Investigation of the Epigenetic Regulators and Signaling Pathways influencing Pluripotency Inducing Transcription Factors mediated Tumorigenesis

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Investigation of the Epigenetic Regulators and Signaling Pathways influencing Pluripotency Inducing Transcription Factors mediated Tumorigenesis

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by

Swayamsiddha Kar
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and

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This is to certify that the work presented in the thesis entitled "*Investigation of the epigenetic regulators and signaling pathways influencing pluripotency inducing transcription factors mediated tumorigenesis*" submitted by *Swayamsiddha Kar,* Roll Number 511LS103, is a record of original research carried out by her under our supervision and guidance in partial fulfillment of the requirements of the degree of *Doctor of Philosophy* in *Life Science.* Neither this thesis nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

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I dedicate this thesis to my Mother

Mrs. Sasmita Mishra

Because for being the friend, the support and the mom that you are,

I cannot thank you enough…..

Swayamsiddha Kar

January 20, 2017

Declaration of Originality

I, *Swayamsiddha Kar*, Roll Number 511LS103 hereby declare that this thesis entitled "*Investigation of the epigenetic regulators and signaling pathways influencing pluripotency inducing transcription factors mediated tumorigenesis"* presents my original work carried out as a doctoral student of NIT Rourkela and, to the best of my knowledge, contains no material previously published or written by another person, nor any material presented by me for the award of any degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the thesis. Works of other authors cited in this thesis have been duly acknowledged under the sections "Reference". I have also submitted my original research records to the scrutiny committee for evaluation of my thesis.

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Abstract

Pluripotency inducing transcription factors (TFs) Oct4, Sox2 and Nanog under the influence of epigenetic modifications (especially, DNA methylation and histone H3 modifications) and signaling pathways work stringently to guard stem cell pluripotency and smoothly manoeuvre transition between differential gene expression states during both normal and pathological conditions. The present work is undertaken to investigate the influence of epigenetic regulators and signaling pathways on pluripotency inducing TFs during tumorigenesis. The expression profile of Oct4, Sox2 and Nanog in breast and prostate cancer along with epigenetic regulatory enzymes (DNA methyltransferases (DNMTs), histone methyltransferases (HMTs) and histone deacetylases (HDACs) predominantly active in controlling the expression of these TFs are studied. Furthermore, hedgehog (HH) signaling pathway mediated regulation of Oct4, Sox2 and Nanog is also examined. Oct4, Sox2 and Nanog are over-expressed both at transcript (mRNA) and protein level in a stage-specific manner in both cancers. The over-expression of Oct4, Sox2 and Nanog is associated with enhanced tumorigenic potential as is evident from reduction in cell proliferation, decrease in cell migration and invasive potential, cell cycle arrest at G_1 phase and increase in apoptotic population upon silencing of these factors via si-RNA. Upon investigating the epigenetic regulatory mechanism controlling their over-expression, it was found that active histone modifications H3K4me3 and H3K9AcS10p in promoters of Oct4 and Sox2 predominantly up-regulate expression of these genes in both cancers whereas promoter DNA methylation is not effective. Alongside these marks, it is also seen that HH-Sox2 axis is active in prostate cancer and mediates androgen independent prostate cancer. As researchers and oncologists are struggling to find a successful treatment approach for metastatically aggressive malignancies, unravelling the epigenetic machinery and allied signaling pathways controlling transcriptional network of a cancer cell will be one step forward in this endeavour.

Keywords: Pluripotency; Epigenetics; Transcription Factors; Breast Cancer; Prostate Cancer; Signaling Pathway

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Abbreviations

ABCB1	:	ATP-Binding Cassette Sub-Family B Member 1
ABCG2	:	ATP-Binding Cassette Sub-Family G Member 2
ACF	:	Aberrant Crypt Foci
AMPK	:	Adenosine Monophosphate-Activated Protein Kinase
APC	:	Adenomatous Polyposis Coli
AZA	:	5-Aza-2'-deoxycytidine
Bax	:	BCL2-associated X protein
BCR–ABL	:	Breakpoint Cluster Region–Abelson Murine Leukemia
BCL-2	:	B-Cell Lymphoma-2
BMP4	:	Bone Morphogenetic Protein 4
BRCA1	:	Breast Cancer 1
CCND1/2	:	Cyclin D1/D2
CDK2AP1	:	Cyclin Dependent Kinase 2 Associated Protein 1
CDKN2A/2B	:	Cyclin Dependent Kinase Inhibitor 2A /2B
CDH1	:	Cadherin 1
CDK1	:	Cyclin Dependent Kinase 1
CDX2	:	Caudal-Related Homeobox 2
CGI	:	CpG Island
CNS	:	Central Nervous System
COUP-TFI/II	:	COUP Transcription Factor 1/2
CSC	:	Cancer Stem Cells
CT	:	Cancer-Testis
DAB	:	3,3′-Diaminobenzidine tetrahydrochloride
DAPI	:	4',6-Diamidino-2-Phenylindole, Di-acetate
DAPK1	:	Death Associated Protein Kinase1
DAX1	:	Dosage Sensitive Sex-Reversal (DSS), Adrenal Hypoplasia Congenita (AHC) Critical Region on the X-Chromosome, Gene 1
DDR	:	DNA Damage Responses
DE	:	Distal Enhancer
DHH	:	Desert Hedgehog
DMEM	:	Dulbecco’s Modified Eagle’s Medium
DMSO	:	Dimethyl Sulphoxide
DNMT1/3A/B	:	DNA Methyltransferase 1/3A/3B
DOT1	:	Disruptor of Telomeric Silencing 1
DP	:	Dermal Papilla
DZNeP	:	3-Deazaneplanocin A

ECC	:	Embryonic Carcinoma Cell
EGFR	:	Epidermal Growth Factor Receptor
EHMT2/G9A	:	Euchromatic Histone-Lysine N-Methyltransferase 2
EMT	:	Epithelial to Mesenchymal Transition
EphB	:	Ephrin Type-B Receptor
esBAF	:	Embryonic Specific BRG1- or HRBM-Associated Factors
ESC	:	Embryonic Stem Cells
ESRRB	:	Estrogen-Related Receptor B
EZH2	:	Enhancer of Zeste Homologue 2
F-12	:	Nutrient Mixture F-12 Ham (Kaighn's Modification) Medium
FACS	:	Fluorescence Activated Cell Sorter
FAK	:	Focal Adhesion Kinase
FBS	:	Fetal Bovine Serum
FFPE	:	Formalin Fixed Paraffin Embedded
FGF4	:	Fibroblast Growth Factor 4
FGF8	:	Fibroblast Growth Factor 8
FITC	:	Fluorescein Isothiocyanate
FOX3A	:	Forkhead Box 3A
FOXA2	:	Forkhead Box A2
FOXB1	:	Forkhead Box B1
FOXD3	:	Forkhead Box Protein D3
FOXJ1	:	Forkhead Box J1
FOXO1	:	Forkhead Box Protein O1
FOXP1	:	Forkhead Box Protein P1
GAPDH	:	Glyceraldehyde-3-Phosphate Dehydrogenase
GATA6	:	GATA-Binding Factor 6
GCNF	:	Germ Cell Nuclear Factor
GDF3	:	Growth Differentiation Factor3
GKM	:	Gate Keeper Mutations
GSK3	:	Glycogen Synthase Kinase 3
GSTP1	:	Glutathione S-Transferase Pi 1
H3K14Ac	:	H3 Lysine14 Acetylation
H3K14Ac2	:	H3 Lysine 14 Di-Acetylation
H3K27me3	:	H3 Lysine 27 Tri-Methylation
H3K36me1/2	:	H3 Lysine36 Mono And Di-Methylation
H3K4me1	:	H3 Lysine4 Mono-Methylation
H3K4me2	:	H3 Lysine 4 Di-Methylation
H3K4me3	:	H3 Lysine 4 Tri-Methylation

H3K4MT	:	H3 Lysine4 Methyl Transferase
H3K64me3	:	H3 Lysine64 Trimethylation
H3K9Ac	:	H3 Lysine 9 Acetylation
H3K9Ac2	:	H3 Lysine 9 Di-Acetylation
H3K9AcS10p	:	H3 Lysine9 Acetylation Serine10 Phosphorylation
H3K9me2	:	H3 Lysine9 Di-Methylation
H4K12Ac	:	H4 Lysine 12 Acetylation
HAT	:	Histone Acetyltransferase
HDAC	:	Histone Deacetylases
HH	:	Hedgehog
HP1	:	Heterochromatin Protein 1
ICM	:	Inner Cell Mass
IDH	:	Isocitrate Dehydrogenase
IGF2	:	Insulin Growth Factor 2
IHH	:	Indian Hedgehog
iPSC	:	Induced Pluripotent Stem Cells
JAK/STAT3	:	Janus Kinase/Signal Transducers And Activators of Transcription 3
KAT2A/6A	:	K(lysine) Acetyltransferase 2A/6A
KDM	:	Lysine (K)-specific Demethylase
KLF4	:	Kruppel-Like Factor 4
KMT	:	Lysine (K)-specific Methyltransferase
L-15	:	Leibovitz's L-15 Medium
LIF	:	Leukemia Inhibitory Factor
LRH1	:	Liver Receptor Homolog 1
LSD1	:	Lysine Specific Demethylase1
MASPIN	:	Mammary Serine Protease Inhibitor
MAGE	:	Melanoma Associated Antigen
MBD3	:	Methyl-Binding Domain 3
MCF-7	:	Michigan Cancer Foundation-7
MDC1	:	Mediator of DNA Damage Checkpoint Protein 1
MEM	:	Minimum Essential Medium
MGMT	:	O-6-Methylguanine–DNA Methyltransferase
MLL	:	Mixed Lineage Leukemia
MLH1	:	MutL Homolog 1
miRNA	:	microRNA
MSP	:	Methyl Specific PCR
MTT	:	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NCCS	:	National Centre for Cell Sciences

Ncoa3	:	Nuclear Receptor Coactivator 3
NPC	:	Neural Precursor Cells
NR5A2	:	Nuclear Receptor Subfamily 5 Group A Member 2
NuRD	:	Nucleosome Remodeling
Oct4	:	Octamer Binding Transcription Factor 4
O-GlcNAc	:	O-Linked β-N-Acetylglucosamine
OGT	:	O-GlcNAc Transferase
Paf1C	:	RNA Polymerase II-Associated Factor 1C
PBS	:	Phosphate Buffered Saline
PBST	:	Phosphate Buffered Saline with Tween-20
PC3	:	Prostate Cancer-3
PDGFR-α	:	Platelet Derived Growth Factor Receptor α
PE	:	Proximal Enhancer
PGC	:	Primordial Germ Cell
PGC-1α	:	Peroxisome proliferator-activated receptor Gamma Coactivator 1-Alpha
PI	:	Propidium Iodide
PI3K	:	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
Pin1	:	Prolyl Isomerase
PKCε	:	Protein Kinase Cε
PNS	:	Peripheral Nervous System
POU	:	Pit-Oct-Unc
POU5F1	:	POU Domain, Class 5, Transcription Factor 1
PP	:	Proximal Promoter
PRDM14	:	PR Domain Zinc Finger Protein 14
PRMT	:	Protein Arginine Methyltransferase
PTCH	:	Patched
PTM	:	Post-Translational Modification
RAR	:	Retinoic Acid Receptor
Rb	:	Retinoblastoma
RBPJ	:	Recombining Binding Protein Suppressor Of Hairless
RIF1	:	Rap1 Interacting Factor 1
RM	:	Restriction-Modification
R-Ras	:	Related-RAS
RT-PCR	:	Real-Time Polymerase Chain Reaction
S100A4	:	S100 Calcium-Binding Protein A4
SALL4	:	Splat-Like Transcription Factor 4
SAM	:	S-Adenosyl Methionine
SATB1/2	:	Special Adenine-Thymine (AT)-Rich DNA-Binding Protein 1/2

SET	:	Su (var), E (z), Trithorax
SETDB1	:	SET Domain Bifurcated 1
SF1	:	Steroidogenic Factor-1
SFRP	:	Secreted Frizzled-Related Protein
SHH	:	Sonic Hedgehog
SIN3A/HDAC	:	SIN3 Homolog A/Histone Deacetylase
SMAD1	:	SMAD Family Member 1
SMO	:	Smoothened
Sox2	:	SRY (Sex determining Region Y)-Box 2
SP1	:	Specificity Protein 1
STAT3	:	Signal Transducer And Activator of Transcription 3
STK40	:	Serine/Threonine Kinase 40
STIM1	:	Stromal Interaction Molecule 1
SUFU	:	Serine–Threonine Kinase Fused and Suppressor of Fused
SWI/SNF	:	SWItch/Sucrose Non-Fermentable
TBS	:	Tris Buffered Saline
TBX2/3	:	T-Box Transcription Factor 2/3
TCF3	:	Transcription Factor 3
TCFCP2L1	:	Transcription Factor CP2-Like 1
TCL1A	:	T-Cell Leukemia/Lymphoma 1A
TET	:	TET Methylcytosine Dioxygenase
TF	:	Transcription Factor
TFIID	:	Transcription Factor II D
TGF-β	:	Transforming Growth Factor-βeta
TMS1	:	Target of Methylation-Induced Silencing 1
TORC2	:	Target of Rapamycin Complex 2
TSA	:	Trichostatin A
TSG	:	Tumour Suppressor Gene
TSS	:	Transcription Start Site
uPA	:	Urokinase-Type Plasminogen Activator
UTF1	:	Undifferentiated Embryonic Cell Transcription Factor 1
WDR5	:	WD repeat-containing protein 5
WWP2	:	WW Domain Containing E3 Ubiquitin Protein Ligase 2
Xi	:	Inactive X-chromosome
YAP1	:	Yes-Associated Protein 1
Zfp281	:	Zinc Finger Protein 281
ZFX	:	Zinc Finger X-Chromosomal
Zic3	:	Zinc Finger of the Cerebellum 3

Chapter 1

Introduction

The overall phenotypic and functional identity of a cell is determined by coordinated crosstalk between transcriptional regulatory networks, epigenetic modifiers and cellular signaling pathways (Patra et al., 2011; Apostolou and Hochedlinger, 2013) (Fig. 1.1). In response to critical developmental signals, genes critical for maintaining pluripotency are switched "on" by core pluripotency inducing transcription factors (TFs) and sustained by permissive chromatin remodelling whereas genes necessary for differentiation are transiently repressed by histone modifications. However, during linage-speciation and commitment, genes crucial for pluripotency are more permanently silenced by DNA methylation whereas differentiation-specific gene expression is activated by lineage-determining factors (Lunyak and Rosenfeld, 2008; Christophersen and Helin, 2010; Watanabe et al., 2013). Thus, integration of extrinsic signaling cues with intrinsic epigenetic factors and allied transcriptional response ensures homeostatic balance between determination, maintenance and expression of pluripotency (Reik, 2007; Pei, 2009).

On the other hand, disruption of this ordered developmental scheme is responsible for genomic instability that drives progenitor cells towards improper malignant phenotype (Easwaran et al., 2014; Feinberg et al., 2016). Constitutively active oncogenic signaling pathways trigger changes in chromatin structure and dynamics in precursor cells and predispose them towards aberrant differentiation by endowing strategic advantageous properties of survival and proliferation (Sever and Brugge, 2015). Secondly, altered epigenetic events result in silencing of prominent tumor suppressor, DNA repair and cell cycle regulatory genes, and even facilitate the emergence of cancer stem cells (CSC) (Scaffidi and Misteli, 2010; Baylin, 2012). Finally, unscheduled expression of pluripotency inducing TFs like octamer binding transcription factor 4 (Oct4), SRY (sex determining region Y) box 2 (Sox2) and Nanog hinders normal maturation programs and removes barriers against dedifferentiation of cancer cells into CSC phenotype (Klimczak, 2015; Muller et al., 2016). Hence, understanding the intricate relationship between these three crucial determinants is necessary to apprehend the molecular floor plan that underlies cellular development as well as fuels oncogenic transformation.

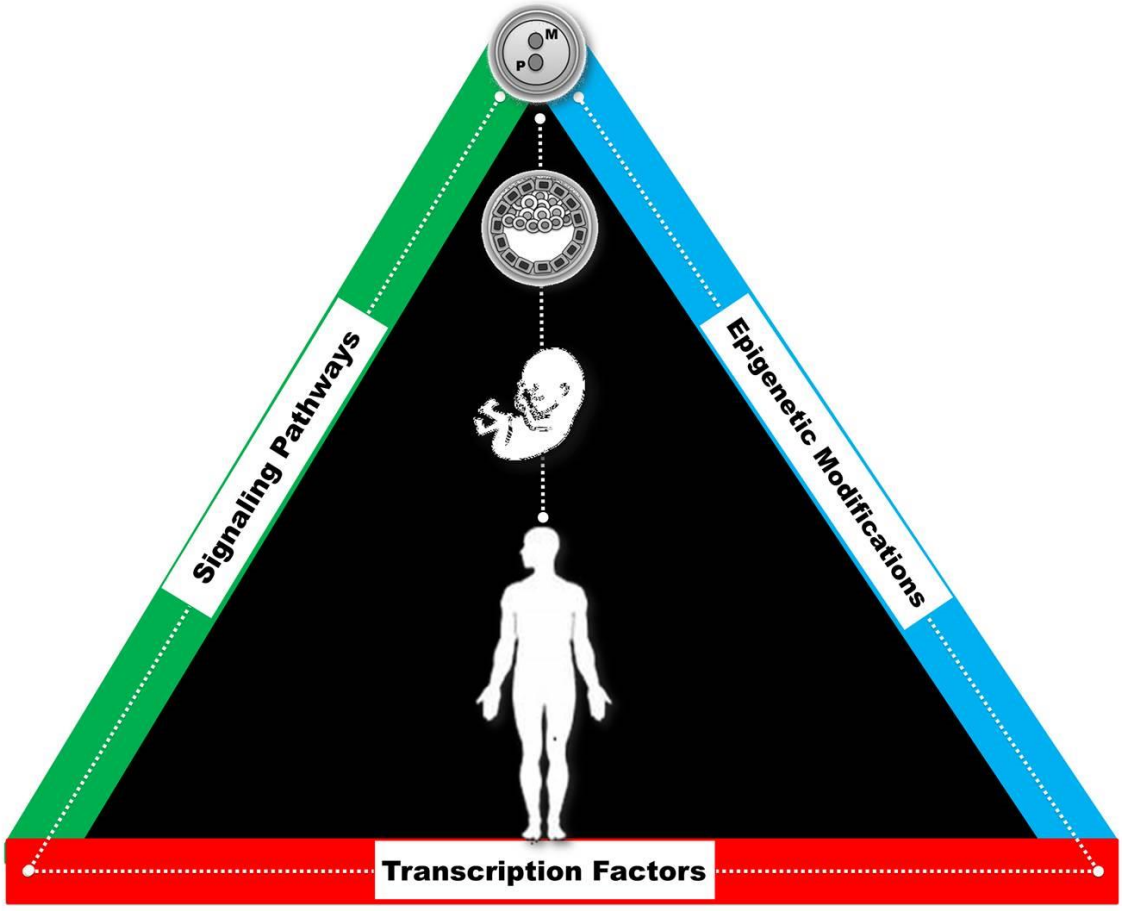


Figure 1.1: The three corner stones of pluripotency and differentiation in mammalian developmental saga. The transition from totipotency in zygote, through pluripotency in blastocyst and multipotency in embryo to unipotency in adults is facilitated by co-ordination between signaling pathways, epigenetic modifications and transcription factors.

1.1 Epigenetic modifications play crucial role in determining transcriptional fate:

The functional template of all eukaryotic genetic information and the associated protein components are subjected to a diverse array of pre- and post-transcriptional and translational modifications. These distinct set of biochemical tags in both DNA (methylation) and histones (methylation, acetylation, phosphorylation etc.) can generate synergistic or antagonistic interaction affinities with chromatin domain binding proteins and dictate dynamic transitions between transcriptionally active or silent states (Jaenisch and Bird, 2003; Meissner, 2010). A harmonious and homeostatic balance between various epigenetic modifications essentially preserves the integrity of the genetic message across several generations. On the molecular level, these signatures include DNA methylation and demethylation of cytosine ($5meC$)[5], covalent post-translational reversible

modifications (PTMs) of all histone proteins, incorporation of specific histone variants as well as RNA mediated interference and gene silencing (Jurkowska et al., 2011; Li et al., 2012). In general, epigenetic manipulation of chromatin landscape impacts almost all transcriptional outcomes and decides cell-specific phenotypic archetype and functional destiny during both normal and pathological development (Hajkova, 2011; Kar et al., 2012).

