mice at a young age (≤14 weeks) (Di Cristofano, et al. 1998; Podsypanina, et al. 1999). Moreover, *PTEN* deletion in various prostate cells has also been carried out to promote PI3K-AKT signal activation in order to determine the prostate cell lineage responsible for subsequent prostate tumour initiation, as previously discussed (Choi et al. 2012; Wang et al. 2009; Wang et al. 2013). Prior to these studies, probasin promoter-driven *PTEN* deletion was carried out, resulting in PI3K-AKT signal activation specifically in both basal and luminal cells of the prostate epithelium (Wang, et al. 2003). By 6 weeks of age, these mice formed murine PIN lesions, developed adenocarcinoma that invades the basement membrane and stromal regions by 9 weeks, and generated prostate cancer metastases in the lymph nodes and lungs by 12 weeks of age (Wang et al. 2003). Moreover, expression of a constitutively-active form of AKT1 (Src myristoylation sequence fused to the N-terminus of AKT1; c-AKT) results in PIN formation in the mouse prostate when expression is driven by the probasin promoter, or after reconstituting ectopically-expressing prostate basal cells with UGSM and grafting *in vivo* (Lawson et al. 2010). Therefore, the mouse prostate epithelium has been shown to be vulnerable to PTEN loss, with the propensity to undergo AKT-driven tumorigenesis.

1.2.5. The role of EGFR, PI3K-AKT and MAPK signalling in cancer stem-like cells

Although the epidermal growth factor receptor (EGFR) is a prognostic marker of various advanced stage carcinomas such as breast (Livasy, et al. 2006), ovarian (Nielsen, et al. 2004), and non-small cell lung cancers (Garcia de Palazzo et al. 1993), the role of EGFR expression and signalling in CSCs has only recently been investigated. In human brain cancer, EGF supplementation significantly enhances the formation of sphere cultures, while EGFR inhibition potently inhibits sphere formation as well as the number of brain tumour stem ($CD133/AC141^{+}$) cells (Soeda, et al. 2008). Furthermore, EGFR expression in glioma CSCs confers enhanced tumorigenic potential to the EGFR-positive ($EGFR^{+}$) fractions obtained from $EGFR^{+}$ CSC lines. Furthermore, EGFR knockdown in $EGFR^{+}$ CSCs decreases their tumorigenicity *in vivo* and promotes differentiation of these cells. In cell sorting experiments, EGFR-high expressing cells displayed reduced proliferative capacity compared to EGFR-low or EGFR-negative cell fractions (Mazzoleni, et al. 2010), an observation that is consistent with stem cells being quiescent or slow cycling in their niches (Morrison and Spradling 2008). Glioma CSCs have been shown to be responsive to EGFR-targeting clinical drugs, erlotinib and gefitinib, with sensitivity to these drugs likely requiring inhibition of PI3K-AKT signalling (Griffero, et al. 2009). In examining the functionality of EGFR in tumorigenesis, EGFRvIII heterogeneously expressed in a minor subpopulation of glioblastoma was shown to enhance the tumorigenicity of the entire tumour cell population in a paracrine-driven manner by recruiting wild-type EGFR-expressing cells into accelerated proliferation (Inda, et al. 2010). EGFRvIII expression has also been detected in primary breast

carcinoma, with ectopic EGFRvIII expression in human breast cancer cell lines increasing the proportion of prospective $CD44^+CD24^-$ breast cancer stem-like cells and enhancing the expression a stem cell-associated genes (Del Vecchio et al. 2012). In addition, ectopic EGFRvIII expression increased the sphere-forming capacity and enhanced the *in vivo* tumorigenicity of breast cancer cells (Del Vecchio et al. 2012). Taken together, this supports the notion that a minor tumour cell population with active EGFR signalling can drive accelerated growth of the entire tumour mass, and yet actively maintain heterogeneity within the solid tumour.

The role of PI3K-AKT signalling in the proliferation and maintenance of cancer stem-like cells has been examined in human prostate (Dubrovska, et al. 2010; Dubrovska et al. 2009), breast (Korkaya, et al. 2009; Zhou, et al. 2007) and brain cancers (Eyler, et al. 2008; Sunayama, et al. 2010). PTEN knockdown in DU145 prostate cancer cells increases *in vivo* xenograft tumour formation and results in an increased capacity of these cells to form primary spheres under EGF and bFGF-supplemented serum-free conditions (Dubrovska et al. 2009). In addition, treatment with the PI3K inhibitor LY294002 reduced DU145 primary sphere formation (Dubrovska et al. 2009) while the PI3K/mTOR inhibitor NVP-BEZ235 reduced the $CD44^+CD133/AC141^+$ DU145 cell population *in vivo* and subsequently delayed xenograft tumour formation (Dubrovska et al. 2010). PTEN knockdown in MCF7 and Sum159 human breast cancer cell lines resulted in increased AKT signal activation and sphere formation, while treatment with the AKT inhibitor perifosine reduced Sum159 xenograft tumour formation in the fat pads of immuno-compromised mice (Korkaya et al. 2009). Furthermore, LY294002 has been shown to

reduce a tumour-initiating subpopulation derived from MCF7 cells, and reduced the formation of subcutaneously-derived MCF7 xenograft tumours (Zhou et al. 2007). The PI3K-AKT pathway is also differentially sensitive in CD133/AC133^{+} compared to CD133/AC133^{-} brain cancer cells. Treatment with an AKT inhibitor (AKT VIII) resulted in inhibition of AKT signal activation and decreased the viability of CD133/AC133^{+} brain cancer cells, as well as reduced their sphere-forming capacity (Eyler et al. 2008). Similarly, NVP-BEZ235 treatment was also shown to reduce the propagation of spheres derived from human glioblastoma stem-like cells (Sunayama et al. 2010). Taken together, these results suggest that PI3K-AKT signalling plays a role in the viability and maintenance of CSCs from solid tumours.

The extent of MAPK signalling in the regulation of CSCs in solid tumours is not well known. In breast cancers, activation of MAPK-β-catenin signalling promotes breast tumour-initiating cell (TIC) expansion, with MAPK inhibition following sorafenib (Raf-1 inhibitor) or AZD6244 (MEK inhibitor) treatment significantly reducing the number of EZH2-induced breast TICs (Chang, et al. 2011). In addition, knockdown of Raf-1 significantly reduced the proportion of these breast TICs by reducing ERK activation (Chang et al. 2011). Furthermore, suppression of MAPK signalling also reduced the self-renewal capacity of cancer stem-like cells from both cell line and patient-derived glioblastoma (Sunayama et al. 2010). Through targeted inactivation of MEK1/2 through silencing RNA (siRNA)-mediated knockdown or using the MEK inhibitor U0126 resulted in reduced sphere formation and induced a pro-differentiation effect (βIII-tubulin expression) in glioblastoma stem-like cells (Sunayama et al. 2010). Although the support

for MAPK signalling in CSC regulation is not extensive, these reports suggest that MAPK signalling is required for mediating the self-renewal of stem-like cells in breast and brain cancers.

1.3. SOX2 and its role in pluripotency, cellular reprogramming and tumorigenesis

Cancer stem cells have been proposed to originate from the transformation of a normal stem cell, or they may arise from mutations attained by restricted progenitors and differentiated cells (Pardal et al. 2003; Reya et al. 2001; Visvader and Lindeman 2008). Recent studies have suggested that non-tumorigenic (differentiated) cells can acquire *de novo* stem-like properties, and that cancer cells can reversibly transition stochastically between tumorigenic and non-tumorigenic states (Chaffer, et al. 2011; Gupta, et al. 2011). Further insight into the common mechanisms associated between cellular plasticity (or 'dedifferentiation') and tumorigenesis have arisen from studies directed at the controlled reprogramming of somatic cells into the pluripotent stem-like state (Daley 2008). This reprogramming approach, which generates induced pluripotent stem (iPS) cells, utilized ectopic expression of transcription factors (Oct4, Sox2, Klf4 and c-Myc) to reset the epigenetic state of differentiated somatic cells to a pluripotent stem-like state (Park, et al. 2008; Takahashi, et al. 2007; Wernig, et al. 2007). Alternatively, pluripotency factors NANOG and LIN28 can be substituted for c-Myc and Klf4 to reprogram somatic cells (Yu, et al. 2007). In addition, OCT4 (Lin, et al. 2012), SOX2 (Chen, et al. 2008; Gangemi, et al. 2009; Rodriguez-Pinilla, et al. 2007), and NANOG (Noh, et al. 2012)

have been implicated in tumorigenesis. Furthermore, an ESC-like gene signature in which Oct4, Sox2, Nanog and c-Myc target genes are more frequently expressed in poorly differentiated tumours compared to well-differentiated tumours (Ben-Porath, et al. 2008) suggests that common molecular circuitry may exist between somatic cell reprogramming and cancer (Suva, et al. 2013).

The transcription factor Sox2/SOX2 (SRY-related HMG-box gene 2) belongs to the SOX (Sry-related HMG Box) family of proteins (Kamachi, et al. 2000). The human *SOX2* gene is a single exon gene located on chromosome 3, which encodes a 317 amino acid protein, while the mouse *Sox2* gene encodes a 319 amino acid protein. The SOX2 protein contains a DNA binding (High Mobility Group; HMG) domain which recognizes DNA sites with the consensus sequence, C(T/A)TTG(T/A)(T/A), often referred to as the HMG motif (Boer, et al. 2006; Chen et al. 2008). Within its HMG domain, SOX2 contains two nuclear localization signals (NLS), with each NLS being partially effective at localizing SOX2 to the nucleus (Li, et al. 2007). SOX2 contains a transactivation domain within its C-terminal portion, which contains a serine-rich region; the serine-rich region and the extreme C-terminus are essential for its transactivational activity (Nowling, et al. 2000).

1.3.1. The role of Sox2 in embryonic development and adult tissues

In embryonic stem cells, pluripotency is maintained by the core transcription factors Oct4, Sox2 and Nanog, which co-occupy the promoters of various target genes (Boyer, et al. 2005; Loh, et al. 2006), self-regulate their transcription levels (Kuroda, et al. 2005; Masui, et al. 2007; Okumura-Nakanishi, et al. 2005), with Sox2 and Oct4 forming a

complex to regulate expression of target genes (Ambrosetti, et al. 1997) and maintain pluripotency (Chew, et al. 2005). Sox2 is expressed in the nuclei of embryonic stem cells, and acts as a transcription factor required for maintaining their pluripotency and self-renewal capacity (Fong, et al. 2008; Li et al. 2007). While Oct4 maintains pluripotency in concert with Sox2 and Nanog (Loh et al. 2006; Masui et al. 2007; Pan, et al. 2006), there is evidence suggesting that Sox2 may play additional roles in fetal and adult progenitors.

The essential role of Sox2 in early embryonic development is demonstrated by the failure of *Sox2*-null embryos to develop beyond the epiblast stage. Sox2 is expressed in the inner cell mass and extraembryonic ectoderm of blastocysts (Avilion, et al. 2003). While maternal Sox2 protein has been shown to mask a phenotype in preimplantation embryos, depletion of maternal and zygotic *Sox2* mRNA transcripts by siRNA-mediated knockdown caused early embryos to arrest at the morula stage and inhibited trophectoderm formation (Keramari, et al. 2010), which is necessary for producing the placenta. This suggests that Sox2 is necessary for the segregation of trophectoderm from the inner cell mass during embryo development (Keramari et al. 2010). During fetal development, Sox2 expression has been detected in proliferating neural progenitors within the neural tube, and its expression has been associated with inhibition of neuronal differentiation (Graham, et al. 2003). Furthermore, Sox2 downregulation has been associated with neurodegeneration in the adult mouse brain (Ferri, et al. 2004). Sox2 is expressed in neural stem cells (Brazel, et al. 2005), with neural precursor cell self-renewal being enhanced through a cellular feedback loop involving mutual regulation of EGFR and Sox2 *in vitro* (Hu, et al. 2010). In addition, $Sox2^{+}$ neural cells can be propagated as

spheres, thereby demonstrating their self-renewal capacity and potential for multipotent differentiation into neurons, astrocytes and oligodendrocytes (Ellis, et al. 2004). Sox2 is also involved in inner ear (Kiernan, et al. 2005), pituitary gland (Kelberman, et al. 2006) and taste bud development (Okubo, et al. 2006). In addition, Sox2 is expressed in retinal progenitor cells and regulates their differentiation (Taranova, et al. 2006), while collagen I promoter-driven conditional Sox2 knockout mice display reduced bone density and bone volume implicating a role for Sox2 in osteoblasts (Basu-Roy, et al. 2010).

In humans, heterozygous *SOX2* mutations are associated with anophthalmia-esophageal-genital (AEG) syndrome. Symptoms associated with AEG syndrome include microphthalmia (small eyes), ocular malformations like bilateral anophthalmia (absence of eye), tracheo-esophageal fistula (trachea and esophagus fail to separate), hearing loss, brain abnormalities associated with learning disabilities, and urogenital defects including micropenis, cryptorchidism and hypospadias (Hagstrom, et al. 2005; Kelberman et al. 2006; Ragge, et al. 2005; Williamson, et al. 2006). Sox2 loss in the early mouse embryo resulted in the esophagus morphologically resembling the trachea, and the forestomach having an abnormal phenotype (Que, et al. 2007). Although *Sox2* heterozygote mice appear normal but smaller than their wild-type littermates, they display reduced pituitary size and hormone production, as well as testicular atrophy and impaired fertility (Kelberman et al. 2006). Through a series of allelic *Sox2* mutations in the mouse, variable levels of Sox2 expression have been detected in the eyes that have resulted in varying degrees of ocular malformations, with conditional deletion of *Sox2* from retinal progenitors resulting in microphthalmia and optic nerve hypoplasia. These results indicate

that eye defect severity is related to *Sox2* gene dosage (Taranova et al. 2006). Therefore, manifestations of the disease in patients caused by heterozygous mutations in the *SOX2* gene are comparable to the dose-dependent loss of function observed in *Sox2* hypomorphic mice.

While Oct4 is dispensable for the self-renewal and maintenance of somatic stem cells in the adult mammal (Lengner, et al. 2007), a systematic survey of Sox2 expression in adult tissues was recently performed in order to elucidate its functional role in adult tissues (Arnold, et al. 2011). Sox2 expression was observed in the subventrical zone of the brain as well as in the retina, tongue, trachea, bronchiolar epithelium of the lungs and dermal papillae of hair follicles in the skin. In addition, SOX2 expression was identified for the first time in testes, cervix, lens epithelium, glandular stomach, and squamous epithelia of the esophagus. The persistence and expansion of Sox2-derived cells, as identified by lineage-tracing experiments, are consistent with long-term stem cells within these tissues (Arnold et al. 2011). $Sox2^+$ adult stem cells can be either unipotent (e.g., germ and lens stem cells) or multipotent as in the glandular stomach where $Sox2^+$ stem cells differentiate into parietal, neuroendocrine, and gastrin-producing cells. In order to address the self-renewal and differentiation potential of $Sox2^+$ stem cells, $Sox2^+$c-kit^- spermatogonial cells were able to recapitulate spermatogenesis in the seminiferous tubules and produce mature sperm in an infertile mouse model. Finally, the function of SOX2-expressing cells in maintaining homeostasis of different adult tissues was addressed by ablating $Sox2^+$ cells, whereby thymidine kinase was driven by the Sox2 promoter in Sox2-expressing cells. Short-term ablation (two weeks) of $Sox2^+$ cells lead to

the death of the mice, with abnormalities being found in the tissue architecture of squamous epithelial tissues. Conversely, acute ablation of $Sox2^+$ cells had long-term effects on spermatogenesis; atrophic spermatogonial tubules were found months following ganciclovir treatment (Arnold et al. 2011). Therefore, Sox2 is expressed and regulates the function of a broad range of stem cells including pluripotent stem cells, various tissue-specific embryonic progenitors and adult stem cells.

1.3.2. SOX2 dysregulation in cancer

Besides its role in embryonic, fetal and adult tissue development and maintenance, emerging studies are revealing an association between SOX2 and various cancers. Amplification of a chromosomal segment (3q26.33) containing the *SOX2* gene has been observed in lung (23%) and esophageal (15%) squamous cell carcinomas (SCCs) (Bass, et al. 2009), and in approximately 27% of small cell lung cancers analyzed (Rudin, et al. 2012). Knockdown of SOX2 in cell lines derived from lung and esophageal SCCs and small cell lung cancers reduced their cell proliferation (Bass et al. 2009; Rudin et al. 2012), and reduced the *in vitro* anchorage-independent growth of lung SCC cells (Bass et al. 2009). SOX2 expression was observed to be elevated in glioblastoma specimens (Alonso, et al. 2011; Schmitz, et al. 2007), with its expression being heterogeneous in tumour samples (Schmitz et al. 2007). SOX2 is overexpressed in human breast tumours, with its expression levels being correlated with higher tumour grade (Chen et al. 2008; Rodriguez-Pinilla et al. 2007). Moreover, SOX2 expression was observed in 16.7% of breast cancer cases, particularly in basal-like (43.3%), $HER2^+$ (13.3%) and luminal

(10.6%) breast carcinomas (Rodriguez-Pinilla et al. 2007). Furthermore, SOX2 knockdown has been shown to reduce brain tumour (Gangemi et al. 2009) and breast tumour (Chen et al. 2008; Leis, et al. 2012) formation *in vivo*, suggesting that SOX2 has a role in the tumorigenicity of these diseases.

1.3.3. SOX2 and prostate cancer

In the human prostate, nuclear SOX2 expression has been shown in less than 10% of cells within normal prostate glands (in 85% of cases) (Ugolkov, et al. 2011), and elevated in prostate carcinomas (Jia, et al. 2011; Ugolkov et al. 2011). In addition, prostate cancers positive for SOX2 staining have also been observed to be positive for the CD44 cell surface marker (Ugolkov et al. 2011). Given that the $CD44^+$ prostate cancer cell population is heterogeneous with approximately 1% of the $CD44^+$ cells representing cancer stem (tumour-initiating) cells (Patrawala et al. 2006), SOX2 may identify a minor subpopulation of prospective CSCs within $CD44^+$ prostate carcinomas.

The expression of SOX2 has also been implicated in prostate cancer progression. SOX2 expression was demonstrated to be positive in 92% of human prostate tumour specimens, with the intensity of the staining being strongly correlated with increasing tumour histological grade (Jia et al. 2011). In contrast, 8% of normal or hyperplastic tissues were SOX2-positive (Jia et al. 2011). Interestingly, an amplification unit at 3q25-q27 has been detected in prostate carcinomas, with the *SOX2* gene (3q26.3-q27) being located in this amplicon (Sattler, et al. 2000). This suggests that SOX2 may be amplified in prostate cancers. The contribution of SOX2 expression towards prostate tumorigenesis

has been evaluated *in vitro*. Ectopic SOX2 expression increased the proliferation rate and anti-apoptotic properties of DU145 cells, while SOX2 knockdown cells displayed apoptotic sensitivity to cisplatin treatment (Jia et al. 2011). In addition, SOX2 knockdown reduced the tumorigenicity of DU145 monolayer cells *in vivo* (Jia et al. 2011), further suggesting that SOX2 regulates prostate tumorigenesis and may have a functional role in PCSCs.

1.3.4. The role of SOX2 in cancer stem cells

SOX2 is expressed in discrete cells required for maintaining the integrity of multiple tissues (Arnold et al. 2011) and its upregulation in various cancers, including prostate cancer, suggests that it is required in maintaining tissue stem cells and cancer stem cells. In breast cancer, SOX2 expression has been observed in 43% of basal cell-like breast carcinomas and found to be strongly correlated with CK5/6, vimentin and EGFR immunoreactivity, markers which are typically associated with a less differentiated phenotype (Rodriguez-Pinilla et al. 2007). Moreover, SOX2 was upregulated in MCF7 breast cancer spheres compared to their isogenic monolayer (serum-grown) cell population, with SOX2 knockdown reducing *in vitro* sphere formation, reducing *in vivo* tumour formation (Leis et al. 2012) and delaying the growth of existing tumour xenografts following induced SOX2-targeted silencing (Stolzenburg, et al. 2012). SOX2 has also been implicated in maintaining tumour-initiating cells in brain tumours (Alonso et al. 2011; Gangemi et al. 2009), osteosarcomas (Basu-Roy, et al. 2011) and breast cancer cells (Leis et al. 2012). SOX2 is overexpressed in human and murine

osteosarcoma cell lines, with SOX2 knockdown reducing their sphere-forming capacity and inhibiting the *in vivo* tumorigenicity of murine OS-482 osteosarcoma cells (Basu-Roy et al. 2011). Similarly, SOX2 knockdown in human brain tumour-initiating cells reduced their cell proliferation (Gangemi et al. 2009), as well as reduced their sphere-mediated self-renewal capacity (Alonso et al. 2011) and *in vivo* tumorigenic potential (Gangemi et al. 2009). More recently, SOX2 has been identified as a downstream target of Myeloid Elf-1-like factor (MEF) in mouse and human gliomas, with SOX2 overexpression being sufficient to rescue the reduction in stem-like properties observed in *MEF*-null cells (Bazzoli, et al. 2012). Finally, SOX2 knockdown in MCF7 human breast cancer cells reduced their sphere-forming capacity and delayed their ability to initiate tumour formation in immuno-compromised mice (Leis et al. 2012). Although SOX2 has been reported to demonstrate oncogenic properties in various cancers, more importantly, SOX2 may regulate the stem-like properties of tumour-initiating cells present within solid tumours.

1.4. Project Rationale, Hypothesis, Objectives and Thesis Outline

1.4.1. Project rationale and hypothesis

Despite prostate cancer being the most prevalent malignancy in men (Siegel et al. 2012), our understanding of prostate tumorigenesis remains limited, including knowledge of its origin, as well as the factors and signalling pathways that regulate its initation. At the time when this project was initiated, the concept of CSC in solid cancers was just

emerging based on the identification of CSCs in primary breast (Al-Hajj et al. 2003) and brain tumours (Singh et al. 2004), with evidence for the existence of CSCs in prostate cancer having yet to be reported. However, based on the reported tumour-initiating capacities demonstrated for CSCs of breast, brain and colon tumours (O'Brien et al. 2007; Ricci-Vitiani et al. 2007), we hypothesized that PCSCs were also the origin of prostate cancer. Evidence in support of this hypothesis included the existence of prostate stem cells (PSCs) as demonstrated through *in vivo* prostate regeneration following castration and re-administration of androgen (English et al. 1987; Sugimura et al. 1986; Wang et al. 2013), candidate PSCs residing in the basal cell population of prostate glands that survive androgen-depletion (Wang et al. 2013), and the expression of EGFR, a major oncogenic protein in prostate cancer, in basal cells of the prostate epithelium (Maygarden et al. 1992).

EGFR expression in prostate cancer has been shown to be associated with progression towards an androgen-independent state (Di Lorenzo et al. 2002; Hernes et al. 2004; Shah et al. 2006). Moreover, activation of EGFR signalling, through expression of mutant forms of EGFR that promote its activation (Cai et al. 2008; Olapade-Olaopa et al. 2000) or production of secretory EGF-like ligands (Hofer et al. 1991; MacDonald et al. 1990) that promote autocrine/paracrine signalling, have been reported in prostate cancer. EGFR signalling has also been shown to maintain cancer stem-like cells (Griffero et al. 2009; Soeda et al. 2008) and promote their tumorigenicity in various solid tumours (Del Vecchio et al. 2012; Mazzoleni et al. 2010). Based on this knowledge, we further

hypothesized that EGFR contributes to prostate tumorigenesis by promoting stem-like properties, like self-renewal capacity, in prostate cancer stem cells.

Transcriptional factors play critical roles in maintaining the self-renewal of stem cells. In embryonic stem cells, pluripotency is maintained by the core transcription factors Oct4, Sox2 and Nanog (Boyer et al. 2005; Loh et al. 2006), which have also been shown to reprogram somatic cells towards a pluripotent state (commonly referred to as induced pluripotent stem (iPS) cells) (Park et al. 2008; Takahashi et al. 2007; Wernig et al. 2007; Yu et al. 2007). Given that SOX2 has been reported to be expressed, and functionally important, in adult stem cells (Arnold et al. 2011), brain CSCs (Alonso et al. 2011; Gangemi et al. 2009) and breast CSCs (Leis et al. 2012), we hypothesized that SOX2 plays an essential role in PCSC maintenance.

In summary, the hypothesis of my Ph.D. research consists of three layers: 1) PCSCs are the source of prostate tumorigenesis, 2) PCSCs are regulated by the EGFR pathway, and 3) the transcriptional factor SOX2 plays a critical role in maintaining the self-renewal of PCSCs.

1.4.2. Objectives and experimental approach

To investigate the hypothesis, this research focused on the examination of the following objectives:

1. Isolation and characterization of a human prostate cancer stem-like cell (PCSC) subpopulation capable of propagating as non-adherent spheres *in vitro*.

2. Determination of the contribution of EGF and EGFR signalling, as well as downstream PI3K-AKT and MAPK signalling, towards PCSC propagation and maintenance.

3. Examination of the role of SOX2 in PCSC propagation and maintenance, and investigation of the relationship between SOX2 and EGFR signalling in PCSCs.

In order to investigate the hypothesis of my Ph.D. research, the DU145 human prostate cancer cell line was used. DU145 androgen-independent prostate cancer cells were isolated from a 69 year old Caucasian male who had a three-year history of lymphocytic leukemia. This individual underwent bilateral orchiectomy (surgical removal of testicles), transurethral resection of the prostate, and parieto-occipital craniotomy to excise a metastasized prostate tumour mass. These prostate cancer cells derived from the brain metastasis grow as isolated islands in cell culture and form anchorage-independent colonies in soft agar. Furthermore, DU145 human prostate cancer cells demonstrate an aneuploid human karyotype with a chromosome number of 64 (Stone, et al. 1978).

1.4.3. Thesis outline

The thesis objectives were explored in three manuscripts (Chapters 2, 3 and 4), and are presented in this work as follows:

Chapter 2: Characterization of sphere-propagating cells with stem-like properties from DU145 prostate cancer cells.

This journal article, published in *Biochimica et Biophysica Acta (BBA) – Molecular Cell Research* (2011), was accepted for publication on January 18, 2011. This journal article characterizes a subpopulation of DU145 prostate cancer cells that are capable of propagating as non-adherent spheres *in vitro*.

Chapter 3: Propagation of human prostate cancer stem-like cells occurs through EGFR-mediated ERK activation.

This journal article, published in *PLoS One* (2013), was accepted for publication on March 17, 2013. This journal article provides evidence that EGFR signalling promotes self-renewal of prostate cancer stem-like cells (PCSCs), in part, by activating the MEK-ERK pathway.

Chapter 4: SOX2 plays a critical role in EGFR-mediated self-renewal of human prostate cancer stem-like cells.

This manuscript was submitted to *Molecular Oncology* (May 2013) for publication. This manuscript demonstrates that the stem cell transcription factor, SOX2, is required for

EGFR-mediated PCSC self-renewal. Furthermore, the results in the manuscript demonstrate that activation of EGFR signalling promotes SOX2 protein expression in DU145 spheres, while inhibition of EGFR signalling attenuates its expression and PCSC self-renewal. Therefore, SOX2 is a downstream target of EGFR signaling and it is required for PCSC self-renewal.

CHAPTER 2

Characterization of sphere-propagating cells with stem-like properties from DU145 prostate cancer cells

Adrian P. Rybak, Lizhi He, Anil Kapoor, Jean-Claude Cutz and Damu Tang

Biochimica et Biophysica Acta (BBA) - Molecular Cell Research (2011). 1813(5): 683-694. doi: 10.1016/j.bbamcr.2011.01.018.

Preface

A subpopulation of cancer cells with tumor-initiating potential have been identified recently in various solid tumors, including brain (Singh, et al. 2004), breast (Al-Hajj, et al. 2003), and colon cancers (O'Brien, et al. 2007; Ricci-Vitiani, et al. 2007). However, bona-fide PCSCs have not been isolated from primary prostate cancer tissues to date. This is largely due to the challenging nature of isolating prostate cancer cells since there is a limited amount of clinically available prostate cancer tissue material, which itself is highly heterogeneous in nature. Due to the constraints in obtaining a sufficient amount of human prostate cancer material in order to subsequently isolate and characterize prospective PCSCs, established human prostate cancer cell lines have become surrogate sources for investigating PCSCs. Therefore, stem-like cells from the established DU145 human prostate cancer cell line were isolated, propagated in a defined serum free medium (SFM) and subsequently characterized. One of the methods used to isolate and expand somatic (Reynold and Weiss, 1996; Dontu et al., 2003) and cancer stem cells (Ponti et al., 2005; Ricci-Vitiani et al., 2007; Singh et al., 2004) is the sphere assay; a SFM culture supplemented with recombinant growth factors which allows for stem and progenitor-like cells to propagate under non-adherent conditions.

This chapter consists of an author-generated version of the following published article: Adrian P. Rybak, Lizhi He, Anil Kapoor, Jean-Claude Cutz, and Damu Tang (2011) Characterization of sphere-propagating cells with stem-like properties from DU145 prostate cancer cells. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1813(5): 683-694. This article is reprinted with permission from Elsevier Limited (see

Appendix 3 for License Agreement). Please note that American spelling is used throughout the article, and that the formatting and referencing style has changed to meet the journal's criteria. Please note that reverse transcriptase (RT)-PCR and agarose gel electrophoresis of the resulting PCR products was carried out on RNA isolated from high passage (passage 25) DU145 sphere cells (Supplementary Figure 1). This information was not provided in the figure legend of the publication. I, Adrian P. Rybak, generated and established all DU145 sphere cultures and sphere-derived adherent cultures (SDACs) used in this study. I performed all of the experiments, assembled the results, and generated the figures and table in the manuscript. Furthermore, I wrote and revised the manuscript along with Damu Tang. Lizhi He generated the short-hairpin PTEN targeting (shPTEN) vector and assisted with *in vivo* tumor injections in order to generate prostate cancer xenografts in immuno-compromised mice. Anil Kapoor provided human primary prostate cancer tissue which was used in preliminary work. Jean-Claude Cutz made revisions to the manuscript and provided intellectual input.

This manuscript establishes that a minor subpopulation of DU145 cells, capable of forming and propagating as non-adherent spheres *in vitro*, display increased *in vivo* tumorigenic potential compared to the monolayer cells from which they were derived. These sphere-forming DU145 cells express both prostate basal and luminal cytokeratins, along with cell surface markers previously associated with cancer stem cells. Results demonstrated that epidermal growth factor (EGF) treatment enhanced the formation and maintenance of DU145 spheres, while basic fibroblast growth factor (bFGF) inhibited these events. Furthermore, sphere cells proliferate slowly and display reduced activation

of the PI3K-AKT pathway compared to monolayer cells. While knockdown of PTEN enhanced AKT activation, the generation of primary spheres and the propagation of secondary spheres were not affected. This pioneering work involving DU145 sphere formation and maintenance established the rationale for investigating further into the roles of EGFR, downstream MEK-ERK signalling and the SOX2 transcription factor in PCSC self-renewal, as examined in Chapters 3 and 4.

Characterization of Sphere-Propagating Cells with Stem-like Properties from DU145 Prostate Cancer Cells

Adrian P. Rybak,[1, 2, 4, 5] Lizhi He,[1,2,4, 5] Anil Kapoor,[2, 4, 5] Jean-Claude Cutz,[3] and Damu Tang [1, 2, 4, 5#]

[1] Division of Nephrology, Department of Medicine, [2] Division of Urology, Department of Surgery, McMaster University and [3] Department of Pathology and Molecular Medicine, McMaster University, [4] Father Sean O'Sullivan Research Institute, [4] the Hamilton Centre for Kidney Research (HCKR), St. Joseph's Hospital, Hamilton, ON, Canada

#: Corresponding author:
Damu Tang
T3310, St. Joseph's Hospital
50 Charlton Ave East
Hamilton, ON
Canada L8N 4A6
Tel: (905) 522-1155, ext. 35168
Fax: (905) 521-6181
Email: damut@mcmaster.ca

Running title: Characterization of prostate cancer cells with stem-like properties

Key words: Prostate cancer stem cell, PI3K, AKT and PTEN

Abstract

While accumulating evidence demonstrates the existence of prostate cancer stem cells (PCSCs), PCSCs have not been isolated and thoroughly characterized. We report here the enrichment and characterization of sphere-propagating cells with stem-like properties from DU145 PC cells in a defined serum-free medium (SFM). Approximately 1.25% of monolayer DU145 cells formed spheres in SFM and 26% of sphere cells formed secondary spheres. Spheres are enriched for cells expressing prostate basal and luminal cytokeratins (34βE12 and CK18) and for cancer stem cell markers, including CD44, CD24, and integrin α2β1. Upon culturing spheres under differentiating media conditions in the presence of 10% serum, cells positive for CD44 and CD24 were substantially reduced. Furthermore, spheres could be generated from the sphere-derived adherent cell cultures and xenograft tumors, demonstrating the stemness of DU145 spheres. We have maintained spheres for more than 30 passages within 1.5 years without noticeable loss of their "stemness". Sphere cells possess self-renewal capacity, display significant increases in proliferation potential, and initiate xenograft tumors with enhanced capacity compared to monolayer DU145 cells. While EGF promoted the generation and maintenance of these stem-like cells, bFGF inhibited these events. Sphere cells proliferate slowly with a significant reduction in the activation of the PI3K-AKT pathway compared to monolayer DU145 cells. While knockdown of PTEN enhanced AKT activation, this did not affect the generation of primary spheres and the propagation of secondary spheres. Consistent with this observation, we were able to demonstrate the generation and propagation of spheres without the addition of external growth factors.

1. Background

Prostate cancer (PC) is the most common cancer affecting men and the second leading cause of cancer-related deaths in males in the developed world [1, 2]. The disease progresses from intra-epithelial neoplasia or *de novo*, locally invasive carcinoma to metastatic cancer that advances to hormone refractory prostate cancers (HRPCs). HRPCs contribute virtually to all PC associated deaths [3, 4]. Although the exact mechanisms responsible for PC initiation and progression remain elusive, PCSCs are widely regarded to be the origin of PC initiation and progression [5-7].

Several lines of evidence suggest the existence of PCSCs. A small population of primary PC cells expressing the surface antigenic profile $CD44^{+}\alpha_2\beta_1{}^{hi}CD133^{+}$ was suggested to be a candidate for PCSCs [8]. This possibility is supported by several reports. Cancer stem cells are known to share similar surface antigens with their tissue stem cell counterparts [9-13]. Consistent with this concept, human $\alpha_2\beta_1{}^{hi}CD133^{+}$ prostate epithelial cells possess the properties of prostate stem cells [14], and a single cell in the mouse prostate defined as $Lin^{-}Sca\text{-}1^{+}CD133^{+}CD44^{+}CD117^{+}$ is capable of generating an intact prostate [15]. Additionally, primary human prostate epithelial cells and malignant cells that ectopically express human telomerase reverse transcriptase (hTERT) displayed the $CD44^{+}\alpha_2\beta_1{}^{hi}CD133^{+}$ surface profile [16-18]. Furthermore, hTERT-transformed primary prostate epithelial cells derived from a patient with advanced PC were able to generate prostate tumors that resembled the original tumor [17]. These observations are

consistent with a report that $CD44^+$ subpopulations isolated from several cultured PC cell lines were more tumorigenic in nude mice than the isogenic $CD44^-$ population [19]. However, despite these observations supporting the candidacy of $CD44^+\alpha_2\beta_1^{hi}CD133^+$ cells as PCSCs, it remains to be demonstrated whether $CD44^+\alpha_2\beta_1^{hi}CD133^+$ cells isolated from primary PC tissues are capable of initiating tumors in immuno-compromised mice. Therefore, to date, bona-fide PCSCs have not been isolated from primary PC tissues. This is largely due to the challenging nature of PC tissue, limited access to clinical samples, highly heterogeneous PC materials, and frequent infiltration into surrounding non-neoplastic prostatic tissue.

Although *in vitro* PC cells with the properties of cancer stem cells (CSC) have been studied, these cells have not been thoroughly investigated. Different systems have been used to culture PCSCs, including using suspension, low adherence culture on a layer of agar [16, 18], or use of low adherence plates [17, 20]. In general, two types of media are used to culture these stem-like PC cells that contained either fetal bovine serum (FBS) [17, 19], or supplemented with epidermal growth factor (EGF; 10 or 20 ng/ml) and basic fibroblast growth factor (bFGF; 10 or 20 ng/ml) [16, 20]. Although these stem-like PC cells have been demonstrated to have CSC properties, these cells have not been cultured for an extended period of time. Therefore, whether these cells constituted as progenitors or bona-fide PCSCs remains to be determined. Furthermore, the essential pathways that regulate PCSCs have not been examined until very recently. Dubrovska and colleagues recently reported that the PI3K-AKT pathway is activated, which is required for isolation and maintenance of DU145-derived PCSCs [20].

We have characterized a sub-population of DU145 cells that propagate as spheres under defined serum-free conditions and which display stem-like properties. Approximately 1.25% of DU145 monolayer cells are able to form spheres in a serum-free medium supplemented with EGF (10 ng/ml). Spheres are enriched in sphere-forming cells, since approximately 26% of sphere-derived cells are capable of forming secondary spheres. bFGF inhibits the formation of primary spheres and the propagation of secondary spheres. Although EGF significantly promotes the generation and propagation of spheres, these stem-like cells are able to form spheres without external EGF. DU145 sphere cells initiated xenograft tumors with increased ability compared to monolayer cells. Unlike a recent report, the PI3K-AKT pathway does not play a major role in the generation and maintenance of DU145 spheres.

2. Materials and methods

2.1 Tissue cultures and knockdown of PTEN

DU145 prostate cancer cells, 293T, Caco-2 and PC12 cells were obtained from ATCC, and were cultured and maintained according to ATCC instructions.

Short-hairpin PTEN targeting (shPTEN) vector was constructed by inserting the PTEN targeting sequence (GTATAGAGCGTGCAGATAA) into the pRIH retroviral vector as a hairpin, according to our published conditions [21]. High titres of pRIH (empty vector: EV) and pRIH/shPTEN were produced using 293T cell as described previously [21]. DU145 cells were subsequently infected with EV or shPTEN retrovirus. Infected cells

were selected in hygromycin (0.5 mg/ml) and then examined for PTEN expression using western blot.

2.2 Isolation and propagation of PCSC

DU145 monolayer cells were enzymatically-dissociated in trypsin (0.25%)/EDTA and resuspended at clonal (10 cell/μl) or sub-clonal density in a serum-free medium (DMEM/F12; 3:1 mixture) containing 0.4% BSA, 0.2 × B27 lacking Vitamin A (Invitrogen) and supplemented with recombinant EGF at 10 ng/ml (Sigma-Aldrich). When examining the effect of bFGF on sphere formation, recombinant bFGF (Invitrogen) was added at a concentration of 10 ng/ml, in addition to heparin at 4 μg/ml (Sigma-Aldrich). Typical spheres formed in 10 to 12 days. Sphere cells were sub-cultured using trypsin and then resuspended in the above medium at clonal density.

To examine the sphere forming capacity of DU145 monolayer cells, cells from sub-confluent (~80%) cultures were resuspended in the above SFM at a density 5×10^3 or 10^4 cells/ml, followed by dispensing 1 mL of this cell suspension into individual wells of a 24-well culture plate (4 wells per treatment). The number of spheres that formed after 12 days of culture was counted. To assay the sphere forming capacity of secondary or established spheres, sphere cells were individualized by trypsinization and plated in 96-well plates at a density of 1 cell/well (120 wells in total). Wells containing only single cells were monitored. The number of spheres that formed was counted after 12 days of culture. Triplicate experiments were conducted.

2.3 Anchorage-Independent Growth Assay

DU145 monolayer and sphere cell cultures were plated in individual wells of six-well plates at a density of 10^4 cell/well in 1.5 mL of media containing 0.25% agarose. Each treatment was conducted in triplicate wells. After 8 weeks, colonies were counted under a phase-contrast microscope in 5 random fields per well. Digital images of plates were taken using a Sony Cybershot® (DSC-N2) camera, and mean colony area (pixels) was determined using ImagePro Plus 5.0 software. Duplicate experiments were conducted.

2.4 Cell Proliferation assay

DU145 monolayer cells and sphere cells were seeded in triplicate in a 96-well plate at clonal density. Cell number was measured using Roche Cell Proliferation kit 1 (MTT) according to the manufacturer's instructions.

2.5 Western blot analysis

Preparation of cell lysates and performance of western blotting were carried out according to our published procedure [22]. Antibodies used were goat anti-AKT (Santa Cruz, C-20, 1:1000), rabbit anti-phospho-AKT (AKT-P; Ser473 phosphorylation) (Cell Signaling, 1:1000), mouse anti-PTEN (Santa Cruz, clone A2B1, 1:500) and goat anti-β-actin (Santa Cruz, C-11, 1:1000). Densitometric analysis of bands was conducted using Scion Image software.

2.6 Immunofluorescence and Immunohistochemistry

Immunofluorescent (IF) staining of monolayer cell cultures was performed by fixing cells with 4% paraformaldehyde (20 min) or methanol:acetone (3:7) solution for 10 min, followed by staining with the specified primary antibodies. For spheres, intact spheres were fixed, washed three times with PBS and allowed to settle in a 30% sucrose solution overnight at 4^{o}C. Sucrose solution was removed and intact spheres were added to embedding medium for frozen tissue sections (Sakura Tissue-Tek O.C.T. compound). Frozen sections (4 μm) were prepared, and IF staining was carried out using the following antibodies: rabbit polyclonal anti-p16^{INK4A} (Santa Cruz, C-20, 4 μg/ml), rabbit polyclonal anti-BMI1 (Santa Cruz, H-99, 4 μg/ml), mouse anti-CD133/1 (Miltenyi Biotec, clone AC133, 5 μg/ml), mouse anti-human CD44 (BD Biosciences, clone G44-26, 2 μg/ml), mouse anti-CD24 (Neomarkers, clone SN3, 2 μg/ml), mouse anti-cytokeratin 18 (CK18; Santa Cruz, clone DC-10, 2 μg/ml), mouse anti-integrin α2β1 (Chemicon, clone BHA2.1, 0.5 μg/ml), mouse anti-human cytokeratin HMW (DakoCytomation, clone 34βE12, 1 μg/ml), mouse anti-Synaptophysin (Millipore, clone SVP-38, 1:100). IgG was used as a negative control. Slides were subsequently washed in 1×PBS and then incubated with secondary antibodies (donkey anti-mouse IgG-FITC or donkey anti-mouse IgG-PE, 1:200 dilution; Jackson Laboratories) for 1 hour. Slides were mounted with a coverslip using Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc., H-1200), and images were captured using a Zeiss Axiovert 200M inverted fluorescence microscope using AxioVision 3.1 software. Images were processed using CorelDraw 14.

For dual IF staining of DU145 xenografts, paraffin-embedded tissues were sectioned at 3 μm thickness, deparaffinized, rehydrated, subjected to antigen retrieval and endogenous peroxidase quenching as indicated for immunohistochemistry (IHC) staining. Sections were blocked for 1 hour at room temperature in 3% goat serum and 3% BSA in TBST, followed by overnight incubation at 4^{o}C with rabbit anti-human CD44 (Abcam, ab51037, 1:100) and mouse anti-human cytokeratin HMW (Dako, clone 34βE12) antibodies. Slides were subsequently washed in PBS and then incubated with secondary antibodies (goat anti-rabbit IgG Alexa Fluor 594 and goat anti-mouse IgG Alexa Fluor 488, 1:200; Invitrogen) for 1 hour. Sections were counterstained with DAPI.

IHC staining of the DU145 xenografts was performed as described previously [23]. Briefly, sectioned slides were deparaffinized, antigen retrieval was performed and sections were incubated with the following primary antibodies: anti-Chromogranin A (Dako, clone DAK-A3, 1:100), anti-human cytokeratin HMW (Dako, clone 34βE12, 1:100), anti-CK18 (Santa Cruz, clone DC-10, 1:100), anti-E-cadherin (Dako, clone NCH-38, 1:200), and anti–Synaptophysin (Dako, clone SY38, 1:25). Biotinylated goat anti-mouse IgG and avidin-biotin complex (ABC) were then added (Vectastain ABC kit, Vector Laboratories). The chromogen reaction was carried out with diaminobenzidine, and counterstaining was done with hematoxylin. Images were captured using an Olympus BX41 light microscope.

2.7 RNA extraction and Reverse Transcriptase–PCR (RT-PCR)

RNA was extracted from cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR was carried out using SuperScript III reverse transcriptase (Invitrogen) and the designed primers. β-Actin was used as an internal control. Primers and RT-PCR conditions are listed in Supplementary Table 1.

2.8 Xenograft tumor formation

DU145 monolayer and sphere cells were resuspended in MEM/Matrigel mixture (1:1 volume), followed by implantation of 0.1 ml of this mixture subcutaneously (s.c.) into flanks of 8-week-old male NOD/SCID mice (The Jackson Laboratory). Mice were inspected for tumor appearance, by observation and palpation, and tumor growth was measured weekly using a caliper. Tumor volume was determined using the standard formula: $L \times W^2 \times 0.52$, where L and W are the longest and shortest diameters, respectively. The presence of each tumor nodule was confirmed by necropsy. All animal work was carried out according to experimental protocols approved by the McMaster University Animal Research Ethics Board.

2.9 Statistical analysis

Statistical analysis was performed using SPSS 10.0 for Windows software. Data were presented as mean ± SE (standard error) unless otherwise specified. All Student's *t*-tests performed were two-tailed. A p-value < 0.05 was considered statistically significant.

3. Results

3.1 Generation and propagation of DU145 sphere cells

A variety of evidence suggests that human prostate stem cells reside in the basal cell layer of the human prostate [24, 25] and basal cells express the EGF receptor [26]. Based on this knowledge, we formulated a medium composed of DMEM/F12 (3:1), 0.4% BSA, 0.2× B27 supplemented with EGF at 5, 10, or 20 ng/ml and examined their ability to support the growth of DU145 stem-like cells as suspension spheres. Supplementation of 10 ng/ml of recombinant EGF to the serum-free medium (SFM+EGF) was sufficient to support DU145 cells to grow as spheres (Fig 1A). Individual spheres were most likely derived from single cells, as spheres were formed at clonal (10 cells/μl) density (Fig 1A). Neural spheres were routinely generated at the densities of 10 or 50 cell/μl [27] and at 10 cells/μl, neural spheres have been shown to derive from single cell [28]. Approximately 1.25% of DU145 monolayer cells are able to form primary spheres (Fig 1C) and spheres contain an average of 100-150 cells (data not shown).

We subsequently examined whether DU145 sphere cells possess self-renewal capacity using a well-established assay [29]. When sphere cells were seeded at single cell/well in a 96-well plate, typical spheres were formed during the course of 12 days (Fig 1B). In the total of 360 single-cell wells (120 single-cell wells per experiment in the total of three repeats) analyzed, 26.1±2% of sphere cells formed subsequent spheres (Fig 1C). This is consistent with 20% of neural [29] and brain cancer [9] sphere cells being able to form new spheres. Collectively, these observations suggest that DU145 spheres are composed

of a heterogeneous population of cells with (stem-like cells) or without (progenitor cells) self-renewing capacity. In comparison to monolayer cells, sphere cells display high levels of proliferation potential when cultured in SFM+EGF (Fig 1D). This is consistent with the reported high level of proliferation potential associated with brain cancer stem cells when cultured in a defined serum-free medium [9].