1.1.1 DNA methylation mediated epigenetic modulation in transcription:

DNA methylation is a principal epigenetic enforcer that facilitates functional re-orientation of genomic data and effectively modulates gene expression profile via cell-specific regulation of transcriptional activity. Methylated cytosine in the genome was first discovered in calf thymus DNA by Hotchkiss in 1948 (Hermann et al., 2004a). Methylation of DNA is a post-synthetic biochemical process, facilitated by a family of DNA methyltransferases (DNMTs) which methylate C_5 cytosine residue specifically at CpG rich promoter sequences in the presence of cofactor S-adenosyl methionine (SAM) (Patra et al., 2003; Lan et al., 2010; Patra et al., 2011). The methylation signature is generated during early embryonic development and maintained faithfully in each successive cellular division by synchronized interaction between the DNMTs. Addition of new methyl marks to previously unmethylated cytosines is supervised by *de novo* methyltransferases DNMT3A and DNMT3B in the nucleolus, whereas DNMT1, the maintenance methyltransferase ensure that hemi-methylated daughter strands in somatic differentiated cells faithfully maintain and propagate the proper DNA methylation patterns across successive cell generations (Jeltsch, 2008; Jones and Liang, 2009; Denis et al., 2011). DNA methylation obstructs transcriptional activity via two ways; firstly methylated cytosine bases sterically hinder the interaction of transcriptional factors and RNA polymerase II with their cognate DNA recognition sequences (Bogdanovic and Veenstra, 2009). Secondly, methyl-CpG-binding proteins such as methyl-CpG-binding domain (MBD) proteins associate with methylated DNA and result in heterochromatin formation by recruiting histone deacetylases (HDACs) (Klose and Bird, 2006; Clouaire and Stancheva, 2008; Kar et al., 2014).

DNA methylation predominantly acts as a gene silencing mechanism, which assimilates the repression marks previously established via histone modifications, rearrangement of nucleosomes as well as higher-order chromatin restructuring to

permanently turn off unnecessary genes at crucial junctures during development and differentiation. In prokaryotes, DNA methylation takes places in both adenine and cytosine bases, and functions to distinguish between self and non-self DNA as a defense against bacteriophagial infection using the restriction/modification (RM) systems. It also helps in recognizing and repairing replication errors by differentiating unmethylated daughter strand from methylated parent template (Jeltsch, 2002). In eukaryotes, DNA methylation participates in a number of cellular processes to set-up the appropriate framework of transcriptional activity in varied biological settings. It plays an important role in regulation of parental imprinting and stabilization of X-chromosome inactivation (Suzuki and Bird, 2008). It guarantees maintenance of the genome integrity by silencing of repetitive sequences, endogenous retroviruses and selfish genetic elements like transposons (Hermann et al., 2004; Dean et al., 2005). It is also implicated in the development of the immune system, in brain function and behavior as well as in cellular reprogramming and induction of stem cell differentiation (Weber and Schubeler, 2007; Lees-Murdock, and Walsh, 2008).

1.1.2 Histone modifications extend the potential, specificity and diversity of downstream chromosomal processes:

Histone proteins are subjected to an assorted array of covalent modifications such as methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation and glycosylation (Sims et al., 2003; Kouzarides, 2007). These post-translational alterations occur typically on highly accessible N-terminal histone tails which changes the structural dynamics of the nucleosome core and affects accessibility of the underlying DNA to transcription factors and other nucleosomes. Histone modifications can act individually to directly alter chromatin architecture and assembly as well as in combination to reinforce or reverse existing DNA and other histone modifications (Bannister and Kouzarides, 2011). They may also provide a scaffold for binding of chromatin-associated factors or act as signals for nucleosomal repositioning and rearrangement. The reversible histone modifications are fundamentally responsible for regulation of a diverse set of biological processes such as DNA replication, repair and recombination, chromosome condensation (mitosis) and spermatogenesis (meiosis) (Bhaumik et al., 2007; Suganuma and Workman, 2011). Together with DNA methylation, covalent histone modifications carry out the bulk of epigenetic maintenance and control of genetic transcriptional status.

Histone Acetylation and Deacetylation:

Histone acetylation occurs by the enzymatic addition of an acetyl group (-COCH3) to the ε-amino group of lysine side chains from acetyl coenzyme A. Histone acetyltransferases (HAT) and deacetylases (HDACs) are the enzymes responsible for writing and erasing the acetylation of histone tails (Eberharter and Becker, 2002). Lysine residues within histone H3 (H3K9, H3K14, H3K18) and H4 (H4K5, H4K8, H4K12) are preferential targets for acetylation. Addition of an acetyl group reduces electrostatic attraction between histones and negatively charged DNA backbone, thus opening up access to DNA loosening and generating accessible chromatin for transcription factors and polymerases (Wang et al., 2008). The process of histone acetylation is tightly involved in the regulation of many cellular processes including chromatin dynamics and transcription, gene silencing, cell cycle progression, apoptosis, differentiation, DNA replication, DNA repair, nuclear import, and neuronal repression (Verdone et al., 2005; Tamburini and Tyler, 2005). H3K9ac and H3K27ac are normally associated with enhancers and promoters of active genes. H3K56ac stabilizes the genome at replication forks to prevent fork collapse at DNA damage checkpoints after DNA repair is complete, allowing cells to re-enter the cell cycle. H4K16ac mediates chromatin decondensation during G_2/M transition (Shogren-Knaak et al., 2006; Vaquero et al., 2006; Wurtele et al., 2012).

Histone Phosphorylation and Dephosphorylation:

Phosphorylation of histones is highly dynamic and takes place on serine, threonine and tyrosine residues (Oki et al., 2007; Banerjee and Chakravarti, 2011; Rossetto et al., 2012). Phosphorylation of H2A, especially in serine 139 of the H2AX variant histone, commonly referred to as γH2AX is the most noted histone phosphorylation mark. It occurs in all phases of cell cycle during DNA damage responses (DDR) and hence is considered to be an indispensable tag for DNA repair (Pinto and Flaus, 2010). At the site of double strand DNA break, γH2AX results in accumulation and retention of DNA damage response proteins such as mediator of DNA damage checkpoint protein 1 (MDC1) and p53-binding protein 1 (53BP1), thus preventing cell progression through G_1/S phase and cell cycle arrest (Celeste et al., 2003; Fernandez-Capetillo et al., 2004). This modification is also linked to induction of apoptosis by death receptor activation as well as reduced epithelial growth factor receptor (EGFR)-mediated cellular growth and differentiation. Similarly, H2B phosphorylation in serine residues (Ser14 and Ser36) mediates a number of physiological functions such as (1) chromatin condensation and DNA fragmentation

leading to cell death and apoptosis (Fernandez-Capetillo et al., 2004; Solier and Pommier, 2008) (2) cellular response to stress (by facilitating transcriptional elongation of stress-activated genes such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and target of rapamycin complex 2 (TORC2) to support cell survival, and (3) sustaining adenosine monophosphate-activated protein kinase (AMPK-FOXO3) hormone signaling and circadian function so as to connect cellular energy status to genomic responses (Bungard et al., 2010). H3 phosphorylation marks H3S10p and H3S28p are both indicators of cell cycle progression. H3S10p mediates dissociation of heterochromatin protein 1 (HP1) facilitating chromosome decondensation at metaphase and subsequent segregation during telophase. H3S10 phosphorylation is also present at pericentric heterochromatin of cells during G_2 (Nowak and Corces, 2004; Lau et al., 2011). H4S1 phosphorylation is also linked to DNA repair via joining of non-homologous ends in double stranded breaks and stabilizing new nucleosomes by preventing their acetylation (Bird et al., 2002).

Histone Methylation and Demethylation:

Histone methylation is considered to be the most complex of all histone modifications, since its functional output is dependent upon the precise methylation site and degree of modification (Sims et al., 2003). Histone methylation occurs on positively charged amino acid residues such as lysine and arginine. Lysine can undergo monomethylation, dimethylation or trimethylation on its ε-amine group whereas arginine can be monomethylated, symmetrically demethylated or asymmetrically dimethylated on its guanidinyl group. The most extensively studied histone methylation marks among these include histone H3 lysine (K) (H3K4, H3K9, H3K27, H3K36, H3K79 and H4K20. Important residues where arginine (R) is mostly methylated includes H3R2, H3R8, H3R17, H3R26 and H4R3 (Bedford and Clarke, 2009). Three families of enzymes are known to catalyze the addition of methyl groups donated by S-adenosylmethionine (SAM) to histones. While SET-domain containing proteins and disruptor of telomeric silencing 1 (DOT1)-like proteins methylate lysine residues, members of the protein arginine N-methyltransferase (PRMT) family are known to methylate arginines (Lachner and Jenuwein, 2002; Le and Fujimori, 2012).

Transcriptional regulation mediated by histone methylation is carried out in two different contexts; firstly gene-specific expression and secondly at the bulk chromosomal

level. The methylation of histone lysine residues in the promoter regions of individual genes constitutes "gene level" regulation. Similarly, regulation at 'chromosome level' includes formation of heterochromatin and euchromatin via methylation of various large chromatin domains (Zhang and Reinberg, 2001; Black et al., 2012). Histone methylation is a multi-factorial tag with contrasting effects in different settings; while lysine methylation of H3 and H4 is implicated in both transcriptional activation and repression depending on the methylation site, while arginine methylation promotes transcriptional activation. At the chromosomal level, large-scale permissive and transcriptionally competent euchromatin is established by H3K4 methylation (H3K4me2 and H3K4me3) which disrupt large-scale silencing by H3K9 methylation and by H3K79 methylation which prevent spreading of heterochromatin. After large-scale permissive chromatin is established, gene-specific transcriptional activation and elongation is assisted by H3K4 and H3K36 methylation at mRNA coding regions (H3K4me at enhancers and H3K4me3 at gene promoters) and gene bodies respectively (Greer and Shi, 2012; Deb et al., 2014). On the other hand, histone lysine methylation-mediated chromosome level repression is mainly targeted to H3K9, H3K27, H4K20 residues. Highly condensed constitutive heterochromatic regions of chromosomes correspond to H3K9 and H3K27 tri-methylation. H3K9me3 is generally enriched in gene poor regions such as satellite repeats, telomeres and pericentromeres and retrotransposons whereas H3K27me3 is found primarily at promoters in gene-rich regions, closely associated with developmental regulators. Inactive X-chromosome (Xi) is also marked by H3K9me3 and H3K27me3 marks that help in establishment and maintenance of this modification might participate in the initiation of X-chromosome inactivation (Justin et al., 2010). Protein arginine methylation mediates a number of biological processes including nuclear/cytoplasmic shuttling, mRNA splicing, DNA Repair, signal transduction as well as affects transcriptional status by acting as transcriptional co-activator or co-repressor (Lanouette et al., 2014).

In theory, methyl turnover is always believed to be slower than other PTMs, and histone methylation was originally thought to be irreversible. However, the discovery of histone demethylase enzyme capable of demethylating methylated lysines known as lysine-specific demethylase (LSD1A; also KDM1A) established that histone methylation is, in fact, alterable (Bannister and Kouzarides, 2005). Currently, two families of demethylases have been recognized such as amine oxidases and jumonji C (JmjC)-

domain containing, iron-dependent dioxygenases. The first group of histone demethylases consists of amine oxidase-domain containing enzymes represented by LSD1 (also known as AOF2) and LSD2 (also known as AOF1) which specifically remove H3K4me1/me2 marks. The second group of histone demethylases consists of Jumonji domain-containing proteins (JmjC), the prominent members being Fe (II) and 2-oxoglutarate (2OG) dependent oxygenases capable of erasing all the three histone lysine tags (Hou and Yu, 2010). The histone demethylase enzymes contribute significantly to specification of active and repressive chromatin as well as cellular reprogramming events. Stem cell differentiation is affected by histone demethylation where specific epigenetic states during lineage commitment, reprogramming of germline and maintaining epigenetic stability are arbitrated by histone demethylases. Histone demethylation is also essential for cell cycle transitions by re-expressions of cell cycle regulators and segregation of chromosomes. Histone demethylation also protects the genome by specifying DNA damage response and sustaining genomic integrity (Cloos et al., 2008; Dimitrova et al., 2015).

Other Histone Modifications:

In addition to the above major biochemical alterations of the histone proteins, large molecule covalent modifications such as ubiquitylation and sumoylation have also been reported in histones. Histone ubiquitylation occurs within H2A and H2B; H2AK119 mono-ubiquitylation involved in gene silencing and H2BK123 mono-ubiquitylation plays an important role in transcriptional initiation and elongation, nucleosome stability and mediating trans-histone H3 methylation (Weake and Workman, 2008; Kim et al., 2009; Chandrasekharan et al., 2010). This covalent modification is reversed by de-ubiquitin enzyme which also affects gene activity and silencing. Histone sumoylation involves addition of small ubiquitin-like molecules to lysine residues in all four core histones. It acts antagonistically to acetylation and ubiquitylation and is largely responsible for gene silencing events (Shiio and Eisenman, 2003; Ouyang and Gill, 2009). Histones (H2A, H2B, H3 and H4) have also been shown to be modified by β-N-acetylglucosamine (O-GlcNAc) on serine and threonine residues which regulates mitosis-specific phosphorylation and coordinate G_2/M transition of the cell cycle (Zhang et al., 2011; Fong et al., 2012). All the above covalent histone modifications are highly dynamic and cross-talk with each other to constitute a “histone code” that shapes gene-expression patterns by modulating the transcriptional potential of genomic domains.

1.2 Epigenetic definition of oncogenesis:

Cancer has been explained in many different ways. From Hippocrates' depiction of neo-angiogenesis in tumors as the claws of a crab to Laennec's view of malignancy as an inappropriate developmental malady; cancer has always confounded the human mind regarding its amazingly unpredictable nature (Feinberg et al, 2006). The past century witnessed dominance of the genetic model of cancer which perceived cancer as a set of disorders caused by progressive genetic and chromosomal abnormalities including mutations in key tumor suppressor genes and oncogenes. Each activating or silencing mutation resulted in promoting continued growth of clonally selected tumor cells with increasing tumorigenic potential such as invasiveness, metastasis, drug resistance and recurrence (Hanahan and Weinberg, 2000; Baylin and Ohm, 2006; Zhao et al., 2008; Hanahan and Weinberg, 2011). However, in the last two decades, molecular heterogeneity in cancer cells have led to the idea that alongside genetic changes, extensive alterations of chromatin in neoplastic as well as adjacent non-malignant cells play a greater part in mediating malignant conversion and progression (Hassler and Egger, 2012). In recent years, cancer as a disease has acquired more of an epigenetic essence, with researchers acknowledging epigenetics as the more dominant protagonist in oncogenic transformation. In fact, epigenetic alterations are not only recognized as strategic features of cancer cells, but are also considered to herald the onset of carcinogenesis (Pogribny, 2010; Dhanasekaran et al., 2013).

1.2.1 The theory of epigenetic progenitor origin of cancer:

In an attempt to explain oncogenesis in epigenetic language, epigenetic progenitor model of cancer was envisioned. According to this model, early epigenetic alterations in progenitor cells trigger neoplastic changes which together with succeeding genetic lesions drive tumorigenic transformation (Feinberg et al., 2006; Feinberg et al., 2016). Besides initiating pre-neoplastic changes, these epigenetic modifications set the stage for tumor plasticity and genetic variations later during tumor progression. Moreover, by placing precursor/progenitor/stem cells at the epicenter of neoplastic reprogramming, epigenetic changes provide mechanistic evidence for similarities between embryonic differentiation and oncogenic conversion (Baylin, 2012; Sandoval and Esteller, 2012) (Fig. 1.2). Thus, epigenetic progenitor model more effectively explains issues such as tumor recurrence and resistance, acquisition of heterogeneity and aggressive tumor properties in later stages

as well as genetics of cancer risk by precluding epigenetic disruption of stem/progenitor cells as a key step before commonly recognized neoplastic changes.

In the first step, progenitor/precursor/stem cell population in any given anatomical system is exposed to initial epigenetic stress which primes them to be neoplasia-ready. The polyclonal population of precursor cells continuously strives to maintain an extremely delicate balance between competing interests of proliferation and differentiation (Mathews et al., 2009). They are the primary targets of multitude of genetic, age-dependent damage or injury that largely account for their susceptibility to neoplastic distress. Hence, small but profound epigenetic changes which can be due to distubances within the stem cells themselves or environmental stress perturb the equilibrium between undifferentiated progenitor cells and differentiated committed cells (van Vlerken et al., 2012). These early epigenetic changes force the precursor cells to acquire the capacity of aberrant differentiation and progress towards tumorigenic phenotype (Suva et al., 2013).

The second step involves onset of gatekeeper mutations (GKM) within the compartment of rapidly expanding, epigenetically altered progenitor cells that gives rise to earliest stages of neoplasm and subsequent benign lesions (Sawan et al., 2008). These initiating mutations are specific for different tumor types, for example, mutation in tumor suppressor genes that encode adenomatosis polyposis coli (APC) in solid tumors like colorectal cancer whereas for leukaemia and lymphoma, rearrangement of oncogene homologue Breakpoint Cluster Region–Abelson Murine Leukaemia (BCR–ABL) is more crucial. The gatekeeper mutations were initially recognized as the earliest steps in tumorigenesis, but now epigenetic events are perceived as surrogates for mutation-induced oncogene activation or tumor suppressor-gene silencing (Dawson and Kouzarides, 2012).

In the third and final phase, progressive genetic and epigenetic instabilities lead to stable evolution of primary cancer into advanced tumors with enhanced oncogenic properties such as invasion, metastasis and drug resistance. These phenotypic features are inherently present in the progenitor cells and do not require further mutations, however epigenetic influence stimulates their aberrant expression and function in the developing neoplasia (You and Jones, 2012; Hassler and Egger, 2012). Genetic plasticity can be explained in terms of telomere erosion and DNA palindromes formation, wherein chromosomal shortening by both the processes results in a bridge-break-fuse cycle greatly

destabilizing the genome. Epigenetic plasticity on the other hand, results in heterochromatin decondensation and consequent chromosomal rearrangements as well as pleiotropic changes in transcriptional status of chromatin modifiers. For example, chromosomal rearrangements leading to increase or decrease of active or repressive histone modifying marks alter the gene expression status of many cancer causing genes ultimately leading to evolution of aggressive properties in malignant tumors. The ultimate outcome of repetitive epigenetic and genetic stochasticity is alteration of the epigenomic landscape in the tumor micro-environment which allows enhancing properties and aggressive behavior (Feinberg and Irizarry, 2010; Timp and Feinberg, 2013). Epigenetic progenitor model thus propagates epigenetic alterations as main culprit in oncogenic transformation.

1.2.2 Epigenetic choreography of neoplastic transformation:

During tumorigenic progress, the epigenome is subjected to wide-spread alterations that affect nuclear architecture and chromatin compaction. The effects of these altered epigenetic events influence the course of malignant development in more than one ways involving silencing of prominent tumor suppressor, DNA repair and cell cycle regulatory genes, activation of oncogenes, disruption of differentiation programs and even facilitating the emergence of CSCs (Jones and Baylin, 2002; Sadikovic et al., 2008; Iacobuzio-Donahue, 2009). Following Darwin's theory of selective evolution, these heritable gene silencing processes team up with genetic alterations to confer selective growth and proliferative advantages to tumor cells and sustain neoplastic progress (Jones and Baylin, 2007; Pogribny, 2010).

Epigenetic gene-silencing causes loss of function and pre-disposition to mutation:

Gene silencing via promoter DNA hypermethylation is the most prevalent and documented epigenetic change occurring in almost all tumor types (Baylin, 2012). In fact, promoter CpG hypermethylation is so extensively operational in the neoplastic cells that methylation induced epigenetic silencing of tumor-suppressor genes is presently considered to be more effective than genetic mutations in inflicting tumorigenic growth (Bhattacharjee et al., 2016). The transcriptional inhibition as a consequence of promoter hypermethylation causes loss of function of genes involved in important cellular processes such as (i) DNA repair by O-6-methylguanine–DNA methyltransferase (MGMT), MutL homolog 1 (MLH1) and breast cancer 1 (BRCA1), (ii) cell cycle control

by cyclin-dependent kinase Inhibitor 2A (CDKN2A), retinoblastoma (Rb) (iii) apoptosis by target of methylation-induced silencing 1(TMS1), death associated protein kinase1 (DAPK1), (iv) metastasis by cadherin 1 (CDH1), CDH13, (v) detoxification by glutathione S-transferase pi 1 (GSTP1) (Muntean and Hess, 2009; Sharma et al., 2010; Kanwal and Gupta, 2012; Shukla and Meeran, 2014). The lack of functional expression of these tumor-suppressor genes results in faulty mismatch repair and microsatellite instability, thus destabilizing the genome and making it susceptible to genetic mutations. Moreover, methylated cytosines are susceptible to spontaneous hydrolytic deamination which causes transitional mutations such as C to T and CC to TT. Silencing of cell cycle regulatory genes like cyclin dependent kinase inhibitor 2B (CDKN2B) allows defective cells to proliferate abnormally and cause tumor growth. Thus, epigenetic gene silencing can be considered as the primary cause of tumor adaptability and evolution (Wilting and Dannenberg, 2012).

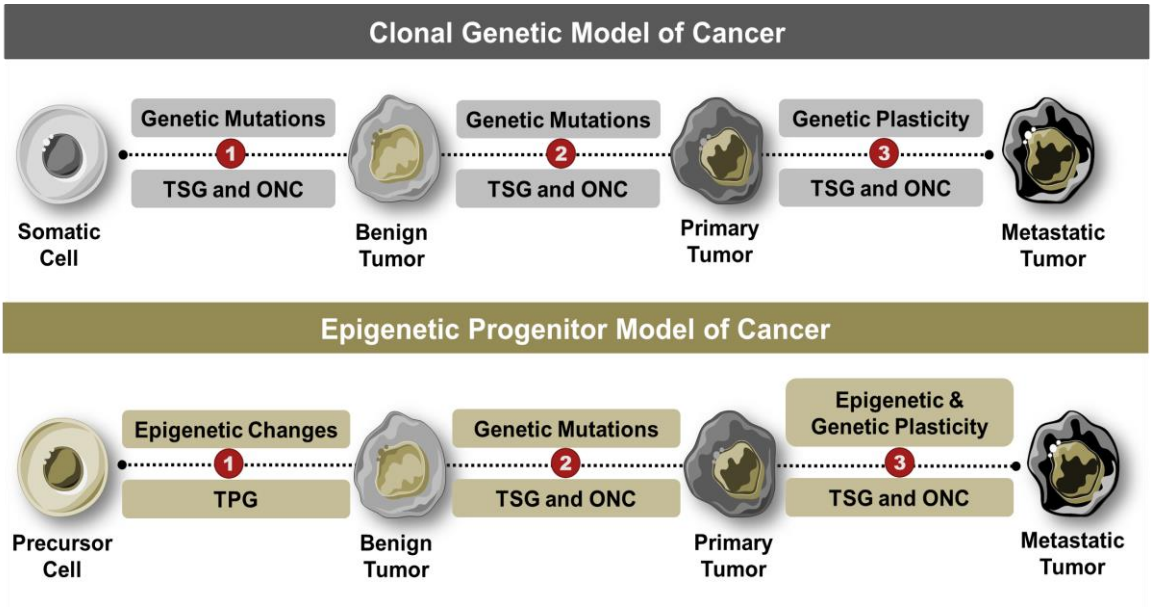


Figure 1.2: The clonal genetic and epigenetic progenitor models of cancer initiation, maintenance and progression. While the clonal genetic model predominantly supported genetic mutations in tumor suppressor genes (TSG) and oncogenes (ONC) as the propagators of neoplastic conversion, epigenetic model gave credit to epigenetic alterations in tumor progenitor genes (TPG), especially pluripotency factors as the main mediators of oncogenesis.

Epigenetic derepressing mechanisms activate unwanted oncogenic activity:

Global loss of methylation occurs mainly at non-coding intergenic and intronic regions like retrotransposons and pericentromeric repeats where it promotes chromosomal rearrangements and insertional mutagenesis to destabilize and predispose the epigenome

to cancer-initiating mutations (Patra et al., 2008; Ehrlich, 2009; De Smet and Loriot, 2010). Additionally, DNA hypomethylation results in transcriptional derepression and over-expression of (i) proto-oncogenes such as c-Myc and related-RAS (R-Ras), (ii) genes associated with metastasis and invasion like mammary serine protease inhibitor (MASPIN), urokinase-type plasminogen activator (uPA) and S100 calcium-binding protein A4 (S100A4), (iii) cancer-testis (CT) antigens like melanoma-associated antigen (MAGE) and (iv) imprinted genes like insulin growth factor 2 (IGF2) that drives malignant cell proliferation, invasion and metastasis (Syzf et al., 2004; Wilson et al., 2007; Wild and Flanagan, 2010). In fact, paradoxical genomic hypomethylation and gene-specific hypermethylation are considered characteristic feature of neoplastic progression.

Epigenetic "addiction" to oncogenic signaling pathways:

Epigenetic gene silencing events in pre-malignant lesions result in over-activation of oncogenic signaling pathways and cell-signaling molecules. The addicted cells are susceptible to genetic mutations during early stages of tumor development that endows them with greater proliferative and survival abilities that ultimately drives neoplastic development (Baylin and Ohm, 2006; Baylin and Jones, 2011). For example, loss of secreted frizzled-related protein (SFRP) expression (due to its promoter hypermethylation) during early stages of colon cancer development results in abnormal activation of the Wnt signaling pathway. The colon epithelial progenitor cells become habituated to over-activity of the Wnt pathway and subsequently accumulate mutations in APC complex. This favors sustained survival and proliferation of early-stage colon lesions into aberrant crypt foci (ACF) (Suzuki et al., 2004; Schepers and Clevers, 2012).

Epigenetic disruption of differentiation programs in cancer:

Aberrant epigenetic silencing events can interfere with cellular differentiation programs in pre-malignant cells and result in continuous self-renewal and inherent tumorigenicity (Scaffidi and Misteli, 2010). One of the most notable oncogene c-Myc induces tumor cell to acquire stem cell properties and interferes with cellular differentiation by histone deacetylation and chromatin remodeling (Liu et al., 2007; Rapp et al., 2008; Varlakhanova and Knoepfler, 2009). In addition, epigenetic signatures found in embryonic stem cells (ESC) is mimicked in various cancers, indicating that epigenetic reprogramming may lead to loss of cellular identity, generation of tumor heterogeneity and finally facilitate the emergence of CSCs (Ben-Porath et al., 2008; Wong et al., 2008).

1.3 Pluripotency inducing nuclear transcription factors act as epigenetic mediators of malignant changes:

Pluripotency is essentially defined by two distinct characteristics, firstly the ability to undergo continuous self-renewal indefinitely and secondly, the capacity to generate differentiated progeny of nearly all lineages in mature organisms. These molecular hallmarks of pluripotency can be explained through a distinct set of markers that outline a unique stemness profile for pluripotent cells (Gonzales and Ng, 2011; Adachi and Scholer, 2012). In concert with signaling axes, transcription factors decide between various states of pluripotency (Liu et al., 2013; Kar et al., 2013). Transcription factors maintain pluripotent ESCs in their undifferentiated state by activating genes necessary for stem cell survival and proliferation while simultaneously repressing genes responsible for lineage commitment and speciation. In contrast, by facilitating expression of cell type-specific genes, transcription factors also participate in establishment of cellular identity (Romito and Cobellis, 2016). Remarkably, the ectopic over-expression of Oct4, Sox2 and Nanog triumvirate has also been shown to induce the reprogramming of differentiated somatic cells back to pluripotent state of ESC (Takahashi and Yamanaka, 2006; Jaenisch and Young, 2008; Welstead et al., 2008; Heng et al., 2010).

1.3.1 A veritable cocktail of transcription factors govern maintenance, expression and acquisition of pluripotency:

The establishment of embryonic pluripotency is carried out by three crucial factors Oct4, Sox2 and Nanog that constitute the core pluripotency network (Silva and Smith, 2008; Smith et al., 2016). Oct4 was the first identified germline specific pluripotency determinant. Though Oct4 null embryos develop blastomeres, yet inner cell mass (ICM) in the blastomeres do not have the power to give rise to pluripotent ESCs and ultimately give rise to trophoectodermal cells. Similarly, Sox2, another HMG-box transcription factor is a close associate of Oct4 and together they decide stem cell pluripotent state. Knockout of Sox2 results in embryonic lethality. Nanog is more involved in preventing differentiation than in maintaining pluripotency (Orkin et al., 2008; Greenow and Clarke, 2012). Embryos lacking Nanog die around implantation due to lack of epiblast and absence of Nanog in ICM results in differentiation into endoderm. Not only are Oct4, Sox2 and Nanog necessary for establishing pluripotency, they also maintain the pluripotent state without allowing differentiation programs to start. Therefore, silencing or

over-expression of Oct4, Sox2, or Nanog initiates ESC differentiation. For example, increase in Oct4 expression induces endodermal and mesodermal lineages, whereas decrease in Oct4 level causes trophoectodermic differentiation (Barrand and Collas, 2010). Oct4, Sox2 and Nanog form auto-regulatory positive feedback circuitry for pluripotency by not only binding to their own promoters, but also activating the transcription of other protein-coding genes and microRNAs (miRNA) involved in pluripotency maintenance. In addition to the core transcriptional circuitry, many auxiliary transcriptional factors, including transcription factor 3 (TCF3), SMAD Family Member 1 (SMAD1), signal transducer and activator of transcription 3 (STAT3), spalt-like transcription factor 4 (SALL4), dosage sensitive sex-reversal (DSS), adrenal hypoplasia congenita (AHC) critical region on the X-chromosome, gene 1 (DAX1), estrogen related receptor beta (ESRRB), nuclear receptor subfamily 5 group a member 2 (NR5A2), T-box transcription factor 3 (TBX3), zinc finger X-chromosomal (ZFX), kruppel-like factor 4 (KLF4), forkhead box protein D3 (FOXD3), forkhead box protein O1 (FOXO1), forkhead box protein P1 (FOXP1), PR domain zinc finger protein 14 (PRDM14) and zinc finger of the cerebellum 3 (Zic3) have been demonstrated to be involved with the complex machinery that directs pluripotency maintenance (Huang and Wang, 2014; Ohnishi et al., 2014). These accessory factors help the core regulators by activating developmentally inclined signaling pathways or by stabilizing the core transcriptional circuitry (Fig. 1.3).

1.3.2 Pluripotency inducing transcription factors as the mechanistic link between embryogenesis and carcinogenesis:

As it became clear that ectopic introduction of core pluripotency factors can revert back cells into ESC like condition, focus of scientific community shifted towards exploring the role of these factors in malignant transformation (Yamada et al., 2014). Elevated expression of Nanog, Oct4 and Sox2 has been reported in several cancers where they are known to actively induce tumorigenesis, contribute towards cancer maintenance and encourage acquisition of more aggressive tumor properties such as metastasis and lymph node invasion (Kim and Orkin, 2011). Cancer cells share many properties in common with early ESCs, especially expression of Oct4, Sox2 and Nanog. Therefore, it is not surprising that over-expression of these factors is found in many somatic cancers including oral squamous cell carcinoma, prostate and breast cancer and their aberrant activity increases inherent tumorigenicity in human malignancies by boosting tumor

transformation, metastasis and post-therapeutic distant recurrence and remission. (Kashyap et al., 2009).

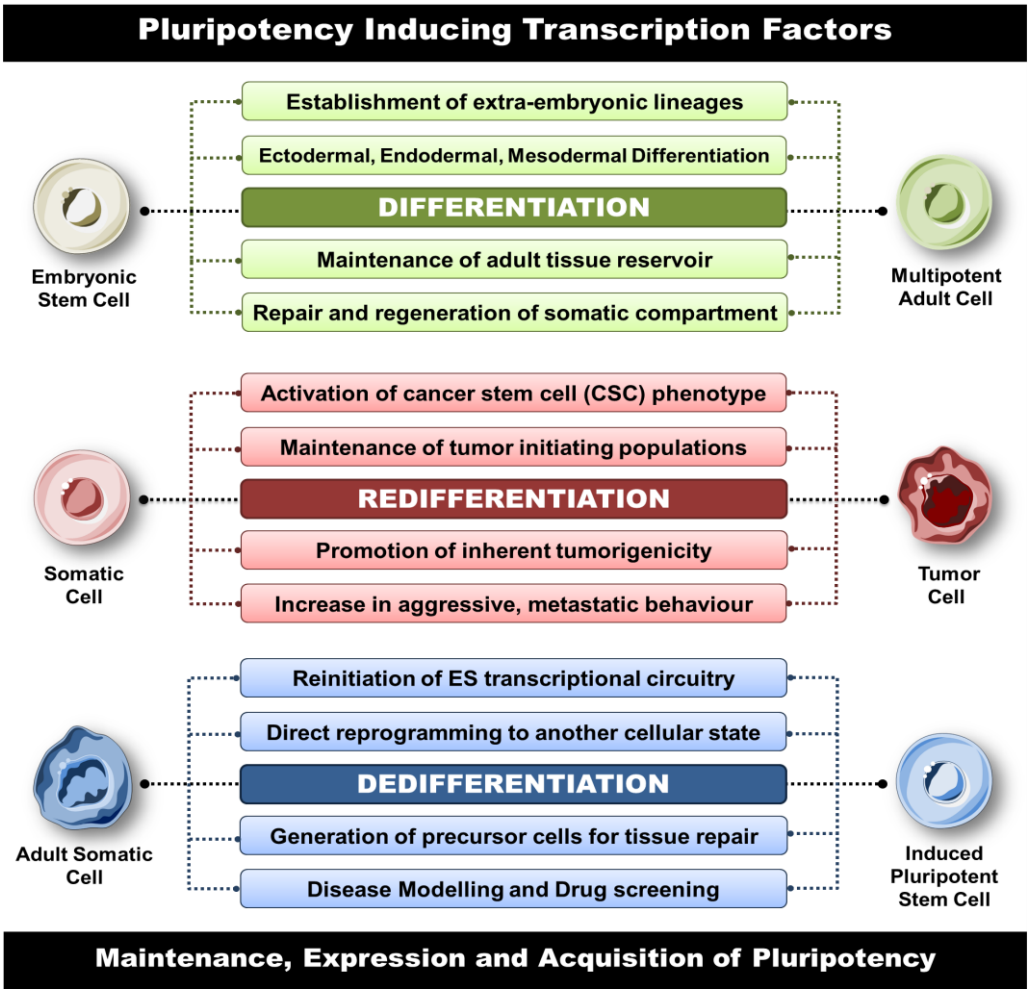


Figure 1.3: Pluripotency inducing transcription factors control three different aspects of stem cell pluripotency via differentiation, redifferentiation and dedifferentiation. Oct4, Sox2 and Nanog form the core pluripotency network that participate in maintenance via self-renewal, expression via differentiation into lineage-committed fates and acquisition via reprogramming into undifferentiated ground state.

The molecular basis of similarity between embryonic development and oncogenic transformation is exemplified by transcription factors as they either target or are targeted by signaling pathways that are active in both ESCs and cancer cells (Semi et al., 2013). Oct4, Sox2 and Nanog in their molecular capacity of pluripotency determinants are modulated by developmentally inclined pathways that help them to ensure self-renewal and maintain pluripotency (Hadjimichael et al., 2015). However, the same signaling pathways are aberrantly hijacked in cancer and drive transcription factors to activate inappropriate expression of genes involved in cell cycle, cell proliferation, apoptosis and

cell death. For example, Oct4 increases transcriptional activity of AKT pathway which encourages abnormal cell growth. Meanwhile, Sox2 participates in the stromal interaction molecule 1 (STIM1) pathway via Sox2/Orail nodule and enhances anti-apoptotic potential in prostate cancer. Nanog is a direct target of the leukemia inhibitory factor (LIF)-STAT3 pathway and maintains self-renewal of CSCs by up-regulating CD133, ATP-binding cassette sub-family G member 2 (ABCG2), aldehyde dehydrogenase 1 family, member A1 (ALDH1A1) and CD44 (Kim and Zaret, 2015; Yilmazer et al., 2015). Pluripotency inducing TFs, otherwise called tumor progenitor genes (TPG) are now considered as the mechanistic link between embryogenesis and oncogenesis.

1.4 Cellular signaling pathways as epigenetic modulators of oncogenic adaptation:

In order to systematically maintain homeostatic balance between survival, proliferation, and differentiation into designated functional identity, cellular systems must respond to a wide variety of intrinsic and extrinsic developmental cues. Precisely timed and rigidly controlled intracellular signaling pathways are largely responsible for amalgamating and translating these developmental cues into cell-type specific transcriptional states and phenotypic status. However, signal transduction pathways utilized by normal cells to ensure equilibrium between cell growth and cell death are often inappropriately manipulated to participate in neoplastic transformation (Patra et al., 2011; Sever and Brugge, 2015). Constitutively active or aberrantly inhibited crucial signaling molecules or pathways encourage malignant conversion by endowing strategic advantageous properties of survival and proliferation on tumor cells. Some of these enabling characteristics include ability to proliferate without exogenous growth stimulation, insensitivity to anti-proliferative signals, capacity to invade adjacent non-tumor niche and migrate to distant sites, evasion of apoptotic death and resistance to replicative senescence as well as to induce neo-angiogenesis (Hanahan and Weinberg, 2000; Martin, 2003; Dongguang and Shaoguang, 2007). Moreover, deregulation in the cellular signaling pathways that regulate self-renewal and differentiation might be a significant contributor to therapy resistance in CSCs (Dreesen and Brivanlou, 2007; Karamboulas and Ailles, 2013). Understanding the abnormal cell signaling events culminating in malignant transgression will provide useful insights to develop effective therapies for treatment of many aggressive and drug resistant cancers (Bianco et al., 2006; Levitzki and Klein, 2010).

1.4.1 Hedgehog signaling pathway in development and cancer:

Hedgehog (HH) pathway is one of the most important developmental signaling pathways controlling many aspects of organogenic patterning and developmental transition from embryonic to adult tissues. Moreover, it also facilitates stem cell maintainenance, repair and regeneration in somatic compartments (Barakat et al., 2010; Teglund and Toftgard, 2010; Kar et al., 2012). The mammalian HH family consists of three members such as sonic hedgehog (SHH), Indian hedgehog (IHH) and desert hedgehog (DHH) (Hatsell and Frost, 2007). The HH ligand reception system is constituted by a 12-span transmembrane protein called patched (PTCH); and a 7-span transmembrane G-coupled receptor protein smoothened (SMO) (Jiang and Hui, 2008). The five-zinc finger containing transcription factors GLI proteins including GLI1, GLI2 and GLI3 are the principal downstream effector molecules of HH signaling pathway (Jacob and Briscoe, 2003; Ingham et al., 2011). In the absence of HH ligand, PTCH blocks SMO activity and retains the GLI proteins in the cytoplasm with other proteins such as kinesin-like COSTAL2, serine–threonine kinase Fused and suppressor of Fused (SUFU). However, when HH ligand binds to PTCH, it enables translocation of SMO to the primary cilium where its associated G protein activity inhibits suppressive kinase action on GLI factors (Ruiz i Altaba et al., 2007). Accumulation of GLI activators in the nucleus results in increased expression of HH target genes such as PTCH, GLI, IGF-2, platelet derived growth factor receptor α (PDGFR-α), cyclin D1 (CCND1) and cyclin D2 (CCND2) for cell cycle acceleration, forkhead box A2 (FOXA2) and T-box transcription factor (TBX2) for cell fate determination, cancer proliferation and invasion-related genes such as CCND, N-Myc, Myc, Snail and B-cell lymphoma-2 (BCL-2) etc. (Katoh and Katoh, 2008).

Given its significant contribution in embryonic development, any aberration in HH pathway components or activities results in a number of pathophysiological disorders and ultimately favors carcinogenic development. In fact, HH pathway is considered to be an oncogenic signaling pathway found constitutively active in many different cancer types such as gastrointestinal cancer, medulloblastoma, breast, lung, skin, gastric, prostate, hepatic and pancreatic cancer. Aberrantly active HH signaling pathway is involved in initiation, proliferation and propagation of many different types of cancers. The constitutive activation of the pathway can be due to multiple genetic and epigenetic aberrations including loss-of-function mutations in PTCH, SUFU, gain-of-function mutations in SMO and missense mutations in GLI1 and GLI3 or manipulation of levels of

HH ligands (SHH, IHH or DHH) (Teglund and Toftgard, 2010; Kar et al., 2012). The dysregulated HH pathway affects tumorigenic properties such as increased metastatic behavior by up-regulation of Snail, enhanced survival capability by the up-regulation of anti-apoptotic mediators Bcl-2, increased proliferative capacity through modulation of the cell cycle machinery and promotion of tumor invasiveness by down-regulating E-cadherin, MMPs and other metalloproteases (Clement et al., 2007; Kasper et al., 2009). Moreover, HH communicates with other signaling pathways active in cancer like Wnt and Notch to co-operatively increase tumorigenicity and inherent malignancy (Cerdan and Bhatia, 2010) (Fig. 1.4). In recent years, studies have implicated HH pathway to synergistically interact with pluripotency factors in mediating tumorigenic progression. Thus, HH pathway can be considered to be a crucial accomplice of the core transcriptional machinery as they arbitrate malignant transformation.

Figure 1.4: HH signaling participates in tumorigenic transformation by controlling various hallmarks of cancer. Constitutively active HH pathway results in initiation, proliferation and sustenance of malignant transformation and tumorigenesis by interaction with different effector and downstream targets (Kar et al., 2012).