3.2 DU145 sphere-propagating cells express stem cell factors, basal and luminal epithelial cell markers

Transcriptional factors Nanog, Oct4 and Sox2 are known to express in stem cells [30-32]. Consistent with this observation, DU145 sphere cells express Nanog, Oct4, and Sox2 (Supplementary Fig 1A). While both Nanog and Oct4 were expressed at comparable levels in both monolayer and sphere cells (Supplementary Fig 1A), Sox2 expression was enhanced in sphere cells (Supplementary Fig 1A). BMI1 plays an important role in the self-renewal of neuronal and haematopoetic stem cells [33, 34]. We have recently shown that BMI1 facilitates prostate tumorigenesis [23], and more recently it has been shown that BMI1 is a critical regulator of murine adult prostate stem cell self-renewal [35]. Consistent with these observations, BMI1 was expressed exclusively in the nuclei of sphere cells (Supplementary Fig 1B). Taken together, these observations support the concept that DU145 spheres contain cells with stem-like properties.

Consistent with the above observations, sphere cells also express candidate cancer stem cell surface markers. PCSCs have been indicated to be $CD44^{+}\alpha_2\beta_1^{hi}CD133^{+}$ [16-18]. CD24 is associated with several CSC or cancer-initiating cells [36]. To determine the cell

surface antigen profile of DU145 sphere cells, we examined the expression of CD44, CD24, integrin α2β1, and CD133. While the majority of sphere cells are positive for CD44, CD24, and integrin α2β1 (Fig 2), we could not convincingly demonstrate the AC133 epitope of CD133 in DU145 spheres (data not shown). Furthermore, in more than 20 primary PC specimens examined with Gleason scores ranging from 6-8, we also failed to detect CD133 using the AC133 antibody (data not shown). This may not result from our staining procedure, as the AC133 antibody clearly detected the AC133 epitope of CD133 in AC133-positive Caco-2 cells [37] (Supplementary Fig 2). The difference between our observation and that of others may be due to the limited specificity of reagents used to detect AC133 (see Discussion for details).

As evidence indicates that prostate stem cells reside in the basal layer of human prostate [24, 25], we have investigated the expression of prostate basal, luminal and neuroendocrine (NE) markers in DU145 spheres. Using immunofluorescence, spheres were shown to be immunoreactive for basal epithelial high molecular-weight cytokeratins (CKs; 34βE12) and the luminal CK18 (Fig 2). No staining was detected in DU145 spheres for the NE marker synaptophysin, although the antibody clearly detected expression in synaptophysin-positive PC12 cells (Supplementary Fig 3). Consistent with these observations, neuroendocrine markers synaptophysin or chromogranin A could not be detected in xenograft tumors that were derived from either DU145 monolayer cells or spheres, while epithelial marker E-cadherin and prostate lineage-specific markers 34βE12 and CK18 were detected (Supplementary Fig 4).

3.3 DU145 spheres exhibit "stemness"

A typical character of SCs and CSCs is their ability to re-populate the original heterogeneous population of cells and from these cells, CSCs can be re-isolated. To demonstrate this property, we found that sphere cells were able to proliferate as monolayer cells in serum (10%) containing medium and furthermore, spheres can be re-generated from sphere-derived adherent cells (SDACs) in SFM+EGF medium. To characterize this process, we have cultured SDACs to passage 20 and analyzed the abilities of SDACs at different passages to form spheres. When 10^3 SDACs were seeded in a 24-well plate at 1 cell/μl, approximately 20% of SDACs formed spheres at passages 0 through 4 and the sphere-forming ability of SDACs significantly declined after passage 4 (Fig 3A). At passage 20, approximately 2% of SDACs were able to form spheres (Fig 3A) in comparison to 1.25% of monolayer cells being able to form spheres (Fig 1C). Therefore, at least 20 passages are required for SDACs to resume a similar sphere-forming frequency as the monolayer cell culture. Furthermore, from these "differentiated" cell populations, spheres could be regenerated and the ability of sphere generation correlates with their degree of differentiation – passage numbers cultured under differentiating media conditions. More importantly, both monolayer and sphere cells formed xenograft tumors in NOD/SCID mice and from these xenograft tumors, spheres could be isolated (Fig 3B).

We further demonstrated that spheres re-populated a heterogeneous cell population based on the profile of cell surface markers of spheres, SDACs, and monolayer DU145 cells. While sphere cells are largely positive for CD44, CD24, integrin α2β1, and CK18

(Fig 2), approximately 40% or less of monolayer cells express these markers (Fig 4, Supplementary Fig 5A). In comparison to sphere cells (Fig 2), the SDACs at passage 4 expressed CD44 and CD24, CK18, and integrin α2β1 at substantially reduced levels (Fig 4, Supplementary Fig 5B). It was surprising to notice that the passage 4 SDAC population consisted of significantly reduced number of CD44-positive or CD24-positive cells compared to the monolayer cell population (Fig 4, Supplementary Fig 5A, B). Although the underlying mechanisms for these observations are not clear, these mechanisms may be specific for CD44 and CD24, as the passage 4 SDAC population contained more CK18- or integrin α2β1-positive cells than the monolayer cell population (Fig 4). The passage 20 population of SDACs consists of increasing numbers of CD44-positive or CD24-positive cells and reduced numbers of CK18-positive or integrin α2β1-positive cells (Fig 4, Supplementary Fig 5B, C). These changes in general make the passage 20 SDACs more closely resemble monolayer cells than the passage 4 SDACs.

Furthermore, the heterogeneity of the sphere-repopulated cell population could also be demonstrated in xenograft tumors. In both monolayer cell- and sphere cell-derived xenograft tumors, CD44 and 34βE12 were clearly heterogeneously expressed (Fig 5A) in comparison to the largely homogenously expression of both markers in sphere cells (Fig 2). Interestingly, sphere cell-derived xenograft tumors contain more cells that are $CD44^+$, $34\beta E12^+$ and $CD44^+34\beta E12^+$ than monolayer cell-derived xenograft tumors (Fig 5A, B) (see Discussion about the significance of this observation).

3.4 bFGF attenuates the isolation and propagation of DU145 spheres

EGF was commonly used together with bFGF in culturing PCSCs [16, 20]. To examine whether bFGF would enhance the numbers of spheres formed, we seeded DU145 monolayer cells in SFM supplemented with EGF, bFGF (10 ng/ml), or EGF+bFGF at clonal (10 cells/μl) or sub-clonal (5 cells/μl) densities. Surprisingly, in comparison to EGF, bFGF not only significantly reduced primary spheres formed but also significantly inhibited EGF-mediated formation of primary spheres (Fig 6A). Similar effects were also observed in sphere propagation (Fig 6B).

A typical characteristic of malignant tumors is their autocrine ability. Since CSCs are responsible for tumor initiation and progression, CSCs may be independent on external growth factors. To investigate this possibility, we performed primary and secondary sphere formation under SFM without addition of EGF or other growth factors (GF). Although medium lacking GF (GF^-) substantially reduced the number of primary and secondary spheres formed compared to the EGF medium, spheres were capable of forming in the GF^- medium (Fig 6A, B). To further consolidate these observations, we took advantage that sphere-derived adherent cells (SDACs) possess a range of sphere-generating capacities (Fig 3A). When seeded at 10^3 cells/ml per well (or 1 cell/μl) in a 24-well plate, SDACs generated spheres, although at reduced levels, compared with the addition of EGF (Fig 6C). Similar to the observations obtained with addition of EGF, the ability of SDACs to form spheres inversely correlates with the length of time (passage) cultured under differentiating media conditions (Fig 6C). Collectively, the above observations demonstrate that external EGF is not required to support DU145 sphere

cultures. Interestingly, when compared to bFGF medium, GF^- medium was superior in the propagation of spheres (Fig 6B). This provides additional evidence that bFGF inhibits the generation and propagation of stem-like DU145 cells, which is consistent with the fact that the basal cells of prostatic glands contain SCs and express EGF receptor [26].

3.5 Sphere cells proliferate slowly with reduced activation of the PI3K-AKT pathway

Stem cells are normally kept in a quiescent stage or proliferate slowly in their niches. It is not clear whether cancer stem cells including PCSCs retain this property. It has been reported that DU145 stem-like cells proliferate more actively than monolayer cells under sphere-forming SFM supplemented with insulin (4 μg/ml), EGF (20 ng/ml), and bFGF (20 ng/ml) [20]. This is consistent with our observation that when cultured in SFM containing only EGF (10 ng/ml), sphere cells show enhanced proliferation potential (Fig 1D). Since approximately 26% of sphere cells are capable of generating spheres in sphere-forming medium (Fig 1C), the majority of sphere cells may not actively proliferate, concordant with the profile of sphere cell proliferation in sphere-forming medium (Fig 1D). Both monolayer and sphere cells proliferate very slowly up to day 4 in sphere-forming medium, but sphere cells begin to proliferate robustly afterwards (Fig 1D), which may be attributable to the initiation of sphere formation. We therefore reasoned that complete medium supplemented with 10% serum (CM) should be used to compare the proliferation rates of monolayer and sphere cells, as the majority of monolayer and sphere cells actively proliferate in this medium.

To minimize potential differentiation of sphere cells, we examined the proliferation of sphere cells immediately cultured in CM along with monolayer DU145 cells (Fig 7A). As expected, both sphere and monolayer cells proliferate in CM (Fig 7A). Monolayer cells exhibit more robust proliferation than sphere cells (Fig 7A). Furthermore, monolayer cells reached a much higher level of confluent density than sphere cells (Fig 7A). Taken together, these observations reveal that sphere cells have an intrinsic property of slow proliferation.

The PI3K-AKT pathway promotes cell proliferation. Our observation that sphere cells proliferate slowly indicates that this pathway is not highly activated in sphere cells. To test this possibility, we briefly cultured sphere cells in CM for 18 hours and then examined for serum-induced AKT activation side-by-side with monolayer DU145 cells (Fig 7B). It is clear that serum induces more robust AKT activation in DU145 monolayer cells than in sphere cells (Fig 7B), demonstrating that DU145 stem-like cells do not display enhanced activation of the PI3K-AKT pathway compared to monolayer DU145 cells. Consistent with this concept, sphere cells do not express reduced levels of PTEN compared to the monolayer cells (Fig 7B). Since PTEN is the dominant PIP3 phosphatase, the aforementioned observation thus supports the notion that DU145 stem-like cells do not have their PI3K-AKT pathway activation elevated. As DU145 sphere cells proliferate more actively than monolayer cells in sphere-forming medium observed by others [20] and ourselves (Fig 1D) and has been reported to display enhanced activation of the PI3K-AKT pathway in sphere-forming medium [20], we compared phosphorylation of Ser 473 of human AKT, a widely used surrogate marker of AKT

activation, in both monolayer and sphere cells that were cultured in sphere-forming medium. When cultured in this medium side-by-side for 24 and 48 hours, sphere cells display significantly reduced levels of AKT activation compared to monolayer cells (Fig 7C). Taken together, the above observations reveal that DU145 sphere cells do not demonstrate elevated activation of the PI3K-AKT pathway.

To further consolidate this notion, we stably knocked down PTEN in monolayer cells using our pRIH retroviral system [21]. As expected, knockdown of PTEN enhances AKT activation (Fig 8A). In comparison to empty vector (EV) retrovirus infected cells, knockdown of PTEN did not enhance the rate of primary sphere formation when cells were seeded at two sub-clonal densities (Fig 8B). Furthermore, we demonstrated that while PTEN remained knocked-down in PTEN shRNA spheres (Fig 8A), formation of secondary spheres was not enhanced compared to EV spheres (Fig 8C), indicating that the PI3K-AKT pathway does not play a major role in maintaining DU145 PCSCs. Taken together, the above observations clearly support the notion that enhanced activation of the PI3K-AKT pathway does not play a major role in the formation and propagation of DU145 sphere cells.

3.6 DU145 spheres display enhanced ability to form xenograft tumors in NOD/SCID mice

CSCs are associated with significantly enhanced tumorigenicity. To examine whether DU145 sphere cells (stem-like cells) possess enhanced capacity of tumorigenesis, we seeded monolayer and sphere cells in soft agar under SFM (without addition of growth factors), SFM+EGF, or CM media (Fig 9). In SFM, sphere cells produced significantly

more numbers and larger colonies than monolayer cells (Fig 9A, B). Even in CM medium, although monolayer cells generated more colonies than sphere cells (Fig 9A), they were substantially smaller in size (Fig 9B). Interestingly, sphere cells grew as efficiently in SFM as in SFM+EGF in soft agar (Fig 9A), which further supports our observation that sphere cells have autocrine capacity. Taken together, the above observations reveal that sphere cells grow much more efficiently under an anchorage-independent environment compared to monolayer cells, indicative of sphere cells having enhanced capacity for tumorigenesis.

To further consolidate this concept, we performed the "gold standard" experiment to examine the ability of DU145 sphere cells to form tumors in NOD/SCID mice. We were able to show that 10^5 DU145 monolayer cells generated tumors efficiently in NOD/SCID mice (Fig 9C), which is consistent with previous reports [38], and that 100 sphere cells efficiently produced xenograft tumors in NOD/SCID mice (Fig 9C). In comparison to monolayer cells, sphere cells exhibited a 100-fold increase in tumor formation in NOD/SCID mice, as 10^3, 10^4, and 10^5 sphere cells generated xenograft tumors as efficient as 10^5, 10^6, and 10^7 monolayer cells, respectively (Fig 9C). This agrees well with our observation that 1.25% of monolayer cells formed spheres in SFM+EGF (Fig 1C). Taken together, we demonstrate that sphere cells have enhanced tumor-initiating capacity compared to monolayer cells.

4. Discussion

Research on PCSCs is the focal point, not only in our understanding of PC tumorigenesis but also in the development of new therapeutic options to control PC [39-41]. The hedgehog pathway, a well known pathway stimulating PC growth, is activated in PCSCs [42]. Despite being heavily investigated, bona-fide PCSCs have not been identified and isolated from primary PCs. While PC cells with CSC properties have been previously demonstrated [16-19, 38], the majority of these cells are unlikely to be PCSCs. This is largely due to the fact that these cells ($CD44^+$ or PC cells ectopically expressing hTERT) consisted of a much larger proportion of the respective cell populations than would be expected for PCSCs [16-19, 38]. While $CD133^+CD44^+$ cells consist of approximately 1% of monolayer DU145 cells and are enriched to 12.2% in primary spheres [20], these cells have not been thoroughly characterized and whether they can be maintained long-term in SFM supplemented with 20 ng/ml EGF and 20 ng/ml bFGF was not clear [20].

We provide here a systematic investigation of sphere-propagating cells that are derived from DU145 monolayer cell cultures. Although bFGF has been widely used in culturing stem-like PC cells [16, 20], we showed that bFGF inhibits the generation and propagation of DU145 spheres. Using bFGF in culturing stem-like PC cells may have been attributed to the lack of long-term maintenance of these cells in published reports. However, we cannot exclude the possibility that our observed inhibition of bFGF on PC sphere formation and propagation is specific for DU145 cell-derived spheres. Although EGF

stimulates sphere generation and propagation, addition of EGF is not required (Fig 6). By using monolayer and sphere-derived adherent cells (SDACs), we were able to produce spheres at different cell densities ranged from 1 cell/μl, 5 cells/μl, and 10 cells/μl (Fig 6). However, we cannot distinguish whether this autocrine-induced effect is attributable to EGF or EGF-mediated autocrine signaling of other growth factors.

Approximately 1.25% of monolayer DU145 cells are able to generate spheres in SFM+EGF and this population is enriched to 26.2±2% in spheres. This is consistent with the percentage of well-characterized neuronal sphere cells and brain cancer stem (sphere) cells [9, 29], and also consistent with the consensus that spheres contain both stem and progenitor cells.

The notion that our spheres display stem-like properties is based on the following observations: 1) Sphere-generating cells in the monolayer cell population are enriched from 1.25% to 26.2±2% in sphere cell culture (Fig 1C); 2) Sphere cells can re-populate a heterogeneous cell population, which can be defined by the profile of cell surface and prostate lineage-specific markers *in vitro* (CD44, CD24, CK18 and integrin α2β1) (Fig 4, Supplementary Fig 5) and in xenograft tumors (CD44 and 34βE12) (Fig 5); 3) Spheres could be regenerated from these heterogeneous cell populations that were produced by culturing *in vitro* in the presence of 10% FCS and *in vivo* (xenograft tumors) (Supplementary Fig 4); 4) Sphere cells initiate xenograft tumors with substantially enhanced ability in comparison to monolayer cells (Fig 9C); 5) In line with the above observation, sphere cell-derived xenograft tumors might be more advanced than monolayer cell-derived xenograft tumors. This is based on the observations that the

former contain more $CD44^+$ cells than the latter (Fig 5) and that $CD44^+$ DU145 cells have been reported to be more tumorigenic compared to DU145 $CD44^-$ cells [19].

The observation that DU145 sphere cells are $CD44^+CD24^+$ further suggests that these cells mark both PC progenitor and stem cell populations. Our results are in line with reports that $CD44^+$ PC cells were more tumorigenic than $CD44^-$ PC cells [19]. Consistent with a report [18], our sphere cells are $CK18^+$. The observation that sphere cells express both prostate basal (CD44, integrin $\alpha2\beta1$, $34\beta E12^+$) and luminal epithelial cell (CK18) markers suggests that these stem-like cancer cells were generated via abnormalities that occurred during the differentiation of a prostate stem/progenitor cell. Although PC cells with enhanced tumor-initiating ability have been shown to be $CD133^+$ [16, 18, 20], evidence also exists that $CD133^+$ DU145 cells are not more tumorigenic than $CD133^-$ cells [43]. This would functionally support our observation that DU145 sphere cells are not CD133-positive. Alternatively, our inability to detect CD133 might be caused by our cell culture condition. Since CD133 (prominin-1) is commonly referred as AC133, a specific glycosylated epitope [44, 45], our culture conditions may prevent this post-translational modification. Furthermore, the differences between our observed and published results regarding CD133 expression in PCSCs may be attributable to the low specificity of anti-CD133 antibodies. While the AC133 epitope of prominin-1 (CD133) is largely associated with stem cells, the protein itself is widely expressed in differentiated cells [45]. During the differentiation of colon cancer stem cells, the AC133 epitope, but not the CD133 protein, is specifically reduced [46]. CD133 can be detected by several monoclonal antibodies, including AC133. These antibodies have been reported to

recognized different CD133 epitopes [47]. Other than AC133, whether other CD133 epitopes associate with the stem cell population remains to be determined [47]. Although the AC133 antibody that recognizes the AC133 epitope was used in our research, this antibody may or may not detect other glycosylated forms of CD133 [46].

It has recently been reported that the PI3K-AKT pathway is activated in DU145 sphere cells and high levels of PI3K-AKT activity are essential for the generation and maintenance of DU145 spheres or the stem-like cell population [20]. Our research, however, is inconsistent with their observations. We have demonstrated, from a variety of perspectives, that DU145 stem-like cells display an intrinsically reduced activation of the PI3K-AKT pathway. This reduction might keep stem-like cells in a quiescent-like stage or cause them to proliferate slowly. Additional evidence supporting this possibility is our observation that sphere cells express very high levels of $p16^{INK4A}$ exclusively in their nuclei (Supplementary Fig 6). Although $p16^{INK4A}$ is mutated in DU145 cells [48], its high level expression in sphere cells supports the concept that sphere cells are operating at a slow proliferative rate. Our observation that sphere cells are in a quiescent-like environment is consistent with stem cells being quiescent or cycling slowly in their niches [49]. The discrepancies between our research and the results of Dubrovska *et al*. (2009) may be attributable to different cell populations being investigated. As their cells were maintained in a high level of bFGF, their cultures might be predominantly composed of heavily proliferating progenitor cells. These cells would be expected to have the activated PI3K-AKT pathway. Our observation that DU145 sphere cells proliferate slowly is

consistent with a recent report showing that $CD24^+$ CSC cells proliferate slower than the $CD24^-$ population [50].

Nonetheless, our research outlines a condition for long-term culture of DU145 stem-like cells. This system will be very useful for the investigation of unique properties of PC stem-like cells in terms of their biology and their specific cell surface marker expression that distinguishes them from normal prostate stem cells. Regarding specific surface markers that are associated with stem-like cells, our current understanding is that potential PCSCs are $CD44^+\alpha_2\beta_1{}^{hi}CD133^+$ [16-18, 51]. As we have shown that majority of DU145 sphere cells, which were prepared by section of embedded spheres, are positive for CD44, CD24, and integrin $\alpha2\beta1$ (Fig 2), it is thus very likely that $CD44^+CD24^+$ $\alpha2\beta1^+$ cells are progenitors as well. Therefore, bona-fide PCSC surface markers remain to be identified. Our system will provide a unique opportunity to identify these markers.

Acknowledgements

The authors would like to thank Catherine Fan for her assistance in IHC staining of CD133 in primary prostate cancer tissues, Dr. Laurie Doering for assistance with sphere culture and assays, and Debra Browning for assistance with immunohistochemistry staining. We also like to acknowledge the financial support from St. Joseph's HealthCare at Hamilton, Ontario, Canada to the Hamilton Centre for Kidney Research (HCKR). L.H. is supported by a postdoctoral fellowship provided by FSORC, St. Joseph's HealthCare at Hamilton. This work was supported by a grant (RMS 79-71) from the Canadian Institutes of Health Research to D.T.

References

[1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu, M.J. Thun, Cancer statistics, 2007, CA: a cancer journal for clinicians, 57 (2007) 43-66.

[2] H. Williams, I.J. Powell, Epidemiology, pathology, and genetics of prostate cancer among African Americans compared with other ethnicities, Methods in molecular biology (Clifton, N.J, 472 (2009) 439-453.

[3] J.S. Ross, The androgen receptor in prostate cancer: therapy target in search of an integrated diagnostic test, Advances in anatomic pathology, 14 (2007) 353-357.

[4] C. Moon, J.C. Park, Y.K. Chae, J.H. Yun, S. Kim, Current status of experimental therapeutics for prostate cancer, Cancer letters, 266 (2008) 116-134.

[5] D.G. Tang, L. Patrawala, T. Calhoun, B. Bhatia, G. Choy, R. Schneider-Broussard, C. Jeter, Prostate cancer stem/progenitor cells: identification, characterization, and implications, Molecular carcinogenesis, 46 (2007) 1-14.

[6] D.A. Lawson, O.N. Witte, Stem cells in prostate cancer initiation and progression, The Journal of clinical investigation, 117 (2007) 2044-2050.

[7] S. Kasper, Stem cells: The root of prostate cancer?, Journal of cellular physiology, 216 (2008) 332-336.

[8] A.T. Collins, P.A. Berry, C. Hyde, M.J. Stower, N.J. Maitland, Prospective identification of tumorigenic prostate cancer stem cells, Cancer research, 65 (2005) 10946-10951.

[9] S.K. Singh, I.D. Clarke, M. Terasaki, V.E. Bonn, C. Hawkins, J. Squire, P.B. Dirks, Identification of a cancer stem cell in human brain tumors, Cancer research, 63 (2003) 5821-5828.

[10] S.K. Singh, C. Hawkins, I.D. Clarke, J.A. Squire, J. Bayani, T. Hide, R.M. Henkelman, M.D. Cusimano, P.B. Dirks, Identification of human brain tumour initiating cells, Nature, 432 (2004) 396-401.

[11] M. Al-Hajj, M.S. Wicha, A. Benito-Hernandez, S.J. Morrison, M.F. Clarke, Prospective identification of tumorigenic breast cancer cells, Proceedings of the National Academy of Sciences of the United States of America, 100 (2003) 3983-3988.

[12] C.A. O'Brien, A. Pollett, S. Gallinger, J.E. Dick, A human colon cancer cell capable of initiating tumour growth in immunodeficient mice, Nature, 445 (2007) 106-110.

[13] L. Ricci-Vitiani, D.G. Lombardi, E. Pilozzi, M. Biffoni, M. Todaro, C. Peschle, R. De Maria, Identification and expansion of human colon-cancer-initiating cells, Nature, 445 (2007) 111-115.

[14] G.D. Richardson, C.N. Robson, S.H. Lang, D.E. Neal, N.J. Maitland, A.T. Collins, CD133, a novel marker for human prostatic epithelial stem cells, Journal of cell science, 117 (2004) 3539-3545.

[15] K.G. Leong, B.E. Wang, L. Johnson, W.Q. Gao, Generation of a prostate from a single adult stem cell, Nature, 456 (2008) 804-808.

[16] J. Miki, B. Furusato, H. Li, Y. Gu, H. Takahashi, S. Egawa, I.A. Sesterhenn, D.G. McLeod, S. Srivastava, J.S. Rhim, Identification of putative stem cell markers, CD133 and CXCR4, in hTERT-immortalized primary nonmalignant and malignant tumor-derived human prostate epithelial cell lines and in prostate cancer specimens, Cancer research, 67 (2007) 3153-3161.

[17] G. Gu, J. Yuan, M. Wills, S. Kasper, Prostate cancer cells with stem cell characteristics reconstitute the original human tumor in vivo, Cancer research, 67 (2007) 4807-4815.

[18] H. Li, J. Zhou, J. Miki, B. Furusato, Y. Gu, S. Srivastava, D.G. McLeod, J.C. Vogel, J.S. Rhim, Telomerase-immortalized non-malignant human prostate epithelial cells retain the properties of multipotent stem cells, Experimental cell research, 314 (2008) 92-102.

[19] L. Patrawala, T. Calhoun, R. Schneider-Broussard, H. Li, B. Bhatia, S. Tang, J.G. Reilly, D. Chandra, J. Zhou, K. Claypool, L. Coghlan, D.G. Tang, Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells, Oncogene, 25 (2006) 1696-1708.

[20] A. Dubrovska, S. Kim, R.J. Salamone, J.R. Walker, S.M. Maira, C. Garcia-Echeverria, P.G. Schultz, V.A. Reddy, The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations, Proceedings of the National Academy of Sciences of the United States of America, 106 (2009) 268-273.

[21] Y. Li, D. Wu, B. Chen, A. Ingram, L. He, L. Liu, D. Zhu, A. Kapoor, D. Tang, ATM activity contributes to the tumor-suppressing functions of p14ARF, Oncogene, 23 (2004) 7355-7365.

[22] D. Tang, H. Okada, J. Ruland, L. Liu, V. Stambolic, T.W. Mak, A.J. Ingram, Akt is activated in response to an apoptotic signal, The Journal of biological chemistry, 276 (2001) 30461-30466.

[23] C. Fan, L. He, A. Kapoor, A. Gillis, A.P. Rybak, J.C. Cutz, D. Tang, Bmi1 promotes prostate tumorigenesis via inhibiting p16(INK4A) and p14(ARF) expression, Biochimica et biophysica acta, 1782 (2008) 642-648.

[24] C. Abate-Shen, M.M. Shen, Molecular genetics of prostate cancer, Genes & development, 14 (2000) 2410-2434.

[25] R.M. Long, C. Morrissey, J.M. Fitzpatrick, R.W. Watson, Prostate epithelial cell differentiation and its relevance to the understanding of prostate cancer therapies, Clin Sci (Lond), 108 (2005) 1-11.

[26] S.J. Maygarden, S. Strom, J.L. Ware, Localization of epidermal growth factor receptor by immunohistochemical methods in human prostatic carcinoma, prostatic intraepithelial neoplasia, and benign hyperplasia, Archives of pathology & laboratory medicine, 116 (1992) 269-273.

[27] V. Tropepe, M. Sibilia, B.G. Ciruna, J. Rossant, E.F. Wagner, D. van der Kooy, Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon, Developmental biology, 208 (1999) 166-188.

[28] C.M. Morshead, A.D. Garcia, M.V. Sofroniew, D. van Der Kooy, The ablation of glial fibrillary acidic protein-positive cells from the adult central nervous system results in the loss of forebrain neural stem cells but not retinal stem cells, The European journal of neuroscience, 18 (2003) 76-84.

[29] B.A. Reynolds, S. Weiss, Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell, Developmental biology, 175 (1996) 1-13.

[30] K. Okita, M. Nakagawa, H. Hyenjong, T. Ichisaka, S. Yamanaka, Generation of mouse induced pluripotent stem cells without viral vectors, Science (New York, N.Y, 322 (2008) 949-953.

[31] M. Stadtfeld, N. Maherali, D.T. Breault, K. Hochedlinger, Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse, Cell stem cell, 2 (2008) 230-240.

[32] T. Brambrink, R. Foreman, G.G. Welstead, C.J. Lengner, M. Wernig, H. Suh, R. Jaenisch, Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells, Cell stem cell, 2 (2008) 151-159.

[33] A.V. Molofsky, R. Pardal, T. Iwashita, I.K. Park, M.F. Clarke, S.J. Morrison, Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation, Nature, 425 (2003) 962-967.

[34] A.V. Molofsky, S. He, M. Bydon, S.J. Morrison, R. Pardal, Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways, Genes & development, 19 (2005) 1432-1437.

[35] R.U. Lukacs, S. Memarzadeh, H. Wu, O.N. Witte, Bmi-1 is a crucial regulator of prostate stem cell self-renewal and malignant transformation, Cell stem cell, 7 (2010) 682-693.

[36] R. Marhaba, P. Klingbeil, T. Nuebel, I. Nazarenko, M.W. Buechler, M. Zoeller, CD44 and EpCAM: cancer-initiating cell markers, Current molecular medicine, 8 (2008) 784-804.

[37] M. Florek, M. Haase, A.M. Marzesco, D. Freund, G. Ehninger, W.B. Huttner, D. Corbeil, Prominin-1/CD133, a neural and hematopoietic stem cell marker, is expressed in adult human differentiated cells and certain types of kidney cancer, Cell and tissue research, 319 (2005) 15-26.

[38] L. Patrawala, T. Calhoun-Davis, R. Schneider-Broussard, D.G. Tang, Hierarchical organization of prostate cancer cells in xenograft tumors: the CD44+alpha2beta1+ cell population is enriched in tumor-initiating cells, Cancer research, 67 (2007) 6796-6805.

[39] A.M. Joshua, A. Evans, T. Van der Kwast, M. Zielenska, A.K. Meeker, A. Chinnaiyan, J.A. Squire, Prostatic preneoplasia and beyond, Biochimica et biophysica acta, 1785 (2008) 156-181.

[40] C.O. Marian, J.W. Shay, Prostate tumor-initiating cells: a new target for telomerase inhibition therapy?, Biochimica et biophysica acta, 1792 (2009) 289-296.

[41] N.J. Maitland, S.D. Bryce, M.J. Stower, A.T. Collins, Prostate cancer stem cells: a target for new therapies, Ernst Schering Foundation symposium proceedings, (2006) 155-179.

[42] S. Teglund, R. Toftgard, Hedgehog beyond medulloblastoma and basal cell carcinoma, Biochimica et biophysica acta, 1805 (2010) 181-208.

[43] M.J. Pfeiffer, J.A. Schalken, Stem cell characteristics in prostate cancer cell lines, European urology, 57 (2010) 246-254.

[44] S. Bidlingmaier, X. Zhu, B. Liu, The utility and limitations of glycosylated human CD133 epitopes in defining cancer stem cells, Journal of molecular medicine (Berlin, Germany), 86 (2008) 1025-1032.

[45] D. Mizrak, M. Brittan, M. Alison, CD133: molecule of the moment, The Journal of pathology, 214 (2008) 3-9.

[46] K. Kemper, M.R. Sprick, M. de Bree, A. Scopelliti, L. Vermeulen, M. Hoek, J. Zeilstra, S.T. Pals, H. Mehmet, G. Stassi, J.P. Medema, The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation, Cancer research, 70 (2010) 719-729.

[47] C.L. Green, M. Loken, D. Buck, H.J. Deeg, Discordant expression of AC133 and AC141 in patients with myelodysplastic syndrome (MDS) and acute myelogeneous leukemia (AML), Leukemia, 14 (2000) 770-772.

[48] S.G. Chi, R.W. deVere White, J.T. Muenzer, P.H. Gumerlock, Frequent alteration of CDKN2 (p16(INK4A)/MTS1) expression in human primary prostate carcinomas, Clin Cancer Res, 3 (1997) 1889-1897.

[49] S.J. Morrison, A.C. Spradling, Stem cells and niches: mechanisms that promote stem cell maintenance throughout life, Cell, 132 (2008) 598-611.

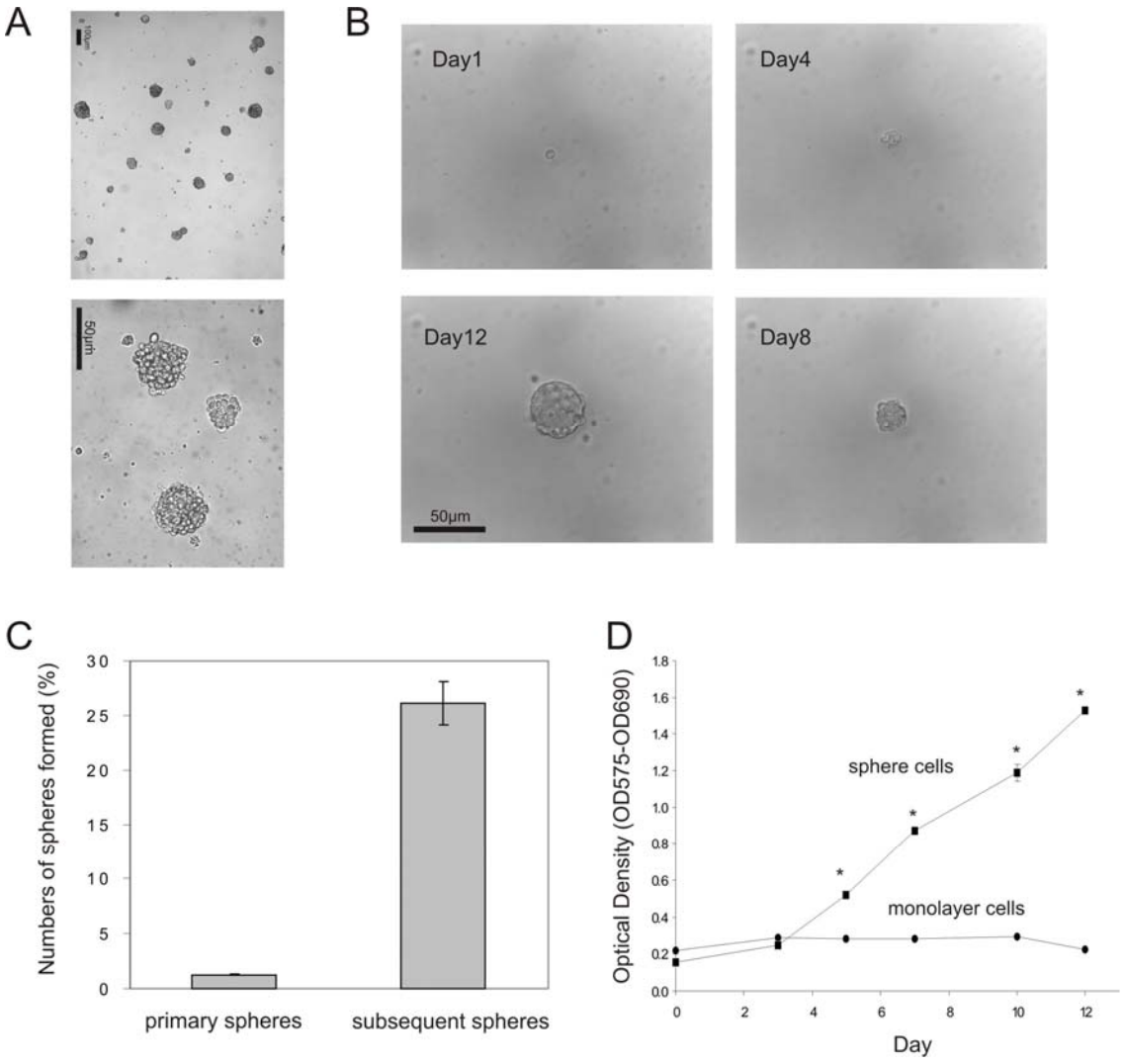
[50] M.Q. Gao, Y.P. Choi, S. Kang, J.H. Youn, N.H. Cho, CD24+ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells, Oncogene, 29 (2010) 2672-2680.

[51] C. Wei, W. Guomin, L. Yujun, Q. Ruizhe, Cancer stem-like cells in human prostate carcinoma cells DU145: the seeds of the cell line?, Cancer biology & therapy, 6 (2007) 763-768.

Figures and Table

Figure 1. Isolation and characterization of a stem-like cell population from DU145 cells. A) Stem-like cells can be cultured as suspension spheres. DU145 cells were in SFM [DMEM/F12 (3:1 mixture), 0.4% bovine serum albumin (BSA), 0.2× B-27 supplement lacking Vitamin A, 1% penicillin/streptomycin (P/S) and 10ng/ml EGF] in a 24-well tissue culture plate for 12 days. Typical floating spheres formed were photographed (bright field images) at 50× (left panel) and 200× magnifications (right panel). **B)** Sphere cells possess self-renewal capacity. Individual sphere cells were seeded at a density of 1 cell per well, which are capable of forming a single sphere in 12 days. **C)** The rate of generation of primary and subsequent spheres from monolayer cells and established spheres (>2 passages). Quantification of primary sphere formation was carried out using 24-well plates (10^4 cells/well seeded). To measure self-renewal capacity, individualized sphere cells were plated in 96-well plates at a density of 1 cell per well. A total of 360 wells were counted for three repeats, with 120 wells recorded per experiment. Data were presented as mean ± SE. **D)** DU145 monolayer cells and spheres were enzymatically-dissociated and plated at a density of 10^3 cells/well (96-well plate) in triplicate. Using the MTT assay, cell proliferation capacity was determined after 0, 3, 5, 7, 10 and 12 days of incubation. Representative image of results (mean ± SE) conducted in three independent experiments. $^{*}p < 0.05$ (two-tailed, independent *t*-test).

Figure 1.


A
B
Day1
Day4
Day12
Day8
50μm
C
Numbers of spheres formed (%)
primary spheres
subsequent spheres
D
Optical Density (OD575-OD690)
sphere cells
monolayer cells
Day

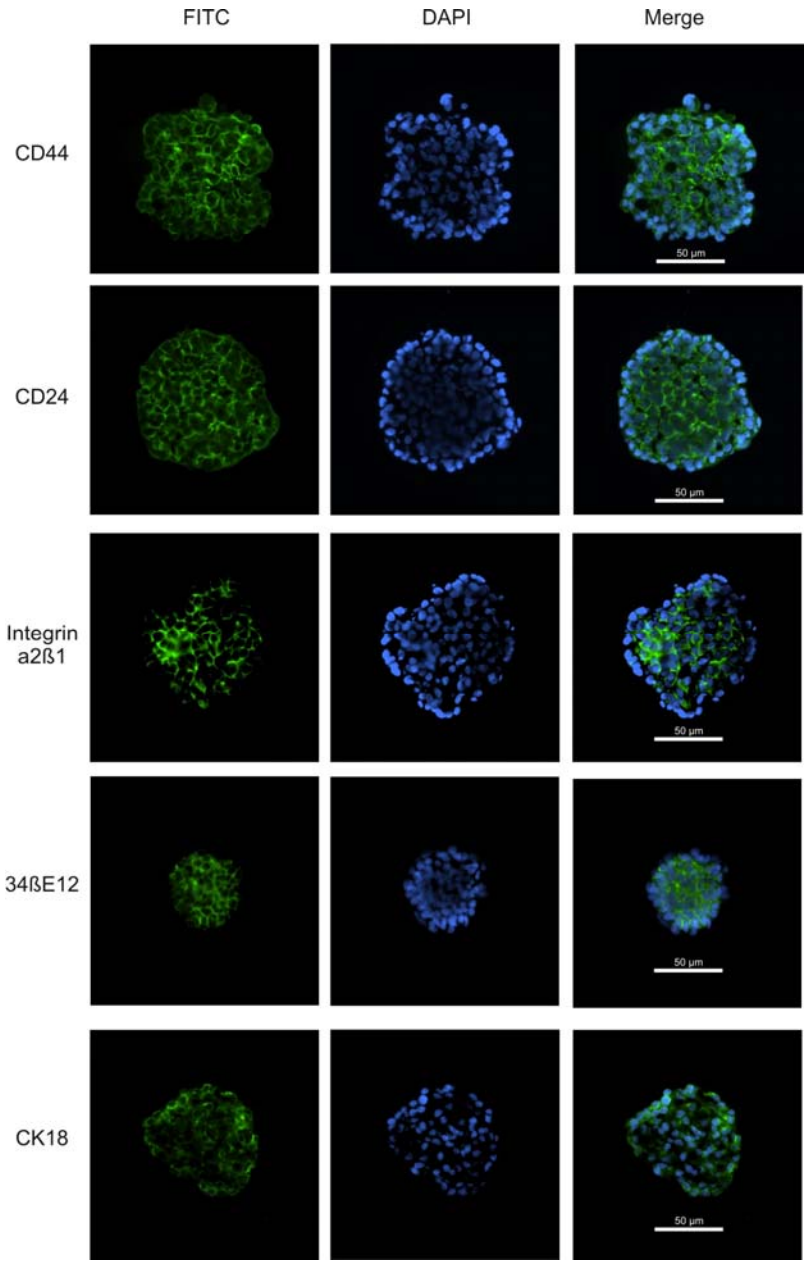
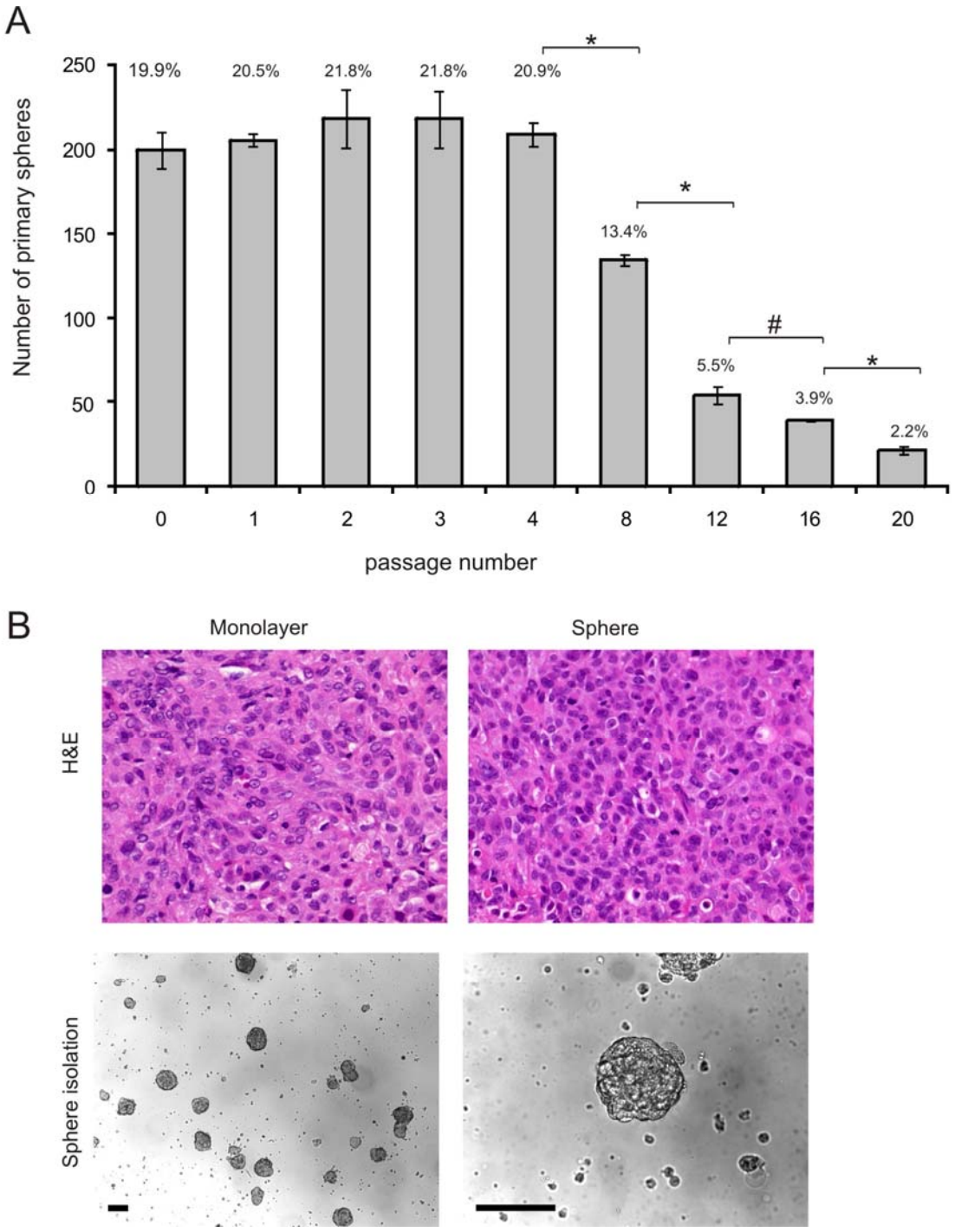


Figure 2. Spheres are positive for CD44, CD24, integrin α2β1, prostate basal cytokeratins (34βE12) and luminal cytokeratin (CK18). DU145 spheres were immunofluorescently stained with the indicated antibodies (see Materials and Methods for details). Nuclei were counter-stained with DAPI. Typical images are shown. Scale bars represent 50 μm.

Figure 3. Sphere cells are able to produce a heterogeneous cell population that can produce spheres. A) The sphere-forming capacity of sphere-derived adherent cells (SDACs). SDACs were cultured in the presence of 10% serum for the indicated passages, at which time their sphere-forming ability was determined by seeding SDACs at 10^3 cells/ml per well in 24-well plates with triplicate replicates. Passage 0 indicates that sphere cells were cultured in the presence of 10% serum for a few days, followed by examination of their sphere-forming ability without passage. The percentages of sphere-forming cells for each passage are also indicated. Data is presented as mean ± SE of three independent experiments [* $p < 0.01$; $^{\#}p < 0.05$ (two-tailed, independent *t*-test)]. **B)** H&E staining of xenograft tumors generated from monolayer DU145 cells and sphere cells (top panel). Isolation of spheres from xenograft tumors derived from monolayer DU145 cells. Similar results were also obtained for sphere-derived xenograft tumors (data not shown). Scale bar represents 50 μm.

Figure 3.