Chapter 2

Review of Literature

2.1 Oct4 as the "gatekeeper" safeguarding ground state embryonic pluripotency:

The pit-oct-unc (POU) domain containing transcription factor Oct4 is a critical regulator of pluripotency in mammals. It is ubiquitously expressed in unfertilized oocytes, in the ICM of blastocyst and epiblasts in pre-implantation embryos as well as in the female germline (Livigni and Brickman, 2013; Jerabek et al., 2014; Wu and Schöler, 2014). One of the most important functions of Oct4 is to prevent early segregation of blastomeres into trophoectodermic lineage, thus it is called as the "gatekeeper" of pluripotent embryonic state (Zuccotti et al., 2011). It is also one of the irreplaceable Yamanaka Factors, meaning that Oct4 is indispensable for somatic reprogramming and cannot be substituted by any other core or auxiliary transcription factor (Sterneckert et al., 2012; Radzisheuskaya and Silva, 2014). Therefore, Oct4 expression, stability and activity is closely monitored and regulated by transcriptional, translational and post-translational modifications (Parfitt and Shen, 2014; Zeineddine et al., 2014). Although Oct4 is not expressed in adult cells, it is re-expressed in different types of cancer where it enhances tumorigenic activity and promotes aggressive behavior of neoplastic cells. Therefore, molecular mechanisms of Oct4 regulation and function and its dynamic interaction with progenitor niche both in stem and tumor cells should be thoroughly investigated.

2.1.1 Oct4 as the master embryonic fate determinant:

Oct4 was discovered as a transcription factor specific to early embryogenesis:

Oct4 was first identified in unfertilized oocytes, in the early embryo, in primordial germ cells (PGCs) and embryonic carcinoma cells (ECCs), but not found in somatic tissues (Schöler et al., 1989 (a)). The existence of Oct4 in almost all cell types having ESC properties identifies it as an ESC-specific transcription factor. Additionally, Oct4 is an indispensable marker for establishment of mammalian germline as its presence in PGCs ensures uninterrupted transfer of genetic information through succeeding generations.

Oct4 is a maternally expressed germline specific factor:

Oct4 is expressed in both male and female PGCs; however it is not detected in sperm or testis implying that Oct4 is a maternally expressed protein essential for specification of female germline during germ cell differentiation. In fact, male and female gametes can be discerned by the presence of Oct4 (Schöler et al., 1989 (b); Schöler et al., 1990 (a)).

Oct4 is essential for zygotic genome activation in pre-implantation embryo:

Maternal Oct4 mRNA level is constantly maintained through unfertilized oocytes to zygotic pronucleus, but is subsequently diminished in the 2- and 4-cell stage of development. Up-regulation of Oct4 again occurs after zygotic genome activation in the blastocyst stage and high levels of Oct4 is found in all blastomeres. Thus, Oct4 is a crucial maternal-derived factor that guides early cell divisions during maternal-to-zygotic transition in early pre-implantation embryo (Schöler et al., 1990 (b); Rosner et al., 1990; Pesce and Schöler, 2000).

Oct4 decides segregation of embryonic lineages in post-implantation blastocyst:

Oct4 is differentially expressed during the first lineage differentiation events in the embryo; while trophoectodermic cells have lower levels of Oct4, while cells of ICM exhibit high protein expression (Palmieri et al., 1994; Nichols et al., 1998). Oct4 recruits SET domain bifurcated 1 (SETDB1) histone methyltransferase and blocks the expression of caudal-related homeobox 2 (CDX2) and other trophoblast associated genes in the blastocyst, thus commencing the first segregation event in embryonic development (Yuan et al., 2011; Blij et al., 2012). In the second part, Oct4 along with Sox2 and Nanog participates in the differentiation of ICM along primitive endoderm lineage by activating fibroblast growth Factor 4 (FGF4) and its downstream target GATA-binding factor 6 (GATA6) which predisposes epiblast cells towards primitive endoderm (Yamanaka et al., 2010). Moreover, Oct4 specifies meso-endodermal commitment with the help of bone morphogenetic protein 4 (BMP4) and suppresses neural ectodermal formation (Zeineddine et al., 2006; Downs, 2008; Thomson et al., 2011; Wang et al., 2012).

Oct4 is restricted to germ cells after gastrulation:

After gastrulation, Oct4 expression is restricted to the male and female germ cells. Although, maturing and ovulated oocytes exhibit Oct4 protein, resting oocytes and sperms are devoid of Oct4, implying that Oct4 is a female germline specific marker and

that Oct4 maintains "totipotency cycle" starting from fertilization to germ cell specification (Yeom et al., 1996).

2.1.2 Multi-tiered regulation of Oct4 expression, stability and activity:

Developmentally restricted auto-regulatory control of Oct4:

The genomic locus of Oct4 in chromosome 6 features regulatory elements like proximal promoter (PP), cis-acting proximal enhancer (PE) and distal enhancer (DE) situated 2 kb upstream of transcription start site (TSS) (Niwa, 2007). The PP region is GC-rich, lacks TATA-box sequence but includes binding sites for specificity protein 1 (SP1), retinoic acid receptor (RAR) as well as for orphan nuclear receptors like germ cell nuclear factor (GCNF) and COUP transcription factor 2 (COUP-TFII/ARP-1) all of which contribute towards POU domain, class 5, transcription factor 1 (*Pou5f1)* silencing during differentiation (Ben-Shushan et al., 1995; Gu et al., 2005 (b)). The cis-acting enhancer elements are functionally restricted to specific developmental stages as PE and DE are differentially active in pluripotent and totipotent cells of embryo respectively. PE, referred to as epiblast enhancer, is stage-and tissue-specific as it controls Oct4 expression in epiblast and epiblast stem cells in the early post-implantation embryo. DE, otherwise called germline enhancer is lineage-specific and active in ICM of preimplantation embryo and embryonic germ cell lines where it specifically drives Oct4 expression in PGCs (Yeom et al., 1996). In addition, each enhancer is enriched with binding sites for numerous active/repressive epigenetic marks and transcription factors suggesting that chromatin remodeling and co-operative transcriptomic network also impact Oct4 expression and function (Gao et al., 2013).

Oct4 transcription is actively maintained in ESCs by Paf1:

Oct4 transcription is actively maintained in ESCs by RNA polymerase II-associated factor 1C (Paf1C), a component of the Paf1 complex, which binds to PP in association with RNA polymerase II and retains active chromatin architecture (Ding et al., 2009). During differentiation, each of the subunits of Paf1 complex is downregulated, followed by decrease in Oct4 transcription. Similarly, Oct4 mRNA is rapidly translated by Lin28 with the help of RNA helicase A in ESCs (Qiu et al., 2010).

DNA methylation and histone modifications silence Oct4 during differentiation:

Epigenetic mechanisms play a very important role in suppressing Oct4 expression although Oct4 gene is not subjected to imprinting. In ESCs, Oct4 activation is necessary for maintaining pluripotency, therefore Oct4 gene locus is hypomethylated and enriched with active histone marks like H3 lysine9 acetylation (H3K9Ac), H3 lysine14 acetylation H3K14Ac and H3 lysine4 di-/tri-methylation (H3K4me2/me3) (Hattori et al., 2004). However, as differentiation sets in, sequential epigenetic changes gradually silences Oct4 activity. Firstly, euchromatic histone-lysine N-methyltransferase 2 (EHMT2/G9A) binds and recruits histone deacetylases (HDACs) to the promoter of Oct4. Secondly, G9A catalyzes repressive histone H3 di-methylation on Lys 9 (H3K9me2) by its Su (var), E (z), Trithorax (SET) domain which results in binding of heterochromatin protein 1 (HP1) and formation of less accessible, highly condensed heterochromatin (Feldman et al., 2006). Following transient repression by histone marks, Oct4 is permanently silenced in somatic cells by DNA methylation in the promoter and enhancer by *de novo* DNA methyltransferases 3A and 3B (DNMT3A, DNMT3B) (Li et al., 2007; Athanasiadou et al., 2010). Additionally, cyclin-dependent kinase 2-associated protein 1 (CDK2AP1) interacts with methyl-binding domain 3 (MBD3) protein present in the methylated Oct4 promoter in association with DNMT3A, DNMT3B and histone deacetylase/nucleosome remodeling (HDAC/NuRD) complex and augments down-regulation of Oct4 expression (Deshpande et al., 2009).

Oct4 transcription is antagonistically modulated by orphan nuclear factors:

Transcription of Oct4 is modulated both positively and negatively by a host of nuclear factors that bind to regulatory elements of Oct4 gene locus in a context-dependent manner (Mullen et al., 2007). For sustaining pluripotency in ESCs, Oct4 is up-regulated by steroidogenic factor1 (SF1) and ESRRB (Yang et al., 2007; Zhang et al., 2008). Similarly, liver receptor homolog 1 (LRH1) binds to PP and PE of Oct4 and sustain its expression in the epiblast (Gu et al., 2005(a)). On the other hand, GCNF up-regulated by retinoic acid during differentiation, binds to PP and by employing DNMT3A and DNMT3B permanently limits Oct4 expression in the germ cell lineage after gastrulation (Fuhrmann et al., 2001; Gu et al., 2005 (b); Sato et al., 2006). Also, COUP-TFII /ARP-1 and COUP transcription factor 1 (COUP-TFI /EAR-3) also bind to PP and repress Oct4 expression (Sylvester and Scholer, 1994; Ben-Shushan et al., 1995).

miRNA mediated post-transcriptional regulation of Oct4:

Non-coding RNAs like microRNAs regulate post-transcriptional expression of Oct4. miR-145 directly binds to 3′ untranslated region of Oct4 mRNA and causes its decay during differentiation (Xu et al., 2009(a)). Similarly, miR-296, miR-134 and miR-470 bind to the amino acid coding sequence of Oct4 to negatively regulate its expression (Tay et al., 2008). Moreover, ESC specific microRNAs miR-290, miR-291, miR-292, miR-293, miR-294, miR-295 indirectly affect Oct4 by facilitating transcription of DNMT3A and DNMT3B (Sinkkonen et al., 2008).

Alternate splicing generates post-transcriptional structural isoforms of Oct4:

Alternate splicing of *Oct4* generates three different mRNA transcripts referred to as Oct4A, Oct4B and Oct4B1 of which Oct4A is the only functional product which localizes to nucleus as Oct4 and helps in maintenance of pluripotency (Atlasi et al., 2008; Wang and Dai, 2010). The Oct4B transcript contains exon 2a in place of exon 1 sequence in the N-terminal and is alternately translated into Oct4B-265, Oct4B-190 and Oct4B-164 proteins by internal ribosomal entry site (Wang et al., 2009). While Oct4B-265 is expressed during genotoxic stress induced by p53 mediated apoptosis; Oct4B-190 over-expression protects cells against heat shock mediated apoptosis (Gao et al., 2012). Oct4B1 is functionally redundant with Oct4B producing the same gene products as Oct4B (Gao et al., 2010).

Post-translation modifications affect functional capacity of Oct4 protein:

Covalent PTMs of proteins such as methylation, acetylation, phosphorylation, sumoylation and ubiquitination influence their functional potential by affecting their activity, stability and interaction with other proteins (Cai et al., 2012; Kar et al., 2012). Oct4 is subjected to differential phosphorylation in POU domain (Thr235, Ser236, Ser288, and Ser289) as well as in the N- and C-terminal transactivation domains where they sterically hinder DNA-binding affinity, homodimer assembly and transactivation potential (Saxe et al., 2009; Brumbaugh et al., 2012). Moreover, phosphorylation of serine 111 residue interferes in the nuclear trans-localization of Oct4 (Spelat et al., 2012). Oct4 is also tagged with monosaccharide O-linked β-N-acetylglucosamine (O-GlcNAc) by the enzyme O-GlcNAc transferase (OGT), affecting the reprogramming capacity of Oct4 (Jang et al., 2012). Sumoylation of K123 residue of Oct4 increases its stability, DNA-

binding and transactivation potential during primitive endoderm differentiation (Wei et al., 2007; Zhang et al., 2007). Poly-ubiquitination of Oct4 by WW domain containing E3 ubiquitin protein ligase 2 (WWP2) promotes proteosomic degradation of Oct4 drastically reduces its transcriptional activity during early stages of differentiation (Xu et al., 2009 (b); Liao et al., 2013).

2.1.3 Functional Interactomes and transcriptional targets of Oct4:

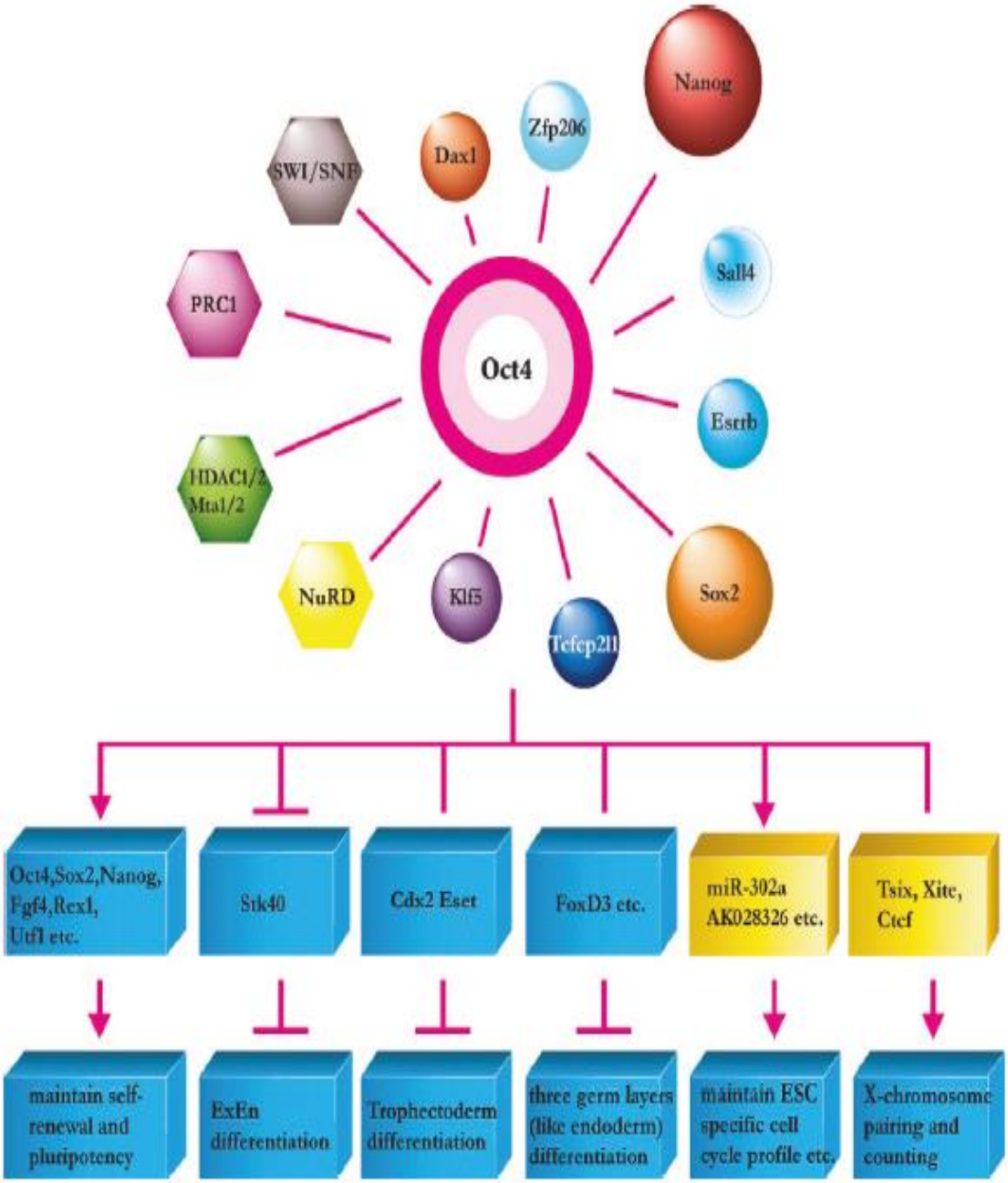


Figure 2.1: Oct4 interacts with multiple partners and accomplices to either activate or repress context-dependent transcriptional program (Adapted from Shi and Jin, 2010).

Interaction with core pluripotency maintaining transcription factors to balance self-renewal and differentiation of ESCs:

The triumvirate of Oct4, Sox2 and Nanog constitute the core pluripotency transcriptional network and synergistically activate the expression of other axillary transcription factors such as ESRRB, SALL4, KLF4, ZFX, STAT3, rap1 interacting factor 1 (RIF1) and undifferentiated embryonic cell transcription factor 1 (UTF1) all of which play important roles in maintaining pluripotency (Matoba et al., 2006; Kim et al., 2008; Chambers and Tomlinson, 2009; Kashyap et al., 2009; Young, 2011). Concurrently, Oct4 directly inhibits serine/threonine kinase 40 (STK40) to prevent extra-embryonic endoderm differentiation (Li et al., 2010). Oct4 also hinders trophoectodermic differentiation by negatively regulating CDX2 and thwarts endoderm formation by repressing FOXD3 (Guo et al., 2002; Yuan et al., 2009) (Fig. 2.1).

Interaction with epigenetic modifiers and chromatin remodelers:

Oct4 is known to interact with many chromatin remodeling complexes such as NuRD/ HDAC complex, polycomb and trithorax group of proteins, SWItch/Sucrose Non-Fermentable (SWI/SNF) and histone lysine specific demethylase 1 (LSD1) to either activate pluripotency genes or generate repressive/poised chromatin environment in ESC during development (Liang et al., 2012) (Fig. 2.1). In concert with stem cell-specific embryonic specific BRG1-or-HRBM-associated factors (esBAF), Oct4 establishes permissive transcriptional state where different other co-factors interact with basal transcriptional machinery to induce pluripotency gene expression. At the same time, Oct4 instructs NuRD complex and polycomb proteins to add repressive H3 lysine27 tri-methylation (H3K27me3) marks and silence developmentally committed genes (Ang et al., 2011; Mansour et al., 2012). These interactions present concrete evidences to support cooperativity between epigenetic modifiers and pluripotency inducing TFs in maintaining ESC-specific gene expression. In addition, during segregation of blastocyst, Oct4 partners with SETDB1 to restrict extraembryonic differentiation in trophoblast lineage (Yuan et al., 2009).

Interaction with ESC exclusive developmental signaling pathways:

In order to regulate stem cell pluripotency, Oct4 cooperates with components of many ESC exclusive developmental signaling pathways. For example, Oct4 associates with recombining binding protein suppressor of hairless (RBPJ), the transcriptional effector of

Notch signaling pathway (Pardo et al., 2010). Similarly, membrane-associated complex of Oct4 with β-catenin results in proteosomal degradation of β-catenin, demonstrating that Oct4 uses Wnt pathway to maintain ground state pluripotency in ESCs (Faunes et al., 2013) (Fig. 2.1).

Interaction with components of basal transcriptional machinery, cell cycle, DNA repair, replication and recombination pathways:

Oct4 target gene ESRRB interacts with the mediator complex (transcription coactivator), RNA polymerase II subunits, TATA-box binding proteins and transcription factor II D (TFIID) in order to control the basal transcriptional machinery during maintenance of pluripotency or initiation of differentiation (Fig. 2.1). One of the direct partners of Oct4, transcription factor CP2-like 1 (TCFCP2L1) interacts with proteins involved in DNA replication and repair (van den Berg et al., 2010). Oct4 positively regulates miR-302a which maintains ESC-specific cell cycle profile by down-regulation of G_1 regulator CCND1 (Card et al., 2008; Sheik Mohamed et al., 2010).

2.2 Sox2 as the transcriptional alchemist conjuring differential stem cell destiny:

Sox2 is one of the most important regulators of mouse and human embryonic stem cell destiny. It is considered as the transcriptional alchemist who arbitrates multiple lineage-speciation and commitment events during embryonic development (Zhang and Cui, 2014). It is also responsible for maintenance of adult stem cells in somatic tissues for homeostatic balance, tissue repair and regeneration and is one of the critical factors for generating induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006). Moreover, ectopic expression and gene amplification of Sox2 has been associated with occurrence and development of cancer in multiple tissues, such as lung and breast (Weina and Utikal, 2014). A dynamic surveillance system consisting of transcription factors, miRNAs, epigenetic regulators and signaling pathways and handled by transcriptional, translational and post-translational manipulations is therefore operational in order to control and regulate the diverse functional roles played by Sox2 (Liu et al., 2013; Sarkar and Hochedlinger, 2013; Feng and Wen, 2015). The intricate network of regulators and modulators controlling Sox2 expression, activity and stability is closely monitored and rigidly preserved so that Sox2 mediated differentiation and reprogramming efficiently maintains pluripotency and cell-specific destiny.

2.2.1 Versatility of Sox2 in regulating progenitor cell fate:

Sox2 is a multi-tasking transcription factor associated with almost all stages of embryonic transition. It is necessary for the establishment of totipotent pre-implantation zygote, maintenance of pluripotent ESCs during blastocyst formation and differentiation of multipotent progenitor ectodermal, endodermal and mesodermal cells into lineage committed byproducts. Further down the lane, Sox2 also takes part in adult tissue homeostasis and regeneration displaying its indispensability during mammalian embryogenesis and organogenesis (Sarkar and Hochedlinger, 2013; Sarlak and Vincent, 2016).