A
Number of primary spheres
250
200
150
100
50
0
19.9%
20.5%
21.8%
21.8%
20.9%
13.4%
5.5%
3.9%
2.2%
*
*
#
*
0
1
2
3
4
8
12
16
20
passage number
B
Monolayer
Sphere
H&E
Sphere isolation

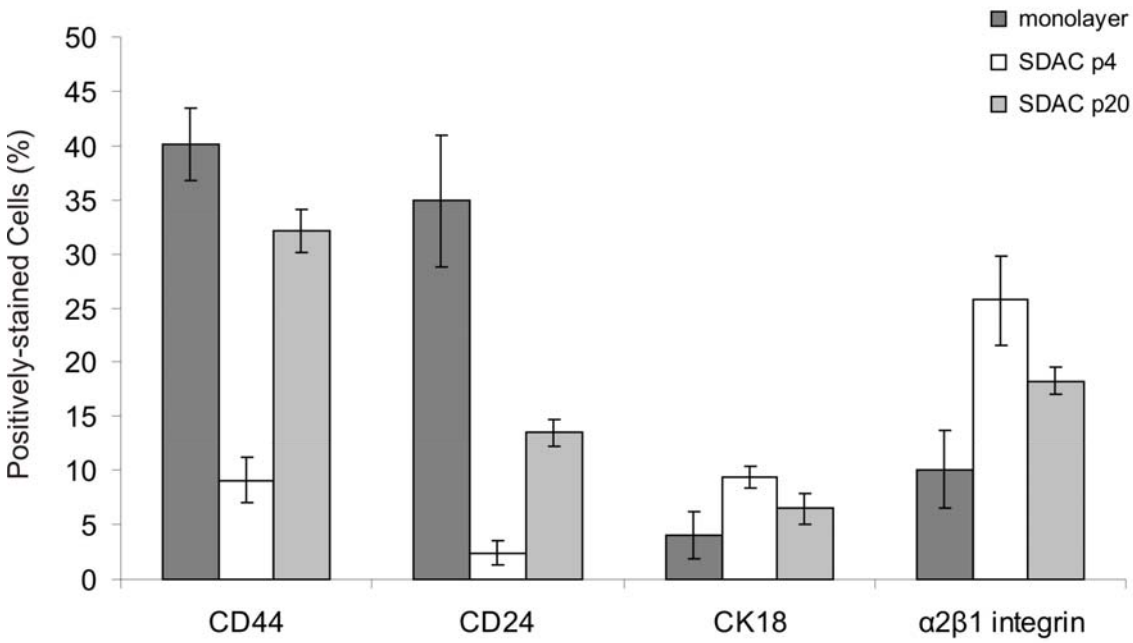
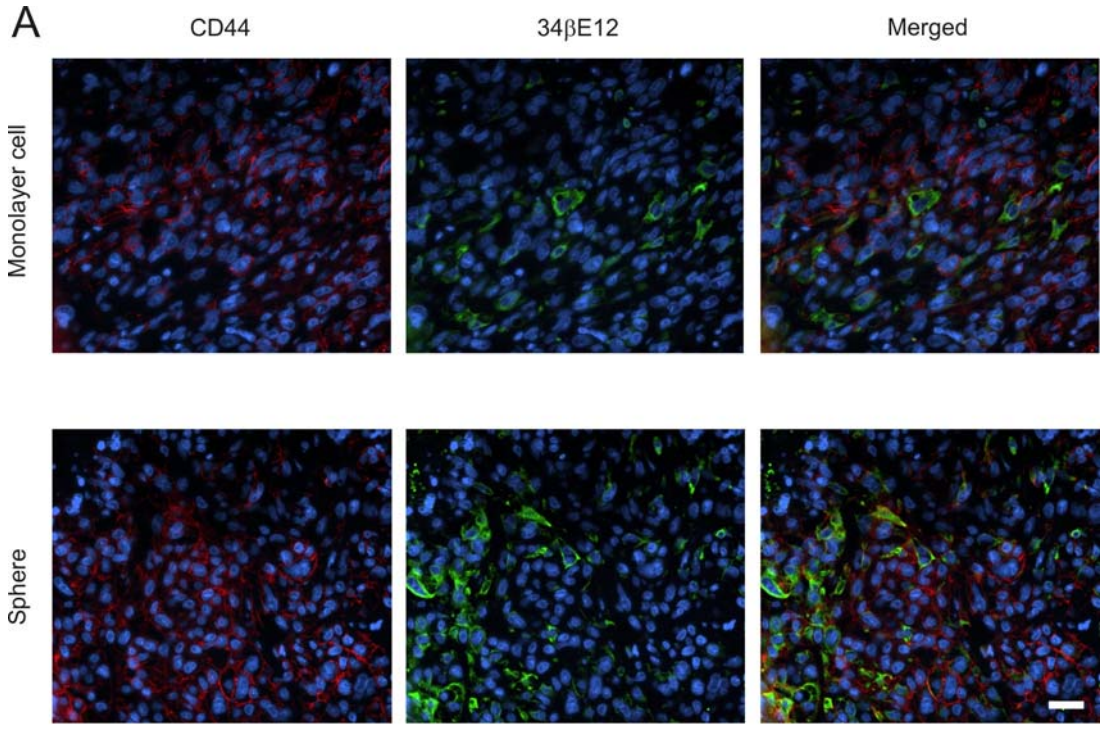


Figure 4. Spheres are able to re-populate a heterogeneous cell population. Spheres were cultured in complete medium (DMEM, 10% FBS) as a monolayer (adherent culture) for 4 and 20 passages (p4 and p20), respectively, followed by examination for the expression of CD44, CD24, CK18 and integrin α2β1 expression in monolayer, p4 and p20 sphere-derived adherent cells (for typical images, see Supplementary Fig 5). The percentages of positively-stained cells for CD44, CD24, CK18 and integrin α2β1 markers were derived from randomly counting a total of 10^3 cells/marker in 5 random fields. Data are presented as mean ± SE. $^{*}p < 0.05$ (two-tailed, independent *t*-test).

Figure 5. Sphere cell-derived xenograft tumors consist of heterogeneous cell populations. A) Expression of CD44 and prostate basal cytokeratin marker 34βE12 in DU145 monolayer cell- and sphere cell-derived xenograft tumors. Detection of CD44 and 34βE12 was performed using anti-CD44 and 34βE12 antibodies. Nuclei were counter-stained with DAPI. Typical images are shown. Scale bars represent 20 μm. **B)** The percentages of $CD44^+$, $34\beta E12^+$ and $CD44^+34\beta E12^+$ cells were derived by randomly counting a total of 10^3 cells in 5 random fields. Data are presented as mean ± SE. $^*p <$ 0.001 (two-tailed, independent *t*-test) in comparison to monolayer-derived xenograft tumors.

Figure 5.



B

Xenograft	Percentage of Cells (Mean ± SE)		
	$CD44^+$	$34\beta E12^+$	$CD44^+34\beta E12^+$
Monolayer (Parental)	54.2 ± 2.5	20.8 ± 1.8	8.8 ± 1.3
Sphere	85.7 ± 1.6*	30.4 ± 1.6*	24.8 ± 1.3*

Figure 6. EGF, but not bFGF, contributes to the formation of DU145 spheres. A) Primary sphere formation. DU145 monolayer cells were seeded at a density of 5×10^3 and 10^4 cells/ml per well, respectively. Triplicate experiments were performed, with quadruplicate replicates in each experiment. $^{\#}$ $p < 0.05$ (For 5,000 cells/ml seeded, EGF group is statistically different from the EGF+bFGF group; two-tailed, independent *t*-test). **B)** Secondary sphere formation. Primary DU145 sphere-derived cells were seeded at a density of 10^3 cells/ml per well, with quadruplicate replicates for each growth condition. Data in both panels are presented as mean ± SE. $^{*}p < 0.01$; $^{\#}$ $p < 0.05$ (two-tailed, independent *t*-test). **C)** SDACs at the indicated passages were seeded at a density of 10^3 cells/ml per well, with quadruplicate replicates for each growth condition. Triplicate experiments were performed. $^{*}p < 0.01$ (two-tailed, independent *t*-test).

Figure 6.

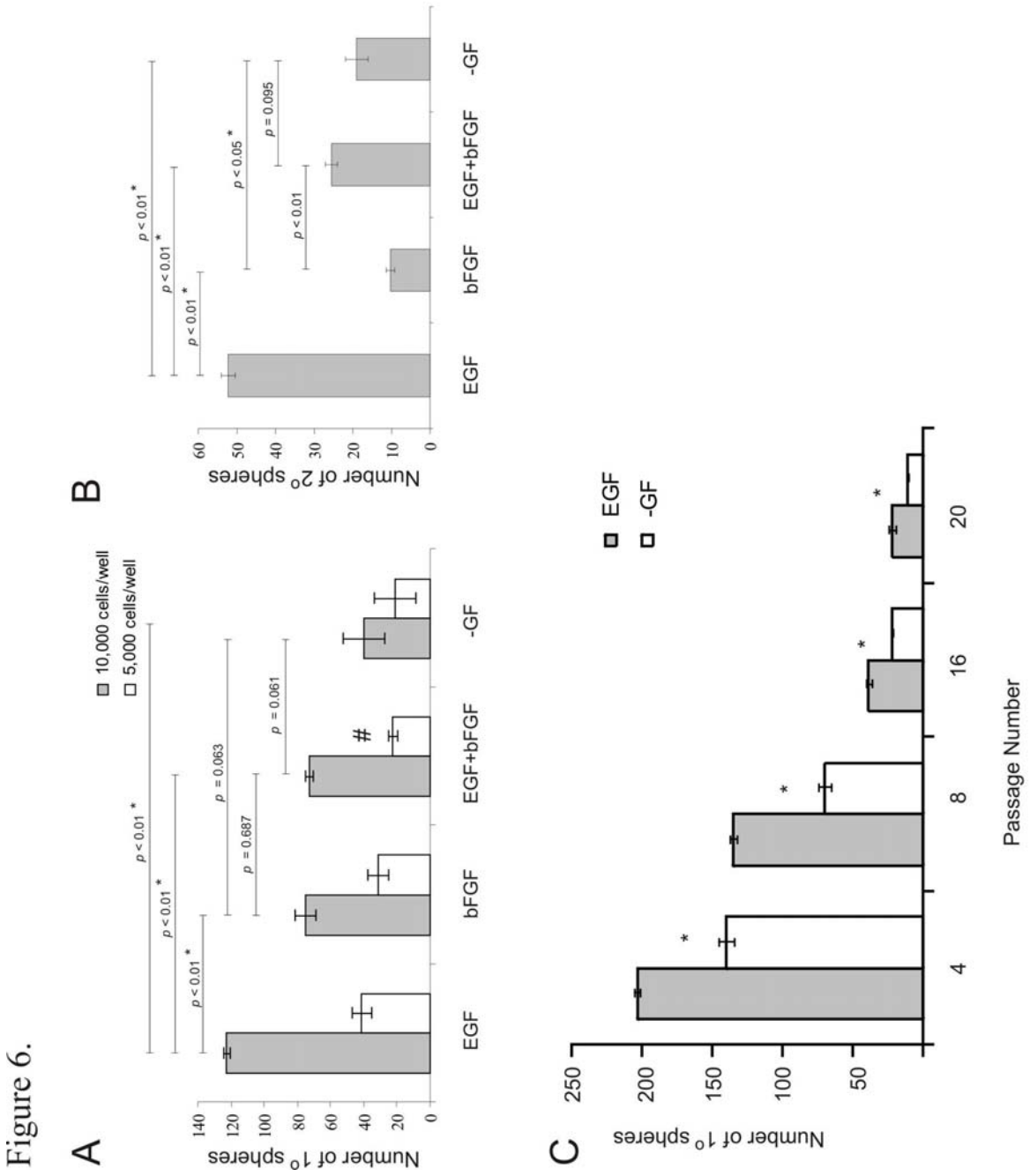
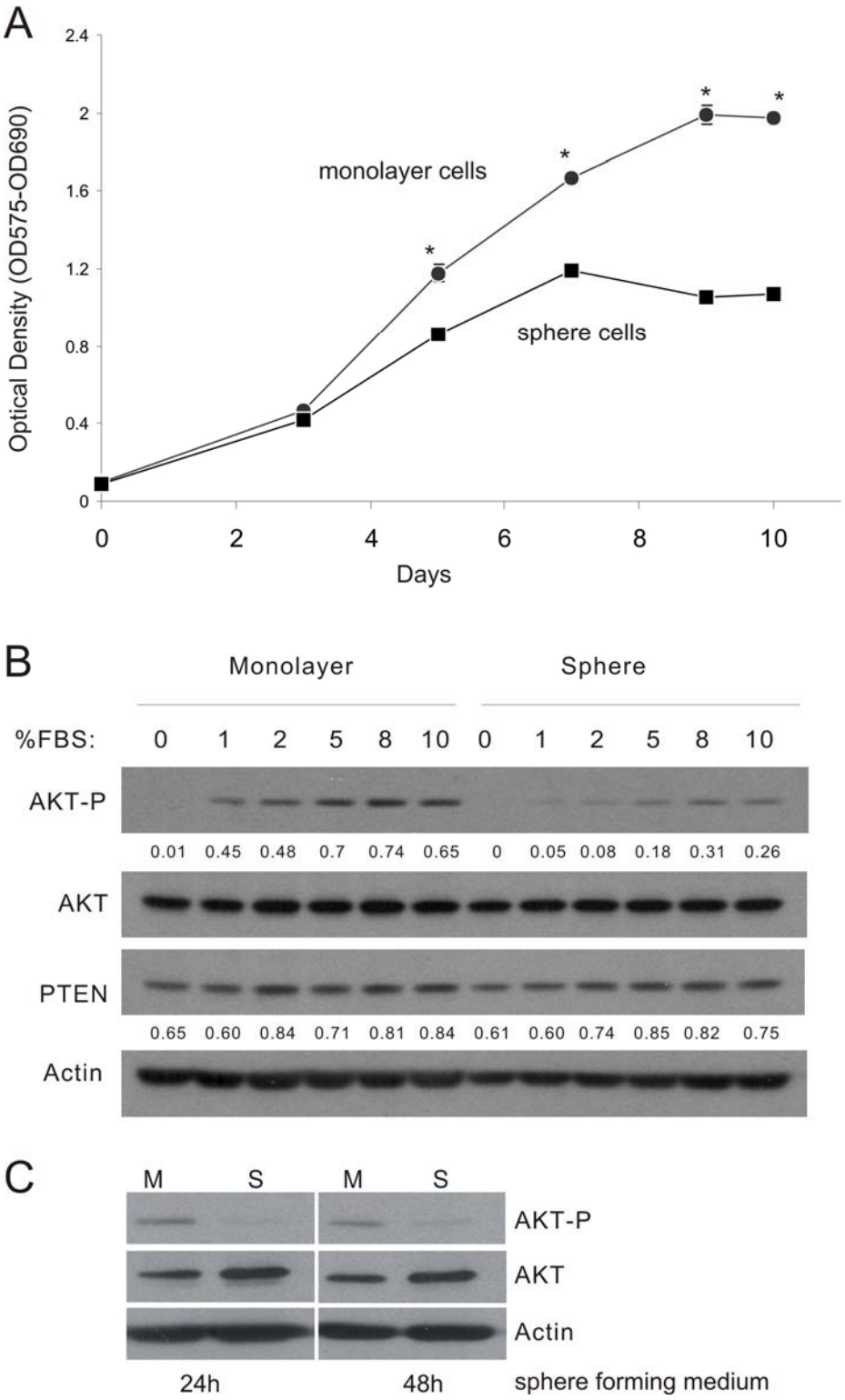

A
B
C
10,000 cells/well
5,000 cells/well
Number of 1° spheres
Number of 2° spheres
EGF
bFGF
EGF+bFGF
-GF
p < 0.01
p = 0.687
p = 0.063
p = 0.061
p < 0.05
p = 0.095
Passage Number

Figure 7. DU145 sphere cells proliferate at a reduced rate with reduced levels of activation of the PI3K-AKT pathway. A) DU145 monolayer and sphere cells were seeded at a density of 1000 cells/well (96 well) in triplicate in CM. Cell proliferation was measured using the MTT assay at the indicated days. Representative results (mean ± SE) of three experiments are shown. $^{*}p < 0.05$ (two-tailed, independent *t*-test). **B)** DU145 monolayer and sphere cells were plated in serum-supplemented media for 18 hours, followed by serum-starvation for 3 hours and then stimulating with indicated doses of serum for 1 hour. AKT activation (phosphorylation of Ser 473, AKT-P), total AKT, PTEN, and actin expression was determined using western blot. AKT-P was quantified by first normalizing AKT against the respective actin and then normalizing AKT-P against the normalized total AKT. PTEN was quantified against the respective actin. Levels of AKT-P and PTEN were presented under the AKT-P and PTEN panels, respectively. Quantification was performed using the Scion Image software on scanned western blot images. **C)** DU145 monolayer (M) and sphere (S) cells were cultured in our sphere-forming medium for the indicated duration, followed by examination of AKT Ser 473 phosphorylation (AKT-P), total AKT, and actin by western blot using specific antibodies.

Figure 7.


A
Optical Density (OD575-OD690)
2.4
2
1.6
1.2
0.8
0.4
0
monolayer cells
sphere cells
*
0
2
4
6
8
10
Days
B
Monolayer
Sphere
%FBS:
0 1 2 5 8 10 0 1 2 5 8 10
AKT-P
0.01 0.45 0.48 0.7 0.74 0.65 0 0.05 0.08 0.18 0.31 0.26
AKT
PTEN
0.65 0.60 0.84 0.71 0.81 0.84 0.61 0.60 0.74 0.85 0.82 0.75
Actin
C
M S M S
AKT-P
AKT
Actin
24h
48h
sphere forming medium

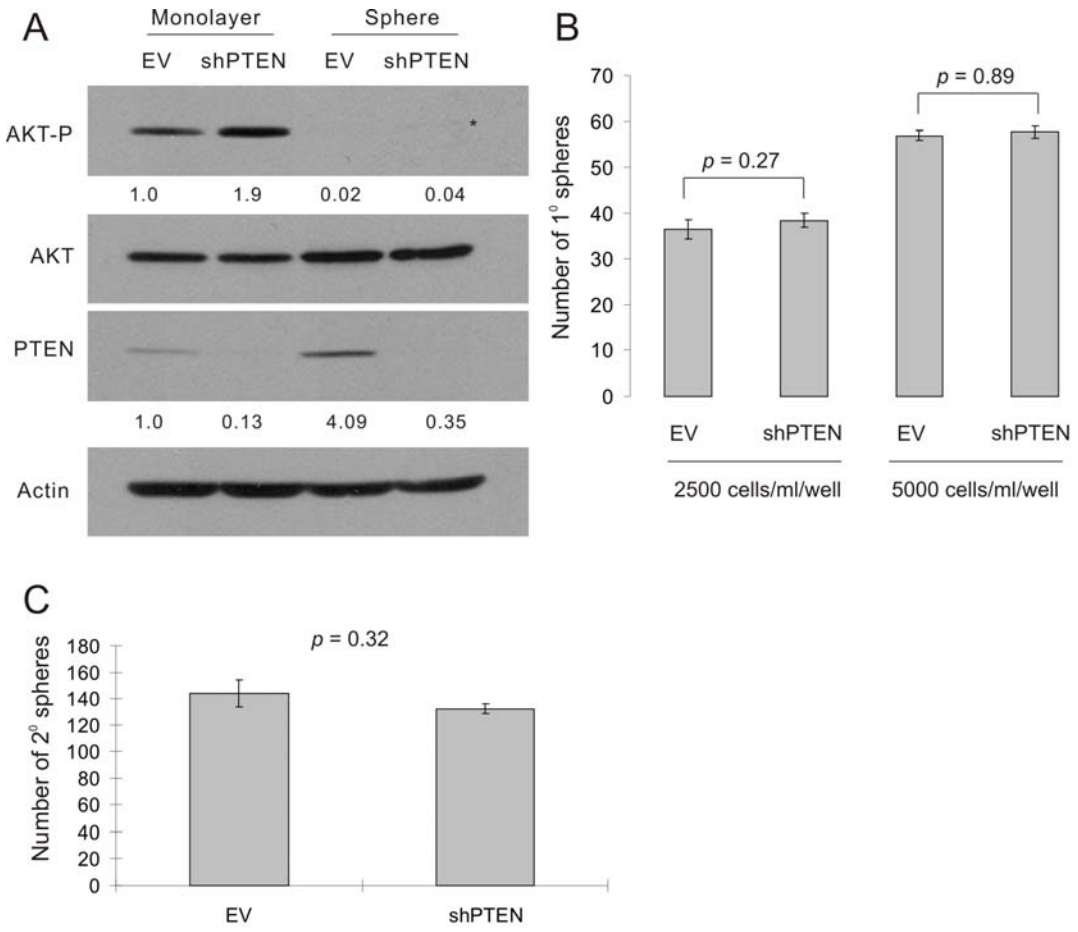
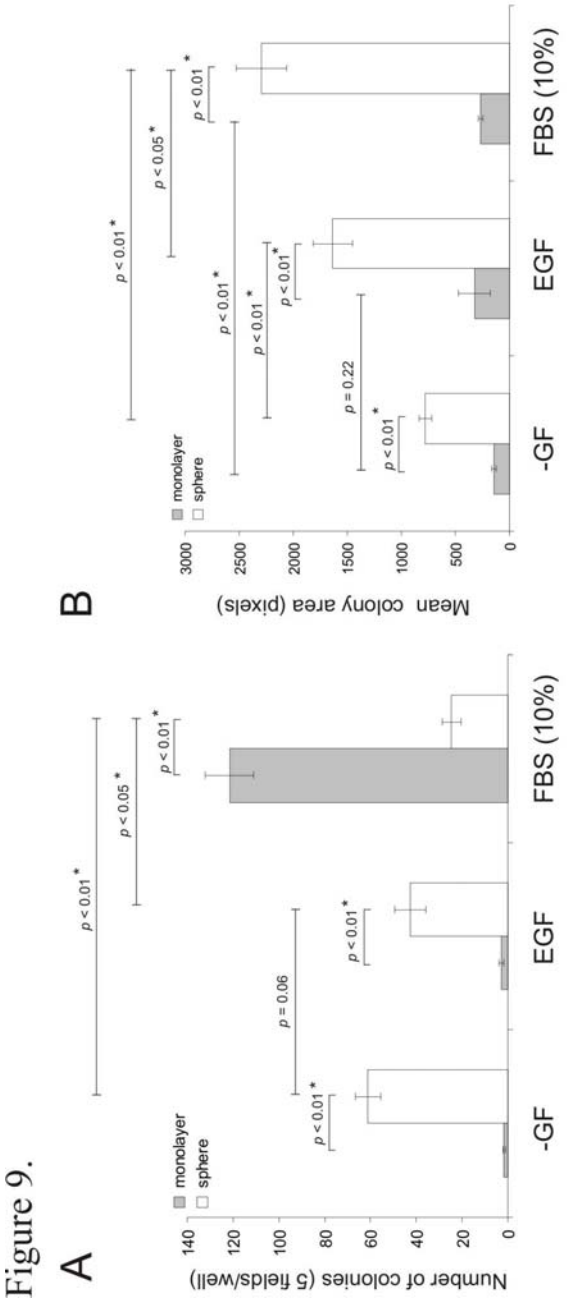


Figure 8. Elevated activation of the PI3K-AKT pathway is not required for the generation and passage of DU145 spheres. A) DU145 monolayer cells were infected with an empty vector (EV) or shPTEN retrovirus, followed by generation of spheres from these lines. AKT activation (AKT-P), total AKT, PTEN, and actin in these cells were determined by western blot. Quantification was performed as described in the Figure 6 legend. * indicates AKT-P band. **B)** Primary spheres were generated using 24-well plates from EV and shPTEN cells at the indicated density (< 10 cell/µl, the clonal density). Experiments were repeated three times. **C**) Generation of secondary spheres from primary EV and shPTEN spheres.

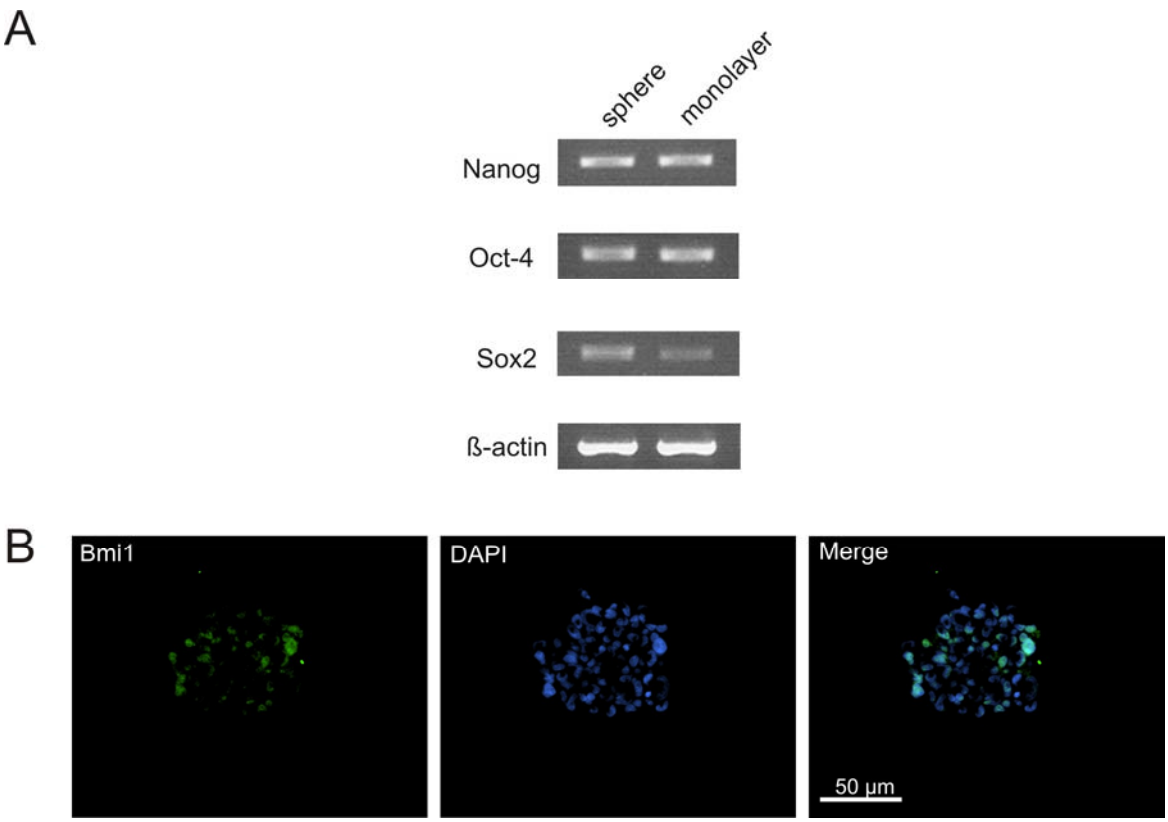
Figure 9. DU145 sphere cells display enhanced tumorigenic ability. 10^4 DU145 sphere or monolayer cells were seeded in soft agar in 6-well plates in serum-free medium without the addition of growth factor (-GF) or with the addition of EGF (10 ng/ml), and in DMEM/F12 media supplemented with 10% FBS. Cell colonies were formed after 8-weeks in soft agar. The number (**A**) of colonies in 5 fields was determined, and the area (**B**) of the colonies was analyzed using ImagePro Plus 5.0 software. Similar results were obtained when cells were seeded at 2×10^4 cells/well (data not shown). $^*p < 0.05$ (two-tailed, independent *t*-test). **C)** The indicated monolayer and sphere cells were subcutaneously implanted into both flanks of NOD/SCID mice at the indicated cell numbers. Tumor incidence (number of tumors formed/number of implantations) and tumor volume (mm^3) at time of sacrifice (day 57 for 10^7 monolayer cells and day 71 for the rest of cell doses) are indicated. Mean tumor volume ± standard error (SE) at time of sacrifice is also indicated.

Figure 9.

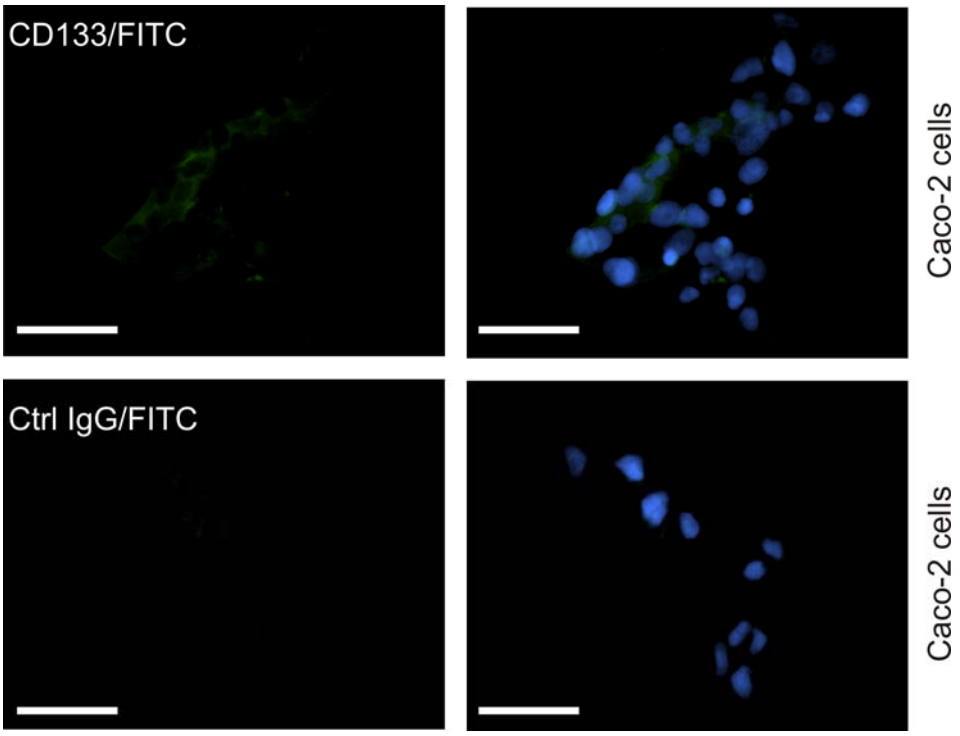


C

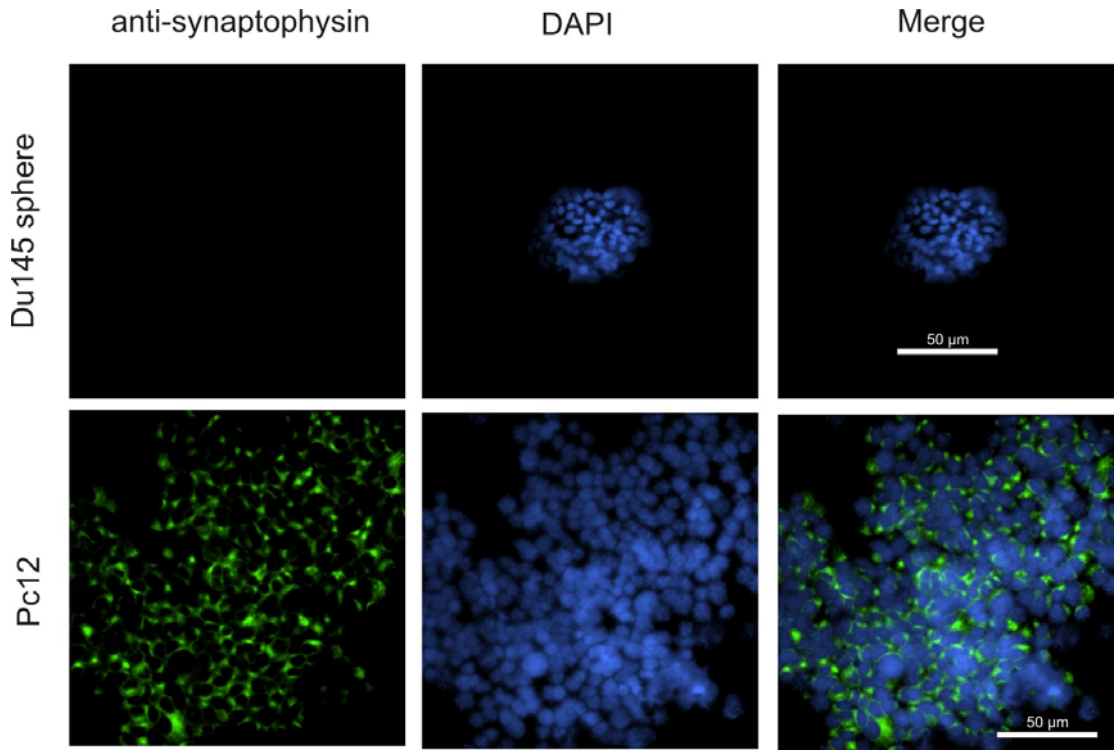
Cell type	Cell dose	Tumour incidence	Mean volume
Monolayer cells	10^7	8/8	1084 ± 379
	10^6	8/8	1101 ± 325
	10^5	8/8	710 ± 301
Sphere cells	10^5	6/6	1392 ± 572
	10^4	8/8	1068 ± 364
	10^3	8/8	681 ± 189
	10^2	6/6	557 ± 232



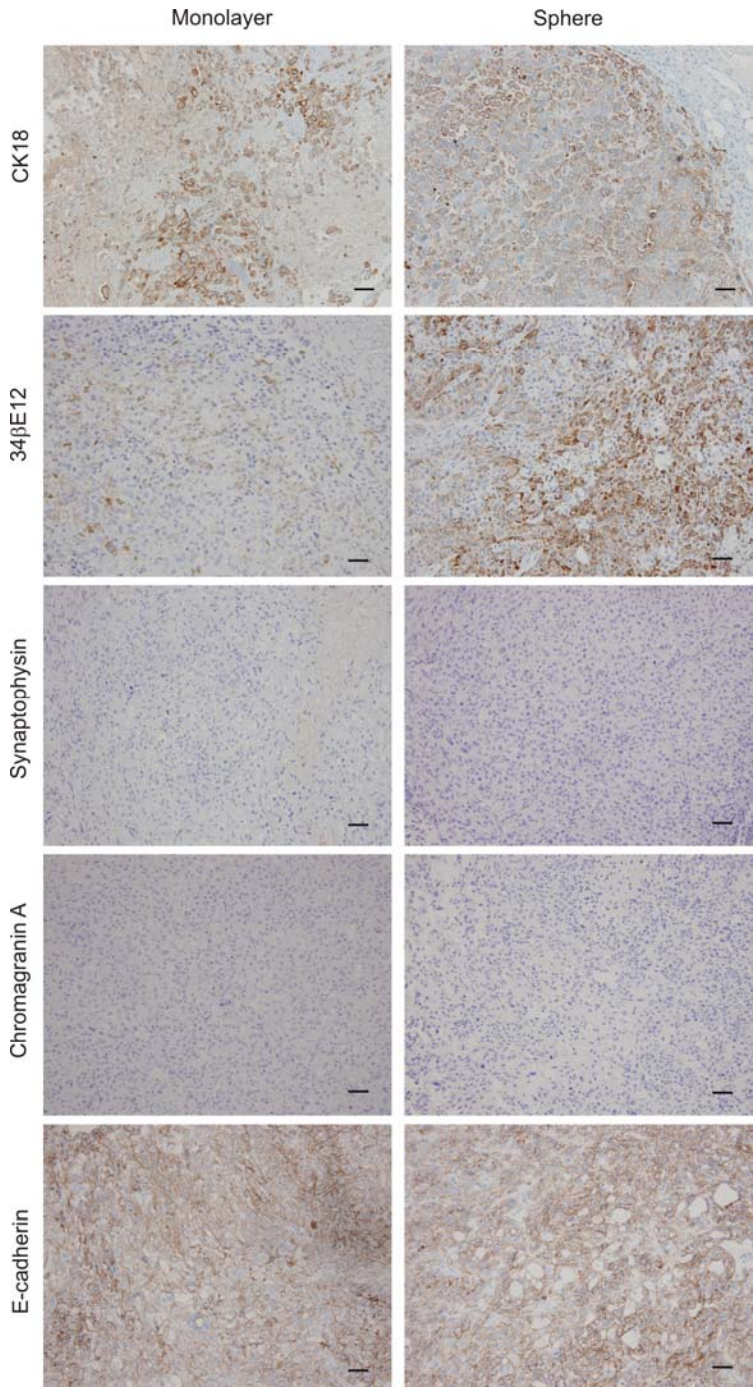
Supplementary Figure 1. DU145 spheres express stem cell factors. (**A**) RT-PCR analysis of NANOG, OCT4 and SOX2. (**B**) DU145 spheres were immunofluorescently stained with anti-BMI1 antibody (4 µg/ml) and counter-stained with DAPI. Typical images are shown. Experiments were repeated at least three times. Scale bar represenst 50 µm.



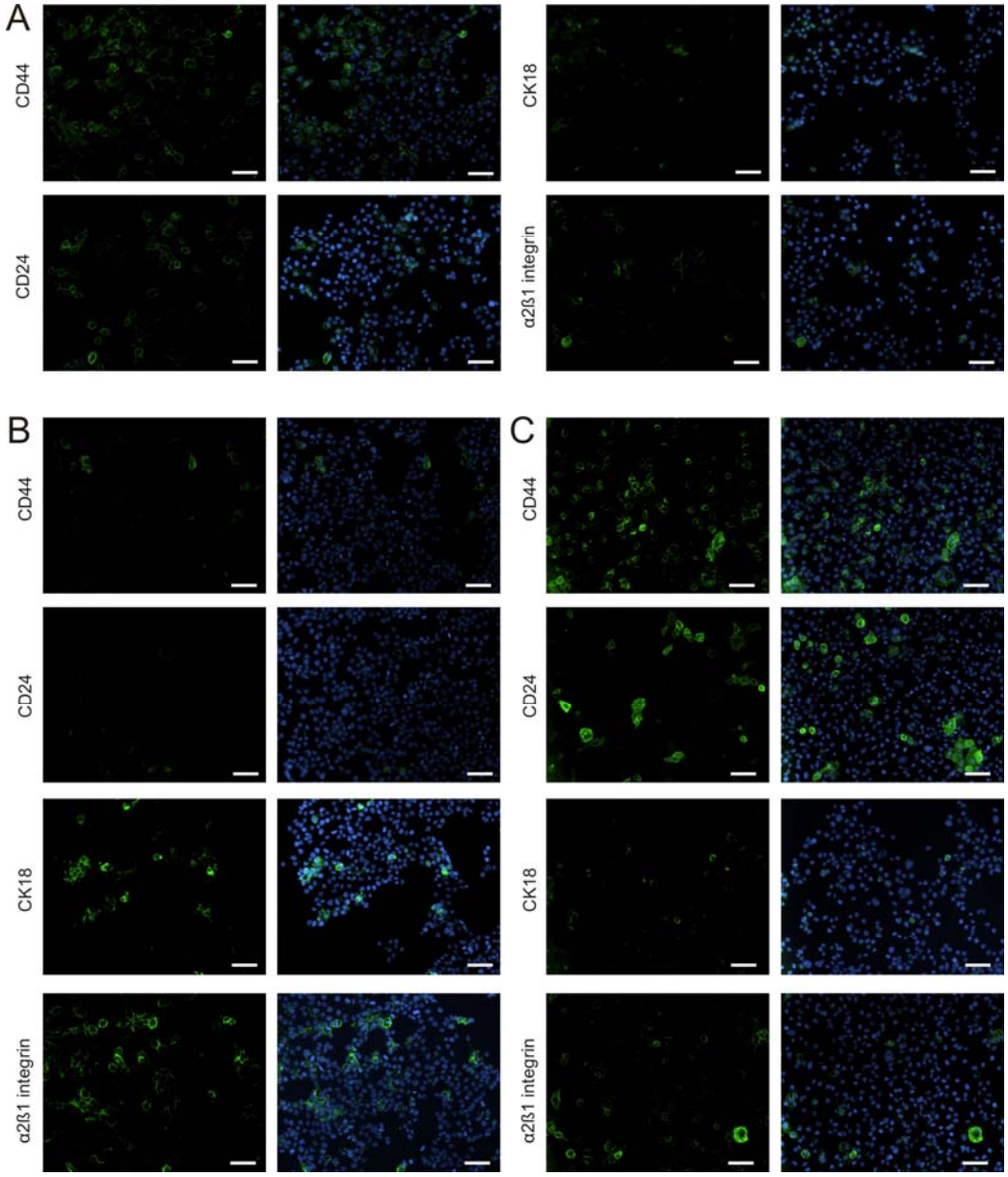
Supplementary Figure 2. DU145 sphere and Caco-2 cells were immunofluorescently stained with AC133 antibody and counter-stained with DAPI. Scale bar represents 50 μm.



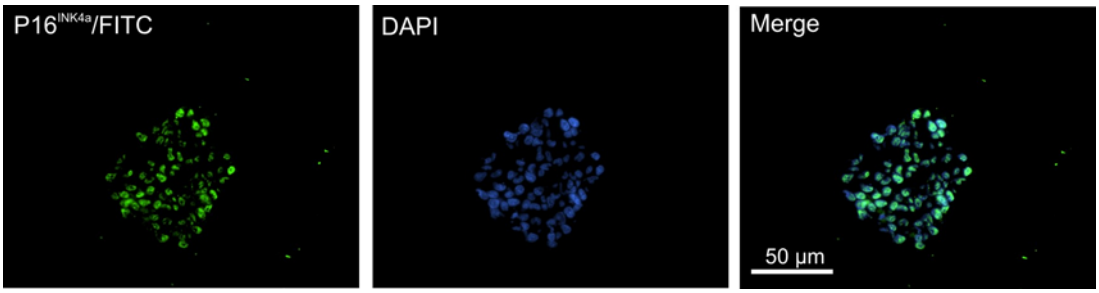
Supplementary Figure 3. DU145 sphere and PC12 cells were immunofluorescently stained with anti-synaptophsin antibody and counter-stained with DAPI. Scale bar represents 50 µm.



Supplementary Figure 4. Monolayer cell or sphere cell-derived xenograft tumors were immunohistochemically stained with the indicated antibodies. Scale bar represents 50 μm.



Supplementary Figure 5. Sphere-derived adherent cells (SDACs) express reduced CD44, CD24, CK18, and integrin α2β1. DU145 monolayer cells (A), passage 4 (B) and passage 20 SDACs (C) were immunofluorescently stained for the indicated surface antigens. Nuclei were counter-stained with DAPI. Typical images are shown. Scale bar represents 50 μm.



Supplementary Figure 6. DU145 sphere cells express p16^{INK4A}. DU145 spheres were immunofluorescent stained with anti-16^{INK4A} antibody (4 µg/ml) and counter-stained with DAPI. Typical images are shown. Scale bar represents 50 µm.

Supplementary Table 1. Primer sets, amplicon sizes and PCR conditions for pluripotent stem cell markers and actin.

	Forward (F) / Reverse (R)	Sequence (5'~3')	Amplicon Size (bp)	Annealing Temperature (T)	PCR conditions
NANOG†	F	TGCCTCACACGGAGACTGTC	354bp	55.4℃	94℃ 3min 94℃ 45sec T ℃ 45sec 72℃ 45sec 35 cycles 72℃ 15min
	R	TGCTATTCTTCGGCCAGTTG			
OCT4†	F	GACAACAATGAAAATCTTCAGGAGA	216bp	56.4℃	
	R	CTGGCGCCGGTTACAGAACCA			
SOX2†	F	CACCTACAGCATGTCCTACTC	384bp	57.7℃	
	R	CATGCTGTTTCTTACTCTCCTC			
β-actin	F	ACCAACTGGGACGACATGGAGAA	852bp	55℃	94℃ 3min 94℃ 45sec T ℃ 45sec 72℃ 1min 30 cycles 72℃ 15min
	R	CTGCTTGCTGATCCACATCTGCTG			

†primer sequences published in [17].

CHAPTER 3

Propagation of human prostate cancer stem-like cells occurs through EGFR-mediated ERK activation

Adrian P. Rybak, Alistair J. Ingram, and Damu Tang

PLoS One (2013). 8(4):e61716. doi: 10.1371/journal.pone.0061716.

Preface

EGFR is a prognostic marker of various advanced stage carcinomas such as breast (Livasy, et al. 2006), ovarian (Nielsen, et al. 2004), and non-small cell lung cancers (Garcia de Palazzo, et al. 1993). In addition, EGFR expression (Di Lorenzo, et al. 2002; Hernes, et al. 2004; Shah, et al. 2006) is associated with prostate cancer progression towards a hormone-refractory (androgen-independent) state. However, the role of EGFR expression and signalling in cancer stem cells (CSCs) has only recently been investigated. In human brain cancer, exogenous EGF significantly enhances the formation of sphere cultures, while EGFR inhibition potently inhibits sphere formation as well as the number of brain tumor stem ($CD133^{+}$) cells (Soeda, et al. 2008). EGFR expression identifies tumor-initiating cells with different tumorigenic capacities in human glioblastoma multiforme (Mazzoleni, et al. 2010). A constitutively-active mutant form of EGFR, known as EGFR variant III (EGFRvIII), has been detected in primary breast carcinoma (Del Vecchio, et al. 2012). Ectopic EGFRvIII expression has been shown to increase the proportion of cancer stem-like ($CD44^{+}CD24^{-}$) breast cancer cells, enhance their *in vitro* sphere formation as well as increase their *in vivo* tumor formation (Del Vecchio, et al. 2012).

This chapter consists of an author-generated version of the following published article: Adrian P. Rybak, Alistair J. Ingram, and Damu Tang (2013) Propagation of human prostate cancer stem-like cells occurs through EGFR-mediated ERK activation. *PLoS One* (2013). 8(4):e61716. PLOS applies the Creative Commons Attribution License (CCAL) to all works published. Under the CCAL, authors retain ownership of the

copyright for their journal article. Please note that American spelling is used throughout the article, and that the formatting and referencing style has changed to meet the journal's criteria. In addition, ERK1 and ERK2 (ERK1/2) activation was abbreviated to Thr202/Tyr204 phosphorylation (ERK1/2-P) for space constraints in the *PLoS One* journal article. However, to be technically correct, ERK1/2 activation was demonstrated by examining human ERK1 phosphorylation at residues Thr202 and Tyr204, and on conserved residues Thr185 and Tyr187 on human ERK2. I, Adrian P. Rybak, generated and established all DU145 sphere cultures, stable cell lines, and the ERK knockdown constructs used in this study. I performed all of the experiments, assembled the results, and generated the figures in the manuscript. Furthermore, I wrote and revised the manuscript along with Damu Tang. Alistair J. Ingram contributed resources towards carrying out experiments and provided intellectual input.

In Chapter 2, the results demonstrated that epidermal growth factor (EGF) treatment enhanced the generation and propagation of DU145 spheres. Sphere cells proliferated slowly and displayed reduced activation of the PI3K-AKT pathway compared to monolayer cells. While knockdown of PTEN enhanced AKT activation, primary sphere formation and secondary sphere maintenance were not affected. This manuscript demonstrates that propagation and maintenance of prostate cancer stem-like cells (PCSCs) is dependent on EGFR signalling, with EGFR activation promoting PCSC self-renewal, in part, by activating the MEK-ERK pathway. This work expands on the observations revealed in the previous manuscript (Chapter 2), and provides further

mechanistic evidence of the contributions of EGFR and downstream MEK-ERK signalling towards PCSC self-renewal.