Sox2 in pre-implantation zygotic development:

Following fertilization, maternal Sox2 protein initiates maternal-to-zygotic transition by zygotic gene activation during morula to blastocyst transition resulting in totipotent blastomeres of the early preimplantation embryo (Pan and Schultz, 2011). Sox2 facilitates the first lineage speciation process involving segregation of blastocyst into ICM and trophoectoderm where it gets confined to the ICM and subsequently promotes maintenance of ESCs (Avilion et al., 2003; Keramari et al., 2010). In addition, deletion of Sox2 from ICM results in trophoblastic differentiation of ESCs demonstrating the significance of Sox2 in maintenance and stabilization of pluripotency (Masui et al., 2007) (Fig. 2.2).

Sox2 in post-implantation lineage speciation and commitment:

Post-gastrulation, Sox2 participates in lineage differentiation process in developing ectodermal, mesodermal and endodermal cell lineages (Fig. 2.2).. In ectodermal speciation, Sox2 adopts two contrasting methods; where firstly, it sustains proliferation of fetal progenitor cells into neuroectodermal fate and secondly induces terminal differentiation into central and peripheral nervous systems (CNS and PNS). Sox2 contributes towards differentiation of ESCs into early neuroectodermal neural fate by suppressing meso-endodermal commitment via inhibition of brachyury (Thomson et al., 2011; Wang et al., 2012). Sox2 decides the fate of early neural progenitor cells into either paraxial mesoderm in alliance with T-box 6 (Tbx6) or neural tubes in the absence of any other competing transcriptional regulator (Takemoto et al., 2011). Further, Sox2 mediates terminal differentiation of CNS and retinal progenitors into brain and eye respectively by

direct interaction with Notch1 (Taranova et al., 2006). Additionally, Sox2 is involved in proliferation of Schwann cell precursors and in maturation of neural crest progenitor cells into sensory ganglia (Le et al., 2005; Cimadamore et al., 2011). Sox2 expression induced by fibroblast growth factor 8 (FGF8) in dental epithelial stem cells during early phases of tooth morphogenesis results in their segregation into enamel-producing ameloblasts and other differentiated cells of dental stem cell niche (Juuri et al., 2012). Moreover, Sox2 is necessary for the formation of cochlear prosensory domain within the inner ear (Dabdoub et al., 2008).

Similar to its role in ectoderm, Sox2 is also implicated in mesodermal lineage commitment during skin and bone development (Fig. 2.2). During skin development, Sox2 expression initiates formation of dermal sheath and dermal papilla (DP) from mesenchymal somatic dermal condensates. Continuous expression of Sox2 in DP niche induces differentiation of hair shaft progenitors by specifying hair follicle type and controlling BMP-mediated mesenchymal-epithelial crosstalk (Driskell et al., 2009; Clavel et al., 2012). Also, Sox2 is found to help in proliferation of osteoblast progenitors. Extending Sox2 function from ectodermal and mesodermal patterning into endodermal derivatives, it is seen that Sox2 helps in organogenic specification of foregut by giving esophagus and anterior stomach (Que et al., 2007). Sox2 also endorses morphogenic differentiation of embryonic tongue into taste bud sensory cells (Okubo et al., 2009) and lung and tracheal formation from respective embryonic counterparts (Gontan et al., 2008; Que et al., 2009). Thus, Sox2 is extensively associated with remodeling of ectodermal, mesodermal and endodermal progenitor populations into desired committed cells during lineage speciation events.

Sox2 in sustaining adult tissue homeostasis and regeneration:

Sox2 expression persists in the adult progenitor pool in those tissues and organs that are dependent upon Sox2 for development. For example, Sox2 maintains neural precursor cells (NPCs) in neurogenic regions of lateral ventricle and adult hippocampus where it mediates neurogenesis (Favaro et al., 2009). Moreover, Sox2 also enables propagation of adult unipotent stems cells of retina, trachea, tongue epithelium and dermal papilla of the hair follicle (Taranova et al., 2006; Que et al., 2009; Okubo et al., 2009; Driskell et al., 2009). Also, adult testes, forestomach, glandular stomach, trachea, anus, cervix, esophagus, lens, and dental epithelium also depend upon Sox2 for constant tissue homeostasis (Arnold et al., 2011). In addition, Sox2 is reactivated in case of tissue

damage and assist in the repair process by promoting dedifferentiation or expansion of resident adult progenitor cells. As in case of peripheral nerve damage, Sox2 is re-expressed in mature adult Schwann cells by ephrin type-B receptor 2 (EphB2) signaling where it mediates demyelination and clustering of Schwann cells for regrowing axons across the site of injury (Parrinello et al., 2010)(Fig. 2.2).

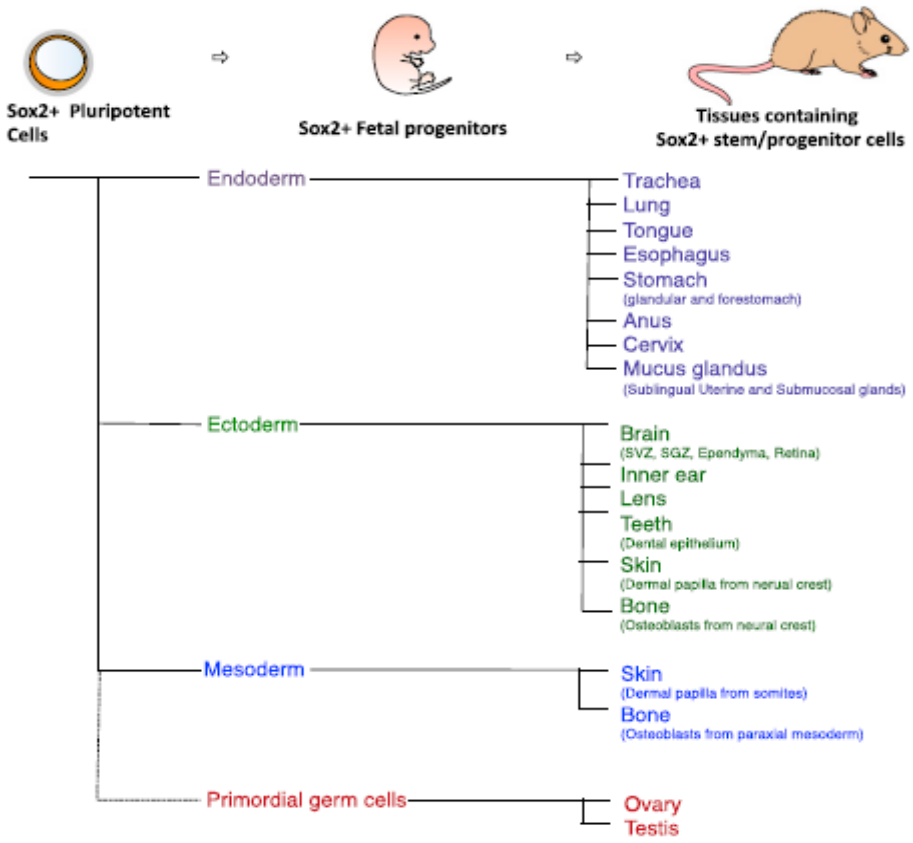


Figure 2.2: Versatile role of Sox2 in maintaining and expressing pluripotency states during development. Sox2 helps in determination and establishment of pluripotency and cell-fate in pre-implantation zygotic, post-gastrulation embryonic and fetal as well adult progenitor and somatic stem cells (Adapted from Sarkar and Hochedlinger, 2013).

2.3 Nanog as the transcriptional rheostat against differentiation and speciation:

Nanog is a more recently identified transcription factor which participates in determination of cell fate in the pluripotent ICM, maintains stem cell phenotype in the epiblast, thwarting impromptu endoderm differentiation (Wang et al., 2013). Nanog expression is regulated by Oct4/Sox2 heterodimers which bind to its proximal promoter and regulate its transcriptional activity. In conjunction with Oct4 and Sox2, Nanog forms auto-regulatory feedback circuitry by binding to their own promoters and maintaining

pluripotency (Pan and Thomson, 2007; Saunders et al., 2013). Amazingly, Oct4, Sox2 and Nanog simultaneously target many secondary transcription factors that stabilize the ESC state and subdue differentiation and lineage-specific genes. Most notably, Nanog also contributes towards malignant transformation in a variety of cancers and results in tumor growth and recurrence, increased drug-resistance, enhanced proliferation and up-regulation of invasion and metastatic ability (Du et al., 2012).

2.3.1 Regulatory networks controlling Nanog expression and function:

Auto-regulatory positive and negative feedback control of Nanog:

In order to compensate for the extensive fluctuations in its level in ESCs, Nanog can bind to its own promoter and positively or negatively impact its own mRNA and protein levels. Although, Nanog increases expression of its own protein generally in conjunction with Oct4 and Sox2, it can also interact with Zic3 at its promoter and drive its transcription even in the absence of Oct4 and Sox2 (Lim et al., 2010; Navarro et al., 2012). Similarly, auto-repression of endogenous Nanog production is achieved by interaction of Nanog with zinc finger protein 281 (Zfp281) associated with NuRD/HDAC repressor complex (Fidalgo et al., 2011; Fidalgo et al., 2012).

Regulation of Nanog by key transcriptional factors of pluripotency network:

Nanog is extensively regulated by a host of transcription factors, including Oct4 and Sox2 in order to maintain balanced Nanog expression and functional capacity. Oct4, Sox2 and Nanog co-regulate each other through positive feedback loop by binding to enhancer elements in their respective promoters and modulate their expression (Rodda et al., 2005; Kuroda et al., 2005). The proximal promoter region of Nanog is mostly occupied by a battery of transcription factors that either activate or repress Nanog. For example, FoxD3 binds to enhancer located 270 kb upstream to Nanog promoter and activates Nanog gene transcription. ESSRB, bound to nuclear receptor coactivator 3 (Ncoa3) is recruited to Nanog promoter by Oct4 where it associates with RNA polymerase II and activates the basal transcriptional machinery and Nanog expression (van den Berg et al., 2008; Festuccia et al., 2012). Another core transcriptional factor KLF4 also binds to the proximal promoter of Nanog to increase its expression (Chan et al., 2009). Negative regulators that reduce Nanog levels during differentiation include CDX2 and GCNR that bind to promoter of Nanog and down-regulate it in order to enable differentiation to start

(Chen et al., 2009). Further, TCF3 also suppresses Nanog levels by binding to its regulator region and allowing proper differentiation (Pereira et al., 2006).

Regulation by epigenetic modifiers and chromatin remodeling complexes:

Epigenetic modifications are already known to influence transcriptional activity of Oct4 and Sox2, hence it is not surprising that expression and activity of Nanog is also affected by chromatin modifiers. For example, SIN3 homolog A/histone deacetylase (SIN3A/HDAC) co-repressor complex is recruited by p53 to the promoter of Nanog during differentiation to reduce acetylation and create a compacted chromatin structure so as to inhibit transcription of Nanog (Lin et al., 2005; Baltus et al., 2009). On the other hand, active gene expression of Nanog is supported by histone acetyltransferases and methyltransferases. The histone acetyltransferase, males absent on the first (MOF) directly binds to the promoter of Nanog and increases its mRNA and protein levels (Li et al., 2012). WD repeat-containing protein 5 (WDR5), a histone methyltransferase belonging to the mixed-lineage leukemia (MLL) complex is instructed by Oct4 to enrich H3K4 tri-methylation at Nanog promoter to stimulate its expression (Ang et al., 2011). Another histone methyltransferase enhancer of zeste homolog 2 (EZH2) is also known to influence expression of Nanog by H3K27me3 marks in the Nanog promoter and balances the equilibrium between self-renewal and differentiation in ESCs (Villasante et al., 2011; Herberg and Roeder, 2011). Higher order chromatin organizers like special adenine-thymine (AT)-rich DNA-binding protein 1 (SATB1) and SATB2 are also respectively involved in positive and negative regulation of Nanog expression (Savarese et al., 2009).

Post-translational modulation of the stability and function of Nanog:

Post-translational phosphorylation of Nanog Serine/Threonine residues is known to increase Nanog stability by promoting its interaction with prolyl isomerase (Pin1) and preventing its proteosomal degradation (Moretto-Zita et al., 2010). Interestingly, phosphorylation of Nanog by focal adhesion kinase (FAK) and protein kinase Cε (PKCε) promotes nuclear translocation of Nanog where it activates oncomiR-21 and mediates tumor growth (Bourguignon et al., 2012). In addition, Nanog is ubiquitinated at Lys26 and Lys48 residues which targets it for proteosomal degradation (Ramakrishna et al., 2011).

Signaling pathways control Nanog expression and activity:

Signal transduction pathways are crucial to maintaining homeostatic gene expression programs during self-renewal and pluripotency or differentiation and lineage-commitment. Nanog expression is also modulated by several signaling cascades, notable among them being LIF pathway which works via two parallel effectors Janus kinase/signal transducers and activators of transcription 3 (JAK/STAT3) and phosphatidylinositol-4,5-bisphosphate 3-kinase/AKT (PI3K/AKT) (Niwa et al., 2009). LIF-STAT3 pathway also activates Nanog expression by interacting with Brachyury at STAT3 binding site in the enhancer region 5 kb upstream of the TSS in Nanog promoter. BMP pathway is also involved in the regulation of Nanog in association with transforming growth factor-β/SMAD1 (TGFβ /SMAD1) (Suzuki et al., 2006; Xu et al., 2008). FGFR2 down-regulates Nanog gene transcription by activating MEK pathway and subsequently mediates differentiation into primitive endoderm (Santostefano et al., 2012). Similarly, glycogen synthase kinase 3 (GSK3) is also involved in regulation of Nanog in order to specify pluripotency (Luo et al., 2013).

2.3.2 Role of Nanog in pro-tumorigenic reprogramming:

Over-expression of Nanog is indicative of poor survival and prognosis:

Expression of Nanog has been detected in germ cell tumors as well as other tumors, including breast, cervix, oral, kidney, prostate, lung, gastric, brain, and ovarian cancer. Strong expression of Nanog is shown as an indicator of a poor prognosis for ovarian serous carcinoma, colorectal, and breast cancer patients. In oral squamous cell and lung adenocarcinoma, higher expression of Nanog, along with Oct4, was associated with advanced cancer stage and shorter patient survival rate (Fig. 2.3).

Nanog promotes increased cell proliferation and cell cycle in cancer:

In cancers, over-expression of Nanog mediates increased tumor cell proliferation and growth (Fig. 2.3). Nanog interacts with members of cell cycle machinery such as CCND1, CCNE1, cyclin dependent kinase 1(CDK1), growth differentiation factor3 (GDF3) and transcriptionally activates them to accelerate proliferation of neoplastic cells by over-riding cell cycle checkpoints. While gain-of Nanog expression studies demonstrated increase in colony formation, loss-of-function of Nanog resulted in G_1 arrest and lower proliferation and survival in lung, breast and ECC (Choi et al., 2012; Han et al., 2012; Park et al., 2012).

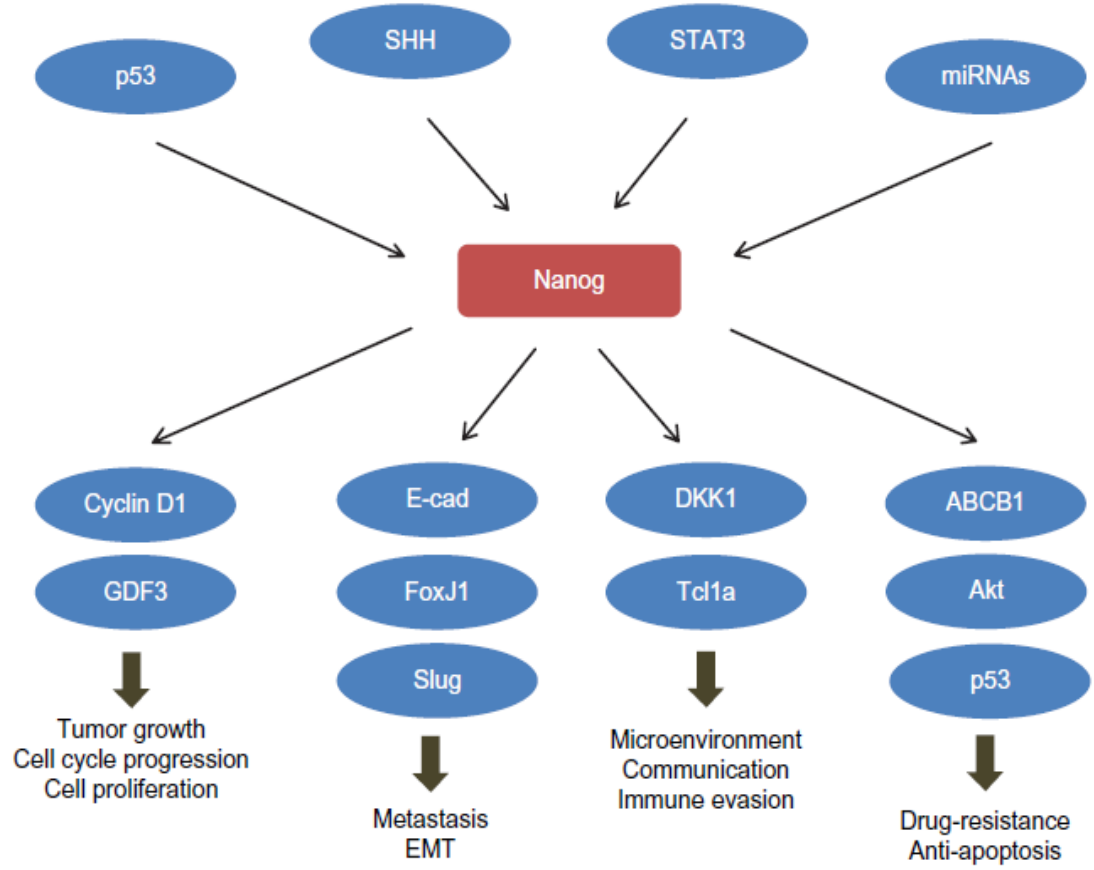


Figure 2.3: Role of Nanog in oncogenic reprogramming and malignant adaption. Nanog is regulated by several signaling pathways and in turn interacts with multiple protein partners to encourage tumorigenic properties and cause malignancy (Adapted from Wang et al., 2013).

Nanog enhances tumor metastasis and invasive potential of tumor cells:

Up-regulation of Nanog results in increase of transcript levels of epithelial to mesenchymal (EMT) specific genes such as E-cadherin, FOXO1, FOXO3A, forkhead box J1 (FOXJ1), forkhead box B1 (FOXB1) and SLUG, thus improving metastatic ability of tumor cells and encouraging EMT and invasiveness in higher grade, advanced stages of cancer (Chiou et al., 2010; Siu et al., 2013) (Fig. 2.3).

Nanog provides resistance against apoptosis and chemotherapeutic drugs:

Increase in stemness marker Nanog promotes resistance to chemotherapeutic drugs like cisplatin (Tsai et al., 2011). Nanog associates with STAT3 to increase the expression of multi-drug resistance transporter ATP-binding cassette sub-family B member 1 (ABCB1) which excludes drugs from tumor niche (Bourguignon et al., 2012). In addition, Nanog interacts with p53 and AKT pathways and makes tumor cells resilient to apoptotic death.

Nanog improves tolerance of tumor cells to immune attack and surveillance:

In conjunction with hyperactive T-cell leukemia/lymphoma 1A/AKT (TCL1A/AKT) pathway, Nanog helps neoplastic cells to evade CD8+ cytotoxic T lymphocytes employed by host immune system. By increasing stem-cell like properties of cancer cells, Nanog empowers the tumor cells to avoid detection and elimination by immune system (Noh et al., 2012 (a); Noh et al., 2012(b)).

Nanog interacts with oncogenic pathways to encourage malignant growth:

Crosstalk between Nanog and oncogenic signaling pathways such as ESRRB, PI3K/AKT, HH, STAT3, p53 etc. boosts inherent tumorigenicity by enhancing CSC-like properties, cell survival and self-renewal and improving cross-communication between cancer associated stromal cells in the tumor niche (Zbinden et al., 2010; Lee et al., 2011; Hawkins et al., 2012; Yi et al., 2012) (Fig. 2.3).

2.5 Objectives of the thesis:

Crosstalk between pluripotency factors and epigenetic modifiers is one of the fundamental forces behind pluripotent state of stem cells and their gradual progression towards specialized cellular identity. The Holy Trinity of "Oct4, Sox2 and Nanog" are regarded as the connecting bridge between embryonic development and malignant evolution and are increasingly considered as novel targets for chemotherapeutic interventions in cancer. Hence, it is necessary to investigate the epigenetic regulatory network and signaling pathways controlling expression of pluripotency inducing TFs in cancer. Keeping the above views in mind, objectives of the thesis are as follows:

1. To investigate the expression profile of pluripotency inducing TFs in breast and prostate cancer.
2. To evaluate the role of pluripotency inducing TFs in promoting inherent tumorigenicity in breast and prostate cancer.
3. To elucidate the epigenetic modification marks regulating pluripotency inducing TFs in breast and prostate cancer.
4. To explore the connection between oncogenic Hedgehog signaling pathway and pluripotency inducing TFs in breast and prostate cancer.