Propagation of Human Prostate Cancer Stem-like Cells Occurs through EGFR-mediated ERK Activation

Adrian P. Rybak,[1,2,3] Alistair J. Ingram,[1,2,3] and Damu Tang [1,2.3#]

[1] Division of Nephrology, Department of Medicine, McMaster University, Hamilton, Ontario, Canada, [2] Father Sean O'Sullivan Research Institute, Hamilton, Ontario, Canada, [3] the Hamilton Centre for Kidney Research (HCKR), St. Joseph's Hospital, Hamilton, Ontario, Canada

#: Corresponding author:
Damu Tang
Juravinski Innovation Tower, room T3310
St. Joseph's Hospital
50 Charlton Avenue East
Hamilton, Ontario
Canada
L8N 4A6
Tel: (905) 522-1155, ext. 35168
Fax: (905) 521-6181
Email: damut@mcmaster.ca

Keywords: Prostate cancer stem cell, EGFR, MEK, ERK and MAPK

Abstract

Prostate cancer stem-like cells (PCSCs) are being intensely investigated largely owing to their contributions towards prostate tumorigenesis, however, our understanding of PCSC biology, including their critical pathways, remains incompletely understood. While epidermal growth factor (EGF) is widely used in maintaining PCSC cells in vitro, the importance of EGF-dependent signaling and its downstream pathways in PCSC self-renewal are not well characterized. By investigating DU145 sphere cells, a population of prostate cancer cells with stem-like properties, we report here that epidermal growth factor receptor (EGFR) signaling plays a critical role in the propagation of DU145 PCSCs. Activation of EGFR signaling via addition of EGF and ectopic expression of a constitutively-active EGFR mutant (EGFRvIII) increased sphere formation. Conversely, inhibition of EGFR signaling by using EGFR inhibitors (AG1478 and PD168393) and knockdown of EGFR significantly inhibited PCSC self-renewal. Consistent with the MEK-ERK pathway being a major target of EGFR signaling, activation of the MEK-ERK pathway contributed to EGFR-facilitated PCSC propagation. Modulation of EGFR signaling affected extracellular signal-related kinase (ERK) activation. Inhibition of ERK activation through multiple approaches, including treatment with the MEK inhibitor U0126, ectopic expression of dominant-negative MEK1(K97M), and knockdown of either ERK1 or ERK2 resulted in a robust reduction in PCSC propagation. Collectively, the present study provides evidence that EGFR signaling promotes PCSC self-renewal, in part, by activating the MEK-ERK pathway.

Introduction

Prostate cancer is the most common male malignancy and the second leading cause of cancer-related deaths in males in Western countries [1,2]. During the process of prostate tumorigenesis, oncogenic signaling pathways promote the progression of hormone-dependent carcinomas to hormone refractory prostate cancer (HRPC), the major contributing factor in prostate cancer fatalities [3,4]. Although the exact mechanisms responsible for the initiation and progression of prostate cancer remain largely unknown, prostate cancer stem-like cells (PCSCs) are widely regarded as being critical in prostate tumorigenesis and its development towards HRPC disease [5-7].

Despite the mounting evidence suggesting the existence of PCSCs, identification of human PCSCs in vivo has appeared to be a challenging task. This challenge is largely due to the heterogeneous nature of prostate cancer and the limited samples available from clinical sources. Our limited understanding of PCSCs has also contributed to the inability to isolate and propagate PCSCs from human primary carcinomas. To advance our knowledge of PCSCs, several research groups, including ours, have enriched for PCSCs from human prostate cancer cell lines. This is largely attributable to the demonstration that cancer stem cells (CSCs) can be studied using the sphere culture assay under serum-free (SF) media conditions. This assay has been used to derive and propagate CSCs from brain [8], breast [9], colon [10] and prostate cancer cells [11-16] in vitro. More importantly, the sphere culture approach has allowed the propagation of prostate cancer

stem-like cells that display CSC properties of self-renewal and the ability to initiate tumor formation in vivo [11,12,15,17].

Sphere culture commonly involves propagating stem-like cells in SF media supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [8-13]. Although the presence of both EGF and bFGF allows the generation of spheres from DU145 cells [11,12,17], whether this is the ideal condition for sphere generation and PCSC maintenance for a prolonged period of time remains unclear. In our recent investigation, we have shown that EGF plays a critical role in long-term propagation of DU145 PCSCs, and that these stem-like cells were capable of initiating tumors with a significantly enhanced ability in non-obese, diabetic/severe combined immunodeficient (NOD/SCID) mice [11]. However, the role of EGFR signaling, along with its downstream pathways that are required for DU145 PCSC propagation remain to be characterized.

In our effort to advance this knowledge, we demonstrate here that the EGFR-ERK connection plays an important role in the propagation of DU145 PCSCs. Although these PCSCs are able to propagate in the absence of exogenous EGF, activation of EGFR signaling is critical for the maintenance of DU145 PCSCs as experimental manipulation of EGFR signaling affected DU145 PCSC propagation. Additionally, modulation of EGFR signaling in DU145 PCSCs profoundly affected ERK activation. Furthermore, inhibition of ERK activation through the use of a MEK inhibitor, ectopic expression of dominant-negative MEK1(K97M), and knockdown of endogenous ERK1 or ERK2,

collectively reduced the propagation of DU145 PCSCs. Taken together, these results highlight a contribution of MEK-ERK signaling for EGFR-mediated PCSCs self-renewal.

Materials and Methods

Cell culture and propagation of DU145 PCSCs

DU145 human prostate cancer cells and 293T human embryonic kidney cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and were cultured according to ATCC instructions.

DU145 human prostate cancer stem-like cells were isolated and propagated as previously published [11]. Briefly, DU145 monolayer cells were enzymatically-dissociated into single cells using phenol red-free TrypLE Express solution (Life Technologies, Calsbad, CA, USA) and 40 μm cell strainers (BD Biosciences, Franklin Lakes, NJ, USA), and subsequently resuspended at a sub-clonal (5 cells/μl) density in serum-free (SF) media (DMEM/F12 at a 3:1 mixture) (Life Technologies) containing 0.4% bovine serum albumin (BSA) (Bioshop Canada Inc., Burlington, ON, Canada) and 0.2× B27 lacking Vitamin A (Life Technologies) in T75 flasks (15 ml total volume) designated for suspension cell culture (Sarstedt Inc., Newton, NC, USA). When examining the effect of EGF treatment on sphere formation, recombinant EGF (Sigma-Aldrich, St. Louis, MO, USA) was added at a concentration of 10 or 20 ng/ml. Typical spheres formed in 10 to 12 days. Sphere cells were sub-cultured by enzymatic dissociation, strained, and then resuspended in the above medium at sub-clonal density.

Sphere Formation Assays

To evaluate the formation of primary spheres, DU145 cells from sub-confluent (~80%) monolayer cultures were resuspended in the above SF media at a density of 2.5×10^3 cells into individual wells of a 24-well culture plate (3 wells per treatment) (Corning, Corning, NY, USA). Cells were seeded in a volume of 0.5 mL/well, unlike 1 ml/well as previously published [11]. The spheres that formed after 12 days of culture were counted. To measure the sphere-forming capacity of secondary or established spheres, spheres cells were individualized by enzymatic dissociation, strained, and then plated at a density of 5×10^2 or 1×10^3 cells/well in 24-well plates (3 wells per treatment), unless otherwise specified. The spheres that formed were counted after 12 days of culture.

Plasmids and Virus Production

EGFRvIII/pLNCX was kindly provided by Dr. Khalid Al-Nedawi (Hamilton Centre for Kidney Research, Hamilton, ON, Canada). EGFR(K721A)/pLHCX was kindly provided by Dr. Joan Krepinsky (Hamilton Centre for Kidney Research). Construction of MEK1(K97M)/pLHCX has been previously described [18]. Short-hairpin ERK1 and ERK2 targeting (shERK1 and shERK2, respectively) vectors were constructed by annealing and subcloning the previously published targeting sequences (ERK1: GCCATGAGAGATGTCTACA; ERK2: GAGGATTGAAGTAGAACAG) [19] as a hairpin into the pRIH retroviral vector, according to our published conditions [18]. The short-hairpin control (shLacZ) vector contains a sequence targeting *Escherichia coli* β-galactosidase (shLacZ target sequence: GCAGTTATCTGGAAGATCA). High titres of

empty vector (EV)/pLNCX, EGFRvIII/pLNCX, EV/pLHCX, EGFR(K721A)/pLHCX, MEK1(K97M)/pLHCX, shLacZ/pRIH, shERK1/pRIH and shERK2/pRIH retrovirus were produced using 293T cells, as previously described [18]. DU145 cells were subsequently infected with the specific retrovirus, and infected cells were selected in hygromycin (0.5 mg/ml; Life Technologies) for pLHCX and pRIH-based infections, or G418 (1 mg/ml; Sigma-Aldrich) for pLNCX-based infections. Ectopic EGFRvIII and MEK1(K97M) expression, or knockdown of endogenous ERK1 or ERK2, were verified by Western blot analysis.

Short-hairpin EGFR targeting lentiviral vectors were purchased from Sigma-Aldrich, with the targeting sequences (shEGFR1: GCTGCTCTGAAATCTCCTTTA; shEGFR2: GCCACAAAGCAGTGAATTTAT) cloned as a hairpin in the pLKO.1 vector, while a non-specific shRNA (plasmid 1864; Addgene, Cambridge, MA, USA) was used as a control (shCTRL). Production of shEGFR and shCTRL lentivirus was carried out by co-transfecting each shRNA plasmid (10 μg) with plasmids (10 μg each) necessary for third-generation lentiviral production (pRSV-REV, pCMV-VSV-G, pMDLg/pRRE) [20], kindly provided by Dr. Bryan E. Strauss (University of São Paulo School of Medicine, São Paulo, Brazil), into 293T cells using the calcium phosphate method. Sixty hours (h) post-transfection, the supernatants containing VSV-G pseudotyped, replication-incompetent lentiviral particles were collected, filtered through a 0.45 μm filter and centrifuged for 2 h at 48,000 g. Viral pellets were resuspended in 1 ml of media containing 10 μg/ml polybrene (Sigma-Aldrich) and added to monolayer cells for 2 h in a

humidified 37°C incubator, with periodic mixing at 20 min intervals, to allow for cell infection. Infection was selected with puromycin (1 μg/ml; Sigma-Aldrich).

For infection of sphere cells, the shEGFR2 and shCTRL lentivirus pellets were resuspended in 2 ml of serum-free media containing 0.4% BSA, 0.2× B27 (lacking Vitamin A) and 10 μg/ml polybrene and added to individualized (3×10^5) cells for 2 h in a humidified 37°C incubator, with periodic mixing at 20 min intervals, to allow for cell infection. Sphere cells were subsequently seeded at a density of 10^4 cells/ml in T75 flasks designated for suspension cell culture (Sarstedt Inc.). Puromycin (1 μg/ml) was added to the sphere cultures at 48 hours post-infection.

Anchorage-Independent Growth Assay

DU145 sphere cell cultures were plated in individual wells of six-well plates at a density of 10^4 cells/well as previously described [11]. After 8 weeks, phase contrast images were taken in 5 random fields per well using the Zeiss Axiovert 200M microscope (AxioVision 3.1 software) at 25X magnification, and the colonies consisting of ≥50 cells were counted. Phase contrast images were subsequently processed using CorelDRAW Graphics Suite X4 software (Corel, Ottawa, ON, Canada).

EGFR and MEK inhibition studies

The EGFR inhibitors AG1478 and PD168393 (Calbiochem, Darmstadt, Hesse, Germany) and the MEK inhibitor U0126 (Promega, Madison, WI, USA) were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) and used at the concentrations indicated.

DMSO was administered at an identical volume as a control (mock treatment). EGFR inhibitor (AG1478 or PD168393), MEK (U0126) or DMSO (mock) treatment was administered at the time of seeding sphere cells. Sphere formation was quantitated as previously indicated. Inhibition of EGFR and MEK1 kinase activity (as determined by EGFR and ERK1/2 phosphorylation status, respectively) was verified by Western blot analysis.

Antibody-based EGFR function blocking experiments on sphere cells were carried out as previously described [21]. Briefly, azide-free EGFR blocking mouse monoclonal (Ab-1, clone 528) antibody (Thermo Fisher Scientific, Waltham, MA, USA) or mouse IgG2a isotype control antibody (R&D Systems, Minneapolis, MN, USA) were added to sphere cells (at the time of seeding) at a concentration of 4 μg/ml in the presence or absence of recombinant EGF (10 ng/ml). Spheres were allowed to form and were quantitated as previously indicated.

Western blot analysis

Whole cell lysates were prepared in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml leupeptin and 10 μg/ml aprotinin. For each sample, a total of 50 μg of whole cell lysate was used unless otherwise specified. Resolved lysates (10% SDS-polyacrylamide gels) were transferred onto Amersham Hybond-ECL membranes (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The membranes were

blocked with 5% w/v non-fat dry skim milk at room temperature for 1 h, and then incubated with the indicated primary antibodies in 10 ml of antibody dilution buffer (1× Tris-buffered saline containing 0.1% Tween-20 (TBST), and 5% BSA) with gentle rocking at 4^{o}C overnight. Primary antibodies used for probing were as follows: rabbit anti-human phospho-EGFR (Tyr1068) (Sigma-Aldrich, E6154, 1:1000), rabbit anti-human EGFR (#4267, 1:1000), rabbit anti-human phospho-AKT (Ser473) (#4058, 1:1000), rabbit anti-human phospho-ERK1/2 (Thr202/Tyr204) (#9101, 1:1000), rabbit anti-human ERK1/2 (#9102, 1:1000), rabbit anti-human phospho-STAT3 (Tyr705) (#9145, 1:2000), mouse anti-human STAT3 (#9139, 1:1000) from Cell Signaling Technology (Danvers, MA, USA), goat anti-human AKT (C-20, 1:1000), mouse anti-human MEK1 (H-8, 1:1000) and mouse anti-human α-tubulin (TU-02, 1:500) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were subsequently washed with 1× TBST solution and incubated (1 h at room temperature) with either donkey anti-goat-IgG-horse radish peroxidase (HRP) (Santa Cruz Biotechnology, sc-2033, 1:3000), donkey anti-mouse-IgG-HRP (GE Healthcare, NA931V, 1:3000) or donkey anti-rabbit-IgG-HRP (GE Healthcare, NA934V, 1:3000) conjugated secondary antibodies. Immunoblot signals were detected using Amersham ECL Western blotting detection reagents (GE Healthcare) and then exposed to x-ray film (Thermo Fisher Scientific). The quantification of immunoblot band densities was determined using ImageJ software (version 1.43u; W. Rasband, National Institute of Health).

Statistical analysis

All quantitative data are presented as mean ± S.E.M. and comparisons were made using two-tailed independent Student's *t*-tests. A p-value < 0.05 was considered statistically significant.

Results

DU145 spheres propagated in the absence of exogenous mitogens display EGFR signal activation

We have previously reported that while EGF supplementation significantly promoted the generation and propagation of DU145 spheres, the formation of spheres did not require the addition of exogenous EGF [11] suggesting that EGFR signaling may be activated in the absence of exogenous EGF. To investigate this possibility, we have generated spheres from DU145 cells in the absence (-EGF) and presence of EGF (10 or 20 ng/ml EGF; 10EGF or 20EGF, respectively) and subsequently propagated these established spheres for three passages, under the identical conditions in which primary spheres were generated, prior to evaluating their sphere-forming capacity. Spheres generated in the -EGF media condition can be maintained in a consistent manner for at least three passages. Furthermore, the ability to generate and maintain spheres in the -EGF condition (Figure 1A) suggests that propagation under EGF-free (also without the addition of other external growth factors) media conditions is an intrinsic property of DU145 PCSCs. This possibility is supported by the fact that the number of spheres

maintained after seeding 5×10^2 cells/well was approximately half of that propagated after seeding 1×10^3 cells/well (Figure 1A). Furthermore, this possibility is also in line with our recent observation that -EGF spheres have been propagated in -EGF media for more than 15 consecutive passages, in the consistent manner as indicated in Figure 1A, by the time this manuscript was prepared (data not shown). Although EGF enhanced sphere propagation compared to sphere cells grown in -EGF media (10EGF and 20EGF compared to -EGF), 20EGF did not further stimulate sphere number in comparison to the 10EGF treatment. This suggests that an EGF-induced threshold exists (Figure 1A).

To examine whether spheres generated and maintained in the -EGF media condition respond to EGFR signal activation, we have cultured established, EGF-free spheres in the presence of exogenous EGF. Not only did these EGF-free sphere cells respond to exogenous EGF treatment when seeded at sub-clonal densities of 5×10^2 and 1×10^3 cells/well (1×10^3 and 2×10^3 cells/ml, respectively) (Figure 1B), but they also respond to EGF to the same level as those spheres generated and maintained in EGF-containing media (comparing Figure 1B with Figure 1A). These observations indicate that in the absence of exogenous EGF, spheres may be able to activate EGFR signaling. In support of this possibility, phosphorylation of EGFR at tyrosine (Y) 1068 (EGFR-P), a commonly used surrogate marker of EGFR signal activation [22], was readily detected in -EGF and +EGF (10EGF and 20EGF) sphere cells (Figure 1C). Taken together, the above observations support the notion that EGFR signaling plays an important role in the propagation of DU145 PCSCs.

Enforced EGFR signal activation renders DU145 PCSCs non-responsive to EGF treatment

To further ascertain the role of EGFR signal activation in DU145 PCSCs, the constitutively-active EGFR mutant, EGFR variant III (EGFRvIII) [23], was overexpressed in DU145 monolayer cells (Figure 2A). EGFRvIII-expressing cells displayed elevated levels of Tyr1068 phosphorylation (EGFR-P) and ERK activation (Thr202/Tyr204 phosphorylation on ERK1/2; ERK1/2-P) in SF media (Figure 2A), demonstrating that ectopic EGFRvIII expression resulted in constitutive EGFR signaling. In comparison to empty vector (EV) transduced DU145 cells, DU145 EGFRvIII-expressing cells generated more primary spheres in -EGF media (Figure 2B). Furthermore, EGF supplementation promoted the generation of primary spheres from DU145 EV cells but not EGFRvIII-expressing cells (Figure 2B). However, EGFRvIII-expressing sphere cells can form primary spheres in -EGF media at a similar level as DU145 EV sphere cells propagated in the presence of exogenous EGF (Figure 2B). Therefore, DU145 EGFRvIII-expressing cells possess a comparable capacity for primary sphere generation as DU145 EV cells stimulated with recombinant EGF (Figure 2B). Similar observations were also obtained in sphere propagation (i.e. the production of secondary spheres upon seeding 10^3 cells/well) (Figure 2C). Collectively, these results support our hypothesis that EGFR signaling contributes to the propagation of DU145 PCSCs.

EGFR blockade reduces the self-renewal capacity of DU145 PCSCs

To further investigate the requirement of EGFR signaling in the propagation (self-renewal) of DU145 PCSCs, we examined the effect of EGFR signal inhibition on sphere maintenance. Sphere cells were treated with increasing concentrations of AG1478 (Tyrphostin), a selective inhibitor of EGFR (ErbB1) kinase activity that functions by sequestering it in its inactive form [24]. AG1478 treatment was examined at 24 h post-treatment, as EGF treatment achieved the highest levels in EGFR-P in sphere cells at this time point (Figure S1). AG1478 treatment reduced EGFR activation in a dose-dependent fashion in sphere cells, with and without the addition of exogenous EGF (Figure 3A). Furthermore, AG1478 dose-dependently reduced sphere propagation, as the number of spheres generated from established sphere cell cultures was reduced by AG1478 treatment, irrespective of whether exogenous EGF was present (Figure 3B). Similar observations were also obtained using the EGFR inhibitor PD168393 [25] (Figure 3C). This demonstrates that inhibition of EGFR signaling blocks the self-renewal capacity of DU145 PCSCs.

To further address whether loss of EGFR signaling affects the ability of DU145 PCSCs to maintain themselves, DU145 monolayer cells were infected with shRNA constructs targeting EGFR. These constructs efficiently knocked-down EGFR, with slightly variations in the levels of EGFR knockdown within the respective stable cell lines (Figure 4A). The shEGFR construct #2 (shEGFR2) achieved a greater knockdown than shEGFR1 (95% vs 70%, respectively) (Figure 4A). Stable EGFR knockdown cell lines could be maintained in complete media. However, ectopic expression of EGFR(K721A),

an EGFR mutant lacking protein tyrosine kinase activity [26], resulted in cell death and prevented the generation of a stable cell line (data not shown). By using these stable EGFR knockdown cell lines, we were able to show that EGFR knockdown in DU145 cells reduced their capacity to generate primary spheres compared to shCTRL cells (Figure 4B). The ability of EGFR knockdown to affect sphere maintenance was also examined. Stable EGFR knockdown in sphere cells (Figure 4C) reduced the number of subsequent spheres formed in the -EGF media condition (Figure 4D).

An important feature of cellular transformation in vitro, which closely correlates with in vivo tumorigenicity in immunocompromised mice [27-29], is the ability of tumorigenic cells to propagate under anchorage-independent conditions. To address whether the loss of EGFR expression affects the anchorage-independent growth of DU145 PCSCs, established EGFR knockdown sphere cells were seeded in serum-supplemented, soft agar-containing media. EGFR knockdown in sphere cells resulted in fewer soft agar colonies compared to shCTRL sphere cells under differentiating (ie. serum-supplemented) conditions (Figure 4E). Taken together, we demonstrated that EGFR signaling is critical for maintaining DU145 PCSC propagation, which is consistent with the observed reduction in soft agar colony numbers following EGFR knockdown.

MEK-ERK signaling regulates the self-renewal properties of DU145 PCSCs

After the demonstration that EGFR signaling is critical in the generation and maintenance of PCSCs, we further examined the downstream mechanisms responsible for the observed EGFR activity. EGFR signaling is well known to initiate signaling events

involving the activation of the PI3K-AKT and Raf-MEK-ERK (mitogen activated protein kinases; MAPK) pathways [30]. In addition, EGFR signaling has been shown to activate signal transducer and activator of transcription proteins (STAT) [31], particularly STAT3-mediated signaling [32]. Activation of STAT3 involves phosphorylation at Tyr705 [33], and STAT3 has been shown to be activated in prostate cancers [34,35]. Our previous work has suggested that in our system, other downstream signaling proteins, rather than AKT, must be critical for EGF-enhanced DU145 sphere formation [11]. While AKT may contribute to DU145 sphere propagation, we have further expanded on our previous report to examine the contributions of the ERK pathway towards EGFR-dependent propagation of DU145 PCSCs.

In this effort, we were able to show that EGF stimulation of EGF-free DU145 sphere cells resulted in ERK activation, but not STAT3 activation, in a time-dependent manner (Figure S1). Furthermore, the MEK inhibitor, U0126 [36], dose-dependently reduced the self-renewal capacity of DU145 PCSCs (Figure 5A). As expected, U0126 inhibited ERK activation in DU145 PCSCs (Figure S2). To consolidate the results implicating MEK-ERK signaling in DU145 PCSC propagation, we have expressed the dominant-negative MEK1(K97M) [37] in DU145 cells (Figure 5B). MEK1(K97M) cells displayed reduced ERK activation compared to empty vector (EV) control cells following FBS-stimulation (10%) of serum-deprived cells (Figure 5B). In comparison to EV cells, MEK1(K97M) cells displayed a significant reduction in generating primary spheres (Figure 5C). In support of MEK-ERK signaling being critical to EGFR-mediated generation of DU145

PCSCs, exogenous EGF was incapable of further enhancing primary sphere formation of MEK1(K97M) cells (Figure 5C).

To further consolidate the notion that ERK signaling directly influences PCSC propagation, we have knocked-down either ERK1 or ERK2 in DU145 cells using specific shRNAs (Figure 6A). These ERK1 and ERK2 shRNA target sequences have previously been verified to efficiently knockdown either ERK1 or ERK2 in human multiple myeloma cells [19] and MCF7 breast cancer cells [38]. Knockdown of endogenous ERK1 or ERK2 was capable of significantly reducing primary sphere formation compared to shRNA control (shLacZ) cells (Figure 6B). Moreover, EGF treatment was still capable of enhancing sphere formation when either ERK1 or ERK2 was knocked-down, but not when MEK1 kinase activity was compromised (Figure 5). This suggests that redundancy in ERK1 and ERK2 function likely compensates in EGF-mediated enhancement of sphere formation when either ERK1 or ERK2 is knocked-down.

Discussion

Cancer stem cells (CSCs) are widely regarded to play a major role in cancer progression. In order to better understand CSCs, an essential aspect of CSC research is to develop the capacity to faithfully propagate CSCs. The established system in this domain is to culture CSCs as spheres under SF media conditions supplemented with specific growth factors (GFs) [39]. In terms of PCSC propagation, the commonly used GFs are EGF and bFGF. While the combination of both of these GFs was capable of generating

spheres from DU145 cells [11,12,17], the presence of bFGF actually reduced EGF's ability to promote the propagation of DU145 spheres [11]. Therefore, evidence suggests that EGFR signaling is critical in maintaining the self-renewal capacity of DU145 PCSCs.

Our research reported here further supports the critical contribution of EGFR signaling to DU145 PCSC self-renewal. While propagation of DU145 PCSCs does not require the presence of exogenous EGF, activation of EGFR signaling occurred in this condition and more importantly, the activation of EGFR was also functionally important. This was based on the observation that inhibition of EGFR signaling reduced DU145 sphere cell propagation in the absence of exogenous EGF (Figures 3 and 4). Despite the mechanism leading to the activation of EGFR signaling without the addition of exogenous EGF requires further investigation, multiple possibilities can be envisaged, including the production of EGF or other ligands competent in activating EGFR in an autocrine fashion, as well as ligand-independent activation of EGFR. In our effort to determine whether autocrine-derived EGF-like ligands are responsible for the activation of EGFR in PCSCs, we examined the effect of treating sphere cells with an EGFR blocking antibody [40]. This antibody has been used to reduce EGFR-mediated self-renewal in human brain tumor stem cells [21]. While this antibody reduced EGF-enhanced formation of DU145 spheres, the antibody did not effect DU145 sphere formation under the -EGF media condition (Figure S3). In conjunction with the observation that the EGFR inhibitor AG1478 potently inhibited sphere formation (Figure 3B), evidence suggests the involvement of ligand-independent EGFR activity in DU145 sphere propagation. Although we could not exclude the possibility of autocrine production of EGF or other

EGFR-activating ligands (i.e. TGF-α), it is tempting to suggest that ligand-independent activation of EGFR may contribute towards EGFR activation in the -EGF media condition. Mutations in EGFR resulting in its ligand-independent activation were reported in prostate cancer. For instance, the EGFRvIII mutant [41] and a number of EGFR tyrosine kinase domain mutants [42] that display activated EGFR signaling have been detected in prostate cancer. These mutants enhanced EGFR signaling, cell proliferation and transforming ability in the absence of EGF stimulation [42]. However, future research is needed to identify the mechanisms responsible for EGFR activation in DU145 PCSCs under the -EGF media condition.

Our finding that EGFR signaling is critical for DU145 PCSCs is consistent with the emergence of results that support EGFR in playing an essential role in CSCs. Although EGFR is a prognostic marker of various advanced stage carcinomas such as breast [43], ovarian [44], prostate [45,46] and non-small cell lung cancers [47], the role of EGFR expression and signaling in CSCs has only recently been investigated. Enhanced levels of EGFR expression have been associated with the stem cell population in prostate cancer tissue [48]. In human brain cancer, exogenous EGF significantly enhances the formation of neurosphere cultures, while inhibition of EGFR tyrosine kinase activity potently inhibits sphere formation [49]. EGFR expression identifies tumor-initiating cells with different tumorigenic capacities in human glioblastoma multiforme [50]; enforced EGFR expression in CSCs enhanced their tumorigenic potential, while ectopic EGFR expression increased the in vivo tumorigenic capacity of EGFR-negative CSC lines. In addition, EGFR knockdown in $EGFR^+$ CSCs decreased their tumorigenicity in vivo and promoted

differentiation of these cells. Furthermore, there is evidence suggesting that EGFR-high expressing cells display stem cell characteristics [50].

While downstream of EGFR lies the PI3K-AKT, MEK-ERK and STAT3 pathways, we define here that the MEK-ERK pathway contributes to EGFR's role in maintaining the "stemness" of DU145 PCSCs. This is consistent with the inhibition of self-renewal observed following MEK inhibition in human glioblastoma cancer stem-like cells [51] and human breast tumor-initiating cells [52]. While MEK-ERK activation is necessary and sufficient to mediate Raf-induced androgen receptor (AR) downregulation in prostate cancer cell lines irrespective of AR sensitivity [53], patients who have failed hormone ablation therapy display elevated ERK1/2 signal activation in recurrent tumors [54]. However, we like to stress that our research does not exclude the possibility that the PI3K-AKT pathway also contributes to the self-renewal of DU145 PCSCs.

Acknowledgements

The authors would like to thank Anthony Bruce for his assistance with the interpretation of results and constructive input during the preparation of this manuscript. We would also like to thank Dr. Khalid Al-Nedawi (Hamilton Centre for Kidney Research, Hamilton, ON, Canada) for providing the EGFRvIII/pLNCX construct, Dr. Joan Krepinsky (Hamilton Centre for Kidney Research) for providing the EGFR(K721A)/pLHCX construct, Dr. David Sabatini (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) for providing the non-specific shRNA construct (Addgene plasmid 1864), and Dr. Bryan E. Strauss (University of São Paulo School of Medicine, São Paulo, Brazil) for providing the third-generation lentiviral packaging plasmids used in this study.

References

1. Siegel R, Naishadham D, Jemal A (2012) Cancer statistics, 2012. CA Cancer J Clin 62: 10-29.

2. Williams H, Powell IJ (2009) Epidemiology, pathology, and genetics of prostate cancer among African Americans compared with other ethnicities. Methods Mol Biol 472: 439-453.

3. Ross JS (2007) The androgen receptor in prostate cancer: therapy target in search of an integrated diagnostic test. Adv Anat Pathol 14: 353-357.

4. Moon C, Park JC, Chae YK, Yun JH, Kim S (2008) Current status of experimental therapeutics for prostate cancer. Cancer Lett 266: 116-134.

5. Tang DG, Patrawala L, Calhoun T, Bhatia B, Choy G, et al. (2007) Prostate cancer stem/progenitor cells: identification, characterization, and implications. Mol Carcinog 46: 1-14.

6. Lawson DA, Witte ON (2007) Stem cells in prostate cancer initiation and progression. J Clin Invest 117: 2044-2050.

7. Kasper S (2008) Stem cells: The root of prostate cancer? J Cell Physiol 216: 332-336.

8. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, et al. (2004) Identification of human brain tumour initiating cells. Nature 432: 396-401.

9. Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, et al. (2005) Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 65: 5506-5511.

10. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, et al. (2007) Identification and expansion of human colon-cancer-initiating cells. Nature 445: 111-115.

11. Rybak AP, He L, Kapoor A, Cutz JC, Tang D (2011) Characterization of sphere-propagating cells with stem-like properties from DU145 prostate cancer cells. Biochim Biophys Acta 1813: 683-694.

12. Dubrovska A, Kim S, Salamone RJ, Walker JR, Maira SM, et al. (2009) The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. Proc Natl Acad Sci U S A 106: 268-273.

13. Miki J, Furusato B, Li H, Gu Y, Takahashi H, et al. (2007) Identification of putative stem cell markers, CD133 and CXCR4, in hTERT-immortalized primary nonmalignant and malignant tumor-derived human prostate epithelial cell lines and in prostate cancer specimens. Cancer Res 67: 3153-3161.

14. Li H, Zhou J, Miki J, Furusato B, Gu Y, et al. (2008) Telomerase-immortalized non-malignant human prostate epithelial cells retain the properties of multipotent stem cells. Exp Cell Res 314: 92-102.

15. Rajasekhar VK, Studer L, Gerald W, Socci ND, Scher HI (2011) Tumour-initiating stem-like cells in human prostate cancer exhibit increased NF-kappaB signalling. Nat Commun 2: 162.

16. Yamamoto H, Masters JR, Dasgupta P, Chandra A, Popert R, et al. (2012) CD49f Is an Efficient Marker of Monolayer- and Spheroid Colony-Forming Cells of the Benign and Malignant Human Prostate. PLoS One 7: e46979.

17. Salvatori L, Caporuscio F, Verdina A, Starace G, Crispi S, et al. (2012) Cell-to-cell signaling influences the fate of prostate cancer stem cells and their potential to generate more aggressive tumors. PLoS One 7: e31467.

18. Li Y, Wu D, Chen B, Ingram A, He L, et al. (2004) ATM activity contributes to the tumor-suppressing functions of p14ARF. Oncogene 23: 7355-7365.

19. Chatterjee M, Stuhmer T, Herrmann P, Bommert K, Dorken B, et al. (2004) Combined disruption of both the MEK/ERK and the IL-6R/STAT3 pathways is required to induce apoptosis of multiple myeloma cells in the presence of bone marrow stromal cells. Blood 104: 3712-3721.

20. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, et al. (1998) A third-generation lentivirus vector with a conditional packaging system. J Virol 72: 8463-8471.

21. Kelly JJ, Stechishin O, Chojnacki A, Lun X, Sun B, et al. (2009) Proliferation of human glioblastoma stem cells occurs independently of exogenous mitogens. Stem Cells 27: 1722-1733.

22. Rojas M, Yao S, Lin YZ (1996) Controlling epidermal growth factor (EGF)-stimulated Ras activation in intact cells by a cell-permeable peptide mimicking phosphorylated EGF receptor. J Biol Chem 271: 27456-27461.

23. Ekstrand AJ, Longo N, Hamid ML, Olson JJ, Liu L, et al. (1994) Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. Oncogene 9: 2313-2320.

24. Gan HK, Walker F, Burgess AW, Rigopoulos A, Scott AM, et al. (2007) The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor AG1478 increases the formation of inactive untethered EGFR dimers. Implications for combination therapy with monoclonal antibody 806. J Biol Chem 282: 2840-2850.

25. Fry DW, Bridges AJ, Denny WA, Doherty A, Greis KD, et al. (1998) Specific, irreversible inactivation of the epidermal growth factor receptor and erbB2, by a new class of tyrosine kinase inhibitor. Proc Natl Acad Sci U S A 95: 12022-12027.

26. Honegger AM, Dull TJ, Felder S, Van Obberghen E, Bellot F, et al. (1987) Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. Cell 51: 199-209.

27. Colburn NH, Bruegge WF, Bates JR, Gray RH, Rossen JD, et al. (1978) Correlation of anchorage-independent growth with tumorigenicity of chemically transformed mouse epidermal cells. Cancer Res 38: 624-634.

28. Shin SI, Freedman VH, Risser R, Pollack R (1975) Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro. Proc Natl Acad Sci U S A 72: 4435-4439.

29. Freedman VH, Shin SI (1974) Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. Cell 3: 355-359.

30. McCubrey JA, Steelman LS, Abrams SL, Lee JT, Chang F, et al. (2006) Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Adv Enzyme Regul 46: 249-279.

31. Quesnelle KM, Boehm AL, Grandis JR (2007) STAT-mediated EGFR signaling in cancer. J Cell Biochem 102: 311-319.

32. Grandis JR, Drenning SD, Chakraborty A, Zhou MY, Zeng Q, et al. (1998) Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor- mediated cell growth In vitro. J Clin Invest 102: 1385-1392.

33. Kaptein A, Paillard V, Saunders M (1996) Dominant negative stat3 mutant inhibits interleukin-6-induced Jak-STAT signal transduction. J Biol Chem 271: 5961-5964.

34. Ni Z, Lou W, Leman ES, Gao AC (2000) Inhibition of constitutively activated Stat3 signaling pathway suppresses growth of prostate cancer cells. Cancer Res 60: 1225-1228.

35. Dhir R, Ni Z, Lou W, DeMiguel F, Grandis JR, et al. (2002) Stat3 activation in prostatic carcinomas. Prostate 51: 241-246.

36. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, et al. (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273: 18623-18632.

37. Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, et al. (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 265: 966-970.

38. Wei F, Xie Y, Tao L, Tang D (2010) Both ERK1 and ERK2 kinases promote G2/M arrest in etoposide-treated MCF7 cells by facilitating ATM activation. Cell Signal 22: 1783-1789.

39. Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8: 755-768.

40. Gill GN, Kawamoto T, Cochet C, Le A, Sato JD, et al. (1984) Monoclonal anti-epidermal growth factor receptor antibodies which are inhibitors of epidermal growth factor binding and antagonists of epidermal growth factor binding and antagonists of epidermal growth factor-stimulated tyrosine protein kinase activity. J Biol Chem 259: 7755-7760.

41. Olapade-Olaopa EO, Moscatello DK, MacKay EH, Horsburgh T, Sandhu DP, et al. (2000) Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer. Br J Cancer 82: 186-194.

42. Cai CQ, Peng Y, Buckley MT, Wei J, Chen F, et al. (2008) Epidermal growth factor receptor activation in prostate cancer by three novel missense mutations. Oncogene 27: 3201-3210.

43. Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, et al. (2006) Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Mod Pathol 19: 264-271.

44. Nielsen JS, Jakobsen E, Holund B, Bertelsen K, Jakobsen A (2004) Prognostic significance of p53, Her-2, and EGFR overexpression in borderline and epithelial ovarian cancer. Int J Gynecol Cancer 14: 1086-1096.

45. Shah RB, Ghosh D, Elder JT (2006) Epidermal growth factor receptor (ErbB1) expression in prostate cancer progression: correlation with androgen independence. Prostate 66: 1437-1444.

46. Schlomm T, Kirstein P, Iwers L, Daniel B, Steuber T, et al. (2007) Clinical significance of epidermal growth factor receptor protein overexpression and gene copy number gains in prostate cancer. Clin Cancer Res 13: 6579-6584.

47. Garcia de Palazzo IE, Adams GP, Sundareshan P, Wong AJ, Testa JR, et al. (1993) Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. Cancer Res 53: 3217-3220.

48. Mimeault M, Johansson SL, Batra SK (2012) Pathobiological implications of the expression of EGFR, pAkt, NF-kappaB and MIC-1 in prostate cancer stem cells and their progenies. PLoS One 7: e31919.

49. Soeda A, Inagaki A, Oka N, Ikegame Y, Aoki H, et al. (2008) Epidermal growth factor plays a crucial role in mitogenic regulation of human brain tumor stem cells. J Biol Chem 283: 10958-10966.

50. Mazzoleni S, Politi LS, Pala M, Cominelli M, Franzin A, et al. (2010) Epidermal growth factor receptor expression identifies functionally and molecularly distinct tumor-initiating cells in human glioblastoma multiforme and is required for gliomagenesis. Cancer Res 70: 7500-7513.

51. Sunayama J, Matsuda K, Sato A, Tachibana K, Suzuki K, et al. (2010) Crosstalk between the PI3K/mTOR and MEK/ERK pathways involved in the maintenance of self-renewal and tumorigenicity of glioblastoma stem-like cells. Stem Cells 28: 1930-1939.

52. Chang CJ, Yang JY, Xia W, Chen CT, Xie X, et al. (2011) EZH2 promotes expansion of breast tumor initiating cells through activation of RAF1-beta-catenin signaling. Cancer Cell 19: 86-100.

53. Hong SK, Kim JH, Lin MF, Park JI (2011) The Raf/MEK/extracellular signal-regulated kinase 1/2 pathway can mediate growth inhibitory and differentiation signaling via androgen receptor downregulation in prostate cancer cells. Exp Cell Res 317: 2671-2682.

54. Gioeli D, Mandell JW, Petroni GR, Frierson HF, Jr., Weber MJ (1999) Activation of mitogen-activated protein kinase associated with prostate cancer progression. Cancer Res 59: 279-284.

Figures

Figure 1. Formation and maintenance of DU145 spheres occurs independently of exogenous mitogens, with EGF treatment enhancing sphere formation. A) EGF enhances sphere formation independent of the cell seeding density. DU145 spheres were generated and maintained in serum-free media containing 0.4% BSA and 0.2× B27 (SF), and supplemented with or without EGF (10 or 20 ng/ml; 10EGF or 20EGF, respectively) for three passages to establish EGF-free (-EGF) and EGF-stimulated sphere cultures prior to experimentation. Sphere cells were seeded at a density of 5×10^2 and 1×10^3 cells/well (1×10^3 and 2×10^3 cells/ml, respectively) in 24-well plates (three replicates per treatment). Six experiments were conducted. Spheres maintained in EGF-supplemented SF media display enhanced sphere numbers compared to -EGF sphere cultures. Spheres maintained in SF media containing >10 ng/ml EGF do not display additional increases in sphere-forming capacity. B) Treatment of -EGF spheres with increasing concentrations of EGF (+10EGF or +20EGF, respectively) increased sphere formation, and C) enhanced activation of EGFR signaling (EGFR phosphorylation at Tyr1068; EGFR-P). Sphere numbers are displayed as mean ± S.E.M. of four independent experiments (*$p < 0.05$ in comparison to the respective -EGF media condition; two-tailed independent Student's *t*-test).

Figure 1.

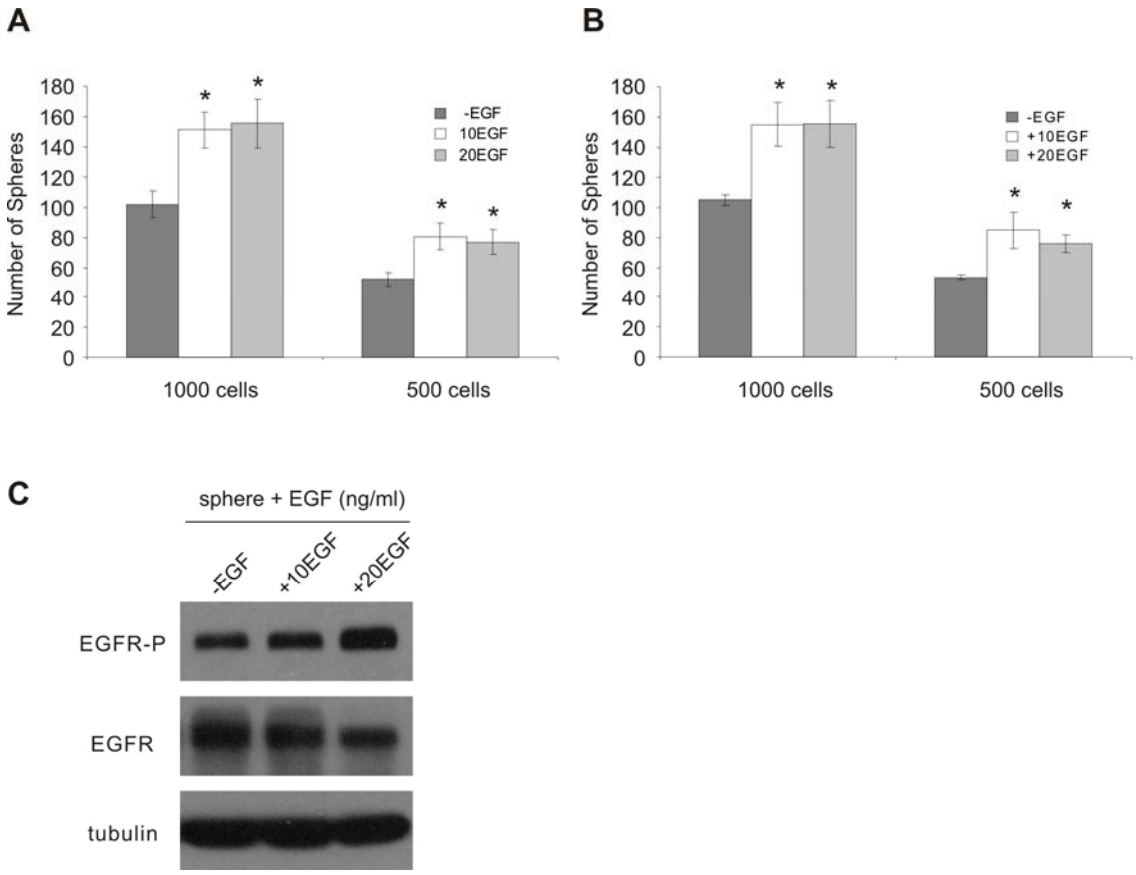

A
B
Number of Spheres
180
160
140
120
100
80
60
40
20
0
-EGF
10EGF
20EGF
+10EGF
+20EGF
1000 cells
500 cells
C
sphere + EGF (ng/ml)
-EGF
+10EGF
+20EGF
EGFR-P
EGFR
tubulin

Figure 2. **Expression of constitutively-active EGFRvIII enhances DU145 sphere formation and maintenance.** A) Western blot analysis of EGFRvIII expression and activation (Tyr1068 phosphorylation; EGFR-P) in DU145 parent (monolayer) cells grown in serum-supplemented (10% FBS) and serum-free (SF) media. EGFRvIII expression in DU145 cells activates ERK signaling. ERK activation was determined by examining the phosphorylation of ERK1 and ERK2 proteins at Thr202 and Tyr204 residues, respectively (ERK1/2-P). B) Primary (1^o) and C) secondary (2^o) sphere formation following EGFRvIII expression in DU145 cells compared to empty vector (EV) control cells. Cells were seeded in serum-free media containing 0.4% BSA, 0.2× B27 and lacking exogenous growth factors (-EGF), or with EGF supplemented (10 ng/ml; 10EGF), at a density of 2.5×10^3 (1^o sphere formation) or 1×10^3 cells/well (2^o sphere formation) in 24-well plates (0.5 ml/well; three replicates per treatment). Experiments were conducted in triplicate. Sphere numbers are displayed as mean ± S.E.M. (the *p* values for the indicated comparisons were obtained by two-tailed independent Student's *t*-test).

Figure 2.

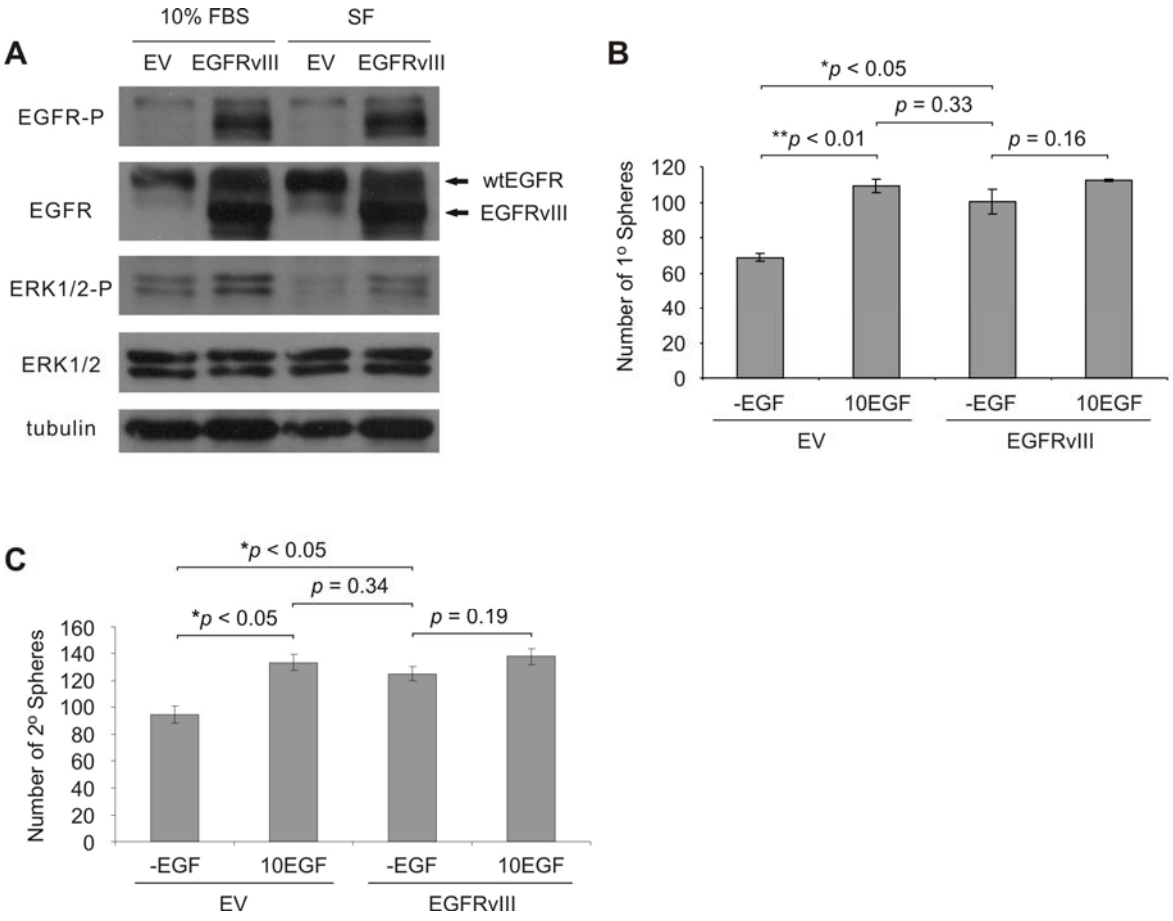

A
10% FBS
SF
EV
EGFRvIII
EV
EGFRvIII
EGFR-P
EGFR
wtEGFR
EGFRvIII
ERK1/2-P
ERK1/2
tubulin
B
*p < 0.05
p = 0.33
**p < 0.01
p = 0.16
Number of 1° Spheres
120
100
80
60
40
20
0
-EGF
10EGF
-EGF
10EGF
EV
EGFRvIII
C
*p < 0.05
p = 0.34
*p < 0.05
p = 0.19
Number of 2° Spheres
160
140
120
100
80
60
40
20
0
-EGF
10EGF
-EGF
10EGF
EV
EGFRvIII

Figure 3. **EGFR signaling blockage reduces the self-renewal activity of DU145 PCSCs.** A) Western blot analysis of whole cell lysates following 24 hour treatment of DU145 spheres with DMSO (D) or increasing concentrations of AG1478 (0.1 - 20 μM concentration range), in the presence or absence of exogenous EGF (10 ng/ml; 10EGF). Sphere formation of EGF-free sphere cells was inhibited following treatment with EGFR inhibitors B) AG1478 or C) PD168393 at various doses in the presence (10EGF) or absence (-EGF) of exogenous EGF (10 ng/ml). Sphere cells were seeded at a density of 2×10^3 cells/well (0.5 ml/well; three replicates per treatment). EGFR inhibitor (AG1478 or PD168393) or DMSO (mock) treatment was administered at the time of seeding. Spheres that formed were counted 12 days post-seeding. Sphere numbers are displayed as mean ± S.E.M. (the *p* values for the indicated comparisons were obtained by two-tailed independent Student's *t*-test).

Figure 3.

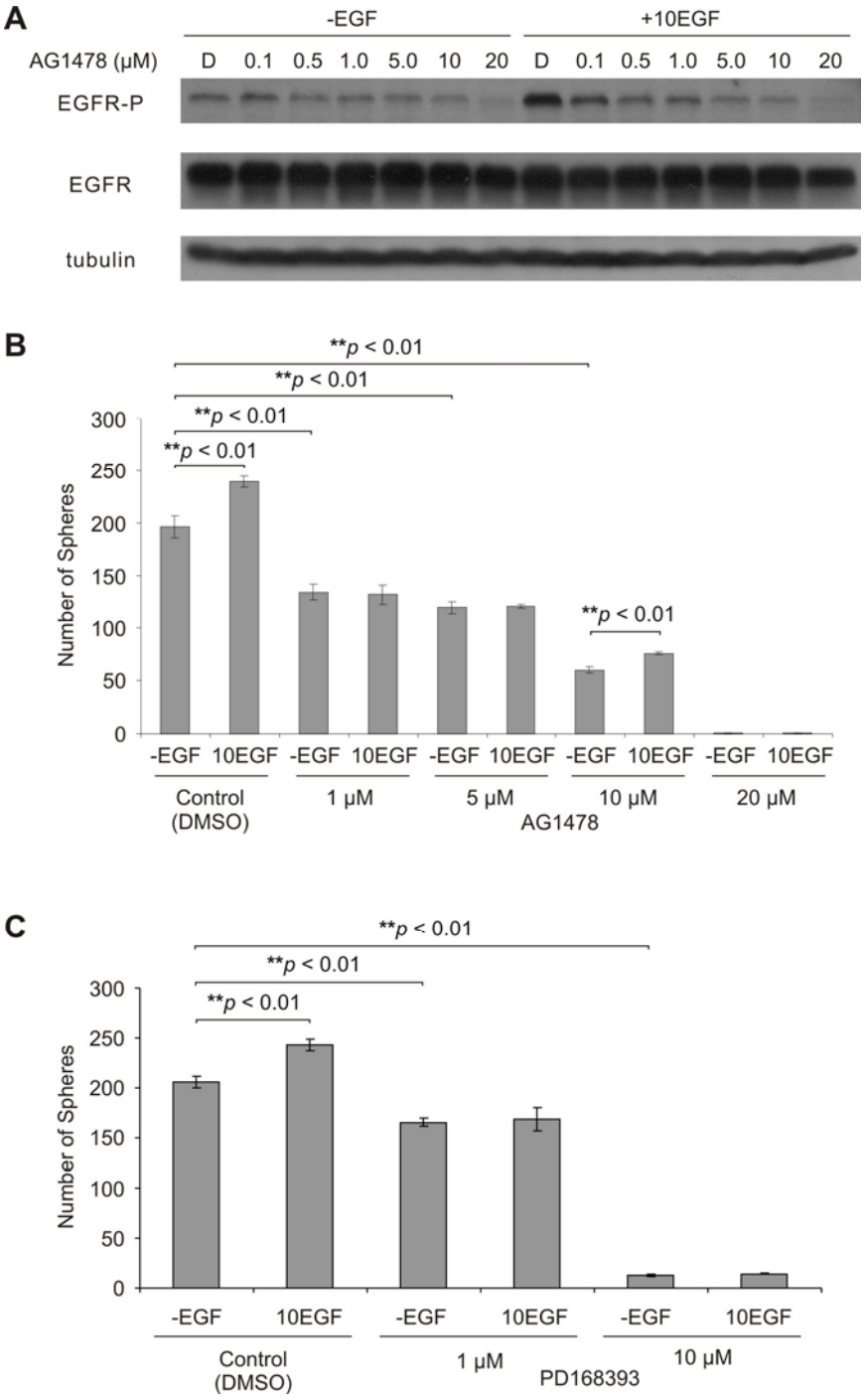

A
-EGF
+10EGF
AG1478 (μM)
D 0.1 0.5 1.0 5.0 10 20 D 0.1 0.5 1.0 5.0 10 20
EGFR-P
EGFR
tubulin
B
**p < 0.01
**p < 0.01
**p < 0.01
**p < 0.01
**p < 0.01
Number of Spheres
300
250
200
150
100
50
0
-EGF 10EGF -EGF 10EGF -EGF 10EGF -EGF 10EGF -EGF 10EGF
Control (DMSO)
1 μM
5 μM
10 μM
20 μM
AG1478
C
**p < 0.01
**p < 0.01
**p < 0.01
Number of Spheres
300
250
200
150
100
50
0
-EGF 10EGF -EGF 10EGF -EGF 10EGF
Control (DMSO)
1 μM
10 μM
PD168393

Figure 4. EGFR knockdown reduces the sphere-forming capacity and self-renewal activity of DU145 PCSCs. A) DU145 monolayer cells were infected with EGFR shRNA lentiviral constructs and selected with puromycin in order to generate stable cell lines. Western blot analysis of various EGFR knockdown (shEGFR) cells was carried out to evaluate the EGFR knockdown efficiency relative to the non-specific shRNA control (shCTRL) cell line. B) Stable EGFR knockdown cells form fewer primary (1^o) spheres compared to shCTRL cells. Cells were seeded at a density of 2.5×10^3 cells/well (0.5 ml/well; three replicates per treatment). Sphere numbers are displayed as mean ± S.E.M. of three independent experiments (*$p < 0.05$ in comparison to the respective -EGF media condition for each cell line; two-tailed independent Student's *t*-test). C) Efficient EGFR knockdown in established DU145 spheres can also be achieved. Cells from EGF-free (-EGF) spheres were infected with shCTRL and shEGFR2 lentivirus. Loss of EGFR protein expression significantly reduces the D) number of DU145 spheres grown under EGF-free media conditions. EGFR knockdown (shEGFR2) and shCTRL sphere cells were seeded at a density of 1×10^3 cells/well (three replicates per cell line). The number of spheres that formed was counted 12 days post-seeding. Sphere number is displayed as mean ± S.E.M. of three independent experiments (**$p < 0.01$; two-tailed independent Student's *t*-test). E) EGFR knockdown in DU145 sphere cells significantly reduced their in vitro anchorage-independent growth. The total number of colonies in 5 random fields (at 25X magnification) was counted and represented as mean ± S.E.M. of three independent experiments (**$p < 0.01$; two-tailed independent Student's *t*-test).

Representative phase contrast images of colonies are shown at 50X magnification (inset). Scale bar is equal to 100 μm.

Figure 4.

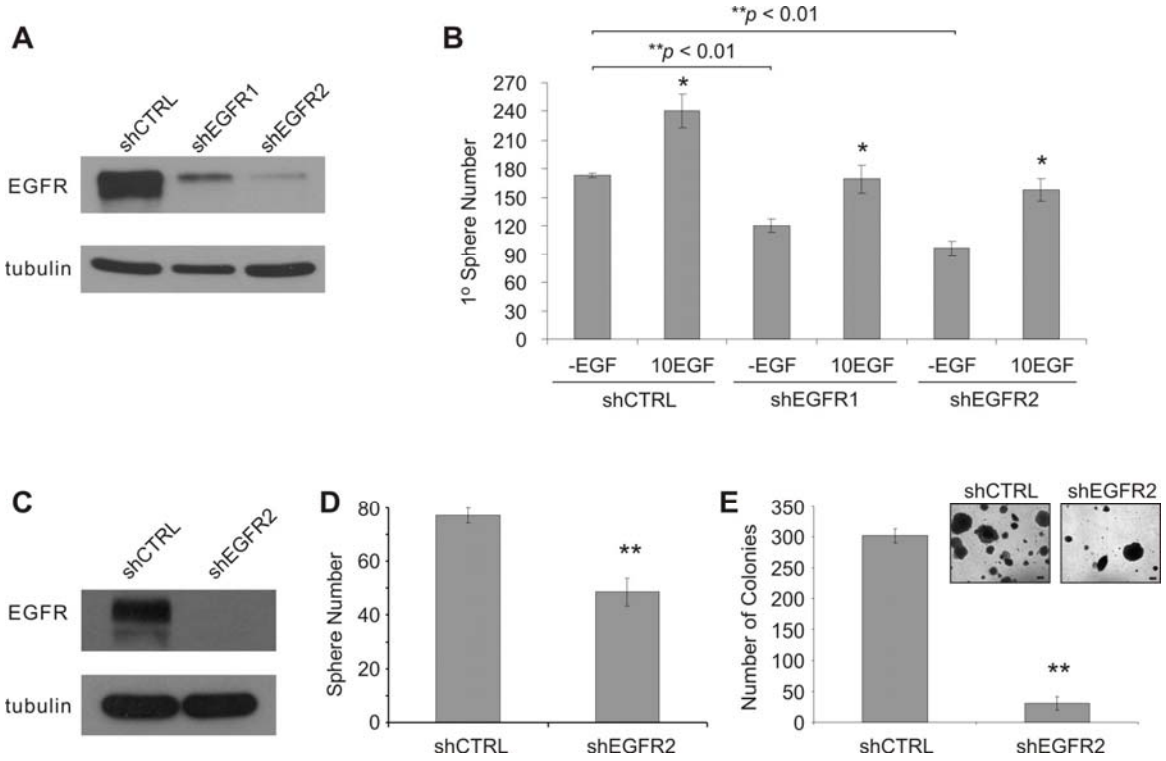
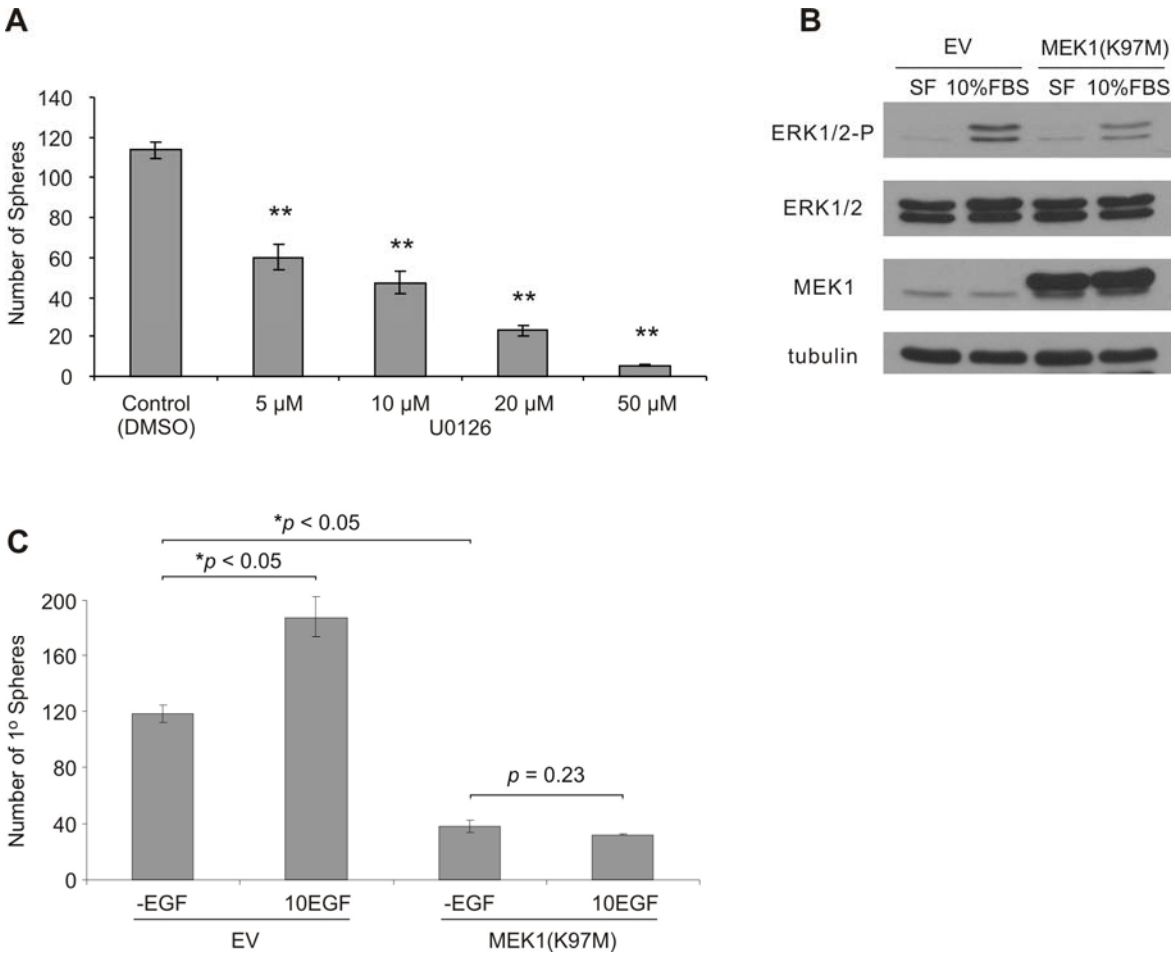

A
shCTRL
shEGFR1
shEGFR2
EGFR
tubulin
B
**p < 0.01
**p < 0.01
1º Sphere Number
270
240
210
180
150
120
90
60
30
0
-EGF
10EGF
-EGF
10EGF
-EGF
10EGF
shCTRL
shEGFR1
shEGFR2
C
shCTRL
shEGFR2
EGFR
tubulin
D
Sphere Number
80
60
40
20
0
shCTRL
shEGFR2
E
shCTRL
shEGFR2
Number of Colonies
350
300
250
200
150
100
50
0
shCTRL
shEGFR2

Figure 5. MEK1-dependent signal blockage inhibits DU145 sphere formation. A) Inhibition of ERK signaling following treatment of DU145 sphere cells with increasing concentrations (μM) of U0126 (MEK inhibitor) reduces their capacity to form subsequent spheres. U0126 or DMSO (mock) treatment was administered at the time of seeding. Sphere cells were seeded at a density of 1×10^3 cells/well (0.5 ml/well; three replicates per treatment). The number of spheres (mean ± S.E.M.) that formed after 12 days of culture was counted (**$p < 0.01$ compared to DMSO control; two-tailed independent Student's *t*-test). B) Dominant-negative MEK1(K97M) (pLHCX) cells display reduced ERK1/2 activation compared to empty vector (EV; pLHCX) control cells. DU145 EV and MEK1(K97M) cells were serum-starved for 12 h, and subsequently stimulated with serum-supplemented (10% FBS) or serum-free (SF) media for 60 min. C) MEK1(K97M) cells display reduced primary (1^o) sphere-forming capacity compared to EV cells when seeded in serum-free media lacking EGF (-EGF) or containing 10 ng/ml EGF (10EGF). Cells were seeded at a density of 2.5×10^3 cells/well (three replicates per treatment). Sphere numbers are displayed as mean ± S.E.M. of three independent experiments (the *p* values for the indicated comparisons were obtained by two-tailed independent Student's *t*-test).

Figure 5.


A
Number of Spheres
140
120
100
80
60
40
20
0
**
**
**
**
Control (DMSO)
5 μM
10 μM
20 μM
50 μM
U0126
B
EV
MEK1(K97M)
SF 10%FBS SF 10%FBS
ERK1/2-P
ERK1/2
MEK1
tubulin
C
*p < 0.05
*p < 0.05
Number of 1º Spheres
200
160
120
80
40
0
p = 0.23
-EGF
10EGF
-EGF
10EGF
EV
MEK1(K97M)

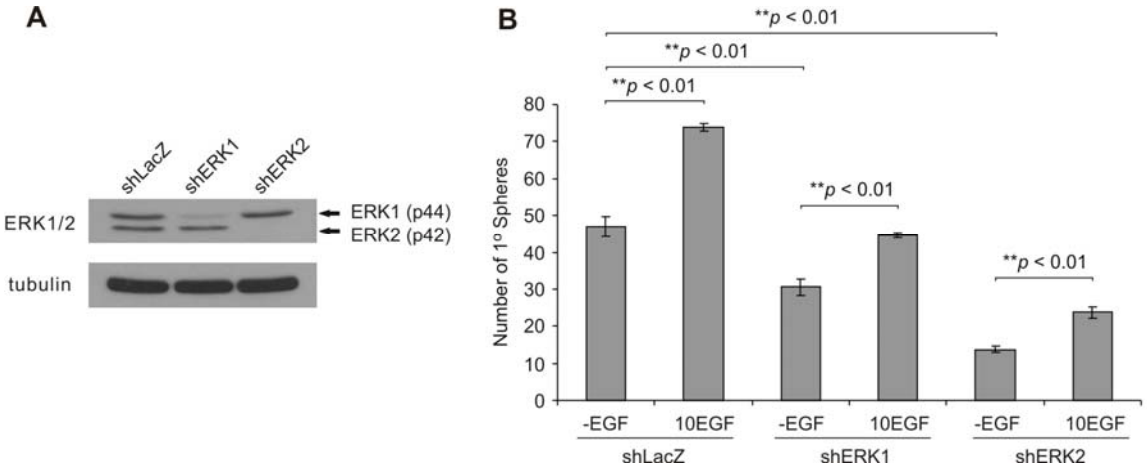
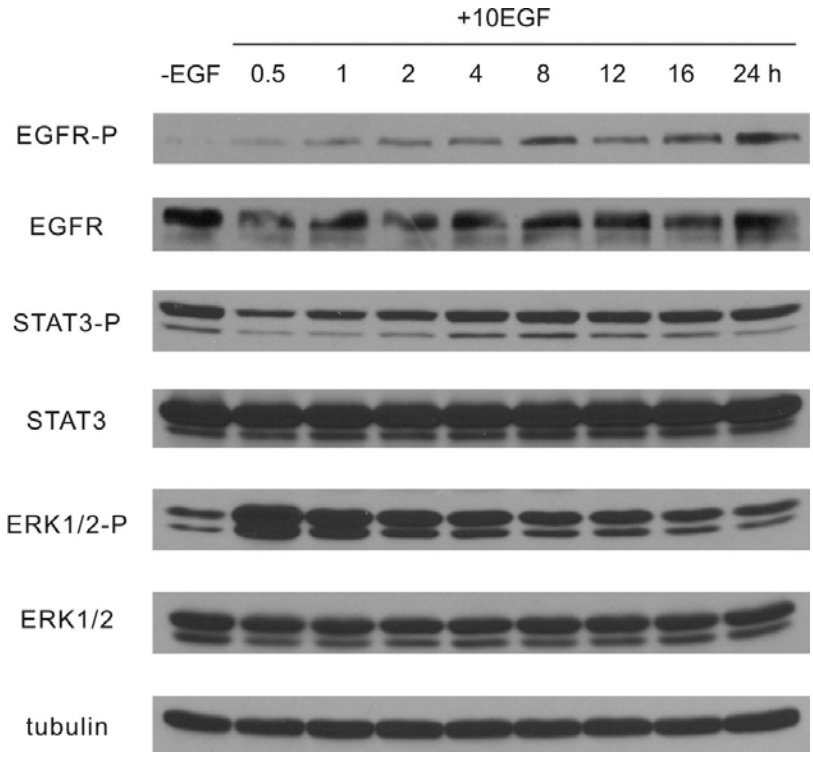
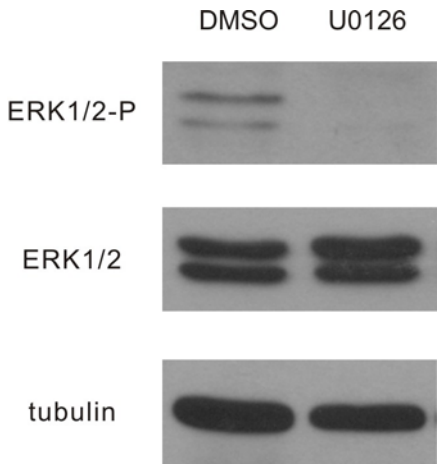


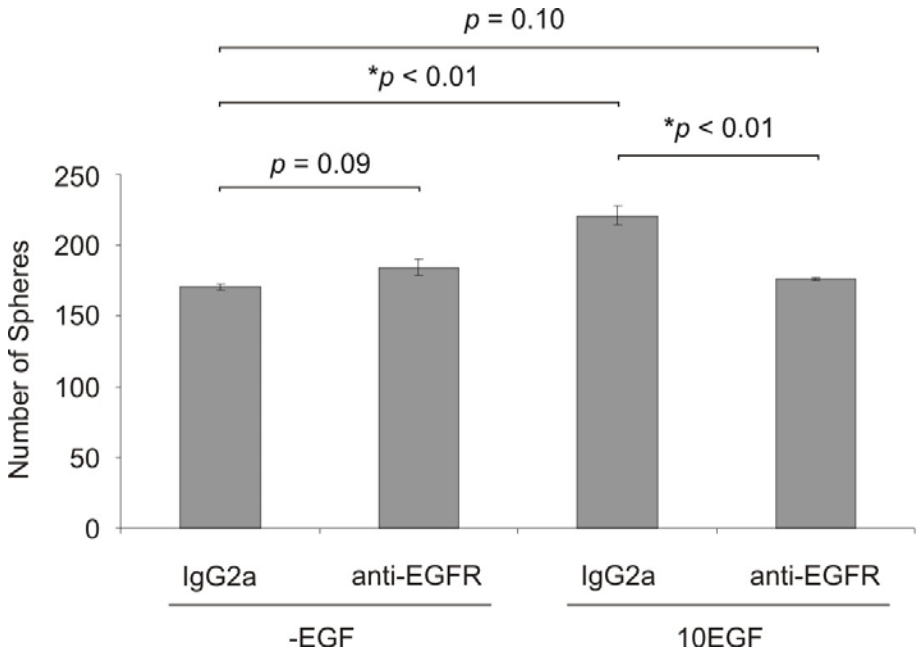
Figure 6. DU145 sphere formation is significantly reduced by ERK1 and ERK2 knockdown. A) Western blot analysis of endogenous ERK1 and ERK2 (ERK1/2) protein levels following ERK1 or ERK2 knockdown in DU145 monolayer cells. B) ERK1 or ERK2 knockdown results in a significant reduction in primary sphere formation compared to shRNA control (shLacZ) cells. Cells were seeded at a density of 2.5×10^3 cells/well (0.5 ml/well; three replicates per treatment). The number of primary (1^o) spheres formed in the absence (-EGF) or presence of 10 ng/ml EGF (10EGF) is displayed as mean ± S.E.M. of three independent experiments (the *p* values for the indicated comparisons were obtained by two-tailed independent Student's *t*-test).



Supplementary Figure S1. EGF treatment of DU145 PCSCs promotes EGFR signal and downstream MAPK (ERK) signal activation. Low passage DU145 spheres, which were cultured and maintained in EGF-free serum-free media containing 0.4% BSA and 0.2× B27 (-EGF), were treated with serum-free media containing 10 ng/ml EGF (10EGF) and whole cell lysates were prepared at different time points (hours; h) post-treatment. Western blot analysis of EGF treatment of spheres results in EGFR (Tyr1068 phosphorylation; EGFR-P), ERK (Thr202/Tyr204 phosphorylation; ERK1/2-P) and STAT3 (Tyr705 phosphorylation; STAT3-P) signal activation. For each sample, a total of 100 µg of whole cell lysate was used.



Supplementary Figure S2. U0126 treatment reduces ERK activation in DU145 PCSCs. Western blot analysis of whole cell lysates (50 μg of lysate was used for each sample) following 24 hour treatment of DU145 spheres with a 50 μM dose of U0126 (MEK inhibitor) or dimethylsulfoxide (DMSO; mock treatment) at an equal volume. ERK activation was determined by examining the phosphorylation of ERK1 and ERK2 proteins at Thr202 and Tyr204 residues, respectively (ERK1/2-P).



Supplementary Figure S3. Treatment of DU145 PCSCs with an EGFR function blocking antibody inhibits EGF-enhanced sphere formation. Individualized cells from EGF-free DU145 spheres were treated (at the time of seeding) with azide-free anti-EGFR blocking mouse monoclonal antibody, or mouse IgG2 isotype control antibody, at a concentration of 4 μg/ml. Sphere cells were seeded at a density of 2×10^3 cells/well (0.5 ml/well; three replicates per treatment) in serum-free media lacking EGF (-EGF) or containing 10 ng/ml EGF (10EGF). The number of spheres that formed was counted 12 days post-seeding. Sphere numbers are displayed as mean ± S.E.M. (*$p < 0.05$; two-tailed independent Student's *t*-test).

CHAPTER 4

SOX2 plays a critical role in EGFR-mediated self-renewal of human prostate cancer stem-like cells

Adrian P. Rybak, and Damu Tang

Molecular Oncology (submitted in May 2013).

Preface

SOX2 is a stem cell transcription factor involved in maintaining pluripotency (Boyer, et al. 2005; Loh, et al. 2006), and it has been shown to contribute towards reprogramming terminally-differentiated adult cells into pluripotent stem-like cells (Park, et al. 2008; Takahashi, et al. 2007; Wernig, et al. 2007). SOX2 has also been implicated in serving a functional role in somatic stem cells and cancer stem cells. In prostate cancer, SOX2 expression is correlated with higher tumor grade (Jia, et al. 2011; Kregel, et al. 2013), while SOX2 knockdown reduced prostate tumor formation *in vivo* (Jia et al. 2011). Furthermore, EGFR signalling regulates the self-renewal of neural precursor cells (Hu, et al. 2010) and maintains cancer stem-like properties in breast cancer cells (Yang, et al. 2013) in a SOX2-dependent fashion. Therefore, the relationship between EGFR signalling and SOX2 in regulating the self-renewal of prostate cancer stem-like cells (PCSCs) was examined in the current manuscript.

This chapter consists of an author-generated version of a manuscript submitted in May, 2013 for peer-review to *Molecular Oncology*: Adrian P. Rybak, and Damu Tang (2013) SOX2 plays a critical role in EGFR-mediated self-renewal of human prostate cancer stem-like cells. Please note that American spelling is used throughout the article, and that the formatting and referencing style has changed to meet the journal's criteria. In addition, *SOX2* mRNA transcript data, which is not included in the manuscript but is presented in the text as "data not shown", has been included in Appendix 1. EGF treatment of EGF-free DU145 sphere cells resulted in increased EGFR signal activation in a time dependent manner, along with increased MAPK (ERK) and AKT signal

activation and increased SOX2 protein levels (Appendix 2). I, Adrian P. Rybak, generated and established all DU145 sphere cultures, stable cell lines, as well as the N-terminal FLAG-tagged SOX2 construct used in this study. I performed all of the experiments, assembled the results, and generated the figures in the manuscript. Furthermore, I wrote and revised the manuscript along with Damu Tang.

Passaging of DU145 spheres resulted in increased expression of the stem cell reprogramming factor SOX2, which is heterogeneously expressed in spheres. Activation of EGFR signalling, following epidermal growth factor (EGF) treatment or ectopic expression of a constitutive-active EGFR mutant in spheres, resulted in increased SOX2 protein expression. SOX2 knockdown reduced the number and size of spheres, with EGF treatment no longer capable of enhancing their number and size. Furthermore, SOX2 knockdown reduced the anchorage-independent growth of PCSCs under differentiating conditions. Ectopic SOX2 expression alone was insufficient in promoting DU145 PCSC self-renewal; enhanced self-renewal capacity was observed following EGF treatment of SOX2-expressing DU145 sphere cells. Taken together, these findings suggest that SOX2 is a downstream target of EGFR signalling in PCSCs, and that it is required for EGFR-mediated PCSC self-renewal.

SOX2 Plays a Critical Role in EGFR-mediated Self-Renewal of Human Prostate Cancer Stem-like Cells

Adrian P. Rybak,[1,2,3] and Damu Tang [1,2,3#]

[1]Division of Nephrology, Department of Medicine, McMaster University, [2]Father Sean O'Sullivan Research Institute, [3]The Hamilton Centre for Kidney Research (HCKR), St. Joseph's Hospital, Hamilton, ON, Canada.

#: Corresponding author:
Damu Tang
Juravinski Innovation Tower, room T3310
St. Joseph's Hospital
50 Charlton Ave East
Hamilton, ON
Canada
L8N 4A6
Tel: (905) 522-1155, ext. 35168
Fax: (905) 521-6181
Email: damut@mcmaster.ca

Running title: SOX2 regulates EGFR-mediated PCSC Self-Renewal

Abstract

SOX2 is a transcription factor that is essential for embryonic stem cell development, stem cell maintenance and reprogramming of differentiated cells towards a stem-like state. More recently, the expression of SOX2 has also been demonstrated in discrete cells in multiple tissues and in various human cancers including prostate cancer, implicating its requirement towards maintaining tissue stem cells and cancer stem cells. Consistent with this possibility, SOX2 protein is significantly upregulated in DU145 prostate cancer stem-like cells (PCSCs) propagated as suspension spheres *in vitro*. The expression of SOX2 in DU145 PCSCs is positively regulated by epidermal growth factor receptor (EGFR) signaling. Activation of EGFR signaling, following the addition of epidermal growth factor (EGF) or ectopic expression of a constitutively-active EGFR mutant (EGFRvIII), increases SOX2 expression and the self-renewal of PCSCs. Conversely, EGFR inhibitor (AG1478) treatment reduced SOX2 expression and inhibited PCSC self-renewal activity, implicating SOX2 in mediating EGFR-dependent self-renewal of sphere cells. In line with this notion, ectopic SOX2 expression enhances EGF-induced self-renewal of PCSCs, while knockdown of SOX2 renders PCSCs non-responsive to EGF-induced self-renewal. Furthermore, SOX2 knockdown reduces the capacity of sphere cells to grow under anchorage-independent conditions. Finally, DU145 sphere cells form xenograft tumors more aggressively with elevated levels of SOX2 expression compared to monolayer cell-derived xenograft tumors. Collectively, we provide evidence that SOX2 plays a critical role in EGFR-mediated PCSC self-renewal.

Keywords: SOX2, EGFR, Self-Renewal, Prostate cancer stem cell

1. Introduction

Prostate cancer, like many other carcinomas, exhibits a functional hierarchy of cells with tumor-initiating cells positioned at the apex and having the ability to develop (or differentiate) into a spectrum of more mature progenies (Patrawala et al., 2007; Patrawala et al., 2006). The cancer stem cell hypothesis postulates that only a subset of cells within the tumor are capable of sustaining tumorigenesis and driving disease progression, while establishing the cellular heterogeneity that constitutes the primary tumor (Visvader and Lindeman, 2012). Stem-like cancer cells, often referred to as cancer stem cells (CSCs) or tumor-initiating cells, have been experimentally defined by their ability to initiate tumors upon implanting in immunocompromised mice (Pardal et al., 2003; Reya et al., 2001; Visvader and Lindeman, 2008). These stem-like cancer cells generate non-tumorigenic progeny, resulting in intrinsically different populations of tumorigenic and non-tumorigenic cells within the tumor (Magee et al., 2012). As CSCs do not necessarily originate from the transformation of a normal stem cell, they may arise from mutations attained by restricted progenitors and differentiated cells that have subsequently acquired self-renewal capacity (Pardal et al., 2003; Reya et al., 2001; Visvader and Lindeman, 2008). Recent studies have suggested that non-tumorigenic (differentiated) cells can acquire *de novo* stem-like properties, and that cancer cells can reversibly transition stochastically between tumorigenic and non-tumorigenic states (Chaffer et al., 2011; Gupta et al., 2011).

Insights into common mechanisms of cellular plasticity and cancer have arisen following studies that have achieved reprogramming of somatic cells into the pluripotent stem-like state (Daley, 2008). This reprogramming method, a process that produces induced pluripotent stem (iPS) cells, utilizes the transcription factors of OCT4, SOX2, KLF4 and c-MYC to reset the epigenetic state of differentiated cells to a pluripotent state (Park et al., 2008; Takahashi et al., 2007; Wernig et al., 2007). The functions of c-MYC and KLF4 can be substituted by NANOG and LIN28A (Yu et al., 2007). In embryonic stem cells (ESCs), pluripotency is maintained by the core transcription factors OCT4, SOX2 and NANOG, which co-occupy the promoters of various target genes (Boyer et al., 2005; Loh et al., 2006). In addition, OCT4 (Lin et al., 2012), SOX2 (Chen et al., 2008; Gangemi et al., 2009; Rodriguez-Pinilla et al., 2007), and NANOG (Noh et al., 2012) have been implicated in tumorigenesis. Furthermore, an ESC-like gene signature in which OCT4, SOX2, NANOG and c-MYC target genes are more frequently expressed in poorly differentiated tumors compared to well-differentiated tumors (Ben-Porath et al., 2008) suggests that common molecular circuitry may exist between cellular reprogramming and cancer (Suva et al., 2013).

Prostate cancer is the most common male malignancy and the second leading cause of cancer-related deaths in males in the Western world (Siegel et al., 2012; Williams and Powell, 2009). In the prostate, nuclear SOX2 [SRY (sex determining region Y)-box 2] expression has been shown in less than 10% of cells within normal prostate glands (Ugolkov et al., 2011) and elevated in prostate carcinomas (Jia et al., 2011; Ugolkov et al., 2011). Interestingly, an amplicon at 3q25-q27, which includes the *SOX2* gene

(3q26.3-q27), has been detected in prostate carcinomas (Sattler et al., 2000), implicating *SOX2* gene amplification in prostate cancers. Additionally, SOX2 expression correlates with prostate tumor grade, as the percentage of SOX2-positive tumor cells increases with Gleason score (Jia et al., 2011; Kregel et al., 2013) and metastasis (Kregel et al., 2013). While an ESC gene signature in prostate tumors was reported to be associated with poor patient prognosis (Markert et al., 2011), SOX2 expression observed in advanced prostate carcinomas supports its role in promoting PCSCs within these tumors (Jia et al., 2011; Kregel et al., 2013). Given that SOX2 expression is repressed by the androgen receptor (AR) (Kregel et al., 2013), the mechanism governing its oncogenic potential in prostate cancer remains unclear.

In evaluating the contribution of SOX2 towards prostate tumorigenesis, we provide evidence that SOX2, which is heterogeneously expressed in DU145 spheres, promotes the self-renewal of the PCSC population by acting downstream of EGFR. Activation of EGFR signaling via addition of epidermal growth factor (EGF) or ectopic expression of a constitutively-active EGFR mutant (EGFRvIII) increased DU145 PCSC self-renewal and SOX2 protein levels, while inhibition of EGFR activation through the use of an EGFR inhibitor reduced SOX2 expression. Furthermore, SOX2 downregulation via short hairpin RNA (shRNA)-targeted knockdown reduced the sphere-forming capacity of DU145 PCSCs, with EGF treatment no longer capable of enhancing their propagation. Taken together, these results highlight a requirement for SOX2 in EGFR-mediated self-renewal of DU145 PCSCs.

2. Material and Methods

2.1 Cell culture and propagation of DU145 PCSCs

DU145 human prostate cancer cells, 293T human embryonic kidney cells and NTERA-2 cl. D1 (NT2/D1) human embryonic carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and were cultured according to ATCC instructions.

DU145 stem-like cells were isolated and propagated as previously described (Rybak et al., 2011). Briefly, DU145 monolayer cells were enzymatically-dissociated into single cells using phenol red-free TrypLE Express solution (Life Technologies, Calsbad, CA, USA) and 40 μm cell strainers (BD Biosciences, Franklin Lakes, NJ, USA), and subsequently resuspended at a sub-clonal (5 cells/μl) density in serum-free medium (SFM; DMEM/F12 at a 3:1 mixture) (Life Technologies) containing 0.4% bovine serum albumin (BSA) (Bioshop Canada Inc., Burlington, ON, Canada) and 0.2× B27 lacking Vitamin A (Life Technologies) in T75 flasks (15 ml total volume) designated for suspension cell culture (Sarstedt Inc., Newton, NC, USA). When examining the effect of EGF on sphere formation, recombinant EGF (Sigma-Aldrich, St. Louis, MO, USA) was added at a concentration of 10 ng/ml. Typical spheres formed in 10 to 12 days. Sphere cells were sub-cultured by enzymatic dissociation, strained, and then resuspended in the above medium at sub-clonal density.

2.2 Self-renewal assays

To examine the sphere-forming capacity of DU145 monolayer cells, cells from sub-confluent (~80%) cultures were resuspended in the above SFM at a density of 2.5×10^3 cells (0.5 mL/well) into individual wells of a 24-well culture plate (3 wells per treatment) (Corning, Corning, NY, USA). The spheres that formed after 12 days of culture were counted. To measure the sphere-forming capacity of secondary or established spheres, spheres cells were individualized by enzymatic dissociation, strained, and then plated at a density of 1×10^3 cells/well, unless otherwise specified. The spheres that formed were counted after 12 days of culture.

To determine sphere size, images of spheres were captured using a Zeiss Axiovert 200M inverted fluorescence microscope using AxioVision 3.1 software (Carl Zeiss, Oberkochen, Baden-Württemberg, Germany), and the mean diameter (in microns) of individual spheres was determined using ImagePro Plus 5.0 software (MediaCybernetics, Bethesda, MD, USA). Spheres that were ≥50 μm in diameter were included in the analysis.

Assessment of the self-renewal capacity of established spheres was carried out as previously described (Rybak et al., 2011). Briefly, sphere cells were individualized by enzymatic dissociation and plated in 96-well plates at a density of 1 cell/well (120 wells in total). The number of spheres that formed was counted after 12 days of culture.

2.3 Plasmids and retrovirus production

EGFRvIII/pLNCX was kindly provided by Dr. Khalid Al-Nedawi (Hamilton Centre for Kidney Research, Hamilton, ON, Canada). FLAG-SOX2/pLHCX was constructed by PCR using the pSIN-EF2-Sox2-Pur construct (plasmid 16577; Addgene, Cambridge, MA, USA) as a template, with subsequent ligation of the transgene. The PCR primers used to generate FLAG-SOX2/pLHCX were as follows: CCCAAGCTTATGTACAACATGATGGAG (5' primer) and GCTCTAGATCACATGTGTGAGAGGGG (3' primer). Empty vector (EV)/pLHCX and NFLAG-SOX2/pLHCX constructs were each co-transfected into 293T cells along with VSV-G and GP retroviral packaging plasmids (Stratagene) using the calcium phosphate transfection method. Sixty hours (h) post-transfection, the supernatants containing high-titre VSV-G pseudotyped, replication-incompetent retroviral particles were collected, filtered through a 0.45 μm filter and centrifuged for 2 h at 48,000 g. Viral pellets were resuspended in media containing 10μg/ml polybrene (Sigma-Aldrich) and added to cells for 2 h in a 37^{o}C humidified incubator, with periodic mixing at 20 min intervals, to allow for cell infection. Infection was selected in hygromycin (0.5 mg/ml; Life Technologies) to generate stable cell lines. Ectopic FLAG-SOX2 expression was verified by Western blot analysis.

Short-hairpin SOX2 targeting lentiviral vector was purchased from Sigma-Aldrich, with the targeting sequences (shSOX2: CTGCCGAGAATCCATGTATAT) cloned as a hairpin in the pLKO.1 vector, while a non-specific shRNA (plasmid 1864; Addgene, Cambridge, MA, USA) was used as a control (shCTRL). Production of shSOX2 and

shCTRL lentivirus was carried out by co-transfecting each shRNA plasmid (10 μg) with plasmids (10 μg each) necessary for third-generation lentiviral production (pRSV-REV, pCMV-VSV-G, pMDLg/pRRE) (Dull et al., 1998), kindly provided by Dr. Bryan E. Strauss (University of São Paulo School of Medicine, São Paulo, Brazil), into 293T cells using the calcium phosphate transfection method. Sixty hours post-transfection, the supernatants containing VSV-G pseudotyped, replication-incompetent lentiviral particles were collected, filtered through a 0.45 μm filter and centrifuged for 2 h at 48,000 g. For infection of sphere cells, the shSOX2 and shCTRL lentivirus pellets were resuspended in 2 ml of serum-free media containing 0.4% BSA, 0.2× B27 (lacking Vitamin A) and 10 μg/ml polybrene and subsequently added to individualized (3×10^5) cells for 2 h in a humidified 37^{o}C incubator, with periodic mixing at 20 min intervals, to allow for cell infection. Sphere cells were subsequently seeded at a density of 10^4 cells/ml in T75 flasks designated for suspension cell culture (Sarstedt Inc.). Puromycin (1 μg/ml) was added to the sphere cultures at 48 h post-infection.

2.4 Anchorage-independent growth assay

DU145 sphere cell cultures were plated in soft agar in individual wells of six-well plates at a density of 10^4 cells/well as previously described (Rybak et al., 2011). After 8 weeks, phase contrast images were taken in 5 random fields per well using the Zeiss Axiovert 200M microscope (AxioVision 3.1 software) at 25X magnification, and the colonies consisting of ≥50 cells were counted. Digital images of wells were taken using a Sony Cyber-shot (DSC-W220) digital camera (Sony Corporation, Tokyo, Japan) and

subsequently processed using CorelDRAW Graphics Suite X4 software (Corel, Ottawa, ON, Canada).

2.5 EGFR inhibition studies

The EGFR inhibitor AG1478 (Calbiochem, Darmstadt, Hesse, Germany) was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich) and used at the concentrations indicated. DMSO was used at an identical volume as a control. Inhibition of EGFR activity (as determined by EGFR Tyr1068 phosphorylation status) was verified by Western blot analysis.

2.6 Immunofluorescence and immunohistochemistry

Immunofluorescent staining of spheres was performed by fixing and preparing spheres for frozen sectioning as previously described (Rybak et al., 2011). Frozen sections (4 μm) were stained using rabbit monoclonal anti-SOX2 antibody (Cell Signaling, D6D9, 2 μg/ml). Rabbit IgG was used at an equal concentration as a negative control. Slides were subsequently washed in 1 × PBS and then incubated with donkey anti-rabbit IgG-FITC secondary antibody (1:200 dilution; Jackson Laboratories) for 1 h. Slides were mounted with a coverslip using Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc., H-1200), and images were captured using a Zeiss Axiovert 200 M inverted fluorescence microscope using AxioVision 3.1 software. Images were processed using CorelDraw 14.

Immunohistochemistry (IHC) on DU145 xenograft tumor sections was performed as previously described (Rybak et al., 2011). Briefly, sectioned slides were deparaffinized, antigen retrieval was performed and sections were incubated with the rabbit anti-SOX2 (D6D9, 1.5 µg/ml) antibody, and subsequent processing of slides were carried out according to the manufacturer's instructions. Rabbit IgG was used at an equal concentration as a negative control. Biotinylated goat anti-rabbit IgG and avidin-biotin complex (ABC) were then added (Vectastain ABC kit, Vector Laboratories). The chromogen reaction was carried out with diaminobenzidine (DAB; Vector Laboratories), and counterstaining was done with hematoxylin (Sigma-Aldrich). Images were captured using an Olympus BX41 light microscope.

2.7 *In vivo* tumor xenograft formation

DU145 monolayer and sphere (10^2) cells were resuspended in MEM/Matrigel mixture (1:1 volume), followed by implantation of 0.1 ml of this mixture subcutaneously (s.c.) into flanks of 8-week-old male NOD/SCID mice (The Jackson Laboratory). Mice were inspected for tumor appearance, by observation and palpation, and tumor growth was measured weekly using a caliper. Tumor volume was determined using the standard formula: $L \times W^2 \times 0.52$, where L and W are the longest and shortest diameters, respectively. The presence of each tumor nodule was confirmed by necropsy. All animal work was carried out according to experimental protocols approved by the McMaster University Animal Research Ethics Board.

2.8 Western blot analysis

Whole cell lysates were prepared in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml leupeptin and 10 μg/ml aprotinin. For each sample, a total of 50 μg of whole cell lysate was used unless otherwise specified. Resolved lysates (10% SDS-polyacrylamide gels) were transferred onto Amersham Hybond-ECL membranes (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The membranes were blocked with 5% w/v non-fat dry skim milk at room temperature for 1 h, and then incubated with the indicated primary antibodies in 10 ml of antibody dilution buffer (1× Tris-buffered saline containing 0.05% Tween-20 (TBST), and 5% BSA) with gentle rocking at 4^{o}C overnight. Primary antibodies used for probing were as follows: rabbit anti-human phospho-EGFR (Tyr1068) (Sigma-Aldrich, E6154, 1:1000), rabbit anti-human EGFR (D38B1, #4267, 1:1000), rabbit anti-human SOX2 (D6D9, #3579, 1:1000), rabbit anti-human NANOG (D73G4, #4903, 1:2000), from Cell Signaling Technology (Danvers, MA, USA), mouse anti-human OCT4 (C-10, sc-5279, 1:200) and mouse anti-human α-tubulin (TU-02, sc-8053, 1:500) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were subsequently washed with 1× TBST solution and incubated (1 h at room temperature) with either donkey anti-mouse-IgG-HRP (GE Healthcare, NA931V, 1:3000) or donkey anti-rabbit-IgG-HRP (GE Healthcare, NA934V, 1:3000) conjugated secondary antibodies. Immunoblot signals were detected using Amersham ECL blotting detection reagents (GE Healthcare) and then exposed to x-ray film (Thermo

Fisher Scientific). The quantification of immunoblot band densities was determined using ImageJ software (version 1.43u; W. Rasband, National Institute of Health).