Chapter 3

To investigate the expression profile of pluripotency inducing TFs in breast and prostate cancer

3.1 Introduction:

Breast and prostate cancers are the most frequent cause of cancer-related deaths in females and males respectively. Both the solid tumors are heterogeneous diseases, with distinct morphologies, metastatic behaviour and therapeutic responses (Ferlay et al., 2010). Despite advances in early detection, approximately 30% of patients with early stage breast and prostate cancer show recurrence (Gonzalez-Angulo et al., 2007). Initially, patients subjected to chemotheraphy or radiation treatment responded favourably to systemic therapy, but post-therapeutic recurrence occurs in majority of cases leading to increased mortality rates (Ben-Porath et al., 2008). Cancer cells are one of the most adaptive entities which continuosly develop better alternatives to escape immune action and treament regimes; hence in order to improve the patient survival rate and develop better therapeutic strategies, understanding the diversity and versatility that underlie the molecular origin and maintenance of oncogeneis is a necessity.

As oncologists are struggling to unravel the molecular heterogenity that defines neoplastic progress, commonalities between embryonic differentiation and malignant transformation are considered to be of prime importance. It is now assumed that factors mediating embryogenic pluripotency and differentiation are important contributors to malignant reprogramming where they facilitate the breaking of differentiation barriers and reset oncogenic potential. These factors are none other than pluripotency inducing TFs especially Oct4, Sox2 and Nanog who together function as the gatekeepers of pluripotency in ESCs. These TFs are now considered as the mechanistic link connecting embyrogenesis and oncogenesis by encouraging similar molecular re-configuration of the epigenomic landscape that supports neoplatic adaption (Klimczak, 2015). In many recent reports, Oct4, Sox2 and Nanog have found to be over-expressed in different types of cancers wherein by multiple mechanisms and interaction with numerous partners they arbitrate oncogenic progress (Ezeh et al., 2005). Considerable evidences have implicated

Oct4 over-expression in neoplastic growth in breast, bladder, non-small cell lung, gastric, esophageal as well as germ cell tumors (Zhang et al., 2010; Rijlaarsdam et al., 2011; Huang et al., 2012; Zhao et al., 2012). Moreover, elevated Oct4 expression in these cancers has been associated with poor prognosis and shorter survival (Cheng et al., 2012; He et al., 2012). Similarly, Sox2 amplification has been reported in many human tumor types including glioblastoma, lung, colon, cervix, gastric, hepatic, breast and prostate cancer as well as in germ cell tumors (Weina and Utikal, 2014). Aberrant up-regulation of Nanog expression has been detected by several groups in germ cell tumors as well as other tumors, including breast, cervix, oral, kidney, prostate, lung, gastric, brain, and ovarian cancer (Choi et al., 2012; Han et al., 2012). The over-expression of Nanog is an indicator of a poor prognosis for ovarian serous carcinoma, colorectal, and breast cancer patients. Higher expression of Nanog is known to indicate advanced cancer stage and results in shorter patient survival rate in oral squamous cell and lung adenocarcinoma (Rodda et al., 2005; Wang et al., 2013).

The present objective was designed to investigate and establish the expression profile of pluripotency inducing TFs- Oct4, Nanog and Sox2 in human breast and prostate cancer tissues and cancer cell lines. In this regard, two breast cancer cell lines, MCF7 and MDA-MB-231 and two androgen independent prostate cancer cell lines DU145 and PC3 were selected as tumorigenic cell lines along with immortalized human keratinocytes HaCaT cell line chosen as the normal counterpart. *In silico* analysis using publicly available databases and experimental validation of transcript and protein level expression of Oct4, Nanog and Sox2 was done in order to elucidate the diverse expressions of these TFs in breast and prostate cancer. A clear and transparent picture regarding the status of Oct4, Nanog and Sox2 in breast and prostate cancer will be helpful in constructing the molecular roadmap by which these TFs mediate and support carcinogenic phenotype. Establishing the expression pattern and functional activity of pluripotency inducing transcription factors Oct4, Sox2 and Nanog in breast and prostate cancer will help in generating new prognostic and diagnostic biomarkers for early detection and identification of aberrant stem-cell signatures in neoplastic samples and provide opportunities for better treatment procedures.

3.2 Materials and Methods:

3.2.1 *In silico* analyses from publicly available databases:

Pre-experimental *in silico* analyses of expression of Oct4, Sox2 and Nanog was done prior to wet lab experiments by employing publicly available online bioinformatics tools cBioPortal having links to TGCA database (Cerami et al., 2012; Gao et al., 2013). From this database, copy number alteration (CNA – amplification, deletion, mutation) and genomic profile (mRNA up-regulation and down-regulation) of Oct4, Sox2 and Nanog from patient samples present in TGCA were evaluated for breast and prostate adenocarcinoma cancer. Further, mutually exclusive relationship between Oct4, Sox2 and Nanog in breast and prostate cancer was also computed.

3.2.2 Immunohistochemistry of breast and prostate tissue samples:

A total of 65 breast (primary=35, metastatic=30) and 33 prostate (benign prostate hyperplasia (BPH)=15, malignant=18) formalin fixed parafilm embedded (FFPE) tissue samples from cancer tissue library were selected and analyzed. The tissue embedded in parafilm blocks were cut into 0.5µm sections and subjected to antigen retrieval with tris-EDTA buffer, endogenous peroxidase blocking and rinsed with tris-buffered saline (TBS) containing 0.025% Triton X-100 (Himedia-RM845) (TBS-T). Rabbit polyclonal anti-Oct4 (Abcam-ab19857), rabbit polyclonal anti-Sox2 (Abcam-ab59776) and rabbit polyclonal anti-Nanog (Abcam-ab21624) were used as primary antibodies. After incubation with primary antibody at 4° C for overnight, tissue sections were rinsed with TBS and incubated at room temperature for 1 h with appropriate HRP conjugated secondary antibody (Goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology- sc-2004). All specimens were developed with 3, 3′-Diaminobenzidine tetrahydrochloride (DAB, Sigma-D3939). Immunoreactive Score (IRS) was calculated using ImmunoRatio Software wherein the percentage of staining was taken as three different scores: 1: no staining, 2: medium staining and 3: high staining.

3.2.3 Cell culture:

Human breast carcinoma cell lines MCF7 and MDA-MB-231, human prostate cancer cell lines PC3 and DU145 and immortalized human keratinocytes HaCaT were obtained from National Centre for Cell Science (NCCS), Pune. MCF7 was cultured and maintained in Minimum Essential Medium (MEM, Himedia-AL047A), MDA-MB-231 in Leibovitz's

medium (L-15, Himedia-AL011S), PC3 was cultured in Nutrient Mixture F-12 Ham medium (F-12, Himedia-AL106S) whereas DU145 and HaCaT in Dulbecco's Modified Eagle's Medium (DMEM, Himedia-AL007A) supplemented with 10% (v/v) fetal bovine serum (FBS, Himedia-RM1112) and 100 IU/mL penicillin and 0.1 mg/mL streptomycin (Himedia-A002A) in a humified atmosphere of 5% CO_2 at 37° C. The cells were harvested by trypsinization (Trypsin, Sigma-T4049) and the number of living cells was calculated by trypan blue (Himedia-TC193) staining (0.2% v/v) using hemocytometer before seeding for future experiments.

3.2.4 Relative mRNA expression analysis by real-time PCR:

Total cellular RNA from each of the five cell lines was extracted with TRI Reagent (Sigma-T9424) according to the manufacturer's instructions. cDNA was prepared from 1 µg of total RNA by using RevertAid^{TM} First Strand cDNA Synthesis Kit (Thermo Scientific-K1622) followed by quantitative real-time PCR (RT-PCR) using Maxima SYBR Green/ROX qPCR Master Mix (2X, Thermo Scientific-K0221) in the Realplex4 Eppendorf system according to the manufacturer's instructions. The mRNA level was normalized to house-keeping gene GAPDH used as an endogenous control and calculations were done using $2^{-\Delta\Delta Ct}$ method, as described earlier (Schmittgen and Livak, 2008).

3.2.5 Western Blotting:

2 X 10^5 cells of each cell line were seeded and incubated till 80% confluency. Cells were harvested and total cellular protein was extracted using lysis buffer (RIPA Buffer, Sigma-R01278) supplemented with protease inhibitor cocktail (PIC, Sigma-P8340). Bradford method was used to quantify the protein concentration and equal amount of cell lysate was loaded and separated in 10% SDS-PAGE and subsequently transferred onto PVDF membrane (Millipore, USA-ISEQ00010). Protein containing membranes were blocked in 3% bovine serum albumin (BSA, Himedia-5RMI05) in PBS containing 0.1% Tween 20 (Himedia-MB067) (PBST), followed by overnight incubation with primary antibodies -- rabbit polyclonal anti-Oct4 (Abcam-ab19857), rabbit polyclonal anti-Sox2 (Abcam-ab59776) and rabbit polyclonal anti-Nanog (Abcam-ab21624), then incubated with appropriate HRP conjugated secondary antibody (Goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology, sc-2004) for 2 h at 37° C in dark. The membranes were thoroughly washed with PBST buffer and were developed by Supersignal^{TM} West Femto

Chemiluminescent substrate kit (ThermoScientific-34095). β-Actin (Primary-Mouse IgG_1 (Monoclonal),Santa Cruz Biotechnology-sc-47778 and Secondary-Goat Anti-Mouse IgG-HRP, Santa Cruz Biotechnology-sc-2005) protein levels were used as control for equal protein loading. Relative protein expression was analyzed from the blots obtained using ImageJ quantification software.

3.3 Results:

3.3.1 Oct4, Sox2 and Nanog are amplified in breast and prostate cancer cell lines:

Pre-experimental *in silico* analysis by cBioportal database (http://www.cbioportal.org/) linked to Cancer Genome Atlas (TCGA) using a cohort of clinical (patient-derived) tissue samples is typically an assessment of the expression profile of a gene set (e.g. Oct4, Sox2 and Nanog) and its associated functional characterization in different cancer types.

Copy Number Alteration (CNA) and genomic profiling:

CNA (amplification, deletion and mutation) and genomic profile (mRNA up-and down-regulation) of Oct4, Sox2 and Nanog in breast and prostate cancer tissue samples was analyzed from generated Oncoprint. In breast cancer, alterations for Oct4 was observed in 17 (1.8%, amplification in 1.2%) samples, for Sox2 in 37 (3.8%, amplification in 3.6%) cases and for Nanog in 30 (3.0%, amplification in 2.7%) cases (Fig. 3.1). In prostate cancer, Oct4 and Sox2 were amplified in 0.4% and 1.9% cases, whereas Nanog showed allelic deletion in 1.5% cases (Fig. 3.1). From genomic profiling it is evident that mRNA up-regulation of Oct4 occurs in both breast (5.54%) and prostate cancer (6.0%) which is higher that gene amplification (Fig. 3.1). Similarly, Sox2 shows higher mRNA up-regulation in prostate cancer (6.6%) than in breast cancer (1.84%) in comparison to gene amplification (Fig. 3.1). In case of Nanog, gene amplification is more prevalent that mRNA up-regulation in breast cancer, but not in prostate cancer (Fig. 3.1). The above results indicate that mRNA up-regulation of Oct4, Sox2 and Nanog play a predominant role in over-expression of these factors in breast and prostate cancer.

Mutual exclusivity and co-occurrence:

When alterations in any one of the genes in a gene set and same cancer sample (breast or prostate cancer), is sufficient to deregulate the entire process and lead to cancer progression, the alteration events are considered to be mutually exclusive. On the other

hand, if alterations occur in multiple genes in the same cancer sample, then the alteration events are considered to be co-occurent. As is evident from table 3.1, in breast adenocarcinoma (TCGA, Cell, 2015; n= 974), CNA in gene set - Oct4, Sox2 and Nanog is observed in total of 84 (8.6%) samples, however these alterations have a tendency towards co-occurrence, with statistically significant occurrence between Oct4 and Nanog. In case of prostate cancer (TCGA, Cell, 2015; n= 333), overall alterations (20) have a tendency towards mutual exclusivity. The results indicate that while genetic alterations in Oct4, Sox2 and Nanog are co-occurent in breast cancer (where gene alterations occur simultaneously for cancer progression) and mutually exclusive in prostate cancer (where alteration in any of the genes can cause cancer).

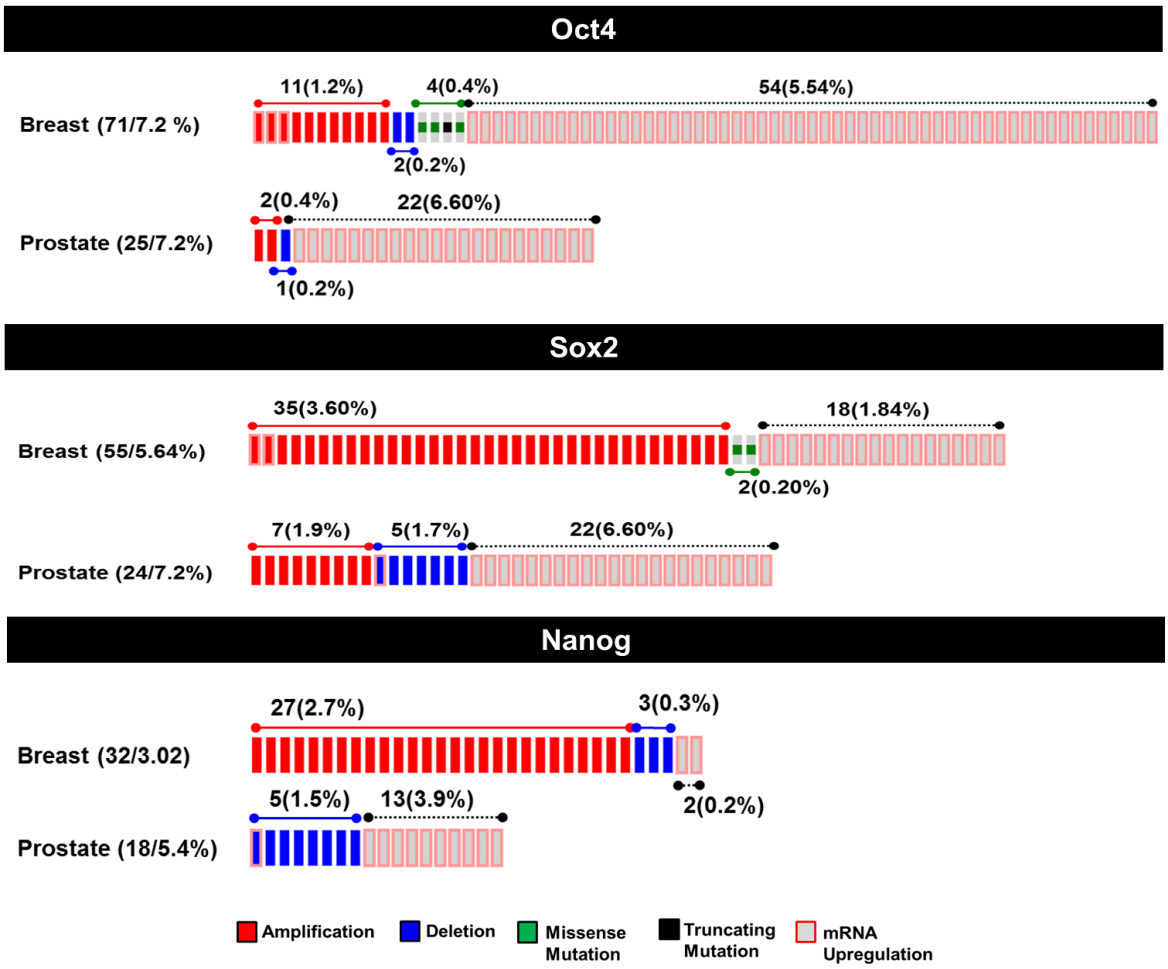


Figure 3.1: Oncoprint of Oct4, Sox2 and Nanog in breast and prostate adenocarcinoma samples using cBioportal. In breast adenocarcinoma patients (n=974), CNA was detected in 1.8% samples and mRNA up-regulation in 5.54% for Oct4, Sox2 showed genetic amplification in 3.6% and mRNA up-regulation in 1.84% and Nanog showed amplification in 2.7% and mRNA up-regulation in 0.2%. In prostate adenocarcinoma patients (n=333), Oct4 and Sox2 showed amplification in 1% and 1.8% cases, while Nanog was delected in 1.5% of cases. mRNA up-regulation is the more dominant reason of over-expression of Oct4, Sox2 and Nanog in both breast and prostate cancer.

Table 3.1 Mutual exclusivity and co-occurrence of CNA of Oct4, Sox2 and Nanog

Breast adenocarcinoma (n= 974)				**Alterations in 84 (8.6%) cases**
Gene 1	**Gene 2**	**p-value**	**Log odds ratio**	**Association**
Oct4	Nanog	<0.001	**2.347***	Tendency towards co-occurrence
Oct4	Sox2	0.358	0.278	Tendency towards co-occurrence
Sox2	Nanog	0.452	-0.630	Tendency towards mutual exclusivity
Prostate adenocarcinoma (n= 333)				**Alterations in 20 (6.0%) cases**
Gene 1	**Gene 2**	**p-value**	**Log odds ratio**	**Association**
Oct4	Sox2	0.929	<-3	Tendency towards mutual exclusivity
Oct4	Nanog	0.970	<-3	Tendency towards mutual exclusivity
Sox2	Nanog	0.169	1.975	Tendency towards co-occurrence

3.3.2 Analysis of Oct4, Sox2 and Nanog expression in breast and prostate cancer tissues:

In order to establish the expression profile of Oct4, Sox2 and Nanog in breast and prostate cancer, protein level expressiion in FFPE tissue samples were analyzed. All the three proteins showed higher expression in metastatic stage breast tissue samples in comparison to primary stage tissues, indicating that over-expression of these factors is associated with advanced stages of cancer and mediates cancer progression (Fig. 3.2a, Table 3.2).

The immunoreactive score for Oct4, Sox2 and Nanog staining was found to be in case of metastatic samples- 4.56 (Oct4), 4.2 (Sox2) and 3.8 (Nanog) indicating that Oct4 has the highest expression among the three factors in breast cancer (Fig. 3.2b).