2.9 Statistical analysis

All quantitative data are presented as mean ± S.E.M. and comparisons were made using two-tailed independent Student's *t*-tests. For repeated measures analysis, a two-way ANOVA was conducted. A p-value < 0.05 was considered statistically significant.

3. Results

3.1 Specific expression of SOX2 in DU145 spheres

SOX2 has been reported to be expressed in prostate cancer (Jia et al., 2011; Kregel et al., 2013), and promote prostate tumorigenesis (Jia et al., 2011). As SOX2 plays a major role in stem cell maintenance (Avilion et al., 2003; Fong et al., 2008; Li et al., 2007; Masui et al., 2007), its expression and oncogenic role in prostate tumorigenesis implicates a role for SOX2 in regulating PCSC self-renewal. To investigate this possibility, we took advantage of the existence of DU145 monolayer (MNL) cells and their derived isogenic spheres that display CSC properties of self-renewal and the ability to initiate tumor formation *in vivo* (Dubrovska et al., 2009; Rybak et al., 2011). In comparison to MNL cells, DU145 sphere cells clearly express SOX2 protein (Fig 1A), despite *SOX2* mRNA being detected in both MNL and sphere cells (data not shown). SOX2 was not homogeneously detected in spheres (Fig 1B), which may not be attributable to the issue

of antibody accessibility given that varying degrees of positive staining was distributed throughout the sphere. This heterogeneic pattern of SOX2 staining was observed in multiple DU145 spheres (Supplementary Figure 1), and is consistent with the heterogeneous nature of SOX2 staining observed in MCF7 mammospheres (Leis et al., 2012). In addition, SOX2 is largely a nuclear protein in DU145 sphere cells (Fig 1B), which is consistent with reports that SOX2 regulates stemness as a transcription factor (Fong et al., 2008; Li et al., 2007). To obtain supportive evidence as to whether SOX2 may play a role in regulating DU145 sphere self-renewal, we observed an enhancement in self-renewal capacity (Fig 1C) and sphere propagation (Supplementary Fig 2), along with elevated SOX2 protein levels (Fig 1D), following increased passaging of DU145 sphere cells. Since a positive control was included in the same Western blot, we intentionally reduced the exposure to quantitatively demonstrate an increase in SOX2 expression following passaging of DU145 sphere cells (Fig 1D). While the self-renewal potential in passage 15 (p15) sphere cells was not statistically higher than that of p5 cells, p30 sphere cells possessed significantly higher self-renewal capacity than p5 sphere cells (Fig 1C). These results support a role for SOX2 in regulating the self-renewal of DU145 PCSCs. SOX2 (Avilion et al., 2003; Fong et al., 2008), OCT4 (Masui et al., 2007; Nichols et al., 1998), and NANOG (Mitsui et al., 2003) are critical stem cell transcription factors required for maintaining ES cells however, both OCT4 and NANOG proteins were not detected in spheres (Fig 1D), indicating that SOX2 is important in DU145 PCSCs. Furthermore, our previous work has demonstrated that seeding sphere cells in serum-supplemented (ie. differentiating) media resulted in their adherence to the tissue culture

plate and reduction in their sphere-forming capacity with increased passaging as sphere-derived adherent cells (SDACs) (Rybak et al., 2011). Consistent with this knowledge, we were able to demonstrate a decrease in SOX2 expression along with this reduced sphere-forming capacity by SDACs (Fig 1E). Collectively, these observations support a role for SOX2 in regulating DU145 PCSC self-renewal.

3.2 SOX2 expression is promoted by EGFR activation in DU145 spheres

Since EGF treatment of DU145 PCSCs has been previously reported to promote the generation (Rybak et al., 2011) and propagation of DU145 spheres (Rybak et al., 2013), we investigated whether EGFR signal activation influences SOX2 expression in DU145 spheres. EGFR activation is well known to initiate signaling events (McCubrey et al., 2006) and to promote the tumorigenicity of cancer cells with stem-like characteristics (Del Vecchio et al., 2012; Mazzoleni et al., 2010). In this study, we were able to show that EGF treatment activated EGFR via phosphorylation at Tyr1068 (EGFR-P), and increased SOX2 protein levels in DU145 spheres a time-dependent manner (Fig 2A). As expected, EGF enhanced the self-renewal capacity of DU145 spheres (Fig 2B). To further reaffirm that EGFR signal activation upregulates SOX2 in DU145 PCSCs, the constitutively-active EGFR mutant, EGFR variant III (EGFRvIII) (Ekstrand et al., 1994), was ectopically-expressed in DU145 sphere cells (Fig 2C). EGFRvIII-expressing sphere cells displayed elevated levels of Tyr1068 phosphorylation (EGFR-P) (Fig 2C), which is indicative of EGFR activation (Rojas et al., 1996). In comparison to EV cells, EGFRvIII-expressing sphere cells displayed increased SOX2 protein levels (Fig 2C) and were

associated with elevated self-renewal capacity (Fig 2D). Furthermore, inhibition of EGFR activity with the EGFR inhibitors AG1478 and PD168393 reduced DU145 sphere formation (Rybak et al., 2013), along with a decrease in SOX2 expression (Supplementary Fig 3). Taken together, we provide evidence that EGFR promotes self-renewal and SOX2 protein expression in DU145 sphere cells, suggesting that SOX2 may contribute to EGFR-mediated self-renewal of DU145 PCSCs.

3.3 Enforced SOX2 expression promotes DU145 PCSC self-renewal in an EGFR-dependent manner

In order to evaluate the contribution of SOX2 towards DU145 sphere formation and subsequent sphere maintenance, N-terminally FLAG-tagged SOX2 was ectopically-expressed in DU145 monolayer cells (Fig 3A). When FLAG-tagged SOX2 (SOX2)-expressing cells were grown under sphere-forming conditions in the presence of 10 ng/ml EGF, ectopic SOX2 expression did not increase primary sphere formation (Supplementary Fig 4). However, subsequent passaging of sphere cells under EGF-supplemented SFM conditions resulted in an enhancement in sphere generation by SOX2-expressing cells compared to EV cells (Supplementary Fig 4). This suggests that SOX2 expression promotes PCSC self-renewal rather than primary sphere formation. To further evaluate the interplay between EGFR signaling and SOX2 expression in DU145 PCSC self-renewal, spheres were generated from SOX2-expressing and EV cells under EGF-free SFM conditions (-EGF). These established spheres were subsequently propagated for three passages, under the same conditions in which primary spheres were generated, prior

to evaluating their *in vitro* self-renewal capacity. In the absence of exogenous EGF, ectopic SOX2 expression did not promote the sphere-forming capacity (self-renewal) of DU145 PCSCs (Fig 3B). On the other hand, EGF treatment increased the self-renewal of SOX2-expressing DU145 PCSC compared to EV spheres (Fig 3B). Collectively, the above observations reveal that EGFR signaling is required for SOX2 to promote the self-renewal of DU145 PCSCs.

3.4 Knockdown of SOX2 inhibits EGFR-dependent self-renewal of DU145 PCSCs

To further address the role of SOX2 in regulating DU145 PCSC self-renewal, SOX2 was knocked-down in established DU145 sphere cells (Fig 4A) using a previously published shRNA construct (Bass et al., 2009). The efficiency of SOX2 knockdown was greater than 90% (Fig 4A). SOX2 knockdown reduced the self-renewal capacity of DU145 spheres by more than two-fold (Fig 4B), supporting endogenous SOX2 as being critical for DU145 PCSC self-renewal. While EGF treatment promoted sphere propagation, knockdown of SOX2 diminished EGFR-mediated enhancement of DU145 sphere number and size (Fig 4C, D). Intriguingly, knockdown of SOX2 also significantly reduced DU145 sphere propagation in the absence of ectopic EGF (Fig 4C, D). As we have recently demonstrated that EGFR activation, via either an autocrine/paracrine-mediated or ligand-independent mechanism, plays a critical role in DU145 sphere maintenance (Rybak et al., 2013), the aforementioned observation further demonstrates the importance of SOX2 towards EGFR-mediated DU145 PCSC self-renewal.

3.5 SOX2 expression is associated with DU145 sphere cell-mediated tumorigenicity

The ability of tumorigenic cells to propagate under anchorage-independent conditions in soft agar is an *in vitro* surrogate measure of tumorigenicity; an ability which closely correlates with *in vivo* tumorigenicity in immunocompromised mice (Colburn et al., 1978; Freedman and Shin, 1974; Shin et al., 1975). To address whether the loss of SOX2 expression affects the anchorage-independent growth of DU145 PCSCs, established SOX2 knockdown sphere cells were seeded in soft agar-containing, serum-supplemented media. SOX2 knockdown in sphere cells resulted in a significantly fewer number of anchorage-independent colonies compared to shCTRL sphere cells under differentiating conditions (Fig 5A, B).

In support of an association between SOX2 and DU145 sphere-mediated tumorigenesis, we were able to confirm our previous observation (Rybak et al., 2011) that DU145 sphere cells demonstrate increased *in vivo* tumorigenic potential compared to DU145 MNL cells (Fig 5C). More importantly, sphere-derived xenograft tumors contained substantially more SOX2-positive cells than xenograft tumors generated by MNL cells (Fig 5D).

4. Discussion

Cancer stem cells (CSCs) are regarded to play a major role in prostate cancer formation, progression and chemotherapeutic drug resistance (Domingo-Domenech et al., 2012; Liu et al., 2011; Patrawala et al., 2006; Rajasekhar et al., 2011; Rybak et al.,

2011), and therefore, they are the prospective candidate targets for therapeutic intervention. Consistent with this knowledge, accumulating evidence demonstrates that prostate cancer stem cells are responsible for prostate tumorigenesis. Prostate stem cells (PSC) have been identified in both human and mouse prostates (Goldstein et al., 2010; Leong et al., 2008; Wang et al., 2009). Lineage-tracking analysis in mouse models of prostate cancer have revealed that different lineages of prostate epithelial cells capable of regenerating the prostate epithelium are also susceptible to tumorigenesis (Choi et al., 2012; Wang et al., 2013). Furthermore, a castration-resistant sub-population of prostate epithelial cells displaying PSC properties, which is vulnerable towards transformation (Wang et al., 2009), has been shown to be associated with a poor survival prognosis in humans (Wang et al., 2013). Despite these advances in PCSC research, the factors required for PCSC maintenance have not been clearly defined. We have provided evidence that SOX2 plays a critical role in PCSC self-renewal.

SOX2 expression occurs in DU145 sphere cells but not in their isogenic MNL cells (Fig 1A). Furthermore, propagating sphere cells under differentiating conditions decreases their sphere-forming potential (Rybak et al., 2011) as well as reduces SOX2 expression (Fig 1F). Consistent with EGFR signaling being critical for the self-renewal of DU145 sphere cells, SOX2 expression is regulated by EGFR activity. EGFR may regulate SOX2 expression by facilitating its transcription, protein stability, and/or subcellular localization. As SOX2 mRNA transcript levels do not seem to change dramatically compared to SOX2 protein levels in DU145 spheres versus MNL cells, this suggests that EGFR may largely regulate SOX2 expression at the post-transcriptional level. In

supporting this possibility, a variety of post-translational modifications, including acetylation, sumoylation, phosphorylation and methylation have been reported to regulate the transcriptional activity of SOX2 (Baltus et al., 2009; Jeong et al., 2010; Tsuruzoe et al., 2006; Zhao et al., 2011). MicroRNAs have also been reported to inhibit SOX2 expression and function. In HSC43 human gastric cancer cells, miR-126 inhibits SOX2 expression by targeting two sites within its 3'-untranslated region while only modestly reducing SOX2 mRNA levels (Otsubo et al., 2011). In addition, miR-9* has been shown to repress SOX2, with the stem-like features and tumor-initiating capacity of glioma CSCs being regulated in a SOX2-dependent fashion (Jeon et al., 2011). Thus, it is possible that EGFR signaling in DU145 PCSCs may prevent these inhibitory microRNAs from downregulating SOX2 function.

The importance of SOX2 in DU145 PCSCs maintenance is in line with the well-established role of SOX2 in the tumorigenesis of brain (Gangemi et al., 2009; Schmitz et al., 2007), breast (Chen et al., 2008; Rodriguez-Pinilla et al., 2007), lung and esophageal cancers (Bass et al., 2009). SOX2 is expressed heterogeneously in glioblastoma (Gangemi et al., 2009; Schmitz et al., 2007) and is upregulated in breast tumors, with its expression levels being correlated with cancer progression (Chen et al., 2008). Furthermore, SOX2 expression has been observed in 43% of basal cell-like breast carcinomas and found to be strongly correlated with CK5/6, vimentin and EGFR expression, markers which are typically associated with a less differentiated phenotype in these tumors (Rodriguez-Pinilla et al., 2007).

Consistent with this knowledge, we provide evidence that SOX2 is an important downstream target of EGFR in regulating DU145 sphere self-renewal. In support of our research, EGFR signaling has been shown to regulate the self-renewal of neural precursor cells (Hu et al., 2010) and maintain cancer stem-like properties in breast cancer side population (SP) cells (Yang et al., 2013) in a SOX2-dependent fashion. However, the relationship between SOX2 and EGFR is a complex one. In addition to being an important target of EGFR, ectopic SOX2 expression is insufficient to enhance DU145 sphere self-renewal without EGFR signaling (Figs 2, 3). This is consistent with the observation that ectopic SOX2 was incapable of enhancing sphere self-renewal of U87 glioma cells (Alonso et al., 2011), indicating that additional factors are required to contribute towards SOX2-mediated self-renewal in prostate and brain tumor stem-like cells. SOX2 also seems to facilitate sphere cell proliferation, as ectopic SOX2 enlarged sphere size in the absence and presence of EGF (Supplementary Fig 5), while SOX2 knockdown reduced sphere size under the same conditions (Fig 4). Whether these increases in cell proliferation and self-renewal are due to EGFR-mediated post-translational modification(s) of SOX2 or other factors necessary for SOX2 function is currently unclear.

SOX2 has been well studied in embryonic stem cells, development and cellular reprogramming, however, its role in adult stem cells for maintaining the integrity of multiple tissues (Arnold et al., 2011) has only more recently emerged. In addition, SOX2 has been shown to act as an oncogene in various cancers (Chen et al., 2008; Gangemi et al., 2009; Jia et al., 2011; Leis et al., 2012). Our current study supports the critical

contribution of SOX2 towards maintaining the sphere-forming capacity of DU145 PCSCs. This is consistent with the inhibition of self-renewal observed following SOX2 knockdown in human glioblastoma cancer stem-like cells (Alonso et al., 2011; Gangemi et al., 2009), breast tumor-initiating cells (Leis et al., 2012), and tumor-initiating cells in osteosarcomas (Basu-Roy et al., 2011). Given that SOX2 is heterogeneously expressed in DU145 spheres (Fig 1B, Supplementary Fig 1), it is possible that SOX2-expressing cells in DU145 spheres may contribute towards the increased tumorigenicity displayed by sphere cells compared to MNL cells (Fig 5C). Future experiments may be required to isolate SOX2-positive prostate cancer cells and determine their tumor-initiating potential, as well as identify SOX2 target genes that are necessary for maintaining PCSCs.

Acknowledgements

The authors would like to thank Dr. Khalid Al-Nedawi (Hamilton Centre for Kidney Research, Hamilton, ON, Canada) for providing the EGFRvIII/pLNCX construct, Dr. James Thomson (University of Wisconsin, Madison, Wisconsin, USA) for providing the pSIN-EF2-Sox2-Pur construct (Addgene plasmid 16577), Dr. David Sabatini (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) for providing the non-specific shRNA construct (Addgene plasmid 1864), and Dr. Bryan E. Strauss (University of São Paulo School of Medicine, São Paulo, Brazil) for providing the third-generation lentiviral packaging plasmids used in this study. We would also like to thank Dr. Lizhi He for his technical help, as well as Anthony Bruce for his assistance with the interpretation of results, his constructive input during the course of the study, and editing of the prepared manuscript. We would also like to acknowledge the financial support from the Hamilton Centre for Kidney Research (HCKR) and St. Joseph's HealthCare at Hamilton, Ontario, Canada. This work was supported by a grant (RMS 79-71) from the Canadian Institutes of Health Research to D.T.

References

Alonso, M.M., Diez-Valle, R., Manterola, L., Rubio, A., Liu, D., Cortes-Santiago, N., Urquiza, L., Jauregi, P., Lopez de Munain, A., Sampron, N., Aramburu, A., Tejada-Solis, S., Vicente, C., Odero, M.D., Bandres, E., Garcia-Foncillas, J., Idoate, M.A., Lang, F.F., Fueyo, J., Gomez-Manzano, C., 2011. Genetic and epigenetic modifications of Sox2 contribute to the invasive phenotype of malignant gliomas. PloS one 6, e26740.

Arnold, K., Sarkar, A., Yram, M.A., Polo, J.M., Bronson, R., Sengupta, S., Seandel, M., Geijsen, N., Hochedlinger, K., 2011. Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. Cell stem cell 9, 317-329.

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., Lovell-Badge, R., 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes & development 17, 126-140.

Baltus, G.A., Kowalski, M.P., Zhai, H., Tutter, A.V., Quinn, D., Wall, D., Kadam, S., 2009. Acetylation of sox2 induces its nuclear export in embryonic stem cells. Stem cells (Dayton, Ohio) 27, 2175-2184.

Bass, A.J., Watanabe, H., Mermel, C.H., Yu, S., Perner, S., Verhaak, R.G., Kim, S.Y., Wardwell, L., Tamayo, P., Gat-Viks, I., Ramos, A.H., Woo, M.S., Weir, B.A., Getz, G., Beroukhim, R., O'Kelly, M., Dutt, A., Rozenblatt-Rosen, O., Dziunycz, P., Komisarof, J., Chirieac, L.R., Lafargue, C.J., Scheble, V., Wilbertz, T., Ma, C., Rao, S., Nakagawa, H., Stairs, D.B., Lin, L., Giordano, T.J., Wagner, P., Minna, J.D., Gazdar, A.F., Zhu, C.Q., Brose, M.S., Cecconello, I., Jr, U.R., Marie, S.K., Dahl, O., Shivdasani, R.A., Tsao, M.S., Rubin, M.A., Wong, K.K., Regev, A., Hahn, W.C., Beer, D.G., Rustgi, A.K., Meyerson, M., 2009. SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. Nature genetics 41, 1238-1242.

Basu-Roy, U., Seo, E., Ramanathapuram, L., Rapp, T.B., Perry, J.A., Orkin, S.H., Mansukhani, A., Basilico, C., 2011. Sox2 maintains self renewal of tumor-initiating cells in osteosarcomas. Oncogene 31, 2270-2282.

Ben-Porath, I., Thomson, M.W., Carey, V.J., Ge, R., Bell, G.W., Regev, A., Weinberg, R.A., 2008. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nature genetics 40, 499-507.

Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., Gifford, D.K., Melton, D.A., Jaenisch, R., Young, R.A., 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122, 947-956.

Chaffer, C.L., Brueckmann, I., Scheel, C., Kaestli, A.J., Wiggins, P.A., Rodrigues, L.O., Brooks, M., Reinhardt, F., Su, Y., Polyak, K., Arendt, L.M., Kuperwasser, C., Bierie, B., Weinberg, R.A., 2011. Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. Proceedings of the National Academy of Sciences of the United States of America 108, 7950-7955.

Chen, Y., Shi, L., Zhang, L., Li, R., Liang, J., Yu, W., Sun, L., Yang, X., Wang, Y., Zhang, Y., Shang, Y., 2008. The molecular mechanism governing the oncogenic potential of SOX2 in breast cancer. The Journal of biological chemistry 283, 17969-17978.

Choi, N., Zhang, B., Zhang, L., Ittmann, M., Xin, L., 2012. Adult murine prostate basal and luminal cells are self-sustained lineages that can both serve as targets for prostate cancer initiation. Cancer cell 21, 253-265.

Colburn, N.H., Bruegge, W.F., Bates, J.R., Gray, R.H., Rossen, J.D., Kelsey, W.H., Shimada, T., 1978. Correlation of anchorage-independent growth with tumorigenicity of chemically transformed mouse epidermal cells. Cancer research 38, 624-634.

Daley, G.Q., 2008. Common themes of dedifferentiation in somatic cell reprogramming and cancer. Cold Spring Harbor symposia on quantitative biology 73, 171-174.

Del Vecchio, C.A., Jensen, K.C., Nitta, R.T., Shain, A.H., Giacomini, C.P., Wong, A.J., 2012. Epidermal growth factor receptor variant III contributes to cancer stem cell phenotypes in invasive breast carcinoma. Cancer research 72, 2657-2671.

Domingo-Domenech, J., Vidal, S.J., Rodriguez-Bravo, V., Castillo-Martin, M., Quinn, S.A., Rodriguez-Barrueco, R., Bonal, D.M., Charytonowicz, E., Gladoun, N., de la Iglesia-Vicente, J., Petrylak, D.P., Benson, M.C., Silva, J.M., Cordon-Cardo, C., 2012. Suppression of acquired docetaxel resistance in prostate cancer through depletion of notch- and hedgehog-dependent tumor-initiating cells. Cancer cell 22, 373-388.

Dubrovska, A., Kim, S., Salamone, R.J., Walker, J.R., Maira, S.M., Garcia-Echeverria, C., Schultz, P.G., Reddy, V.A., 2009. The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. Proceedings of the National Academy of Sciences of the United States of America 106, 268-273.

Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., Naldini, L., 1998. A third-generation lentivirus vector with a conditional packaging system. Journal of virology 72, 8463-8471.

Ekstrand, A.J., Longo, N., Hamid, M.L., Olson, J.J., Liu, L., Collins, V.P., James, C.D., 1994. Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. Oncogene 9, 2313-2320.

Fong, H., Hohenstein, K.A., Donovan, P.J., 2008. Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells. Stem cells (Dayton, Ohio) 26, 1931-1938.

Freedman, V.H., Shin, S.I., 1974. Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. Cell 3, 355-359.

Gangemi, R.M., Griffero, F., Marubbi, D., Perera, M., Capra, M.C., Malatesta, P., Ravetti, G.L., Zona, G.L., Daga, A., Corte, G., 2009. SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. Stem cells (Dayton, Ohio) 27, 40-48.

Goldstein, A.S., Huang, J., Guo, C., Garraway, I.P., Witte, O.N., 2010. Identification of a cell of origin for human prostate cancer. Science (New York, N.Y 329, 568-571.
Gupta, P.B., Fillmore, C.M., Jiang, G., Shapira, S.D., Tao, K., Kuperwasser, C., Lander, E.S., 2011. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. Cell 146, 633-644.

Hu, Q., Zhang, L., Wen, J., Wang, S., Li, M., Feng, R., Yang, X., Li, L., 2010. The EGF receptor-sox2-EGF receptor feedback loop positively regulates the self-renewal of neural precursor cells. Stem cells (Dayton, Ohio) 28, 279-286.

Jeon, H.M., Sohn, Y.W., Oh, S.Y., Kim, S.H., Beck, S., Kim, S., Kim, H., 2011. ID4 imparts chemoresistance and cancer stemness to glioma cells by derepressing miR-9*-mediated suppression of SOX2. Cancer research 71, 3410-3421.

Jeong, C.H., Cho, Y.Y., Kim, M.O., Kim, S.H., Cho, E.J., Lee, S.Y., Jeon, Y.J., Lee, K.Y., Yao, K., Keum, Y.S., Bode, A.M., Dong, Z., 2010. Phosphorylation of Sox2 cooperates in reprogramming to pluripotent stem cells. Stem cells (Dayton, Ohio) 28, 2141-2150.

Jia, X., Li, X., Xu, Y., Zhang, S., Mou, W., Liu, Y., Liu, Y., Lv, D., Liu, C.H., Tan, X., Xiang, R., Li, N., 2011. SOX2 promotes tumorigenesis and increases the anti-apoptotic property of human prostate cancer cell. Journal of molecular cell biology 3, 230-238.

Kregel, S., Kiriluk, K.J., Rosen, A.M., Cai, Y., Reyes, E.E., Otto, K.B., Tom, W., Paner, G.P., Szmulewitz, R.Z., Vander Griend, D.J., 2013. Sox2 is an androgen receptor-repressed gene that promotes castration-resistant prostate cancer. PloS one 8, e53701.

Leis, O., Eguiara, A., Lopez-Arribillaga, E., Alberdi, M.J., Hernandez-Garcia, S., Elorriaga, K., Pandiella, A., Rezola, R., Martin, A.G., 2012. Sox2 expression in breast tumours and activation in breast cancer stem cells. Oncogene 31, 1354-1365.

Leong, K.G., Wang, B.E., Johnson, L., Gao, W.Q., 2008. Generation of a prostate from a single adult stem cell. Nature 456, 804-808.

Li, J., Pan, G., Cui, K., Liu, Y., Xu, S., Pei, D., 2007. A dominant-negative form of mouse SOX2 induces trophectoderm differentiation and progressive polyploidy in mouse embryonic stem cells. The Journal of biological chemistry 282, 19481-19492.

Lin, Y., Yang, Y., Li, W., Chen, Q., Li, J., Pan, X., Zhou, L., Liu, C., Chen, C., He, J., Cao, H., Yao, H., Zheng, L., Xu, X., Xia, Z., Ren, J., Xiao, L., Li, L., Shen, B., Zhou, H., Wang, Y.J., 2012. Reciprocal regulation of Akt and Oct4 promotes the self-renewal and survival of embryonal carcinoma cells. Molecular cell 48, 627-640.

Liu, C., Kelnar, K., Liu, B., Chen, X., Calhoun-Davis, T., Li, H., Patrawala, L., Yan, H., Jeter, C., Honorio, S., Wiggins, J.F., Bader, A.G., Fagin, R., Brown, D., Tang, D.G., 2011. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nature medicine 17, 211-215.

Loh, Y.H., Wu, Q., Chew, J.L., Vega, V.B., Zhang, W., Chen, X., Bourque, G., George, J., Leong, B., Liu, J., Wong, K.Y., Sung, K.W., Lee, C.W., Zhao, X.D., Chiu, K.P., Lipovich, L., Kuznetsov, V.A., Robson, P., Stanton, L.W., Wei, C.L., Ruan, Y., Lim, B., Ng, H.H., 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nature genetics 38, 431-440.

Magee, J.A., Piskounova, E., Morrison, S.J., 2012. Cancer stem cells: impact, heterogeneity, and uncertainty. Cancer cell 21, 283-296.

Markert, E.K., Mizuno, H., Vazquez, A., Levine, A.J., 2011. Molecular classification of prostate cancer using curated expression signatures. Proceedings of the National Academy of Sciences of the United States of America 108, 21276-21281.

Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A.A., Ko, M.S., Niwa, H., 2007. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nature cell biology 9, 625-635.

Mazzoleni, S., Politi, L.S., Pala, M., Cominelli, M., Franzin, A., Sergi Sergi, L., Falini, A., De Palma, M., Bulfone, A., Poliani, P.L., Galli, R., 2010. Epidermal growth factor receptor expression identifies functionally and molecularly distinct tumor-initiating cells in human glioblastoma multiforme and is required for gliomagenesis. Cancer research 70, 7500-7513.

McCubrey, J.A., Steelman, L.S., Abrams, S.L., Lee, J.T., Chang, F., Bertrand, F.E., Navolanic, P.M., Terrian, D.M., Franklin, R.A., D'Assoro, A.B., Salisbury, J.L., Mazzarino, M.C., Stivala, F., Libra, M., 2006. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Advances in enzyme regulation 46, 249-279.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., Yamanaka, S., 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113, 631-642.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., Smith, A., 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95, 379-391.

Noh, K.H., Kim, B.W., Song, K.H., Cho, H., Lee, Y.H., Kim, J.H., Chung, J.Y., Kim, J.H., Hewitt, S.M., Seong, S.Y., Mao, C.P., Wu, T.C., Kim, T.W., 2012. Nanog signaling in cancer promotes stem-like phenotype and immune evasion. The Journal of clinical investigation 122, 4077-4093.

Otsubo, T., Akiyama, Y., Hashimoto, Y., Shimada, S., Goto, K., Yuasa, Y., 2011. MicroRNA-126 inhibits SOX2 expression and contributes to gastric carcinogenesis. PloS one 6, e16617.

Pardal, R., Clarke, M.F., Morrison, S.J., 2003. Applying the principles of stem-cell biology to cancer. Nature reviews 3, 895-902.

Park, I.H., Zhao, R., West, J.A., Yabuuchi, A., Huo, H., Ince, T.A., Lerou, P.H., Lensch, M.W., Daley, G.Q., 2008. Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451, 141-146.

Patrawala, L., Calhoun-Davis, T., Schneider-Broussard, R., Tang, D.G., 2007. Hierarchical organization of prostate cancer cells in xenograft tumors: the CD44+alpha2beta1+ cell population is enriched in tumor-initiating cells. Cancer research 67, 6796-6805.

Patrawala, L., Calhoun, T., Schneider-Broussard, R., Li, H., Bhatia, B., Tang, S., Reilly, J.G., Chandra, D., Zhou, J., Claypool, K., Coghlan, L., Tang, D.G., 2006. Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. Oncogene 25, 1696-1708.

Rajasekhar, V.K., Studer, L., Gerald, W., Socci, N.D., Scher, H.I., 2011. Tumour-initiating stem-like cells in human prostate cancer exhibit increased NF-kappaB signalling. Nature communications 2, 162.

Reya, T., Morrison, S.J., Clarke, M.F., Weissman, I.L., 2001. Stem cells, cancer, and cancer stem cells. Nature 414, 105-111.

Rodriguez-Pinilla, S.M., Sarrio, D., Moreno-Bueno, G., Rodriguez-Gil, Y., Martinez, M.A., Hernandez, L., Hardisson, D., Reis-Filho, J.S., Palacios, J., 2007. Sox2: a possible driver of the basal-like phenotype in sporadic breast cancer. Mod Pathol 20, 474-481.

Rojas, M., Yao, S., Lin, Y.Z., 1996. Controlling epidermal growth factor (EGF)-stimulated Ras activation in intact cells by a cell-permeable peptide mimicking phosphorylated EGF receptor. The Journal of biological chemistry 271, 27456-27461.

Rybak, A.P., He, L., Kapoor, A., Cutz, J.C., Tang, D., 2011. Characterization of sphere-propagating cells with stem-like properties from DU145 prostate cancer cells. Biochimica et biophysica acta 1813, 683-694.

Rybak, A.P., Ingram, A.J., Tang, D., 2013. Propagation of Human Prostate Cancer Stem-Like Cells Occurs through EGFR-Mediated ERK Activation. PloS one 8, e61716.

Sattler, H.P., Lensch, R., Rohde, V., Zimmer, E., Meese, E., Bonkhoff, H., Retz, M., Zwergel, T., Bex, A., Stoeckle, M., Wullich, B., 2000. Novel amplification unit at chromosome 3q25-q27 in human prostate cancer. The Prostate 45, 207-215.

Schmitz, M., Temme, A., Senner, V., Ebner, R., Schwind, S., Stevanovic, S., Wehner, R., Schackert, G., Schackert, H.K., Fussel, M., Bachmann, M., Rieber, E.P., Weigle, B., 2007. Identification of SOX2 as a novel glioma-associated antigen and potential target for T cell-based immunotherapy. British journal of cancer 96, 1293-1301.

Shin, S.I., Freedman, V.H., Risser, R., Pollack, R., 1975. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro. Proceedings of the National Academy of Sciences of the United States of America 72, 4435-4439.

Siegel, R., Naishadham, D., Jemal, A., 2012. Cancer statistics, 2012. CA: a cancer journal for clinicians 62, 10-29.

Suva, M.L., Riggi, N., Bernstein, B.E., 2013. Epigenetic reprogramming in cancer. Science (New York, N.Y 339, 1567-1570.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861-872.

Tsuruzoe, S., Ishihara, K., Uchimura, Y., Watanabe, S., Sekita, Y., Aoto, T., Saitoh, H., Yuasa, Y., Niwa, H., Kawasuji, M., Baba, H., Nakao, M., 2006. Inhibition of DNA binding of Sox2 by the SUMO conjugation. Biochemical and biophysical research communications 351, 920-926.

Ugolkov, A.V., Eisengart, L.J., Luan, C., Yang, X.J., 2011. Expression analysis of putative stem cell markers in human benign and malignant prostate. The Prostate 71, 18-25.

Visvader, J.E., Lindeman, G.J., 2008. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nature reviews 8, 755-768.

Visvader, J.E., Lindeman, G.J., 2012. Cancer stem cells: current status and evolving complexities. Cell stem cell 10, 717-728.

Wang, X., Kruithof-de Julio, M., Economides, K.D., Walker, D., Yu, H., Halili, M.V., Hu, Y.P., Price, S.M., Abate-Shen, C., Shen, M.M., 2009. A luminal epithelial stem cell that is a cell of origin for prostate cancer. Nature 461, 495-500.

Wang, Z.A., Mitrofanova, A., Bergren, S.K., Abate-Shen, C., Cardiff, R.D., Califano, A., Shen, M.M., 2013. Lineage analysis of basal epithelial cells reveals their unexpected plasticity and supports a cell-of-origin model for prostate cancer heterogeneity. Nature cell biology 15, 274-283.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B.E., Jaenisch, R., 2007. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature 448, 318-324.

Williams, H., Powell, I.J., 2009. Epidemiology, pathology, and genetics of prostate cancer among African Americans compared with other ethnicities. Methods in molecular biology (Clifton, N.J 472, 439-453.

Yang, J., Liao, D., Chen, C., Liu, Y., Chuang, T.H., Xiang, R., Markowitz, D., Reisfeld, R.A., Luo, Y., 2013. Tumor-Associated Macrophages Regulate Murine Breast Cancer Stem Cells Through a Novel Paracrine EGFR/Stat3/Sox-2 Signaling Pathway. Stem cells (Dayton, Ohio) 31, 248-258.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, II, Thomson, J.A., 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science (New York, N.Y 318, 1917-1920.

Zhao, H.Y., Zhang, Y.J., Dai, H., Zhang, Y., Shen, Y.F., 2011. CARM1 mediates modulation of Sox2. PloS one 6, e27026.

Figures

Figure 1. SOX2 is heterogeneously expressed in DU145 spheres, and its expression increases with passaging of sphere cells. A) Western blot analysis of SOX2 expression in DU145 monolayer (MNL) and sphere (passage 5; p5) cells. SOX2 protein levels are increased in spheres compared to parent DU145 cells. B) Immunofluorescence staining demonstrates heterogeneous expression of SOX2 protein in the nuclei of DU145 spheres. C) Self-renewal capacity of sphere cells of increasing passage. Spheres cells were individualized and plated in serum-free media containing 0.4% BSA and 0.2× B27 (SFM) at a density of 1 cell/well in 96-well plates (120 wells). Experiments were conducted in triplicate. Self-renewal capacity of sphere cells increases with increased passaging. Percent self-renewal capacity values are displayed as mean ± S.E.M. (*$p < 0.05$; two-tailed independent Student's *t*-test). D) SOX2 protein levels increase with passaging of DU145 sphere cells. SOX2, OCT4 and NANOG protein levels were examined, however, only SOX2 protein was detected by Western blot analysis. E) Reduced SOX2 expression with increased passaging of sphere cells under differentiating (ie. serum-supplemented) conditions. Sphere cells adhere to the tissue culture plate and proliferate as adherent cell cultures under serum-supplemented conditions; these cells are termed sphere-derived adherent cells (SDACs). Western blot analysis of SOX2 protein levels in DU145 sphere cells (passage 28) plated in serum-free media or in serum-supplemented media to generate SDAC cultures of increasing passage.

Figure 1.

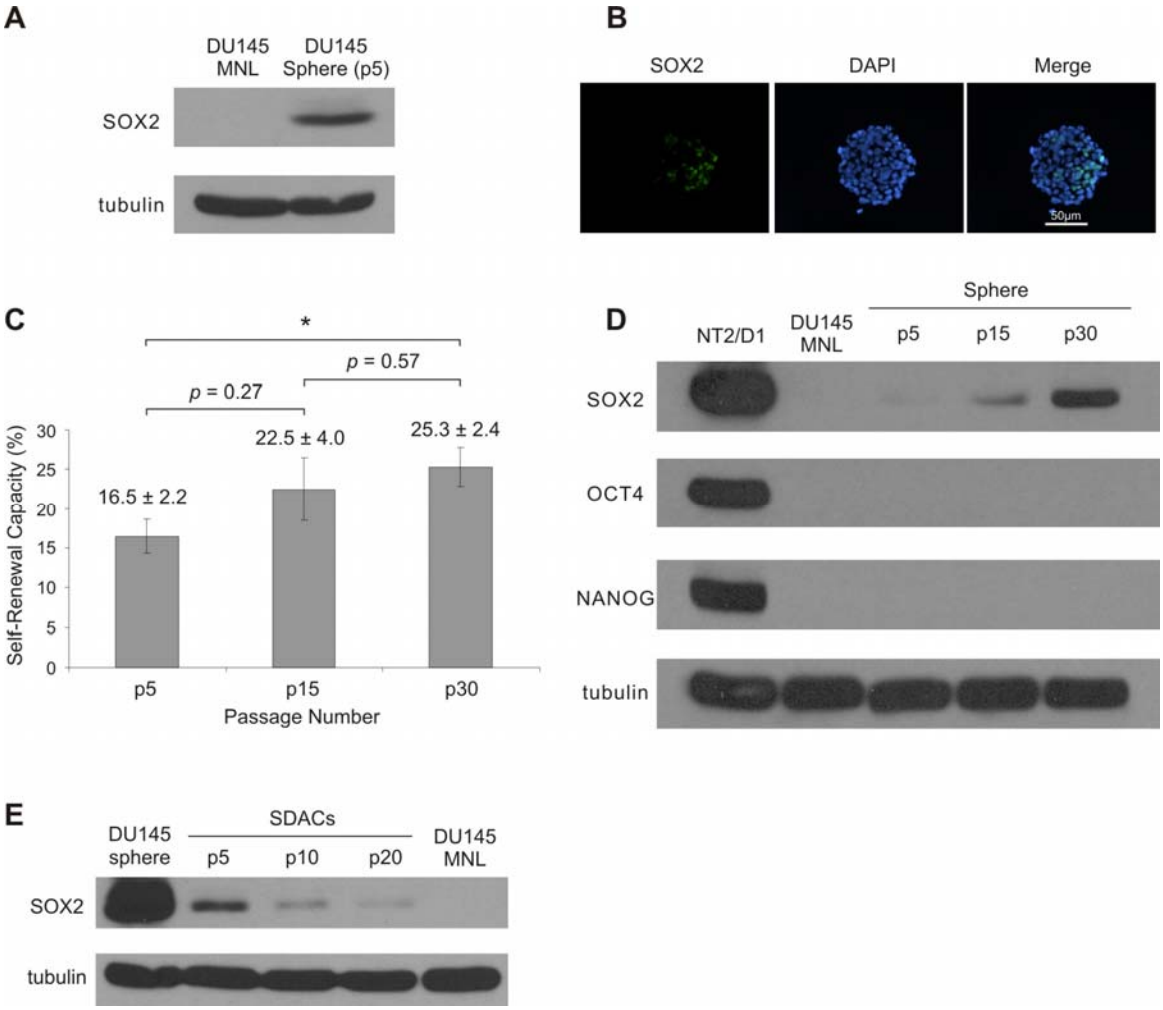
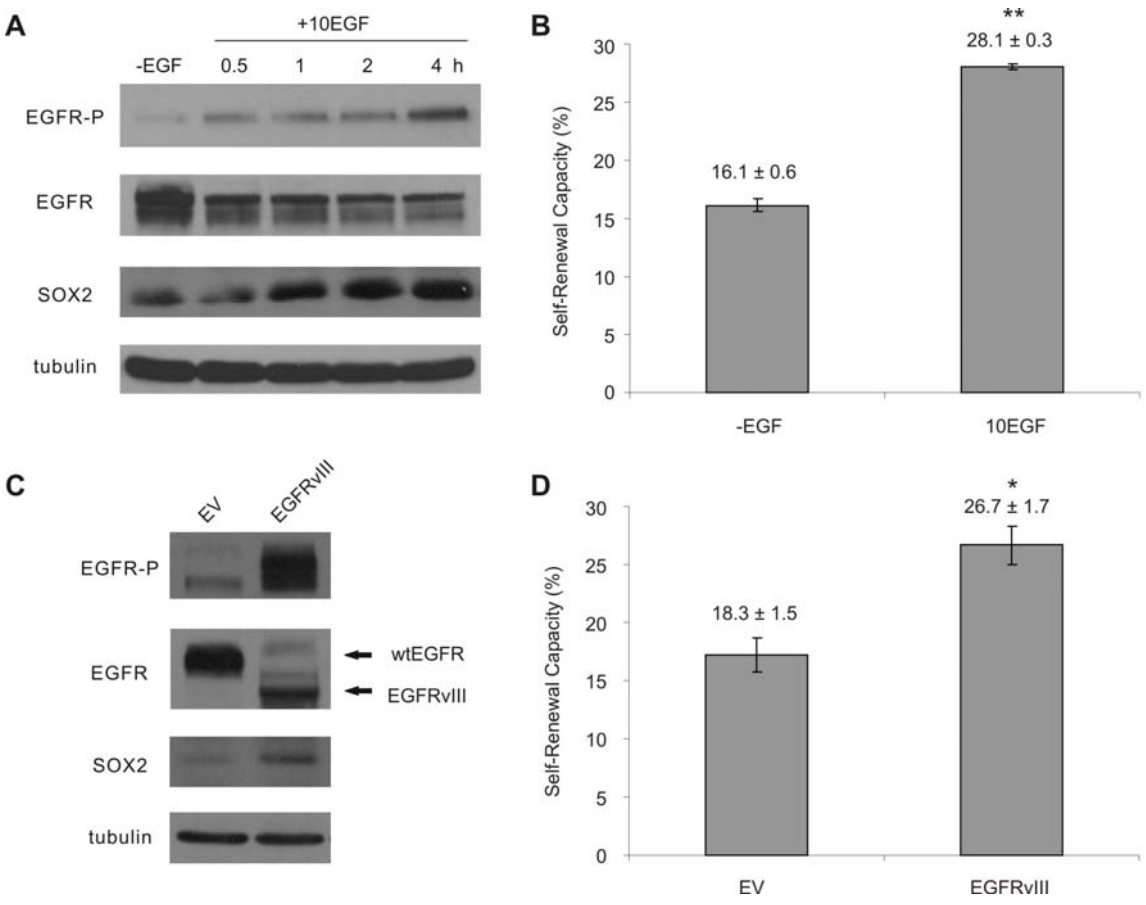

A
DU145 MNL
DU145 Sphere (p5)
SOX2
tubulin
B
SOX2
DAPI
Merge
50μm
C
*
p = 0.57
p = 0.27
22.5 ± 4.0
25.3 ± 2.4
16.5 ± 2.2
Self-Renewal Capacity (%)
30
25
20
15
10
5
0
p5
p15
p30
Passage Number
D
Sphere
NT2/D1
DU145 MNL
p5
p15
p30
SOX2
OCT4
NANOG
tubulin
E
SDACs
DU145 sphere
p5
p10
p20
DU145 MNL
SOX2
tubulin

Figure 2. Activation of EGFR signaling, through EGF treatment or expression of constitutively-active EGFRvIII, increases SOX2 expression and self-renewal of DU145 PCSCs. A) Low passage DU145 spheres (passage 5; p5), which were cultured and maintained in EGF-free serum-free media containing 0.4% BSA and 0.2× B27 (-EGF), were treated with serum-free media containing 10 ng/ml EGF (10EGF) and whole cell lysates were prepared at different time points (hours; h) post-treatment. Western blot analysis of EGF treatment of spheres results in EGFR signal activation (Tyr1068 phosphorylation; EGFR-P) and increased SOX2 protein levels. For each sample, a total of 100 μg of whole cell lysate was used. B) Self-renewal capacity of DU145 sphere cells (p5) examined when in the presence or absence of exogenous EGF (10EGF). C) EGFR variant III (EGFRvIII) expression in DU145 sphere cells (p5) increases SOX2 protein levels. D) Self-renewal capacity of DU145 sphere cells is enhanced following ectopic EGFRvIII expression. Experiments were conducted in triplicate. Percent self-renewal capacity values are displayed as mean ± S.E.M. (*$p < 0.05$, **$p < 0.01$; two-tailed independent Student's *t*-test).

Figure 2.


A
+10EGF
-EGF
0.5
1
2
4 h
EGFR-P
EGFR
SOX2
tubulin
B
**
28.1 ± 0.3
16.1 ± 0.6
Self-Renewal Capacity (%)
30
25
20
15
10
5
0
-EGF
10EGF
C
EV
EGFRvIII
EGFR-P
EGFR
wtEGFR
EGFRvIII
SOX2
tubulin
D
*
26.7 ± 1.7
18.3 ± 1.5
Self-Renewal Capacity (%)
30
25
20
15
10
5
0
EV
EGFRvIII

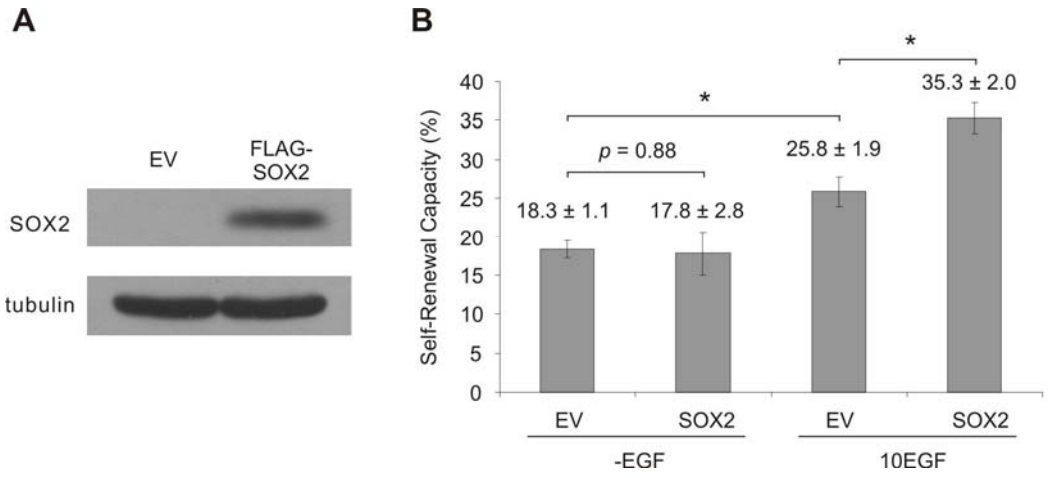


Figure 3. **Ectopic SOX2 expression enhances DU145 PCSC self-renewal in an EGFR-dependent manner.** A) Western blot analysis of whole cell lysates following ectopic FLAG-SOX2 expression in DU145 cells. B) Stable FLAG-SOX2 (SOX2)-expressing DU145 spheres were generated and maintained in serum-free media containing 0.4% BSA and 0.2× B27 (SFM) for three passages to establish EGF-free (-EGF) sphere cultures prior to experimentation. Sphere cells were seeded at a density of 1 cell/well in 96-well plates (120 wells in total per experiment) in SFM supplemented with or without EGF (10 ng/ml; 10EGF). Experiments were conducted in triplicate. SOX2-expressing sphere cells display enhanced self-renewal capacity when treated with recombinant EGF. Percent self-renewal capacity values are displayed as mean ± S.E.M. (*$p < 0.05$; two-tailed independent Student's *t*-test).