Table 3.2 Expression of Oct4, Sox2 and Nanog in FFPE breast cancer tissue samples

Clinical breast cancer tissue samples								
Number of samples	**Age**	**Stage of cancer/ No. of sample**	**Oct4 expression in protein level (%) P-Presence; A-Absence**		**Sox2 expression in protein level (%) P-Presence; A-Absence**		**Nanog expression in protein level (%) P-Presence; A-Absence**	
65	≤50	Primary stage/35	P	28 (10 samples)	P	25 (9 samples)	P	31 (11 samples)
			A	71 (25 samples)	A	75 (26 samples)	A	69 (24 samples)
		Metastasis stage/30	P	83 (25 samples)	P	63(19 samples)	P	57 (17 samples)
			A	17 (5 samples)	A	37 (11 samples)	A	43 (13 samples)

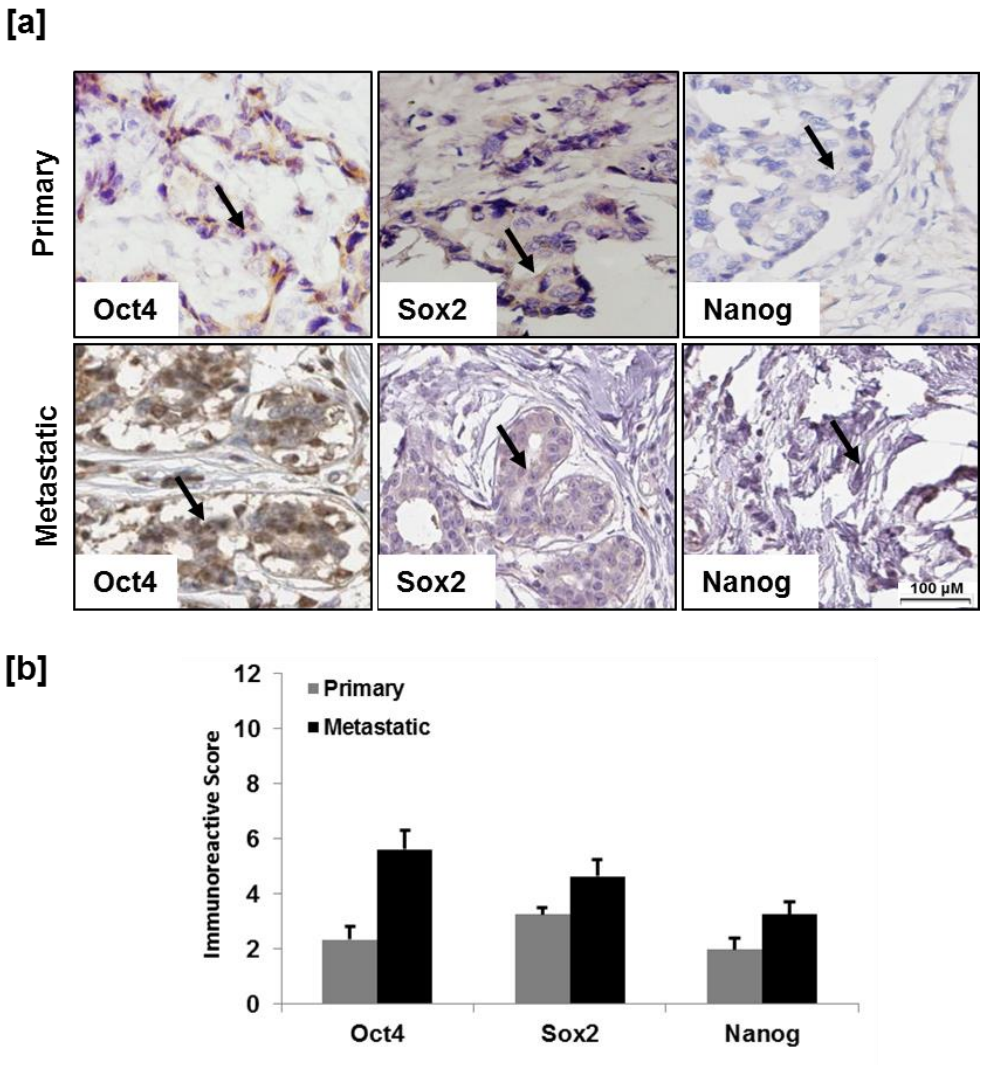


Figure 3.2: Immunohistochemistry of breast cancer tissue samples for analyzing Oct4, Sox2 and Nanog protein expression. [a] Representative immunostained specimens showing Oct4, Sox2 and Nanog expression in primary (n= 35) and metastatic (n=30) FFPE tissue samples. Metastatic tissues show higher expression of all three proteins in comparison to primary stage tissues. Scale Bar = 100 µm, [b] Bar plot shows immunoreactivity score (IRS) for Oct4, Sox2 and Nanog staining in tissues. Oct4 has the highest expression in breast cancer tissue samples. Error bars indicate SD ($P < 0.05$).

Similarly, for prostate cancer, Oct4, Sox2 and Nanog were highly expressed in malignant tissues in comparison to benign hyperplasic samples, which is again indicative of the fact that expression of these pluripotency inducing factors is tissue-stage specific and is involved in progression of cancer stages and mediating aggressive cancer phenotype (Fig. 3.3a, Table 3.3). On computing IRS for Oct4, Sox2 and Nanog, it was seen that Oct4 has 8.45, Sox2 of 11.2 and Nanog of 5.9 in malignant tissues in comparison to BPH where Oct4, Sox2 and Nanog expression is very low (Fig. 3.3b).

Table 3.3 Expression of Oct4, Sox2 and Nanog in FFPE prostate cancer tissue samples

Clinical prostate cancer tissue samples								
Number of samples	**Age**	**Stage of cancer/ No. of sample**	**Oct4 expression in protein level (%) P-Presence; A-Absence**		**Sox2 expression in protein level (%) P-Presence; A-Absence**		**Nanog expression in protein level (%) P-Presence; A-Absence**	
33	≤55	BPH/15	P	20 (3 samples)	P	40 (6 samples)	P	35 (5 samples)
			A	80 (12 samples)	A	60 (9 samples)	A	65 (10 samples)
		Malignant/18	P	72.2(13 samples)	P	83.3 (15 samples)	P	68 (12 samples)
			A	27.8 (5 samples)	A	16.7 (3 samples)	A	31 (6 samples)

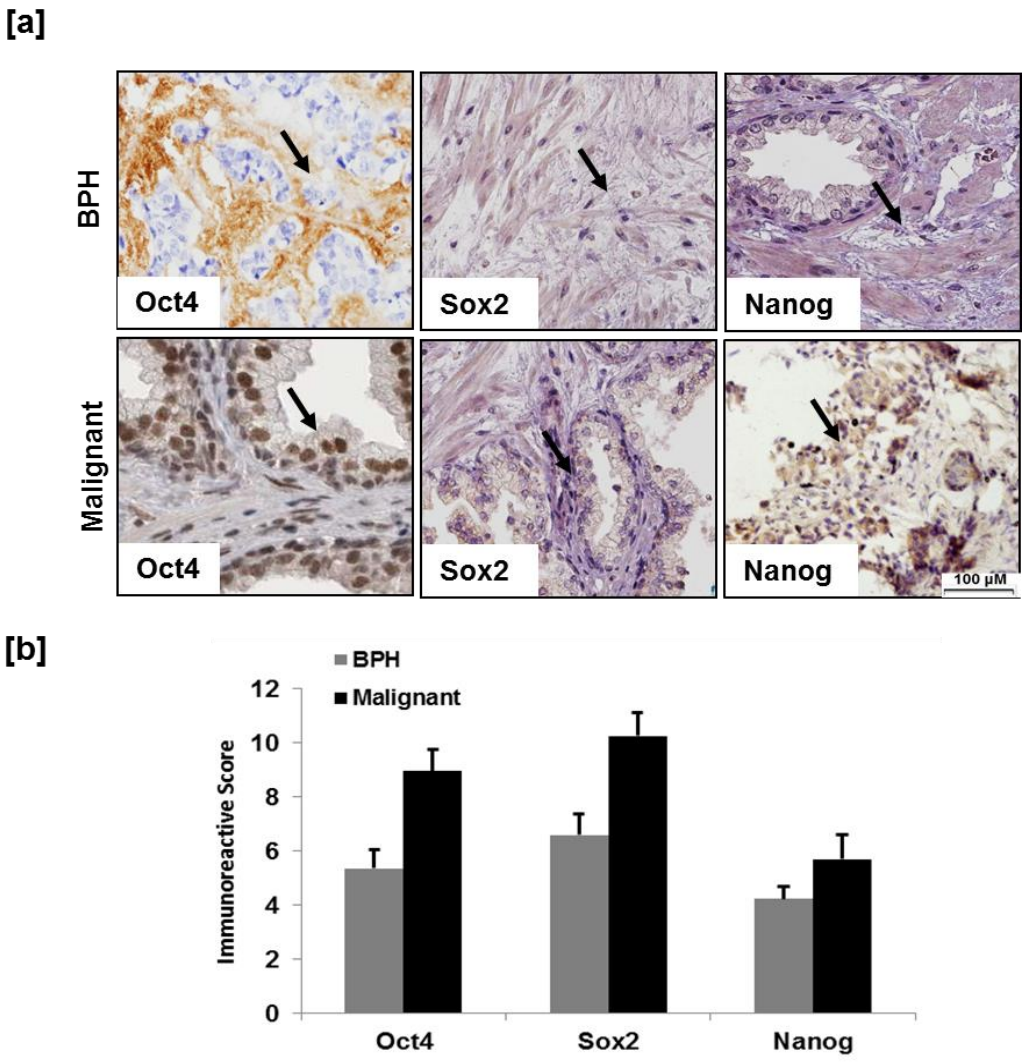


Figure 3.3: Immunohistochemistry of prostate cancer tissue samples for analyzing Oct4, Sox2 and Nanog protein expression. [a] Representative immunostained specimens showing Oct4, Sox2 and Nanog expression in benign prostate hyperplasia (BPH; n= 15) and malignant (n=18) FFPE tissue samples. Metastatic tissues show higher expression of all three proteins in comparison to BPH tissues. Scale Bar = 100 µm, [b] Bar plot shows immunoreactivity score (IRS) for Oct4, Sox2 and Nanog staining in tissues. Sox2 has the highest expression in prostate cancer tissue samples. Error bars indicate SD ($P < 0.05$).

3.3.3 Detection of Oct4, Sox2 and Nanog in breast and prostate cancer cell lines by RT-PCR:

RT-PCR was done to analyze the relative mRNA expression of Oct4, Sox2 and Nanog in breast and prostate cancer cell lines in comparison to house-keeping gene GAPDH. Oct4 showed higher expression in MDA-MB-231 (4.3 folds) than in MCF7 (3.3 folds) , Sox2 expression was higher in MCF7 (2.8 folds) and MDA-MBA-231 (2.1 folds), Nanog showed almost equal expression in both cell lines (2.4 in MCF7 and 2.7 folds in MDA-MB-231) in comparison to HaCaT which was considered as non-tumorigenic phenotype and its expression normalized to 1 (Fig. 3.4).

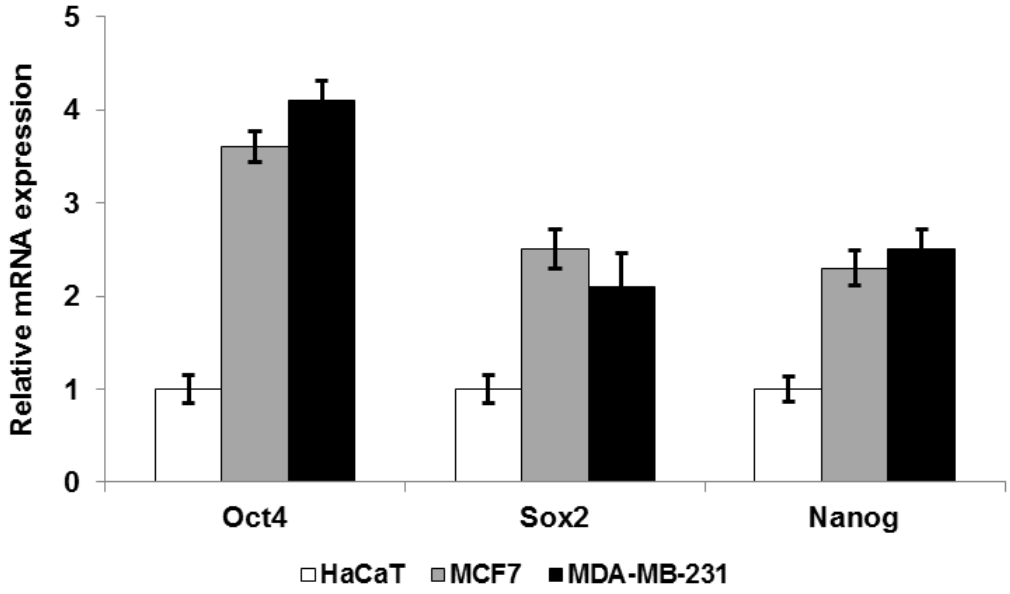


Figure 3.4: Relative mRNA expression level of Oct4, Sox2 and Nanog in breast cancer cell lines MCF7 and MDA-MB-231. The level of Oct4, Sox2 and Nanog in HaCaT cell line was normalized to 1(n=3, mean±S.D, $P < 0.05$). In comparison to HaCaT, Oct4 mRNA expression was highest in MDA-MB-231, Sox2 in MCF7 and Nanog showed almost equalized expression in both the cell lines.

Similarly for prostate cancer, Oct4 and Sox2 expression was higher in PC3 than in DU145, with Sox2 showing highest expression in prostate cancer cells. Nanog expression was more or less uniform in both the cell lines (Fig. 3.5).

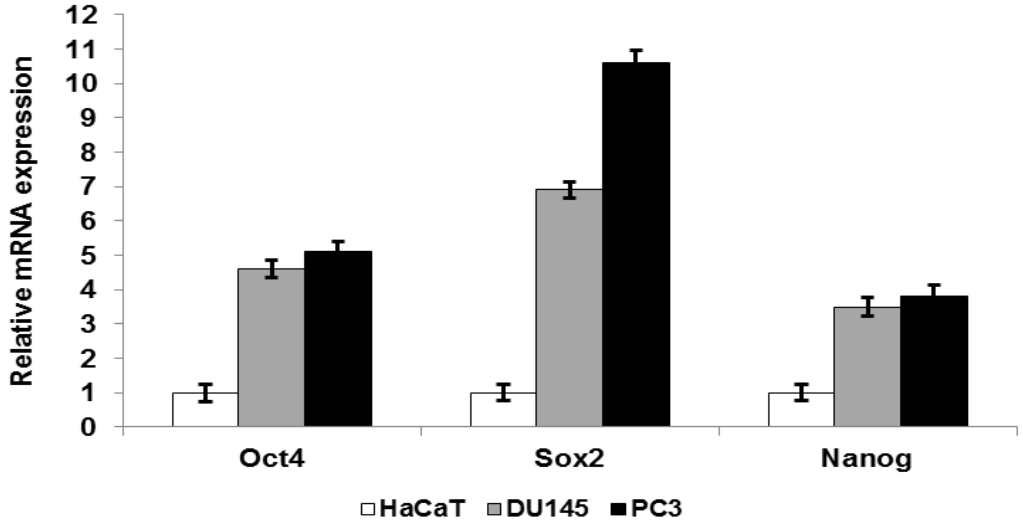


Figure 3.5: Relative mRNA expression level of Oct4, Sox2 and Nanog in prostate cancer cell lines DU145 and PC3. The level of Oct4, Sox2 and Nanog in HaCaT cell line was normalized to 1(n=3, mean±S.D, $P < 0.05$). In comparison to HaCaT, mRNA expression of Oct4, Sox2 and Nanog was higher in PC3 cell line than DU145.

3.3.4 Protein expression corroborated mRNA expression of Oct4, Sox2 and Nanog in breast and prostate cancer cell lines:

Western blotting was done to analyze the protein expression of Oct4, Sox2 and Nanog in breast and prostate cancer cell lines. Oct4 showed highest protein expression in MDA-MB-231 cells consistent with higher mRNA expression in the same cell line. Sox2 expression was uniformly high in both MCF7 and MDA-MB-231 and Nanog was comparatively less in comparison to Oct4 and Sox2 (Fig. 3.6a, 3.6b).

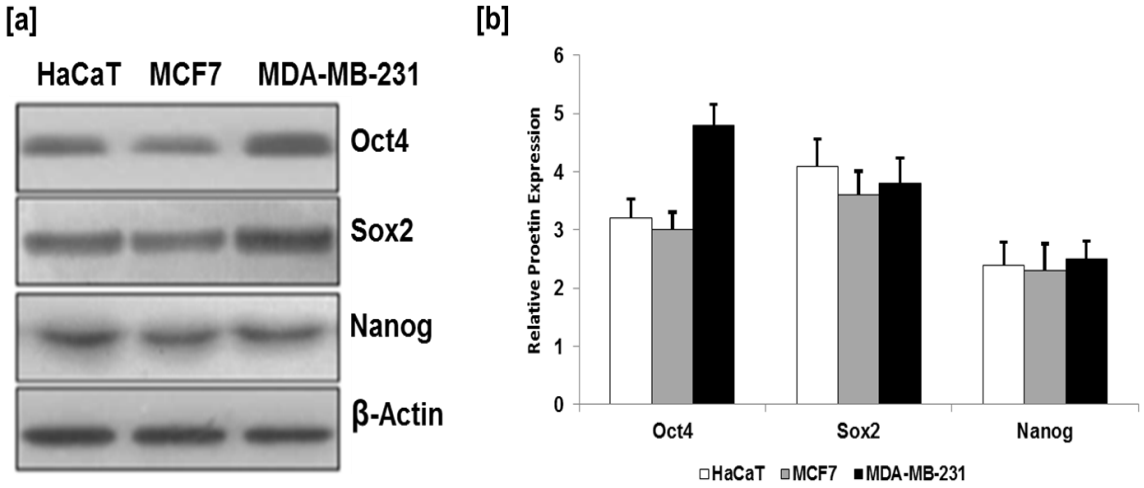


Figure 3.6: Western blot analysis of Oct4, Sox2 and Nanog protein expression in HaCaT, MCF7 and MDA-MB-231 cell lines. [a] HaCaT cells were used as a positive control, and β-Actin served as the loading control. [b] Graphical representation of protein expression after quantification using ImageJ software (n=3, mean±S.D, $P < 0.05$).

Similarly, for prostate cancer, Oct4 expression was highest in DU145 cells, Sox2 expression was higher in PC3 and Nanog showed uniform protein expression in all the three cell lines (Fig. 3.7a, 3.7b).

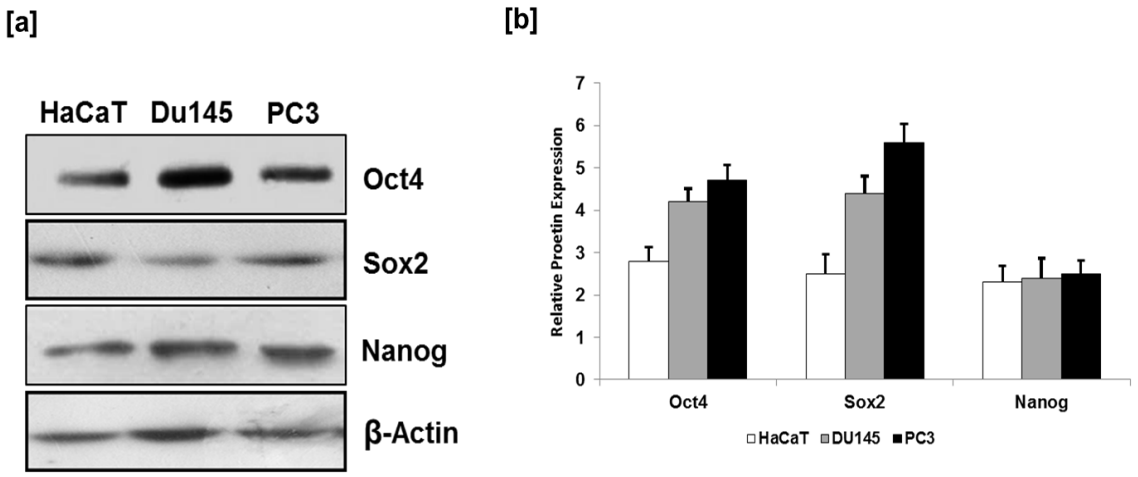


Figure 3.7: Western blot analysis of Oct4, Sox2 and Nanog protein expression in HaCaT, DU145 and PC3 cell lines. [a] HaCaT cells were used as a positive control, and β-Actin served as the loading control. [b] Graphical representation of protein expression after quantification using ImageJ software (n=3, mean±S.D, $P < 0.05$).

3.4 Discussion:

In recent years, striking similarities between embryogenesis and oncogenesis is being increasingly recognized. ESCs, cancer cells and more recently, CSCs arise from the progenitor pool and share numerous key biological properties. All of them are capable of extensive proliferation and can give rise to differentiated cells. Another interesting similarity is the functional and transcriptional role of pluripotency inducing factors like Oct4, Sox2 and Nanog in mediating both the above processes (Visvader and Lindeman, 2008). Transcription factors are critical molecular controllers regulating ESC fate as well as in reprogramming cancer cells. Three pluripotency inducing transcription factors Oct 4, Nanog and Sox2 form the core regulatory network that coordinates to determine the self-renewal and differentiation of ESCs. Hence, it is important to investigate the pluripotency-related genes associated with embryogenesis and tumorigenesis. The aberrant expression of Oct4, Sox2 and Nanog has been shown in numerous types of tumors and might be responsible for encouraging oncogenic progress (Chang et al., 2004). The present work was carried out to investigate the expression profile of pluripotency-associated markers in human breast and prostate cancer tissues and cell lines in order to

establish the link between expression and functional significance of Oct4, Sox2 and Nanog in cancer.

Oct4 is an embryonic transcription factor belonging to the POU domain containing transcription factor family and is highly expressed in ESCs, carcinoma cells and oocytes. It is regarded as essential maternal factor for speciation of female germ line and establishment of pluripotency during pre-implantation embryonic stages. It has been previously proposed that Oct4 acts as a multifunctional factor in cancer biology as it increases the malignant potential of ESCs in a dose-dependent manner (Pan et al., 2002). The over-expression of Oct4 has also been documented in some tumors and is thought to play a role in tumorigenic aggression (Matin et al., 2004). The current study sought to examine the expression of Oct4 at the mRNA and protein levels in human breast and prostate cancer tissues and cancer cell lines. The results revealed that Oct4 was expressed in all four human cancer cell lines, although differential expression patterns were noted in comparison to immortalized HaCaT cell line taken as control. Oct4 was detected in breast cancer and prostate cancer tissues, with higher expression evident in metastatic stages suggesting that Oct4 promotes breast and prostate cancer (Fig. 3.2 and 3.3). Furthermore, Oct4 expression levels were significantly up-regulated in MDA-MB-231 and MCF7 cells as well as in PC3 and DU145 in comparison to HaCaT at both mRNA and protein levels (Fig. 3.4, 3.5, 3.6 and 3.7). Oct4 expression was highest in case of MDA-MB-231 cell line at both mRNA and protein level. MDA-MB-231, MCF7, PC3 and DU145 are all adenocarcinoma cell lines derived from metastatic sites, implying that over-expression of Oct4 contribute towards advanced tumor phenotypes. Nanog is a recently identified transcription factor crucial as a negative regulator of differentiation programs. The constitutive expression of Nanog during development maintains the pluripotent nature of stem cells and allows them to continuously replenish the stem cell compartment while simultaneously suppressing differentiation into lineages (Darr et al., 2006; Zaehres et al., 2005). Studies in tumors have suggested the tumorigenic potential of Nanog and its role in regulation of tumor development (Siu et al., 2008; Wang et al., 2009). The current study demonstrated that Nanog was detected to be over-expressed at both the mRNA and protein levels in the breast and prostate cancer cell lines and tissues respectively in comparison to HaCaT; however, the expression is not as significantly high as in case of Oct4 and Sox2 (Fig. 3.4, 3.5, 3.6 and 3.7). The data is in support of the fact that over-expression of Nanog in breast and prostate cancer cell lines and tissues may correlate with the tumorigenesis of breast and prostate cancer.