Figure 4. SOX2 is required for the self-renewal of DU145 PCSCs. A) DU145 sphere cells were infected with a SOX2 shRNA lentiviral construct and selected with puromycin in order to generate a stable SOX2 knockdown sphere cell line. Western blot analysis of SOX2 knockdown (shSOX2) sphere cells was carried out to evaluate the SOX2 knockdown efficiency relative to non-specific shRNA control (shCTRL) sphere cells. B) Stable SOX2 knockdown sphere cells display reduced self-renewal capacity compared to shCTRL sphere cells. Cells were seeded at a density of 1 cell/well in 96-well plates (120 wells). Experiments were conducted in triplicate. Percent self-renewal capacity values are displayed as mean ± S.E.M. of three independent experiments (**$p < 0.01$; two-tailed independent Student's *t*-test). Loss of endogenous SOX2 protein expression significantly reduces the C) number and D) size (diameter) of DU145 spheres formed under EGF-free (-EGF) and EGF-supplemented (10 ng/ml EGF; 10EGF) media conditions. SOX2 knockdown (shSOX2) and shCTRL sphere cells were seeded at a density of 1×10^3 cells/well in 24-well plates (three replicates per cell line). The number of spheres that formed was counted 12 days post-seeding. Sphere number is displayed as mean ± S.E.M. of three independent experiments (*$p < 0.05$, **$p < 0.01$; two-tailed independent Student's *t*-test).

Figure 4.

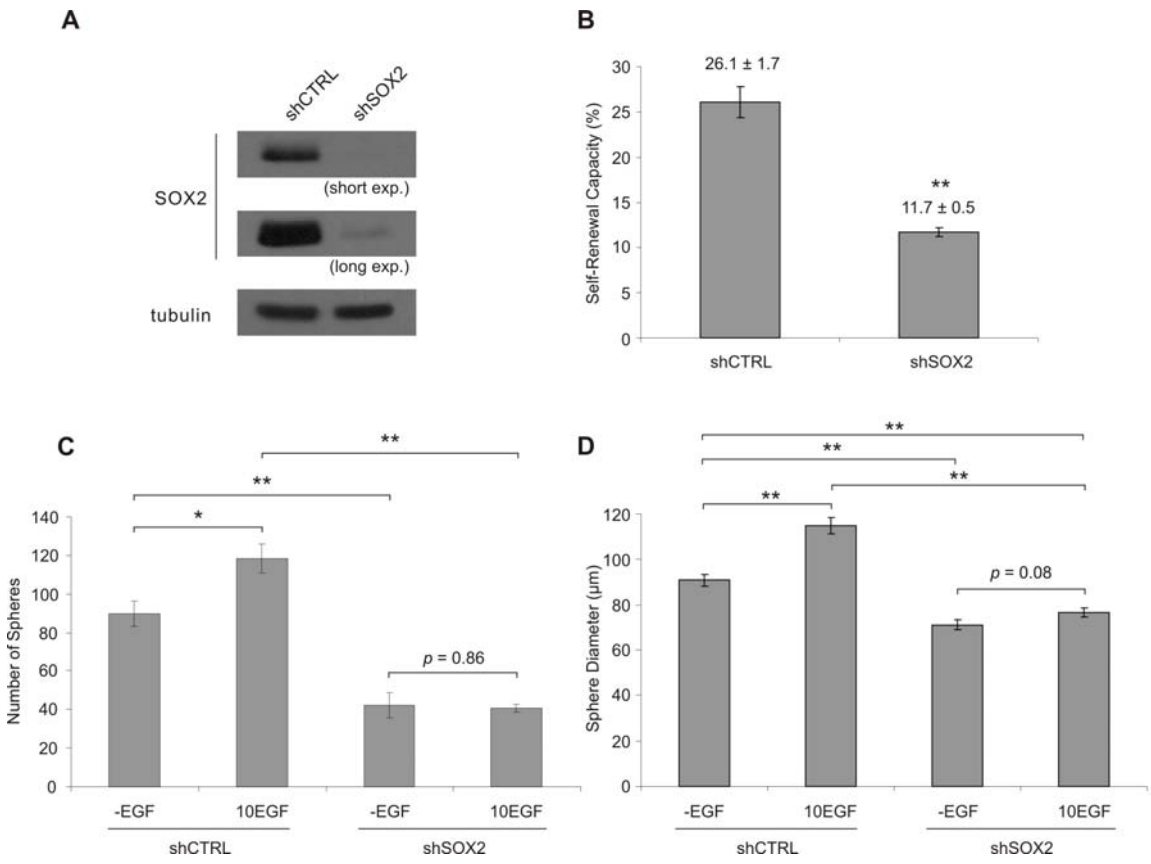
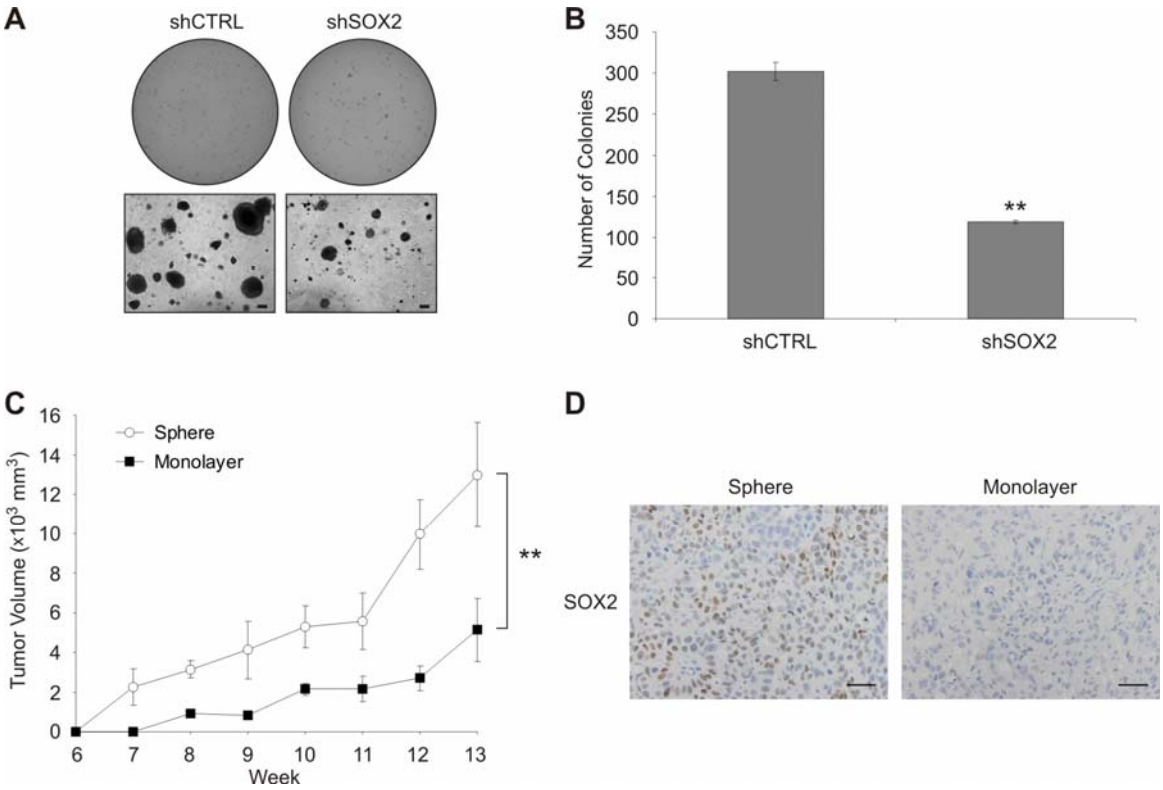
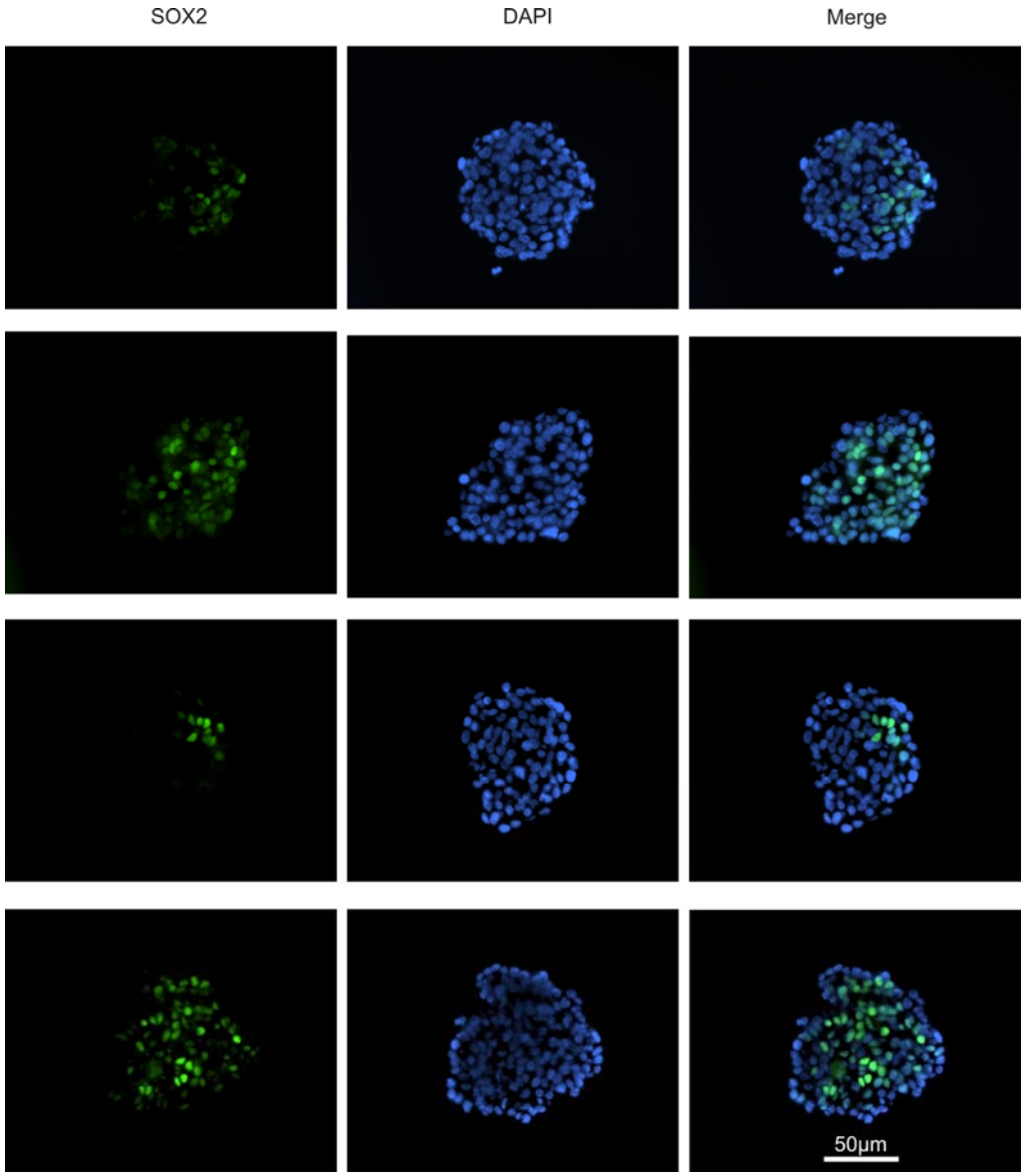

A
shCTRL
shSOX2
SOX2
(short exp.)
(long exp.)
tubulin
B
Self-Renewal Capacity (%)
26.1 ± 1.7
**
11.7 ± 0.5
shCTRL
shSOX2
C
Number of Spheres
*
**
**
p = 0.86
-EGF
10EGF
-EGF
10EGF
shCTRL
shSOX2
D
Sphere Diameter (µm)
**
**
**
**
p = 0.08
-EGF
10EGF
-EGF
10EGF
shCTRL
shSOX2

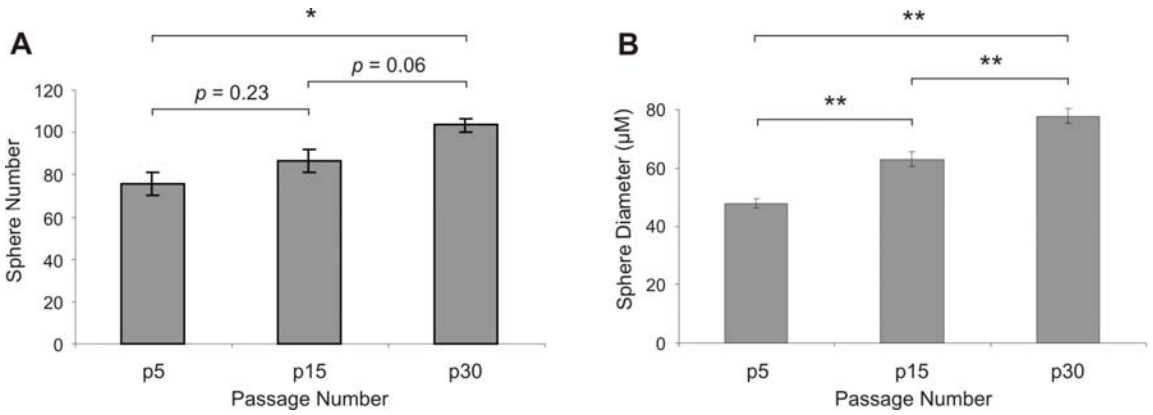
Figure 5. DU145 sphere cells display enhanced *in vivo* tumor-initiating ability and express SOX2 in xenograft tumors, while SOX2 knockdown reduces their *in vitro* anchorage-independent growth. A) SOX2 knockdown (shSOX2) in DU145 sphere cells significantly reduced their *in vitro* anchorage-independent growth compared to the non-specific shRNA control (shCTRL) sphere cell line. Representative images of soft agar wells (top panels), and phase contrast images of colonies are shown at 50X magnification (bottom panels). Scale bar is equal to 100 μm. B) The total number of colonies in 5 random fields (at 25X magnification) was counted and represented as mean ± S.E.M. of three independent experiments (**$p < 0.01$; two-tailed independent Student's *t*-test). C) Tumor-initiating capacity of DU145 monolayer and DU145 sphere (10^2) cells following subcutaneous injection in male NOD/SCID mice. Tumor volume of monolayer and sphere xenograft tumors were measured weekly, and displayed as mean ± S.E.M. (n = 4 for each cell type). Sphere cells display increased tumorigenic capacity (**$p < 0.01$; two-way ANOVA). D) Nuclear SOX2 staining observed in DU145 sphere-derived xenograft tumors, while SOX2 protein cannot be detected in DU145 monolayer cell-derived xenograft tumors.

Figure 5.

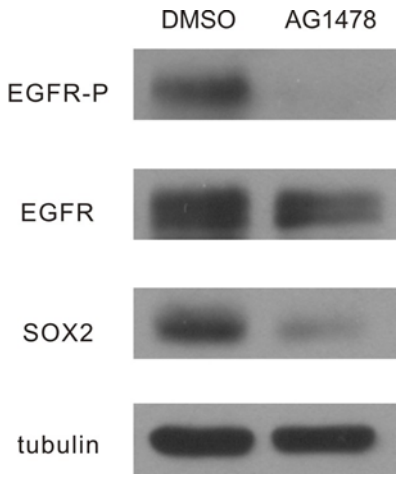

A
shCTRL
shSOX2
B
Number of Colonies
350
300
250
200
150
100
50
0
**
shCTRL
shSOX2
C
Sphere
Monolayer
Tumor Volume (x10^3 mm^3)
16
14
12
10
8
6
4
2
0
**
6
7
8
9
10
11
12
13
Week
D
Sphere
Monolayer
SOX2



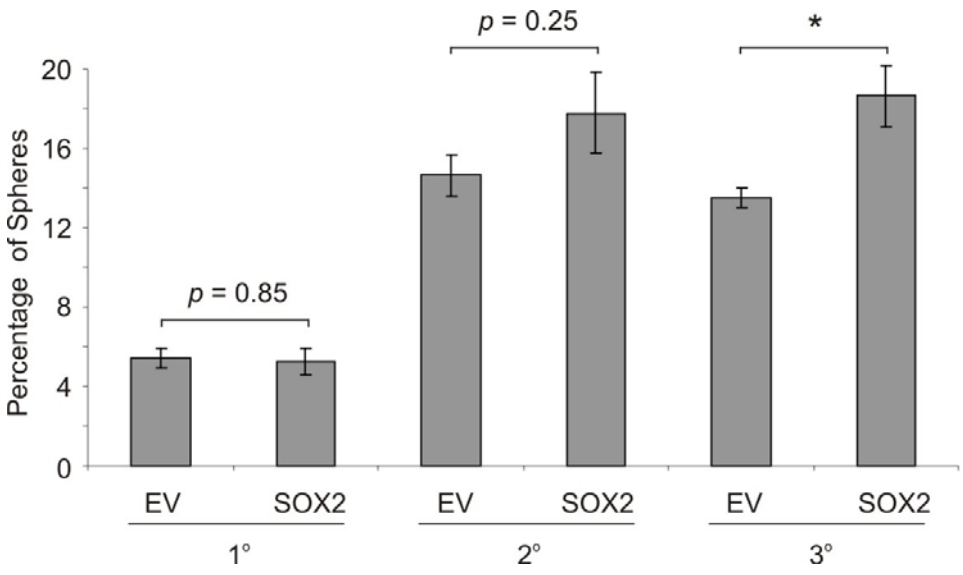
Supplementary Figure 1. Heterogeneous expression of SOX2 in DU145 spheres. Images were taken of various spheres for SOX2 protein expression (400X magnification). Immunofluorescent staining demonstrates heterogeneous expression of SOX2 protein in the nuclei of DU145 spheres. Scale bar represents 50 µm.



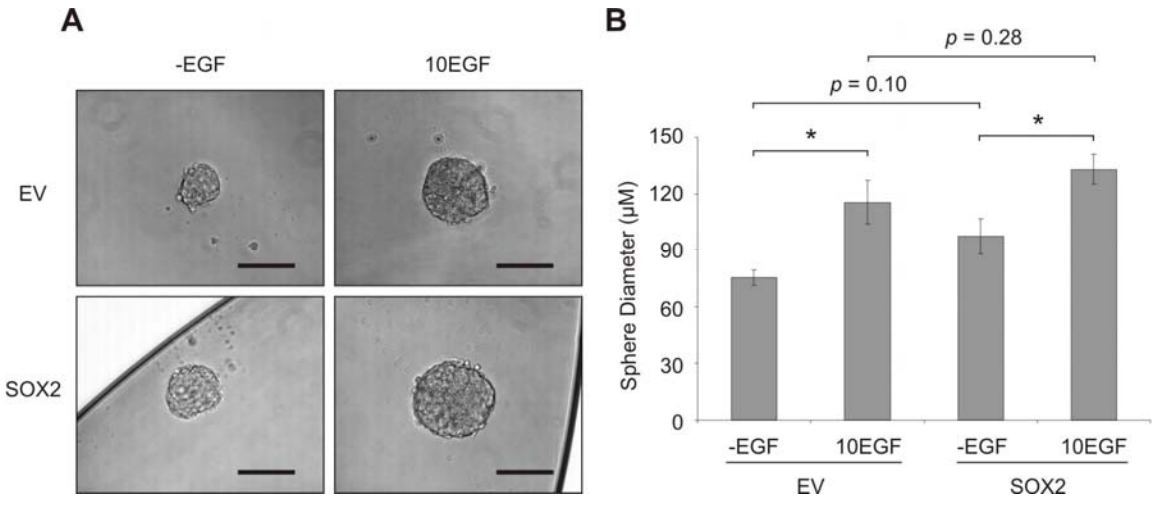
Supplementary Figure 2. Passaging of DU145 sphere cells increases subsequent sphere number and size. A) Sphere number modestly increases with increased passaging of spheres, and B) sphere size (diameter) increases following seeding DU145 sphere cells of different passage in serum-free media containing 0.4% BSA and 0.2× B27 (SFM). Sphere cells were seeded at a density of 1×10^3 cells/well in 24-well plates (0.5 ml/well; three replicates per treatment). Sphere numbers and sphere diameters are displayed as mean ± S.E.M. of three independent experiments (*$p < 0.05$; two-tailed independent Student's *t*-test).



Supplementary Figure 3. EGFR signaling blockage reduces SOX2 expression in DU145 spheres. Western blot analysis of whole cell lysates following 24 hour treatment of DU145 spheres with DMSO (mock treatment) or EGFR inhibitor AG1478 (10 μM). Protein levels of the stem cell factor, SOX2, were reduced following treatment of sphere cells with AG1478.



Supplementary Figure 4. Ectopic SOX2 expression enhances the self-renewal of DU145 PCSCs, but not primary sphere formation. Empty vector (EV) and FLAG-SOX2 (SOX2)-expressing stable DU145 cell lines were seeded in EGF-supplemented (10 ng/ml) serum-free media to evaluate their sphere-forming capacity. The number of spheres was counted 12 days post-seeding. The percentage of primary (1^o), secondary (2^o) and tertiary (3^o) spheres formed (relative to the number of cells seeded) is displayed as mean ± S.E.M. of three independent experiments (*$p < 0.05$; two-tailed independent Student's *t*-test).



Supplementary Figure 5. DU145 sphere size increases following EGF treatment and/or ectopic SOX2 expression. A) Representative phase-contrast images of empty vector (EV) control and FLAG-SOX2 (SOX2)-expressing DU145 spheres (passage 3) formed in the absence (-EGF) or presence of EGF (10 ng/ml; 10EGF). Images were taken at 200X magnification. Scale bar is equal to 100 µm. B) Sphere size (diameter) was evaluated. Sphere diameter is displayed as mean ± S.E.M. of three independent experiments (*$p < 0.05$; two-tailed independent Student's *t*-test).

CHAPTER 5

Discussion and Future Directions

5.1. Discussion

In recent years, a subpopulation of cancer cells with tumour-initiating potential have been identified in various solid tumours, including brain (Singh et al. 2004), breast (Al-Hajj et al. 2003), and colon cancers (O'Brien et al. 2007; Ricci-Vitiani et al. 2007). Cancer stem cells (CSCs) are believed to represent a minor population of cells present within the solid tumour cells (Reya et al. 2001), capable of propagating *in vitro* by forming non-adherent sphere colonies in serum-free media (SFM) conditions while maintaining their stem-like properties and tumour-initiating capacities (Visvader and Lindeman 2008). The first supportive demonstration of a hierarchical organization of prostate cancer cells of varying tumorigenic potential was discovered following the isolation of prostate cancer cells based on CD44 expression. In prostate cancer, CD44 is heterogeneously expressed, however, CD44-positive cells display increased tumour-initiating capacity compared to CD44-negative cells (Patrawala et al. 2006). Approximately 1% of CD44-positive prostate cancer cells were able to asymmetrically divide and generate CD44-negative cells during *in vitro* clonal analyses. Furthermore, CD44-positive cells were capable of generating isogenically-derived CD44-negative cells *in vivo*, suggesting that these cells were capable of reconstituting the heterogeneic nature of the original tumour (Patrawala et al. 2006). Since this initial discovery, additional markers have been identified, such as integrin α2β1 (Patrawala et al. 2007) and CD133 (Collins et al. 2005), which have been useful in further characterizing prospective PCSCs and instrumental in re-classifying the hierarchical nature of prostate cancer cells.

Although the tumour-initiating capacity of prospective PCSCs has been examined (Dubrovska et al. 2009; Rajasekhar et al. 2011), the molecular mechanisms responsible for regulating PCSC maintenance have not been extensively studied. In this dissertation, I have characterized a subpopulation of DU145 cells that are capable of propagating as spheres for an extended period of time, and demonstrated that the self-renewal capacity of DU145 PCSCs is dependent on EGFR-mediated ERK activation as well as SOX2 expression.

5.1.1. Activation of EGFR signalling mediates the propagation and self-renewal of PCSCs

Exogenous mitogens serve as important stimuli to drive symmetric cell division and ensure self-renewal of the stem cell population (Campos 2004). While approximately 1% of monolayer DU145 cells contain a population of sphere-forming cells (Dubrovska et al. 2009) (Chapter 2), these stem-like cells can be further enriched from 12.2% (Dubrovska et al. 2009) to approximately 26.2% (Chapter 2) through sphere culture, depending on whether exogenous EGF is provided alone (Chapter 2) or in combination with bFGF (Dubrovska et al. 2009). Although bFGF has been widely used in culturing PCSCs (Dubrovska et al. 2009; Miki et al. 2007), my studies have revealed that bFGF treatment has a negative impact on the propagation of PCSCs displaying the $CD44^+$integrin $\alpha_2\beta_1{}^+CD24^+$ cell surface profile (Chapter 2). Furthermore, the prospective PCSCs ($CD44^+CD133^+$) isolated from prostate cancer cell monolayer cultures have not been characterized beyond a single passage under sphere-forming conditions, bringing

into question as to whether these cells are bona-fide PCSCs given that their capacity for self-renewal has not been thoroughly evaluated (Dubrovska et al. 2009). Taken together, the differences in sphere culture conditions and evaluation of stem-like properties carried out may be critical factors in distinguishing prospective human PCSCs from simply progenitor-like prostate cancer cells.

While propagation of DU145 PCSCs does not require the presence of exogenous EGF, EGFR signalling is active in DU145 sphere cells propagated in the absence of exogenously-administered mitogens, with EGFR activation nonetheless being required for DU145 PCSCs to self-renew; activation of EGFR signalling enhances DU145 PCSC self-renewal, while its inhibition attenuates their self-renewal activity (Chapter 3). The growth and survival of PCSCs as non-adherent spheres, propagated in the absence of exogenously administered mitogens, can be explained by the following possibilities: 1) sustained proliferative signalling through the production of mitogens themselves which stimulate autocrine/paracrine-mediated signalling, and/or 2) growth factor independence via ligand-independent activation of receptor tyrosine kinases (Hanahan and Weinberg 2011) like EGFR. Several mutations of EGFR have been found in prostate cancers, including the constitutive-active EGFRvIII mutant (Olapade-Olaopa et al. 2000) and a number of EGFR tyrosine kinase domain mutants (Cai et al. 2008), which may account for the enhanced EGFR signal activation, cell proliferation and transforming ability of prostate cancer cells in the absence of EGF stimulation (Cai et al. 2008). Although the extent to which EGFR mutations affect the propagation of PCSCs under mitogen-independent conditions has yet to be investigated, the sheer multitude of EGFR receptors

present on the cell surface of DU145 prostate cancer cells ($25.2 \pm 3.9 \times 10^4$ receptors per cell) (Sherwood, et al. 1998) may account for EGFR signal activation in the absence of exogenous mitogens or serum (Chapter 3). Ligand-independent activation has been previously reported in COS cells expressing similar levels of EGFR (3×10^5 molecules per cell) (Sawano et al., 2002), suggesting that ligand-independent EGFR transactivation may occur in DU145 cells. On the other hand, EGF-like ligands have been detected in the conditioned media of DU145 cell cultures (MacDonald et al. 1990), with activation of EGFR signalling having been shown to regulate the autonomous growth of prostate cancer cells *in vitro* (Connolly and Rose 1991; Hofer et al. 1991). Although mitogen-independent PCSC propagation and self-renewal remains to be EGFR-dependent, we cannot distinguish whether this effect is attributable to EGF, other EGFR-specific ligands, or ligand-independent activation of EGFR. Future experiments will be required to elucidate the mechanism of EGFR activation in DU145 PCSCs.

Although EGFR is a prognostic marker of various advanced stage carcinomas such as breast (Livasy et al. 2006), ovarian (Nielsen et al. 2004), and non-small cell lung cancers (Garcia de Palazzo et al. 1993), the role of EGFR expression and signalling in CSCs has only recently been investigated. In human brain cancer, exogenous EGF significantly enhances the formation of sphere cultures, while EGFR inhibition potently inhibits sphere formation as well as the number of brain tumour stem ($CD133^+$) cells (Soeda et al. 2008). EGFR expression identifies tumour-initiating cells with different tumorigenic capacities in human glioblastoma multiforme (Mazzoleni et al. 2010), while glioma CSCs have been shown to be responsive to EGFR-targeting clinical drugs, erlotinib and gefitinib (Griffero

et al. 2009). Enforced EGFR expression in CSCs enhanced their tumorigenic potential, while ectopic EGFR expression increased the *in vivo* tumorigenic capacity of EGFR-negative CSC lines. In addition, EGFR knockdown in $EGFR^+$ CSCs decreased their tumorigenicity *in vivo* and promoted differentiation of these cells (Mazzoleni et al. 2010). The constitutive-active EGFR mutant, EGFRvIII, has also been detected in primary breast carcinoma, with ectopic EGFRvIII expression resulting in an increased proportion of cancer stem-like ($CD44^+CD24^-$) breast cancer cells, while promoting enhanced *in vitro* sphere formation and *in vivo* tumour formation (Del Vecchio et al. 2012). In another investigation examining the functionality of EGFR in tumorigenesis, EGFRvIII heterogeneously expressed in a minor subpopulation of human glioblastoma cells enhanced the tumorigenicity of the entire tumour cell population in a paracrine-driven manner by recruiting wild-type EGFR-expressing cells into accelerated proliferation (Inda et al. 2010). Taken together, these studies support the notion that a minor tumour cell population with active EGFR signalling, such as a cancer stem-like subpopulation, drives accelerated growth of the entire tumour mass while maintaining heterogeneity within the tumour mass.

5.1.2. Contributions of PI3K-AKT and MAPK signalling towards EGFR-mediated PCSC self-renewal

The EGFR pathway is well known to initiate signalling events involving the activation of the PI3K-AKT and Ras-Raf-MEK-ERK (MAPK) pathways. In addition, activation of PI3K-AKT (Ayala et al. 2004; Bedolla et al. 2007; Lotan et al. 2011) and MAPK (ERK)

signalling (Gioeli et al. 1999; Mulholland et al. 2012) has been shown to be associated with prostate progression. DU145 PCSCs display elevated ERK activation and AKT activation following EGF treatment (Appendix 2). Inhibition of ERK activation, due to U0126 treatment, ectopic expression of dominant-negative MEK1(K97M) or knockdown of either ERK1 or ERK2, resulted in reduced sphere formation and sphere size. Furthermore, EGF treatment could not enhance sphere formation following ectopic MEK1(K97M) expression in DU145 cells (Chapter 3). These results highlight the contribution of MEK-ERK signalling towards EGFR-mediated PCSC self-renewal. This is consistent with the inhibition of self-renewal observed following MEK1 inhibition in human glioblastoma cancer stem-like cells, which further elicited a pro-differentiation effect in these cells (Sunayama et al. 2010), and human breast tumour-initiating cells (Chang et al., 2011). On the other hand, PTEN knockdown did not promote DU145 PCSC formation and self-renewal following EGF treatment, despite a slight increase in AKT activation being observed (Chapter 2). This would suggest that PI3K-AKT signalling is low in DU145 sphere cells, and likely does not contribute towards EGF-mediated enhancement in DU145 sphere formation even in the absence of PTEN which antagonizes PI3K activity (Stambolic et al. 1998). This result contrasts the results of a recent publication suggesting that PTEN knockdown promotes DU145 primary sphere formation (Dubrovska et al., 2009). However, the addition of bFGF to culture conditions used in this study may explain for the elevated levels of PI3K-AKT signalling detected in prostate cancer spheres in this study (Dubrovska et al., 2009). Despite these differences, EGFR signalling promotes PCSC self-renewal, in part, by activating the MEK-ERK pathway.

5.1.3. SOX2 as a downstream target of EGFR signalling that is required for PCSC self-renewal

OCT4, SOX2 and NANOG are stem cell transcription factors involved in maintaining pluripotency (Boyer et al. 2005; Loh et al. 2006), and they have been shown to reprogram terminally-differentiated adult cells towards a stem-like state (Park et al. 2008; Takahashi et al. 2007; Wernig et al. 2007). Despite its critical role in ESC maintenance (Fong et al. 2008; Li et al. 2007) and fetal development (Avilion et al. 2003; Keramari et al. 2010), SOX2 is required for the development of various cells of endoderm (Que et al. 2007), ectoderm (Graham et al. 2003; Taranova et al. 2006) and mesoderm lineage (Basu-Roy et al. 2010) (further reviewed by (Sarkar and Hochedlinger 2013)). In addition, Sox2 has been shown to be required by discrete cells in multiple mouse tissues to maintain their integrity and function (Arnold et al. 2011). Furthermore, ectopic SOX2 expression is sufficient to reprogram fibroblasts into neural stem cells which can differentiate into mature neurons, astrocytes and oligodendrocytes (Ring, et al. 2012), demonstrating the importance of SOX2 in the acquisition of stem-like properties (multipotency) by differentiated cells.

The cell-of-origin in malignant transformation may arise from normal stem cells, progenitors or terminally-differentiated adult cells that have gained oncogenic mutations leading to the acquisition of stem-like properties such as self-renewal capacity (Pardal et al. 2003; Reya et al. 2001; Visvader and Lindeman 2008). However, more recent studies have suggested that CSC-like cells can arise *de novo* from non-tumorigenic cells (Chaffer et al. 2011), and that this reversible transition between epigenetically-defined tumorigenic

and non-tumorigenic states occurs in a stochastic manner (Gupta et al. 2011). Whether SOX2 upregulation is critical for the acquisition of tumorigenic and stem-like properties by non-tumorigenic (differentiated) prostate cancer cells remains unknown. During PCSC propagation, endogenous SOX2 protein, but not OCT4 or NANOG, is upregulated and heterogeneously expressed in DU145 spheres compared to monolayer cells propagated in serum-supplemented media conditions (Chapter 4). This suggests that SOX2 is a critical factor in PCSC maintenance, which is induced upon culturing cells under serum-free, non-adherent conditions. However, the tumour-initiating ability of SOX2-positive cells has not been definitively shown. This is due to the limitations of isolating and sorting out viable SOX2-positive prostate cancer cells from the SOX2-negative cell population needed to graft into immuno-compromised mice. Future advances in cell sorting technology, or following the identification of SOX2 target genes that display themselves on the cell surface of PCSCs, may aid in resolving this unanswered question. *In vivo* limiting dilution analysis of sphere-derived PCSCs following SOX2 knockdown would also address the contribution of SOX2 towards PCSC-mediated tumorigenesis. Furthermore, the transcriptional activity of SOX2 and the identity of its target genes in cancer stem-like cells, particularly in PCSCs, remain largely unknown. Therefore, identifying SOX2 target genes will provide novel avenues for deciphering the mechanisms in maintaining the stem-like properties of DU145 PCSCs and serve as exciting targets for prostate cancer treatment.

The role of SOX2 in tumorigenesis is well-documented and expanding, with SOX2 expression being observed in brain (Alonso et al. 2011; Schmitz et al. 2007), breast (Chen

et al. 2008; Rodriguez-Pinilla et al. 2007), lung and esophageal cancers (Bass et al. 2009). SOX2 has been found to be expressed heterogeneously in glioblastoma specimens (Schmitz et al. 2007) and overexpressed in human breast tumours, with its expression levels being correlated with higher grades of breast cancer (Chen et al. 2008). SOX2 amplification also occurs in various squamous cell carcinomas (SCCs) of the lung, esophagus, cervix, skin and penis (Bass et al. 2009; Maier, et al. 2011; Rudin et al. 2012). In prostate cancer, SOX2 expression is correlated with higher tumour grade (Jia et al. 2011; Kregel, et al. 2013), implicating SOX2 in prostate cancer progression. Furthermore, SOX2 knockdown has been shown to reduce glioma (Gangemi et al. 2009), breast (Chen et al. 2008; Leis et al. 2012) and prostate tumour (Jia et al. 2011) formation *in vivo*, thereby implicating SOX2 in serving a functional role in CSCs.

The results of Chapter 4 support the critical contribution of SOX2 towards maintaining the sphere-forming capacity of DU145 PCSCs. Furthermore, SOX2 contributes to EGFR's role in maintaining the "stemness" of DU145 PCSCs. EGFR signalling has been shown to regulate the self-renewal of neural precursor cells (Hu et al. 2010) and maintain cancer stem-like properties in breast cancer cells (Yang, et al. 2013) in a SOX2-dependent fashion. The mechanism by which EGFR signalling regulates SOX2 protein levels in DU145 PCSCs is unclear. Therefore, future work will be necessary to determine whether the MAPK signal cascade, or other signalling pathway, mediates the increases in SOX2 protein levels observed following EGFR activation in DU145 PCSCs. In addition to increasing overall SOX2 protein levels in spheres, it is unclear as to whether EGFR activation increases the number of SOX2-positive cells, in addition to the increases in

sphere number and size observed (Chapters 3 and 4). Addressing this question would further clarify the association between EGFR signalling in SOX2-positive PCSCs and the enhanced self-renewal capacity observed following EGFR activation in these cells.

EGFR expression (Di Lorenzo et al. 2002; Hernes et al. 2004; Shah et al. 2006) and ERK activation (Gioeli et al. 1999; Mulholland et al. 2012) are associated with prostate cancer progression towards a hormone-refractory (androgen-independent) state. In addition, ectopic expression of a Ras-activated mutant in androgen-sensitive LNCaP prostate cancer cells resulted in xenograft tumours that were resistant to castration and displaying robust ERK activation post-castration (Bakin et al. 2003b). Furthermore, SOX2 is an androgen receptor (AR)-repressed gene (Kregel et al. 2013) that is expressed at elevated levels with increasing prostate tumour grade (Jia et al. 2011; Kregel et al. 2013). Ectopic SOX2 expression has been shown to be sufficient in promoting tumour formation of androgen-sensitive prostate cancer cells in castrated immuno-compromised mice (Kregel et al. 2013). Therefore, EGFR-mediated MEK-ERK activation and increased SOX2 protein expression may be mechanisms by which prostate cancer acquires an androgen-independent state. Given that SOX2 is expressed in approximately 10% of cells in prostate basal and luminal cell layers (Ugolkov et al. 2011), SOX2 may in fact label castration-resistant population(s) of cells in the human prostate epithelium that have yet to be characterized, with future studies being required to elucidate their role in prostate regeneration and prostate tumorigenesis.

5.2. Future directions

In this thesis, I have established conditions to isolate and maintain prostate cancer stem-like cells (PCSCs) from DU145 prostate cancer cells in a defined serum-free medium (SFM) as spheres. This culture system is used for propagating and evaluating the stem-like properties of somatic stem cells (Reynold and Weiss, 1996; Dontu et al., 2003) and cancer stem cells (O'Brien et al., 2007; Ponti et al., 2005; Ricci-Vitiani et al., 2007; Singh et al., 2004). By culturing spheres in SFM conditions, I was able to demonstrate the requirement of EGFR signalling for the self-renewal of DU145 PCSCs, through its ability to regulate SOX2 expression and MEK-ERK signalling.

5.2.1. Investigating the contributions of the PI3K-AKT pathway towards PCSC-mediated tumorigenesis

While EGFR regulates the maintenance of DU145 PCSCs through MEK-ERK signalling in accordance with the known roles of EGFR and ERK in tumorigenesis, the observation that EGFR-PI3K-AKT signalling does not playing a major role in PCSC maintenance is rather surprising. The PI3K-AKT pathway plays a major role in prostate tumorigenesis, as conditional deletion of PTEN in the mouse prostate was sufficient to induce the formation of PIN lesions, adenocarcinoma, metastasis and castration-resistant prostate cancer (Wang et al. 2003). Furthermore, PCSCs are regarded to play a major role in prostate cancer formation, progression and chemotherapeutic drug resistance (Domingo-Domenech, et al. 2012; Liu, et al. 2011a; Patrawala et al. 2006; Rajasekhar et

al. 2011). Therefore, an intriguing area of future investigation is to elucidate the relationship between PCSC-mediated tumorigenesis and the PI3K-AKT pathway. Prostate tumorigenesis is a complex process which involves many hallmarks that are important beyond simply maintaining PCSCs within the tumour. While there may be many factors that are necessary for PCSC generation and tumour initiation *in vivo*, it can be envisaged that secondary events govern the formation and sustenance of the subsequent tumour mass. Whether PI3K-AKT signalling plays a critical role in either or both processes during prostate cancer development, plays an important role in PCSCs or simply is necessary for generating the bulk of the less tumorigenic cells present within the prostate tumour, will need to be further addressed in the future.

A feature of stem cells is the ability to maintain themselves in a quiescent state [as reviewed by (Cheung and Rando 2013)], while a similar feature in CSCs is believed to contribute towards chemotherapeutic resistance, allowing them to survive therapeutic intervention and cause tumour recurrence [as reviewed by (Visvader and Lindeman 2008)]. Partial suppression of AKT signalling has been shown to induce asymmetric cell division and produce slow-proliferating cancer cells *in vitro*. This slow-proliferating cancer cell subpopulation has also been shown to be enriched in tumour specimens of breast cancer patients following chemotherapeutic treatment compared to their pretreatment biopsies (Dey-Guha, et al. 2011). The fact that DU145 PCSCs retain a low level of AKT activation irrespective of PTEN (Chapter 2) would suggest that low levels of PI3K-AKT signalling are necessary for maintaining PCSCs in a slow-proliferating state *in vitro*. Additional evidence in support of this possibility was demonstrated upon

plating DU145 PCSCs in serum-supplemented media. DU145 PCSCs proliferate significantly slower and display reduced levels of AKT activation under these conditions compared to the monolayer cells from which they were derived (Chapter 2). To further address this possibility, constitutively-active PI3K and/or AKT can be ectopically expressed in DU145 PCSCs and the effects of these manipulations on the *in vitro* self-renewal capacity, proliferative capacity, and/or other features (chemotherapeutic resistance) associated with PCSCs can be examined.

5.2.2. Examination of the physiological contributions of EGFR and SOX2 towards prostate regeneration and prostate cancer development

A logical extension of my *in vitro* research would be to investigate whether EGFR signalling regulates PCSCs and contributes towards prostate tumorigenesis *in vivo*. Manipulation of EGFR (ectopic expression of constitutively-active EGFRvIII or deletion (knockout) of EGFR) in specific prostate epithelial lineages can be used to address whether murine prostate stem cell (PSC) self-renewal capacity can be affected, and determine whether prostate basal and/or luminal cells are vulnerable to prostate tumorigenesis. Futhermore, a genetic lineage-tracing approach to track EGFR expression in prospective basal and/or luminal PSCs, and in mouse models of prostate cancer, would be useful in addressing whether the activation of EGFR and downstream MEK-ERK signalling, PI3K-AKT signalling, and/or SOX2 expression can be accordingly affected in mouse PSCs and in prostate tumours, respectively. As the classic evidence in support of the existence of PSCs is the regeneration of the prostate epithelium following castration-

induced prostate regression, the impact of modulating EGFR signalling or Sox2 expression on the regenerative capacity of prospective basal and/or luminal PSCs can also be examined.

In a similar fashion, the role of Sox2 in prostate tumorigenesis can be examined using established mouse models of prostate cancer. For instance, Sox2 expression can be examined at different stages following *PTEN* deletion in prostate basal and/or luminal cells (Choi et al. 2012; Wang et al. 2013). Given that *PTEN* heterozygous (+/-) mice have been shown to generate precancerous PIN lesions (Shen and Abate-Shen 2010), crossing them with *Sox2-GFP* heterozygous mice (Arnold et al. 2011) in order to generate *Sox2-GFP*$^{+/-}$ *PTEN*$^{+/-}$ double heterozygotes may provide a preliminary means of evaluating the role of Sox2 in PTEN-deficient prostate tumorigenesis. Therefore, efforts to address the role of Sox2 in prostate tumorigenesis *in situ* can be carried out using existing transgenic mouse models.

References

Abate-Shen C & Shen MM 2000 Molecular genetics of prostate cancer. *Genes Dev* **14** 2410-2434.

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ & Clarke MF 2003 Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* **100** 3983-3988.

Alonso MM, Diez-Valle R, Manterola L, Rubio A, Liu D, Cortes-Santiago N, Urquiza L, Jauregi P, Lopez de Munain A, Sampron N, Aramburu A, Tejada-Solis S, Vicente C, Odero MD, Bandres E, Garcia-Foncillas J, Idoate MA, Lang FF, Fueyo J & Gomez-Manzano C 2011 Genetic and epigenetic modifications of Sox2 contribute to the invasive phenotype of malignant gliomas. *PLoS One* **6** e26740.

Ambrosetti DC, Basilico C & Dailey L 1997 Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. *Mol Cell Biol* **17** 6321-6329.

Anderson NG, Maller JL, Tonks NK & Sturgill TW 1990 Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* **343** 651-653.

Armbruster DA 1993 Prostate-specific antigen: biochemistry, analytical methods, and clinical application. *Clin Chem* **39** 181-195.

Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S, Seandel M, Geijsen N & Hochedlinger K 2011 Sox2(+) adult stem and progenitor cells are important for tissue regeneration and survival of mice. *Cell Stem Cell* **9** 317-329.

Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N & Lovell-Badge R 2003 Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev* **17** 126-140.

Ayala G, Thompson T, Yang G, Frolov A, Li R, Scardino P, Ohori M, Wheeler T & Harper W 2004 High levels of phosphorylated form of Akt-1 in prostate cancer and non-neoplastic prostate tissues are strong predictors of biochemical recurrence. *Clin Cancer Res* **10** 6572-6578.

Bakin RE, Gioeli D, Bissonette EA & Weber MJ 2003a Attenuation of Ras signaling restores androgen sensitivity to hormone-refractory C4-2 prostate cancer cells. *Cancer Res* **63** 1975-1980.

Bakin RE, Gioeli D, Sikes RA, Bissonette EA & Weber MJ 2003b Constitutive activation of the Ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. *Cancer Res* **63** 1981-1989.

Bass AJ, Watanabe H, Mermel CH, Yu S, Perner S, Verhaak RG, Kim SY, Wardwell L, Tamayo P, Gat-Viks I, Ramos AH, Woo MS, Weir BA, Getz G, Beroukhim R, O'Kelly M, Dutt A, Rozenblatt-Rosen O, Dziunycz P, Komisarof J, Chirieac LR, Lafargue CJ, Scheble V, Wilbertz T, Ma C, Rao S, Nakagawa H, Stairs DB, Lin L, Giordano TJ, Wagner P, Minna JD, Gazdar AF, Zhu CQ, Brose MS, Cecconello I, Jr UR, Marie SK, Dahl O, Shivdasani RA, Tsao MS, Rubin MA, Wong KK, Regev A, Hahn WC, Beer DG, Rustgi AK & Meyerson M 2009 SOX2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinomas. *Nat Genet* **41** 1238-1242.

Basu-Roy U, Ambrosetti D, Favaro R, Nicolis SK, Mansukhani A & Basilico C 2010 The transcription factor Sox2 is required for osteoblast self-renewal. *Cell Death Differ* **17** 1345-1353.

Basu-Roy U, Seo E, Ramanathapuram L, Rapp TB, Perry JA, Orkin SH, Mansukhani A & Basilico C 2011 Sox2 maintains self renewal of tumor-initiating cells in osteosarcomas. *Oncogene* **31** 2270-2282.

Bazzoli E, Pulvirenti T, Oberstadt MC, Perna F, Wee B, Schultz N, Huse JT, Fomchenko EI, Voza F, Tabar V, Brennan CW, DeAngelis LM, Nimer SD, Holland EC & Squatrito M 2012 MEF promotes stemness in the pathogenesis of gliomas. *Cell Stem Cell* **11** 836-844.

Bedolla R, Prihoda TJ, Kreisberg JI, Malik SN, Krishnegowda NK, Troyer DA & Ghosh PM 2007 Determining risk of biochemical recurrence in prostate cancer by immunohistochemical detection of PTEN expression and Akt activation. *Clin Cancer Res* **13** 3860-3867.

Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A & Weinberg RA 2008 An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* **40** 499-507.

Boer B, Bernadt CT, Desler M, Wilder PJ, Kopp JL & Rizzino A 2006 Differential activity of the FGF-4 enhancer in F9 and P19 embryonal carcinoma cells. *J Cell Physiol* **208** 97-108.

Bonkhoff H, Stein U & Remberger K 1994 Multidirectional differentiation in the normal, hyperplastic, and neoplastic human prostate: simultaneous demonstration of cell-specific epithelial markers. *Hum Pathol* **25** 42-46.

Bonnet D & Dick JE 1997 Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* **3** 730-737.

Bostwick DG 1989 Prostatic intraepithelial neoplasia (PIN). *Urology* **34** 16-22.

Bostwick DG, Amin MB, Dundore P, Marsh W & Schultz DS 1993 Architectural patterns of high-grade prostatic intraepithelial neoplasia. *Hum Pathol* **24** 298-310.

Bostwick DG, Shan A, Qian J, Darson M, Maihle NJ, Jenkins RB & Cheng L 1998 Independent origin of multiple foci of prostatic intraepithelial neoplasia: comparison with matched foci of prostate carcinoma. *Cancer* **83** 1995-2002.

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R & Young RA 2005 Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* **122** 947-956.

Brazel CY, Limke TL, Osborne JK, Miura T, Cai J, Pevny L & Rao MS 2005 Sox2 expression defines a heterogeneous population of neurosphere-forming cells in the adult murine brain. *Aging Cell* **4** 197-207.

Burger PE, Xiong X, Coetzee S, Salm SN, Moscatelli D, Goto K & Wilson EL 2005 Sca-1 expression identifies stem cells in the proximal region of prostatic ducts with high capacity to reconstitute prostatic tissue. *Proc Natl Acad Sci U S A* **102** 7180-7185.

Cai CQ, Peng Y, Buckley MT, Wei J, Chen F, Liebes L, Gerald WL, Pincus MR, Osman I & Lee P 2008 Epidermal growth factor receptor activation in prostate cancer by three novel missense mutations. *Oncogene* **27** 3201-3210.

Campos LS 2004 Neurospheres: insights into neural stem cell biology. *J Neurosci Res* **78** 761-769.

Chaffer CL, Brueckmann I, Scheel C, Kaestli AJ, Wiggins PA, Rodrigues LO, Brooks M, Reinhardt F, Su Y, Polyak K, Arendt LM, Kuperwasser C, Bierie B & Weinberg RA 2011 Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state. *Proc Natl Acad Sci U S A* **108** 7950-7955.

Chambers I & Smith A 2004 Self-renewal of teratocarcinoma and embryonic stem cells. *Oncogene* **23** 7150-7160.

Chang CJ, Yang JY, Xia W, Chen CT, Xie X, Chao CH, Woodward WA, Hsu JM, Hortobagyi GN & Hung MC 2011 EZH2 promotes expansion of breast tumor initiating cells through activation of RAF1-beta-catenin signaling. *Cancer Cell* **19** 86-100.

Chen Y, Shi L, Zhang L, Li R, Liang J, Yu W, Sun L, Yang X, Wang Y, Zhang Y & Shang Y 2008 The molecular mechanism governing the oncogenic potential of SOX2 in breast cancer. *J Biol Chem* **283** 17969-17978.

Cheung TH & Rando TA 2013 Molecular regulation of stem cell quiescence. *Nat Rev Mol Cell Biol* **14** 329-340.

Chew JL, Loh YH, Zhang W, Chen X, Tam WL, Yeap LS, Li P, Ang YS, Lim B, Robson P & Ng HH 2005 Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. *Mol Cell Biol* **25** 6031-6046.

Choi N, Zhang B, Zhang L, Ittmann M & Xin L 2012 Adult murine prostate basal and luminal cells are self-sustained lineages that can both serve as targets for prostate cancer initiation. *Cancer Cell* **21** 253-265.

Collins AT, Berry PA, Hyde C, Stower MJ & Maitland NJ 2005 Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* **65** 10946-10951.

Collins AT, Habib FK, Maitland NJ & Neal DE 2001 Identification and isolation of human prostate epithelial stem cells based on alpha(2)beta(1)-integrin expression. *J Cell Sci* **114** 3865-3872.

Connolly JM & Rose DP 1991 Autocrine regulation of DU145 human prostate cancer cell growth by epidermal growth factor-related polypeptides. *Prostate* **19** 173-180.

Daley GQ 2008 Common themes of dedifferentiation in somatic cell reprogramming and cancer. *Cold Spring Harb Symp Quant Biol* **73** 171-174.

De Marzo AM, Nelson WG, Meeker AK & Coffey DS 1998 Stem cell features of benign and malignant prostate epithelial cells. *J Urol* **160** 2381-2392.

Del Vecchio CA, Jensen KC, Nitta RT, Shain AH, Giacomini CP & Wong AJ 2012 Epidermal growth factor receptor variant III contributes to cancer stem cell phenotypes in invasive breast carcinoma. *Cancer Res* **72** 2657-2671.

Dey-Guha I, Wolfer A, Yeh AC, J GA, Darp R, Leon E, Wulfkuhle J, Petricoin EF, 3rd, Wittner BS & Ramaswamy S 2011 Asymmetric cancer cell division regulated by AKT. *Proc Natl Acad Sci U S A* **108** 12845-12850.

Di Cristofano A, Pesce B, Cordon-Cardo C & Pandolfi PP 1998 Pten is essential for embryonic development and tumour suppression. *Nat Genet* **19** 348-355.

Di Lorenzo G, Tortora G, D'Armiento FP, De Rosa G, Staibano S, Autorino R, D'Armiento M, De Laurentiis M, De Placido S, Catalano G, Bianco AR & Ciardiello F 2002 Expression of epidermal growth factor receptor correlates with disease relapse and progression to androgen-independence in human prostate cancer. *Clin Cancer Res* **8** 3438-3444.

Domingo-Domenech J, Vidal SJ, Rodriguez-Bravo V, Castillo-Martin M, Quinn SA, Rodriguez-Barrueco R, Bonal DM, Charytonowicz E, Gladoun N, de la Iglesia-Vicente J, Petrylak DP, Benson MC, Silva JM & Cordon-Cardo C 2012 Suppression of acquired docetaxel resistance in prostate cancer through depletion of notch- and hedgehog-dependent tumor-initiating cells. *Cancer Cell* **22** 373-388.

Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ & Wicha MS 2003 In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* **17** 1253-1270.

Dubrovska A, Elliott J, Salamone RJ, Kim S, Aimone LJ, Walker JR, Watson J, Sauveur-Michel M, Garcia-Echeverria C, Cho CY, Reddy VA & Schultz PG 2010 Combination therapy targeting both tumor-initiating and differentiated cell populations in prostate carcinoma. *Clin Cancer Res* **16** 5692-5702.

Dubrovska A, Kim S, Salamone RJ, Walker JR, Maira SM, Garcia-Echeverria C, Schultz PG & Reddy VA 2009 The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. *Proc Natl Acad Sci U S A* **106** 268-273.

Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM & Weinberg RA 1993 Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363** 45-51.

Ekstrand AJ, Longo N, Hamid ML, Olson JJ, Liu L, Collins VP & James CD 1994 Functional characterization of an EGF receptor with a truncated extracellular domain expressed in glioblastomas with EGFR gene amplification. *Oncogene* **9** 2313-2320.

Ellis P, Fagan BM, Magness ST, Hutton S, Taranova O, Hayashi S, McMahon A, Rao M & Pevny L 2004 SOX2, a persistent marker for multipotential neural stem cells derived from embryonic stem cells, the embryo or the adult. *Dev Neurosci* **26** 148-165.

Engelman JA 2009 Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer* **9** 550-562.

Engelman JA, Luo J & Cantley LC 2006 The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* **7** 606-619.

English HF, Santen RJ & Isaacs JT 1987 Response of glandular versus basal rat ventral prostatic epithelial cells to androgen withdrawal and replacement. *Prostate* **11** 229-242.

Eyler CE, Foo WC, LaFiura KM, McLendon RE, Hjelmeland AB & Rich JN 2008 Brain cancer stem cells display preferential sensitivity to Akt inhibition. *Stem Cells* **26** 3027-3036.

Ferri AL, Cavallaro M, Braida D, Di Cristofano A, Canta A, Vezzani A, Ottolenghi S, Pandolfi PP, Sala M, DeBiasi S & Nicolis SK 2004 Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain. *Development* **131** 3805-3819.

Fong H, Hohenstein KA & Donovan PJ 2008 Regulation of self-renewal and pluripotency by Sox2 in human embryonic stem cells. *Stem Cells* **26** 1931-1938.

Gan Y, Shi C, Inge L, Hibner M, Balducci J & Huang Y 2010 Differential roles of ERK and Akt pathways in regulation of EGFR-mediated signaling and motility in prostate cancer cells. *Oncogene* **29** 4947-4958.

Gangemi RM, Griffero F, Marubbi D, Perera M, Capra MC, Malatesta P, Ravetti GL, Zona GL, Daga A & Corte G 2009 SOX2 silencing in glioblastoma tumor-initiating cells causes stop of proliferation and loss of tumorigenicity. *Stem Cells* **27** 40-48.

Garcia de Palazzo IE, Adams GP, Sundareshan P, Wong AJ, Testa JR, Bigner DD & Weiner LM 1993 Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. *Cancer Res* **53** 3217-3220.

Garraway IP, Sun W, Tran CP, Perner S, Zhang B, Goldstein AS, Hahm SA, Haider M, Head CS, Reiter RE, Rubin MA & Witte ON 2010 Human prostate sphere-forming cells represent a subset of basal epithelial cells capable of glandular regeneration in vivo. *Prostate* **70** 491-501.

Gioeli D, Mandell JW, Petroni GR, Frierson HF, Jr. & Weber MJ 1999 Activation of mitogen-activated protein kinase associated with prostate cancer progression. *Cancer Res* **59** 279-284.

Goldstein AS, Huang J, Guo C, Garraway IP & Witte ON 2010 Identification of a cell of origin for human prostate cancer. *Science* **329** 568-571.

Graham V, Khudyakov J, Ellis P & Pevny L 2003 SOX2 functions to maintain neural progenitor identity. *Neuron* **39** 749-765.

Green CL, Loken M, Buck D & Deeg HJ 2000 Discordant expression of AC133 and AC141 in patients with myelodysplastic syndrome (MDS) and acute myelogeneous leukemia (AML). *Leukemia* **14** 770-772.

Griffero F, Daga A, Marubbi D, Capra MC, Melotti A, Pattarozzi A, Gatti M, Bajetto A, Porcile C, Barbieri F, Favoni RE, Lo Casto M, Zona G, Spaziante R, Florio T & Corte G 2009 Different response of human glioma tumor-initiating cells to epidermal growth factor receptor kinase inhibitors. *J Biol Chem* **284** 7138-7148.

Grignon DJ 2004 Unusual subtypes of prostate cancer. *Mod Pathol* **17** 316-327.

Grisanzio C & Signoretti S 2008 p63 in prostate biology and pathology. *J Cell Biochem* **103** 1354-1368.

Gu G, Yuan J, Wills M & Kasper S 2007 Prostate cancer cells with stem cell characteristics reconstitute the original human tumor in vivo. *Cancer Res* **67** 4807-4815.

Gupta PB, Fillmore CM, Jiang G, Shapira SD, Tao K, Kuperwasser C & Lander ES 2011 Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell* **146** 633-644.

Hagstrom SA, Pauer GJ, Reid J, Simpson E, Crowe S, Maumenee IH & Traboulsi EI 2005 SOX2 mutation causes anophthalmia, hearing loss, and brain anomalies. *Am J Med Genet A* **138A** 95-98.

Hanahan D & Weinberg RA 2000 The hallmarks of cancer. *Cell* **100** 57-70.

Hanahan D & Weinberg RA 2011 Hallmarks of cancer: the next generation. *Cell* **144** 646-674.

Harris RC, Chung E & Coffey RJ 2003 EGF receptor ligands. *Exp Cell Res* **284** 2-13.

Hayward SW, Rosen MA & Cunha GR 1997 Stromal-epithelial interactions in the normal and neoplastic prostate. *Br J Urol* **79 Suppl 2** 18-26.

Hemmati HD, Nakano I, Lazareff JA, Masterman-Smith M, Geschwind DH, Bronner-Fraser M & Kornblum HI 2003 Cancerous stem cells can arise from pediatric brain tumors. *Proc Natl Acad Sci U S A* **100** 15178-15183.

Hernes E, Fossa SD, Berner A, Otnes B & Nesland JM 2004 Expression of the epidermal growth factor receptor family in prostate carcinoma before and during androgen-independence. *Br J Cancer* **90** 449-454.

Hofer DR, Sherwood ER, Bromberg WD, Mendelsohn J, Lee C & Kozlowski JM 1991 Autonomous growth of androgen-independent human prostatic carcinoma cells: role of transforming growth factor alpha. *Cancer Res* **51** 2780-2785.

Hong SK, Kim JH, Lin MF & Park JI 2011 The Raf/MEK/extracellular signal-regulated kinase 1/2 pathway can mediate growth inhibitory and differentiation signaling via androgen receptor downregulation in prostate cancer cells. *Exp Cell Res* **317** 2671-2682.

Hu Q, Zhang L, Wen J, Wang S, Li M, Feng R, Yang X & Li L 2010 The EGF receptor-sox2-EGF receptor feedback loop positively regulates the self-renewal of neural precursor cells. *Stem Cells* **28** 279-286.

Humphrey PA 2007 Diagnosis of adenocarcinoma in prostate needle biopsy tissue. *J Clin Pathol* **60** 35-42.

Hunter T 2000 Signaling--2000 and beyond. *Cell* **100** 113-127.

Hynes NE & Lane HA 2005 ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer* **5** 341-354.

Inda MM, Bonavia R, Mukasa A, Narita Y, Sah DW, Vandenberg S, Brennan C, Johns TG, Bachoo R, Hadwiger P, Tan P, Depinho RA, Cavenee W & Furnari F 2010 Tumor heterogeneity is an active process maintained by a mutant EGFR-induced cytokine circuit in glioblastoma. *Genes Dev* **24** 1731-1745.

Jensen JB & Parmar M 2006 Strengths and limitations of the neurosphere culture system. *Mol Neurobiol* **34** 153-161.

Jia X, Li X, Xu Y, Zhang S, Mou W, Liu Y, Liu Y, Lv D, Liu CH, Tan X, Xiang R & Li N 2011 SOX2 promotes tumorigenesis and increases the anti-apoptotic property of human prostate cancer cell. *J Mol Cell Biol* **3** 230-238.

Kamachi Y, Uchikawa M & Kondoh H 2000 Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet* **16** 182-187.

Karnoub AE & Weinberg RA 2008 Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol* **9** 517-531.

Kelberman D, Rizzoti K, Avilion A, Bitner-Glindzicz M, Cianfarani S, Collins J, Chong WK, Kirk JM, Achermann JC, Ross R, Carmignac D, Lovell-Badge R, Robinson IC & Dattani MT 2006 Mutations within Sox2/SOX2 are associated with abnormalities in the hypothalamo-pituitary-gonadal axis in mice and humans. *J Clin Invest* **116** 2442-2455.

Keramari M, Razavi J, Ingman KA, Patsch C, Edenhofer F, Ward CM & Kimber SJ 2010 Sox2 is essential for formation of trophectoderm in the preimplantation embryo. *PLoS One* **5** e13952.

Kiernan AE, Pelling AL, Leung KK, Tang AS, Bell DM, Tease C, Lovell-Badge R, Steel KP & Cheah KS 2005 Sox2 is required for sensory organ development in the mammalian inner ear. *Nature* **434** 1031-1035.

Korkaya H, Paulson A, Charafe-Jauffret E, Ginestier C, Brown M, Dutcher J, Clouthier SG & Wicha MS 2009 Regulation of mammary stem/progenitor cells by PTEN/Akt/beta-catenin signaling. *PLoS Biol* **7** e1000121.

Kregel S, Kiriluk KJ, Rosen AM, Cai Y, Reyes EE, Otto KB, Tom W, Paner GP, Szmulewitz RZ & Vander Griend DJ 2013 Sox2 is an androgen receptor-repressed gene that promotes castration-resistant prostate cancer. *PLoS One* **8** e53701.

Krohn A, Diedler T, Burkhardt L, Mayer PS, De Silva C, Meyer-Kornblum M, Kotschau D, Tennstedt P, Huang J, Gerhauser C, Mader M, Kurtz S, Sirma H, Saad F, Steuber T, Graefen M, Plass C, Sauter G, Simon R, Minner S & Schlomm T 2012 Genomic deletion of PTEN is associated with tumor progression and early PSA recurrence in ERG fusion-positive and fusion-negative prostate cancer. *Am J Pathol* **181** 401-412.

Kurita T, Medina RT, Mills AA & Cunha GR 2004 Role of p63 and basal cells in the prostate. *Development* **131** 4955-4964.

Kuroda T, Tada M, Kubota H, Kimura H, Hatano SY, Suemori H, Nakatsuji N & Tada T 2005 Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. *Mol Cell Biol* **25** 2475-2485.

Lawson DA, Xin L, Lukacs RU, Cheng D & Witte ON 2007 Isolation and functional characterization of murine prostate stem cells. *Proc Natl Acad Sci U S A* **104** 181-186.

Lawson DA, Zong Y, Memarzadeh S, Xin L, Huang J & Witte ON 2010 Basal epithelial stem cells are efficient targets for prostate cancer initiation. *Proc Natl Acad Sci U S A* **107** 2610-2615.

Lee JT, Lehmann BD, Terrian DM, Chappell WH, Stivala F, Libra M, Martelli AM, Steelman LS & McCubrey JA 2008 Targeting prostate cancer based on signal transduction and cell cycle pathways. *Cell Cycle* **7** 1745-1762.

Leis O, Eguiara A, Lopez-Arribillaga E, Alberdi MJ, Hernandez-Garcia S, Elorriaga K, Pandiella A, Rezola R & Martin AG 2012 Sox2 expression in breast tumours and activation in breast cancer stem cells. *Oncogene* **31** 1354-1365.

Lemmon MA & Schlessinger J 2010 Cell signaling by receptor tyrosine kinases. *Cell* **141** 1117-1134.

Lengner CJ, Camargo FD, Hochedlinger K, Welstead GG, Zaidi S, Gokhale S, Scholer HR, Tomilin A & Jaenisch R 2007 Oct4 expression is not required for mouse somatic stem cell self-renewal. *Cell Stem Cell* **1** 403-415.

Leong KG, Wang BE, Johnson L & Gao WQ 2008 Generation of a prostate from a single adult stem cell. *Nature* **456** 804-808.

Li DM & Sun H 1997 TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res* **57** 2124-2129.

Li H, Zhou J, Miki J, Furusato B, Gu Y, Srivastava S, McLeod DG, Vogel JC & Rhim JS 2008 Telomerase-immortalized non-malignant human prostate epithelial cells retain the properties of multipotent stem cells. *Exp Cell Res* **314** 92-102.

Li J, Pan G, Cui K, Liu Y, Xu S & Pei D 2007 A dominant-negative form of mouse SOX2 induces trophectoderm differentiation and progressive polyploidy in mouse embryonic stem cells. *J Biol Chem* **282** 19481-19492.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH & Parsons R 1997 PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275** 1943-1947.

Li N, Batzer A, Daly R, Yajnik V, Skolnik E, Chardin P, Bar-Sagi D, Margolis B & Schlessinger J 1993 Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. *Nature* **363** 85-88.

Lin Y, Yang Y, Li W, Chen Q, Li J, Pan X, Zhou L, Liu C, Chen C, He J, Cao H, Yao H, Zheng L, Xu X, Xia Z, Ren J, Xiao L, Li L, Shen B, Zhou H & Wang YJ 2012 Reciprocal regulation of Akt and Oct4 promotes the self-renewal and survival of embryonal carcinoma cells. *Mol Cell* **48** 627-640.

Liu AY & True LD 2002 Characterization of prostate cell types by CD cell surface molecules. *Am J Pathol* **160** 37-43.

Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, Patrawala L, Yan H, Jeter C, Honorio S, Wiggins JF, Bader AG, Fagin R, Brown D & Tang DG 2011a The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. *Nat Med* **17** 211-215.

Liu J, Pascal LE, Isharwal S, Metzger D, Ramos Garcia R, Pilch J, Kasper S, Williams K, Basse PH, Nelson JB, Chambon P & Wang Z 2011b Regenerated luminal epithelial cells are derived from preexisting luminal epithelial cells in adult mouse prostate. *Mol Endocrinol* **25** 1849-1857.

Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT & Perou CM 2006 Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. *Mod Pathol* **19** 264-271.

Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B & Ng HH 2006 The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet* **38** 431-440.

Lotan TL, Gurel B, Sutcliffe S, Esopi D, Liu W, Xu J, Hicks JL, Park BH, Humphreys E, Partin AW, Han M, Netto GJ, Isaacs WB & De Marzo AM 2011 PTEN protein loss by immunostaining: analytic validation and prognostic indicator for a high risk surgical cohort of prostate cancer patients. *Clin Cancer Res* **17** 6563-6573.

Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, Ullrich A, Skolnik EY, Bar-Sagi D & Schlessinger J 1992 The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. *Cell* **70** 431-442.

MacDonald A, Chisholm GD & Habib FK 1990 Production and response of a human prostatic cancer line to transforming growth factor-like molecules. *Br J Cancer* **62** 579-584.

Macintosh CA, Stower M, Reid N & Maitland NJ 1998 Precise microdissection of human prostate cancers reveals genotypic heterogeneity. *Cancer Res* **58** 23-28.

Magee JA, Piskounova E & Morrison SJ 2012 Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell* **21** 283-296.

Maier S, Wilbertz T, Braun M, Scheble V, Reischl M, Mikut R, Menon R, Nikolov P, Petersen K, Beschorner C, Moch H, Kakies C, Protzel C, Bauer J, Soltermann A, Fend F, Staebler A, Lengerke C & Perner S 2011 SOX2 amplification is a common event in squamous cell carcinomas of different organ sites. *Hum Pathol* **42** 1078-1088.

Mak AB, Blakely KM, Williams RA, Penttila PA, Shukalyuk AI, Osman KT, Kasimer D, Ketela T & Moffat J 2011 CD133 protein N-glycosylation processing contributes to cell surface recognition of the primitive cell marker AC133 epitope. *J Biol Chem* **286** 41046-41056.

Masui S, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, Okochi H, Okuda A, Matoba R, Sharov AA, Ko MS & Niwa H 2007 Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. *Nat Cell Biol* **9** 625-635.

Maygarden SJ, Strom S & Ware JL 1992 Localization of epidermal growth factor receptor by immunohistochemical methods in human prostatic carcinoma, prostatic intraepithelial neoplasia, and benign hyperplasia. *Arch Pathol Lab Med* **116** 269-273.

Mazzoleni S, Politi LS, Pala M, Cominelli M, Franzin A, Sergi Sergi L, Falini A, De Palma M, Bulfone A, Poliani PL & Galli R 2010 Epidermal growth factor receptor expression identifies functionally and molecularly distinct tumor-initiating cells in human glioblastoma multiforme and is required for gliomagenesis. *Cancer Res* **70** 7500-7513.

McCubrey JA, Steelman LS, Abrams SL, Lee JT, Chang F, Bertrand FE, Navolanic PM, Terrian DM, Franklin RA, D'Assoro AB, Salisbury JL, Mazzarino MC, Stivala F & Libra M 2006 Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv Enzyme Regul* **46** 249-279.

McNeal JE & Bostwick DG 1986 Intraductal dysplasia: a premalignant lesion of the prostate. *Hum Pathol* **17** 64-71.

Miki J, Furusato B, Li H, Gu Y, Takahashi H, Egawa S, Sesterhenn IA, McLeod DG, Srivastava S & Rhim JS 2007 Identification of putative stem cell markers, CD133 and CXCR4, in hTERT-immortalized primary nonmalignant and malignant tumor-derived human prostate epithelial cell lines and in prostate cancer specimens. *Cancer Res* **67** 3153-3161.

Miller GJ & Cygan JM 1994 Morphology of prostate cancer: the effects of multifocality on histological grade, tumor volume and capsule penetration. *J Urol* **152** 1709-1713.

Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, Bray RA, Waller EK & Buck DW 1997 A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood* **90** 5013-5021.

Moodie SA, Willumsen BM, Weber MJ & Wolfman A 1993 Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* **260** 1658-1661.

Moon C, Park JC, Chae YK, Yun JH & Kim S 2008 Current status of experimental therapeutics for prostate cancer. *Cancer Lett* **266** 116-134.

Morrison SJ & Spradling AC 2008 Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell* **132** 598-611.

Moscatello DK, Holgado-Madruga M, Godwin AK, Ramirez G, Gunn G, Zoltick PW, Biegel JA, Hayes RL & Wong AJ 1995 Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res* **55** 5536-5539.

Mulholland DJ, Kobayashi N, Ruscetti M, Zhi A, Tran LM, Huang J, Gleave M & Wu H 2012 Pten loss and RAS/MAPK activation cooperate to promote EMT and metastasis initiated from prostate cancer stem/progenitor cells. *Cancer Res* **72** 1878-1889.

Nielsen JS, Jakobsen E, Holund B, Bertelsen K & Jakobsen A 2004 Prognostic significance of p53, Her-2, and EGFR overexpression in borderline and epithelial ovarian cancer. *Int J Gynecol Cancer* **14** 1086-1096.

Noh KH, Kim BW, Song KH, Cho H, Lee YH, Kim JH, Chung JY, Kim JH, Hewitt SM, Seong SY, Mao CP, Wu TC & Kim TW 2012 Nanog signaling in cancer promotes stem-like phenotype and immune evasion. *J Clin Invest* **122** 4077-4093.

Nowling TK, Johnson LR, Wiebe MS & Rizzino A 2000 Identification of the transactivation domain of the transcription factor Sox-2 and an associated co-activator. *J Biol Chem* **275** 3810-3818.

O'Brien CA, Pollett A, Gallinger S & Dick JE 2007 A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* **445** 106-110.

Okubo T, Pevny LH & Hogan BL 2006 Sox2 is required for development of taste bud sensory cells. *Genes Dev* **20** 2654-2659.

Okumura-Nakanishi S, Saito M, Niwa H & Ishikawa F 2005 Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells. *J Biol Chem* **280** 5307-5317.

Olapade-Olaopa EO, Moscatello DK, MacKay EH, Horsburgh T, Sandhu DP, Terry TR, Wong AJ & Habib FK 2000 Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer. *Br J Cancer* **82** 186-194.

Oldridge EE, Pellacani D, Collins AT & Maitland NJ 2012 Prostate cancer stem cells: are they androgen-responsive? *Mol Cell Endocrinol* **360** 14-24.

Pan G, Li J, Zhou Y, Zheng H & Pei D 2006 A negative feedback loop of transcription factors that controls stem cell pluripotency and self-renewal. *Faseb J* **20** 1730-1732.

Pardal R, Clarke MF & Morrison SJ 2003 Applying the principles of stem-cell biology to cancer. *Nat Rev Cancer* **3** 895-902.

Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW & Daley GQ 2008 Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451** 141-146.

Pastrana E, Silva-Vargas V & Doetsch F 2011 Eyes wide open: a critical review of sphere-formation as an assay for stem cells. *Cell Stem Cell* **8** 486-498.

Patrawala L, Calhoun-Davis T, Schneider-Broussard R & Tang DG 2007 Hierarchical organization of prostate cancer cells in xenograft tumors: the CD44+alpha2beta1+ cell population is enriched in tumor-initiating cells. *Cancer Res* **67** 6796-6805.

Patrawala L, Calhoun T, Schneider-Broussard R, Li H, Bhatia B, Tang S, Reilly JG, Chandra D, Zhou J, Claypool K, Coghlan L & Tang DG 2006 Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene* **25** 1696-1708.

Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE & Parsons R 1999 Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc Natl Acad Sci U S A* **96** 1563-1568.

Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA & Daidone MG 2005 Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* **65** 5506-5511.

Que J, Okubo T, Goldenring JR, Nam KT, Kurotani R, Morrisey EE, Taranova O, Pevny LH & Hogan BL 2007 Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development* **134** 2521-2531.

Ragge NK, Lorenz B, Schneider A, Bushby K, de Sanctis L, de Sanctis U, Salt A, Collin JR, Vivian AJ, Free SL, Thompson P, Williamson KA, Sisodiya SM, van Heyningen V & Fitzpatrick DR 2005 SOX2 anophthalmia syndrome. *Am J Med Genet A* **135** 1-7; discussion 8.

Rajasekhar VK, Studer L, Gerald W, Socci ND & Scher HI 2011 Tumour-initiating stem-like cells in human prostate cancer exhibit increased NF-kappaB signalling. *Nat Commun* **2** 162.

Reiter JL, Threadgill DW, Eley GD, Strunk KE, Danielsen AJ, Sinclair CS, Pearsall RS, Green PJ, Yee D, Lampland AL, Balasubramaniam S, Crossley TD, Magnuson TR, James CD & Maihle NJ 2001 Comparative genomic sequence analysis and isolation of human and mouse alternative EGFR transcripts encoding truncated receptor isoforms. *Genomics* **71** 1-20.

Reya T, Morrison SJ, Clarke MF & Weissman IL 2001 Stem cells, cancer, and cancer stem cells. *Nature* **414** 105-111.

Reynolds BA & Rietze RL 2005 Neural stem cells and neurospheres--re-evaluating the relationship. *Nat Methods* **2** 333-336.

Reynolds BA & Weiss S 1996 Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* **175** 1-13.

Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C & De Maria R 2007 Identification and expansion of human colon-cancer-initiating cells. *Nature* **445** 111-115.

Richardson GD, Robson CN, Lang SH, Neal DE, Maitland NJ & Collins AT 2004 CD133, a novel marker for human prostatic epithelial stem cells. *J Cell Sci* **117** 3539-3545.

Ring KL, Tong LM, Balestra ME, Javier R, Andrews-Zwilling Y, Li G, Walker D, Zhang WR, Kreitzer AC & Huang Y 2012 Direct reprogramming of mouse and human fibroblasts into multipotent neural stem cells with a single factor. *Cell Stem Cell* **11** 100-109.

Robbins DJ, Zhen E, Owaki H, Vanderbilt CA, Ebert D, Geppert TD & Cobb MH 1993 Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. *J Biol Chem* **268** 5097-5106.

Rodriguez-Pinilla SM, Sarrio D, Moreno-Bueno G, Rodriguez-Gil Y, Martinez MA, Hernandez L, Hardisson D, Reis-Filho JS & Palacios J 2007 Sox2: a possible driver of the basal-like phenotype in sporadic breast cancer. *Mod Pathol* **20** 474-481.

Roskoski R, Jr. 2012a ERK1/2 MAP kinases: structure, function, and regulation. *Pharmacol Res* **66** 105-143.

Roskoski R, Jr. 2012b MEK1/2 dual-specificity protein kinases: structure and regulation. *Biochem Biophys Res Commun* **417** 5-10.

Ross JS 2007 The androgen receptor in prostate cancer: therapy target in search of an integrated diagnostic test. *Adv Anat Pathol* **14** 353-357.

Rozakis-Adcock M, Fernley R, Wade J, Pawson T & Bowtell D 1993 The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. *Nature* **363** 83-85.

Rudin CM, Durinck S, Stawiski EW, Poirier JT, Modrusan Z, Shames DS, Bergbower EA, Guan Y, Shin J, Guillory J, Rivers CS, Foo CK, Bhatt D, Stinson J, Gnad F, Haverty PM, Gentleman R, Chaudhuri S, Janakiraman V, Jaiswal BS, Parikh C, Yuan W, Zhang Z, Koeppen H, Wu TD, Stern HM, Yauch RL, Huffman KE, Paskulin DD, Illei PB, Varella-Garcia M, Gazdar AF, de Sauvage FJ, Bourgon R, Minna JD, Brock MV & Seshagiri S 2012 Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer. *Nat Genet* **44** 1111-1116.

Sarbassov DD, Guertin DA, Ali SM & Sabatini DM 2005 Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307** 1098-1101.

Sarkar A & Hochedlinger K 2013 The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. *Cell Stem Cell* **12** 15-30.

Sattler HP, Lensch R, Rohde V, Zimmer E, Meese E, Bonkhoff H, Retz M, Zwergel T, Bex A, Stoeckle M & Wullich B 2000 Novel amplification unit at chromosome 3q25-q27 in human prostate cancer. *Prostate* **45** 207-215.

Schmitz M, Temme A, Senner V, Ebner R, Schwind S, Stevanovic S, Wehner R, Schackert G, Schackert HK, Fussel M, Bachmann M, Rieber EP & Weigle B 2007 Identification of SOX2 as a novel glioma-associated antigen and potential target for T cell-based immunotherapy. *Br J Cancer* **96** 1293-1301.

Shah RB, Ghosh D & Elder JT 2006 Epidermal growth factor receptor (ErbB1) expression in prostate cancer progression: correlation with androgen independence. *Prostate* **66** 1437-1444.

Sharpe B, Beresford M, Bowen R, Mitchard J & Chalmers AD 2013 Searching for Prostate Cancer Stem Cells: Markers and Methods. *Stem Cell Rev*.

Shen MM & Abate-Shen C 2010 Molecular genetics of prostate cancer: new prospects for old challenges. *Genes Dev* **24** 1967-2000.

Sherwood ER, Berg LA, Mitchell NJ, McNeal JE, Kozlowski JM & Lee C 1990 Differential cytokeratin expression in normal, hyperplastic and malignant epithelial cells from human prostate. *J Urol* **143** 167-171.

Sherwood ER, Van Dongen JL, Wood CG, Liao S, Kozlowski JM & Lee C 1998 Epidermal growth factor receptor activation in androgen-independent but not androgen-stimulated growth of human prostatic carcinoma cells. *Br J Cancer* **77** 855-861.

Siegel R, Naishadham D & Jemal A 2012 Cancer statistics, 2012. *CA Cancer J Clin* **62** 10-29.

Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J & Dirks PB 2003 Identification of a cancer stem cell in human brain tumors. *Cancer Res* **63** 5821-5828.

Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD & Dirks PB 2004 Identification of human brain tumour initiating cells. *Nature* **432** 396-401.

Sneddon JB, Borowiak M & Melton DA 2012 Self-renewal of embryonic-stem-cell-derived progenitors by organ-matched mesenchyme. *Nature* **491** 765-768.

Soeda A, Inagaki A, Oka N, Ikegame Y, Aoki H, Yoshimura S, Nakashima S, Kunisada T & Iwama T 2008 Epidermal growth factor plays a crucial role in mitogenic regulation of human brain tumor stem cells. *J Biol Chem* **283** 10958-10966.

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP & Mak TW 1998 Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95** 29-39.

Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH & Tavtigian SV 1997 Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* **15** 356-362.

Stolzenburg S, Rots MG, Beltran AS, Rivenbark AG, Yuan X, Qian H, Strahl BD & Blancafort P 2012 Targeted silencing of the oncogenic transcription factor SOX2 in breast cancer. *Nucleic Acids Res* **40** 6725-6740.

Stone KR, Mickey DD, Wunderli H, Mickey GH & Paulson DF 1978 Isolation of a human prostate carcinoma cell line (DU 145). *Int J Cancer* **21** 274-281.

Sugimura Y, Cunha GR & Donjacour AA 1986 Morphological and histological study of castration-induced degeneration and androgen-induced regeneration in the mouse prostate. *Biol Reprod* **34** 973-983.

Sunayama J, Matsuda K, Sato A, Tachibana K, Suzuki K, Narita Y, Shibui S, Sakurada K, Kayama T, Tomiyama A & Kitanaka C 2010 Crosstalk between the PI3K/mTOR and MEK/ERK pathways involved in the maintenance of self-renewal and tumorigenicity of glioblastoma stem-like cells. *Stem Cells* **28** 1930-1939.

Suva ML, Riggi N & Bernstein BE 2013 Epigenetic reprogramming in cancer. *Science* **339** 1567-1570.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K & Yamanaka S 2007 Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131** 861-872.

Taranova OV, Magness ST, Fagan BM, Wu Y, Surzenko N, Hutton SR & Pevny LH 2006 SOX2 is a dose-dependent regulator of retinal neural progenitor competence. *Genes Dev* **20** 1187-1202.

Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, Arora VK, Kaushik P, Cerami E, Reva B, Antipin Y, Mitsiades N, Landers T, Dolgalev I, Major JE, Wilson M, Socci ND, Lash AE, Heguy A, Eastham JA, Scher HI, Reuter VE, Scardino PT, Sander C, Sawyers CL & Gerald WL 2010 Integrative genomic profiling of human prostate cancer. *Cancer Cell* **18** 11-22.

Taylor RA, Toivanen R, Frydenberg M, Pedersen J, Harewood L, Australian Prostate Cancer B, Collins AT, Maitland NJ & Risbridger GP 2012 Human epithelial basal cells are cells of origin of prostate cancer, independent of CD133 status. *Stem Cells* **30** 1087-1096.

Traish AM & Morgentaler A 2009 Epidermal growth factor receptor expression escapes androgen regulation in prostate cancer: a potential molecular switch for tumour growth. *Br J Cancer* **101** 1949-1956.

Ugolkov AV, Eisengart LJ, Luan C & Yang XJ 2011 Expression analysis of putative stem cell markers in human benign and malignant prostate. *Prostate* **71** 18-25.

Visvader JE & Lindeman GJ 2008 Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* **8** 755-768.

Visvader JE & Lindeman GJ 2012 Cancer stem cells: current status and evolving complexities. *Cell Stem Cell* **10** 717-728.

Wagers AJ & Weissman IL 2004 Plasticity of adult stem cells. *Cell* **116** 639-648.

Wang S, Gao J, Lei Q, Rozengurt N, Pritchard C, Jiao J, Thomas GV, Li G, Roy-Burman P, Nelson PS, Liu X & Wu H 2003 Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* **4** 209-221.

Wang X, Kruithof-de Julio M, Economides KD, Walker D, Yu H, Halili MV, Hu YP, Price SM, Abate-Shen C & Shen MM 2009 A luminal epithelial stem cell that is a cell of origin for prostate cancer. *Nature* **461** 495-500.

Wang ZA, Mitrofanova A, Bergren SK, Abate-Shen C, Cardiff RD, Califano A & Shen MM 2013 Lineage analysis of basal epithelial cells reveals their unexpected plasticity and supports a cell-of-origin model for prostate cancer heterogeneity. *Nat Cell Biol* **15** 274-283.

Warne PH, Viciana PR & Downward J 1993 Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. *Nature* **364** 352-355.

Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE & Jaenisch R 2007 In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* **448** 318-324.

Wheeler DL, Dunn EF & Harari PM 2010 Understanding resistance to EGFR inhibitors-impact on future treatment strategies. *Nat Rev Clin Oncol* **7** 493-507.

Williams H & Powell IJ 2009 Epidemiology, pathology, and genetics of prostate cancer among African Americans compared with other ethnicities. *Methods Mol Biol* **472** 439-453.

Williamson KA, Hever AM, Rainger J, Rogers RC, Magee A, Fiedler Z, Keng WT, Sharkey FH, McGill N, Hill CJ, Schneider A, Messina M, Turnpenny PD, Fantes JA, van Heyningen V & FitzPatrick DR 2006 Mutations in SOX2 cause anophthalmia-esophageal-genital (AEG) syndrome. *Hum Mol Genet* **15** 1413-1422.

Wong AJ, Ruppert JM, Bigner SH, Grzeschik CH, Humphrey PA, Bigner DS & Vogelstein B 1992 Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proc Natl Acad Sci U S A* **89** 2965-2969.

Xin L, Ide H, Kim Y, Dubey P & Witte ON 2003 In vivo regeneration of murine prostate from dissociated cell populations of postnatal epithelia and urogenital sinus mesenchyme. *Proc Natl Acad Sci U S A* **100 Suppl 1** 11896-11903.

Xue Y, Smedts F, Debruyne FM, de la Rosette JJ & Schalken JA 1998 Identification of intermediate cell types by keratin expression in the developing human prostate. *Prostate* **34** 292-301.

Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, Andrews NC, Caput D & McKeon F 1998 p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* **2** 305-316.

Yang J, Liao D, Chen C, Liu Y, Chuang TH, Xiang R, Markowitz D, Reisfeld RA & Luo Y 2013 Tumor-Associated Macrophages Regulate Murine Breast Cancer Stem Cells Through a Novel Paracrine EGFR/Stat3/Sox-2 Signaling Pathway. *Stem Cells* **31** 248-258.

Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, Olweus J, Kearney J & Buck DW 1997 AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* **90** 5002-5012.

Yoon S & Seger R 2006 The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions. *Growth Factors* **24** 21-44.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin, II & Thomson JA 2007 Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318** 1917-1920.

Zhang XF, Settleman J, Kyriakis JM, Takeuchi-Suzuki E, Elledge SJ, Marshall MS, Bruder JT, Rapp UR & Avruch J 1993 Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. *Nature* **364** 308-313.

Zhao RC, Zhu YS & Shi Y 2008 New hope for cancer treatment: exploring the distinction between normal adult stem cells and cancer stem cells. *Pharmacol Ther* **119** 74-82.

Zheng CF & Guan KL 1994 Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. *Embo J* **13** 1123-1131.

Zhou J, Wulfkuhle J, Zhang H, Gu P, Yang Y, Deng J, Margolick JB, Liotta LA, Petricoin E, 3rd & Zhang Y 2007 Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance. *Proc Natl Acad Sci U S A* **104** 16158-16163.

APPENDICES

Appendix 1 – Figure 1.

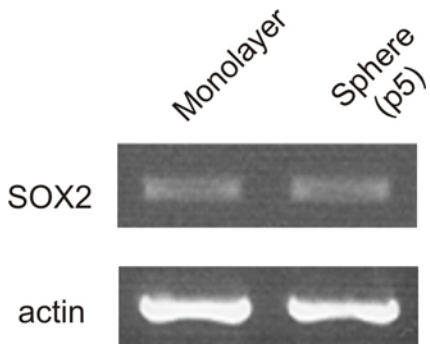


Figure 1. Detection of *SOX2* mRNA in DU145 monolayer cells and low passage DU145 sphere cells. Reverse transcriptase (RT)-PCR was carried out on RNA isolated from DU145 monolayer and low passage (passage 5; p5) DU145 sphere cells. RT-PCR conditions were carried out as described in Chapter 2. Agarose gel electrophoresis was conducted, and the ethidium bromide-stained PCR products were visualized under ultraviolet (UV) light.

Appendix 2 – Figure 2.

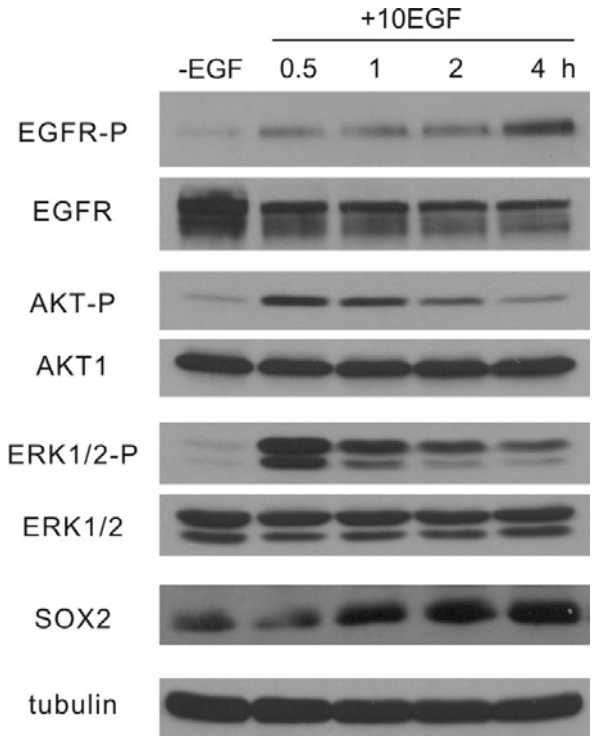


Figure 2. EGF treatment of DU145 PCSCs promotes EGFR signalling and downstream MAPK (ERK) and AKT signal activation. Low passage DU145 spheres, cultured and maintained in EGF-free serum-free media containing 0.4% BSA and 0.2× B27 (-EGF), were treated with serum-free media containing 10 ng/ml EGF (10EGF) and whole cell lysates were prepared at different time points (hours; h) post-treatment. Western blot analysis of EGF-treated spheres demonstrated increased EGFR (Tyr1068 phosphorylation; EGFR-P), ERK1/2 (ERK1 Thr202/Tyr204 phosphorylation, ERK2 Thr185/Tyr187 phosphorylation; ERK1/2-P) and AKT (Ser473 phosphorylation; AKT-P) signal activation occurring in a time-dependent manner, along with increased SOX2 protein levels. For each sample, a total of 100 µg of whole cell lysate was used. Antibodies used for Western blot analysis are described in Chapters 2, 3 and 4.

Appendix 3 – License Agreement.

ELSEVIER LICENSE
TERMS AND CONDITIONS

May 31, 2013

This is a License Agreement between Adrian P. Rybak ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier

Elsevier Limited
The Boulevard,Langford Lane
Kidlington,Oxford,OX5 1GB,UK

Registered Company Number

1982084

Customer name

Adrian P. Rybak

Customer address

273 Glenholme Ave.

Hamilton, ON L8K3M1

License number

3159620427720

License date

May 31, 2013

Licensed content publisher

Elsevier

Licensed content publication

Biochimica et Biophysica Acta (BBA) - Molecular Cell Research

Licensed content title

Characterization of sphere-propagating cells with stem-like properties from DU145 prostate cancer cells

Licensed content author

Adrian P. Rybak,Lizhi He,Anil Kapoor,Jean-Claude Cutz,Damu Tang

Licensed content date

May 2011

Licensed content volume number

1813

Licensed content issue number

5

Number of pages

12

Start Page

683

End Page

694

Type of Use

reuse in a thesis/dissertation

Portion

full article

Format

both print and electronic

Are you the author of this Elsevier article?

Yes

Will you be translating?

No

Order reference number

Title of your thesis/dissertation

Characterization of a population of tumour-initiating cells with stem-like properties in human prostate cancer

Expected completion date

Aug 2013

Estimated size (number of pages)

230

Elsevier VAT number

GB 494 6272 12

Permissions price

0.00 USD

VAT/Local Sales Tax

0.0 USD / 0.0 GBP

Total

0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE

SOCIETY COPYRIGHT OWNER].” Also Lancet special credit - “Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier.”

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website**: The following terms and conditions apply to electronic reserve and author websites:
Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:
This license was made in connection with a course,
This permission is granted for 1 year only. You may obtain a license for future website posting,
All content posted to the web site must maintain the copyright information line on the bottom of each image,
A hyper-text must be included to the Homepage of the journal from which you are licensing athttp://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books athttp://www.elsevier.com , and

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and the permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version. A hyper-text must be included to the Homepage of the journal from which you are licensing athttp://www.sciencedirect.com/science/journal/xxxxx . As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. **Author website** for books with the following additional clauses:
Authors are permitted to place a brief summary of their work online only.
A hyper-text must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. **Website** (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing
at http://www.sciencedirect.com/science/journal/xxxxx. or for books to the Elsevier homepage at http://www.elsevier.com

20. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. **Other Conditions**:

v1.6

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK501033641.
Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support:customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.