Sox2 is essential for the maintenance of stem cell proliferation and has prolific differentiation capabilities as it can give rise to multiple cell lineages. A number of studies have implicated the involvement of transcription factor Sox2 in human cancers (Wilbertz et al., 2011). Sox2 is known to be oncogenic and participates in embryonal carcinoma, teratoma, lung, pancreatic and gastric adenocarcinoma (Dong et al., 2004; Santagata et al., 2007). The present study demonstrated that Sox2 was expressed in all breast and prostate cell lines tested. MCF7, MDA-MB-231, PC3 and DU145 cells expressed significantly higher levels of Sox2 compared to HaCaT cells, thus indicating that Sox2 may be a possible driver of the basal-like phenotype and play an early role in breast and prostate carcinogenesis (Fig. 3.4, 3.5, 3.6 and 3.7). In conclusion, the present study confirms the over-expression of pluripotency-inducing transcription factors Oct4, Sox2 and Nanog in breast and prostate cancer tissues and cell lines. Further experiments are required to explore the complex role of Oct4, Nanog and Sox2 in mediating tumorigenic progression in human breast and prostate cancer.

Chapter 4

To evaluate the role of pluripotency inducing TFs in promoting tumorigenicity in breast and prostate cancer

4.1 Introduction:

Pluripotency inducing TFs Oct4, Sox2 and Nanog are considered as the functional and mechanistic link between embryonic stem-cell state and oncogenic self-renewal potential. Over-expression of Oct4, Sox2 and Nanog has been documented in human cancers and evidences have vindicated them in promoting inherent tumorigenicity. The up-regulation of Oct4 is characteristic of several types of human cancers where it is known to promote pro-tumorigenic properties. As a pluripotency determinant essential for maintaining ESCs, Oct4 recapitulates its function in tumor cells and assists in acquisition of CSC properties by boosting de-differentiation. Some of the enhancing abilities conferred by Oct4 on tumor cells include increased metastatic ability and development of drug resistance. (Zbinden et al., 2010; Choi et al., 2012). Particularly in breast and prostate cancer, Oct4 has been known to sustain the survival of CSCs and support conversion of primary tumors to invasive neoplasia by EMT.

Sox2 amplification and functional over-expression is a characteristic feature of many cancer types where it positively influences tumor initiation, maintenance and evolution into more aggressive types (Weina and Utikal, 2014). Sox2 is known to increase CSC proliferation and augment their growth, support metastatic behavior by improving EMT capacity, develop resistance to therapeutic drugs and finally interact with number of oncogenic signaling pathways such as HH, Wnt and Notch to promote stem-cell like properties (Bourguignon et al., 2012). In fact, Sox2 is considered to be one of the most significant oncogenic transcription factors mediating tumorigenic conversion in many instances. In cancers, over-expression of Nanog mediates increased tumor cell proliferation and growth. Nanog interacts with members of cell cycle machinery transcriptionally activates them to accelerate proliferation of neoplastic cells by over-riding cell cycle checkpoints (Golubovskaya et al., 2013; Siu et al., 2013). Nanog

enhances tumor metastasis and invasive potential of tumor cells, and provides resistance against apoptosis and chemotherapeutic drugs. It also improves tolerance of tumor cells to immune attack and surveillance and interacts with oncogenic pathways to encourage malignant growth. Hence, the following study was undertaken to investigate the role of these pluripotency transcription factors in promoting inherent tumorigenicity in breast and prostate cancer by gene knockdown using si-RNAs against Oct4, Sox2 and Nanog. A number of pro-tumorigenic properties such as cell proliferation, chromatin condensation, metastatic behavior, colony forming ability and cell cycle distribution were analyzed after ectopic silencing of Oct4, Sox2 and Nanog.

4.2 Materials and Methods:

4.2.1 Cell culture:

Human breast carcinoma cell lines MCF7 and MDA-MB-231 and human prostate cancer cell lines PC3 and DU145 were obtained from National Centre for Cell Science (NCCS), Pune. MCF7 was cultured and maintained in Minimum Essential Medium (MEM, Himedia-AL047A), MDA-MB-231 in Leibovitz's medium (L-15, Himedia-AL011S), PC3 was cultured in Nutrient Mixture F-12 Ham medium (F-12, Himedia-AL106S) whereas DU145 and HaCaT in Dulbecco's Modified Eagle's Medium (DMEM, Himedia-AL007A) supplemented with 10% (v/v) fetal bovine serum (FBS, Himedia-RM1112) and 100 IU/mL penicillin and 0.1 mg/mL streptomycin (Himedia-A002A) in a humified atmosphere of 5% CO_2 at 37° C. The cells were harvested by trypsinization (Trypsin, Sigma-T4049) and the number of living cells was calculated by trypan blue (Himedia-TC193) staining (0.2% v/v) using hemocytometer before seeding for future experiments.

4.2.2 si-RNA transfection:

si-RNA against Oct4 (Oct-3/4 si-RNA (h), sc-36123), Sox2 (Sox2 si-RNA (h), sc-38408), Nanog (Nanog si-RNA (h), sc-43958) and control si-RNA (control si-RNA-A, sc-37007) were purchased from Santa Cruz Biotechnology, USA. For determining the optimum concentration of si-RNA that completely abolished gene and protein expression, 5 X 10^5 cells of each of the four cell lines were seeded and after 24 h incubation, were treated with three different concentrations (10, 20, 30 nM) of different si-RNAs using Lipofectamine 2000 (Invitrogen-11668019) and serum free opti-mem (Gibco-31985-070) according to manufacturer's instructions. Six hours after transfection, opti-mem was replaced by

respective cell culture media and cells were left to incubate for next 24 hours. 30 nM of si-RNA efficiently blocked the expression of Oct4, Sox2 and Nanog protein in all the four cell lines. Control si-RNA was also used at the same concentration.

4.2.3 Cell proliferation analysis by colometric MTT assay:

For assaying cellular proliferation after treatment with si-RNAs, 2 X 10^3 cells were seeded in 35mm plates and treated with desired concentrations of si-RNAs. After 24, 48, 72 and 96 h incubation, 100 µL of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT, Himedia-TC191) solution was added to each well and left in the incubator at 37° C for 4 h in dark. Subsequently, 100 µL of di-methyl sulphoxide (DMSO, Himedia-TC185) was added and optical absorbance was measured at 570 nm. Number of extra cells present in comparison to the number of cells seeded earlier i.e. cell number (plotted in the graph) = Number of cells after 24/48/72/96 h – number of cells originally seeded (Santini et al., 2014).

4.2.4 Chromatin condensation assay:

2 X 10^4 cells per well of each cell line were seeded in 6 well plates separately, and then treated with desired concentrations of si-RNAs for 24 h and drugs for 72 h. Cells were then stained with Hoechst 33342 (1 µg/mL, Invitrogen- H1399) dissolved in serum-free media followed by incubation for 10 min at 37° C. Finally, images were taken under UV filter using Epi-fluorescent Microscope (Olympus IX71, excitation wavelength of 355 to 366 nm, emission wavelength of 465 to 480 nm) at 20X magnification. Nuclei with condensed chromatin were counted amongst total number of nuclei in the field, and the percentage of condensed nuclei was calculated and plotted graphically.

4.2.5 Wound healing or scratch assay:

2 X 10^5 cells per well were seeded in 6-well plate and incubated at 37° C until a fully confluent and adherent monolayer is formed. Scratch is then made using sterile 200 µL micropipette tip. Cells were washed thoroughly with PBS to remove the debris and smoothen the edge of the scratch. Treatment with the desired concentrations of si-RNAs for 24 h and drugs for 72 h was done. Images were taken using phase contrast microscope at 0 h (immediately after treatment) and 72 h.

4.2.6 Clonogenic assay:

Briefly, 1000 cells were seeded in six-well plates. After 6 h, cells were treated with different si-RNAs with above mentioned concentrations. After 7 days, colonies were stained with mixture of 6.0% glutaraldehyde (Himedia-RM5927) and 0.5% crystal violet (Himedia-S012) in water. After 30 min staining and cell fixation, colonies were washed with water. Air dried colonies were calculated.

4.2.7 Cell cycle analysis by FACS:

MCF7, MDA-MB-231, DU145 and PC3 cells after treatment with desired concentrations of si-RNAs for 24 h and drugs for 72 h were incubated in their respective media with 5% FBS for 24 h. The cells were then trypsinized, collected by centrifugation (500 × g for 5 min at 4° C), washed twice with PBS and then fixed in 90% ice-cold methanol (Himedia-AS058). After incubation at -20° C for 1 h, cells were centrifuged and resuspended in PBS followed by treatment with RNaseA (500 IU/mL, Fermentas -EN0531) to digest residual RNAs and stained with propidium iodide (PI, 10 µg/mL, Sigma-81845). Samples were incubated for 30 min at 4° C and cell cycle analysis was performed with a Becton-Dickinson fluorescence-activated cell sorter (FACS). The percentage of apoptotic cells was graphically represented.

4.3 Results:

4.3.1 Silencing of Oct4, Sox2 and Nanog inhibits cell growth and reduces rate of cell proliferation in breast and prostate cancer cell lines:

After knockdown of Oct4, Sox2 and Nanog genes, the rate of cell proliferation was examined by MTT assay. It was observed that cells treated with control si-RNA remain unaffected and proliferated vigorously, however, rate of proliferation declined in cells transfected with si-Oct4, si-Sox2 and si-Nanog (Fig. 4.1). In breast cancer cell lines, Oct4, Sox2 and Nanog silencing resulted in slow proliferation with lesser growth in metastatic cell line MDA-MB-231, implying that Oct4, Sox2 and Nanog might be involved in boosting proliferative potential in metastatic cancers (Fig. 4.1a, 4.1b). In prostate cancer also, similar results were obtained in case of PC3 cells. It was observed that silencing of Oct4 and Sox2 have almost identical effect on the rate of proliferation; however, Nanog has significantly lesser effect (Fig. 4.1c, 4.1d). It might imply that initially, Oct4 and Sox2 act concomitantly to regulate cell cycle and survival machinery with Nanog acting

as an accomplish in the later stages of cancer proliferation. However, it is very clear that both breast and prostate cancer cells grow slower after silencing of Oct4, Sox2 and Nanog which proves that these pluripotency factors influence the tumorigenic growth and proliferation potential during oncogenic transformation.

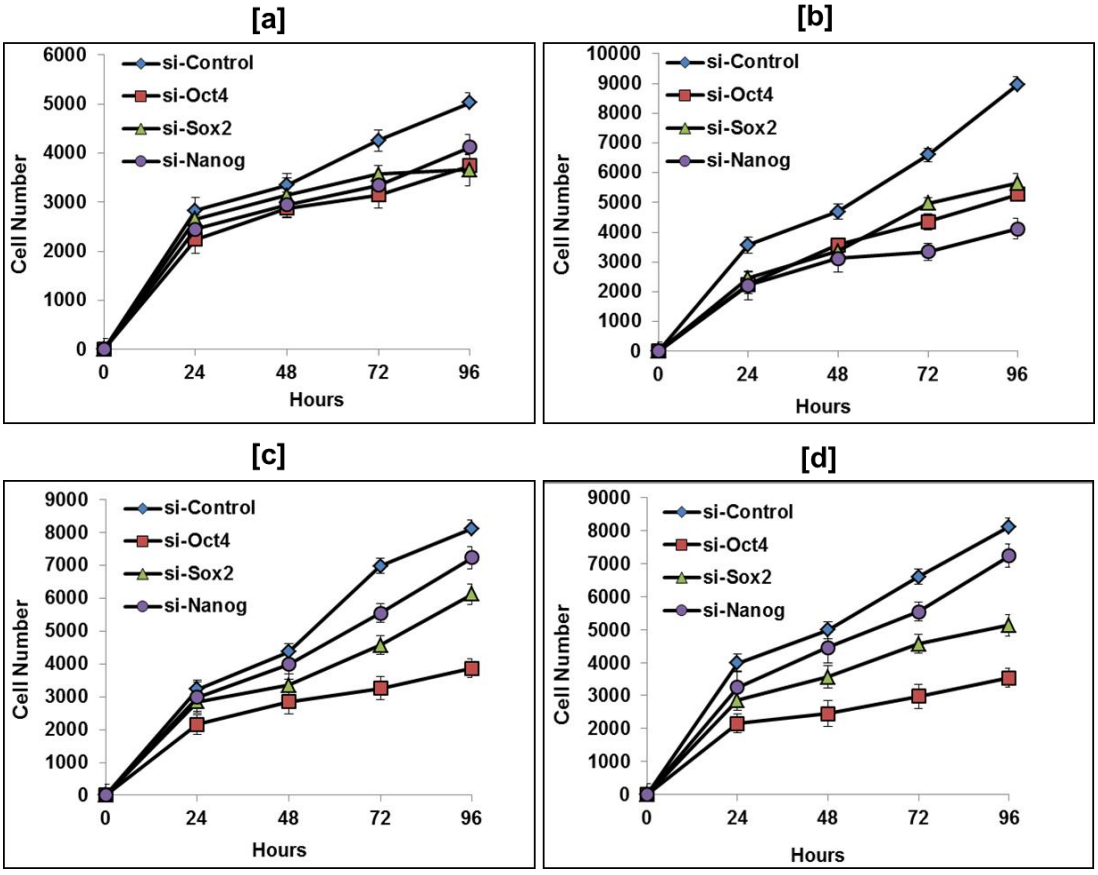


Figure 4.1: Analysis of cell proliferation after si-RNA knockdown of Oct4, Sox2 and Nanog. 30 nM of si-RNA against Oct4, Sox2, Nanog were used for 24 h. Graphical representation of number of proliferated cells in [a] MCF7, [b] MDA-MB-231, [c] DU145 and [d] PC3 after treatment with si-RNA against Oct4, Sox2 and Nanog and control si-RNA (n=3, mean ± S.D, $P < 0.05$).

4.3.2 Silencing of Oct4, Sox2 and Nanog induces chromatin condensation and DNA damage in both breast and prostate cancer cell lines:

Chromatin condensation analysis was done to assess the role of pluripotency factors in inducing DNA damage and chromatin compaction. It was observed that in comparison to si-control treated cells, Oct4, Sox2 and Nanog silenced cells exhibited higher number of condensed chromatin indicating greater DNA damage upon silencing of these factors. In breast cancer cell lines, the percentage of condensed nuclei in MCF7 for si-control, si-Oct4, si-Sox2 and si-Nanog treated cells was 21%, 34.5%, 28.34%, 31.75% and in MDA-MB-231 was 26.43%, 39.63%, 32.13% and 27.45% respectively The above data indicate

that chromatin condensation was higher in metastatic cell lines in comparison to primary cell lines (Fig. 4.2a, 4.2b).

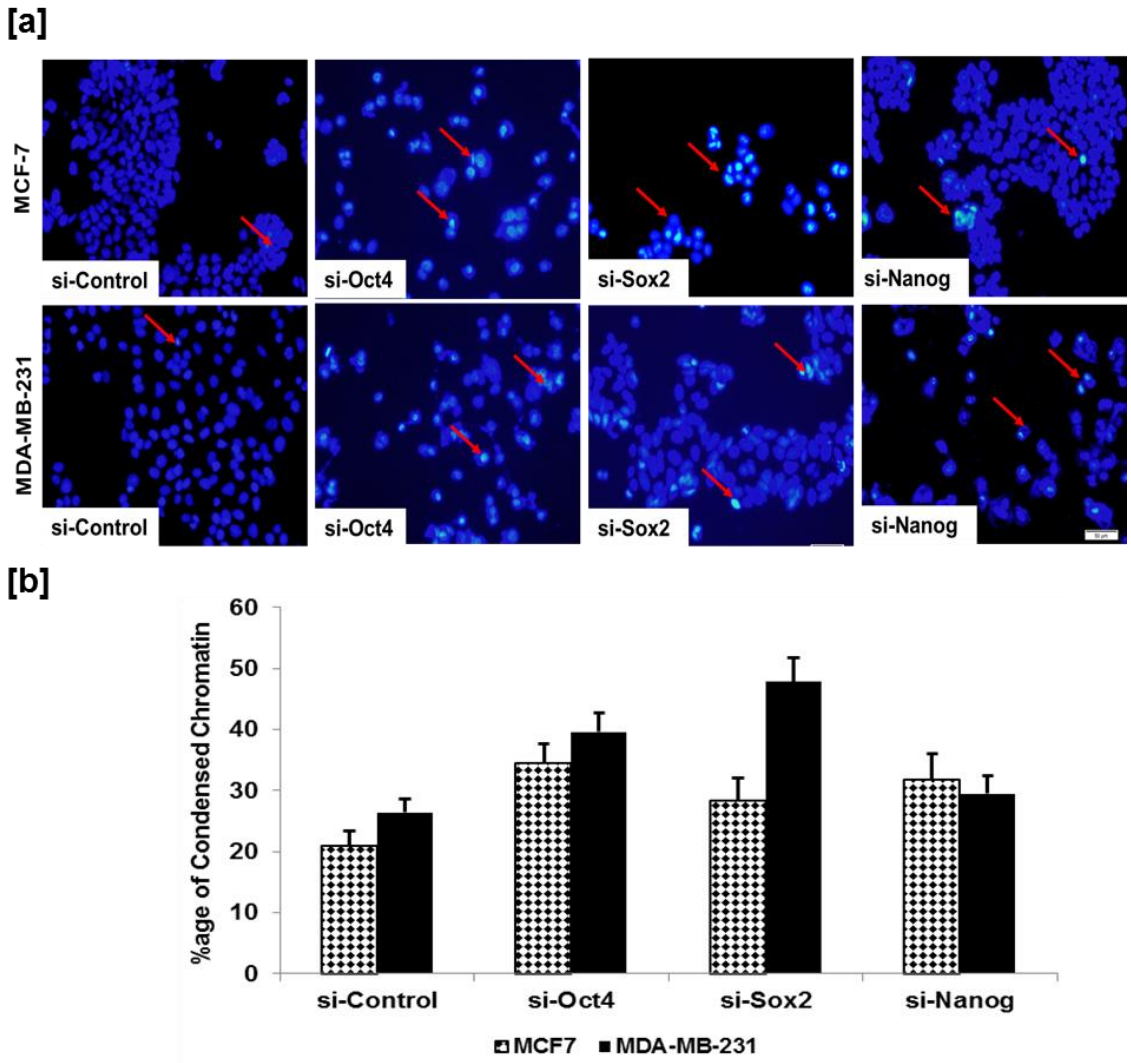


Figure 4.2: Chromatin condensation analysis in breast cancer cell lines after treatment with si-Oct4, si-Sox2 and si-Nanog. 30 nM of si-RNA against Oct4, Sox2, Nanog were used for 24 h. [a] Representative images of Hoechst 33342 stained nuclei in breast cancer cell lines (MCF7 and MDA-MB-231) after treatment with si-RNA against Oct4, Sox2 and Nanog and control si-RNA. Magnification 20X, Scale bar 20 µm. [b] Graphical representation of the percentage of condensed nuclei per field (n=3, mean ± S.D, $P < 0.05$).

Similarly, in prostate cancer cell lines, the percentage of condensed nuclei per field was found to be 19.68%, 28.21%, 34.67%, 23.84% in DU145 cell line and 31%, 42.54%, 51.65%, 37.89% in PC3 cells after treatment with si-contol, si-Oct4, si-Sox2 and si-Nanog respectively (Fig. 4.3a, 4.3b). The results are in agreement with those found in breast cancer wherein knockdown of Oct4, Sox2 and Nanog increased chromatin compaction and apoptotic DNA damage. In both breast and prostate cancer, silencing of

Sox2 resulted in the highest number of condensed chromatin. The percentage of condensed chromatin is higher in advanced stages of both the cancers implying that Oct4, Sox2 and Nanog might participate during metastatic conversion of primary tumors and that they support this behaviour by reducing apoptotic DNA damage and protecting cells from being subjected to cellular processes of death.

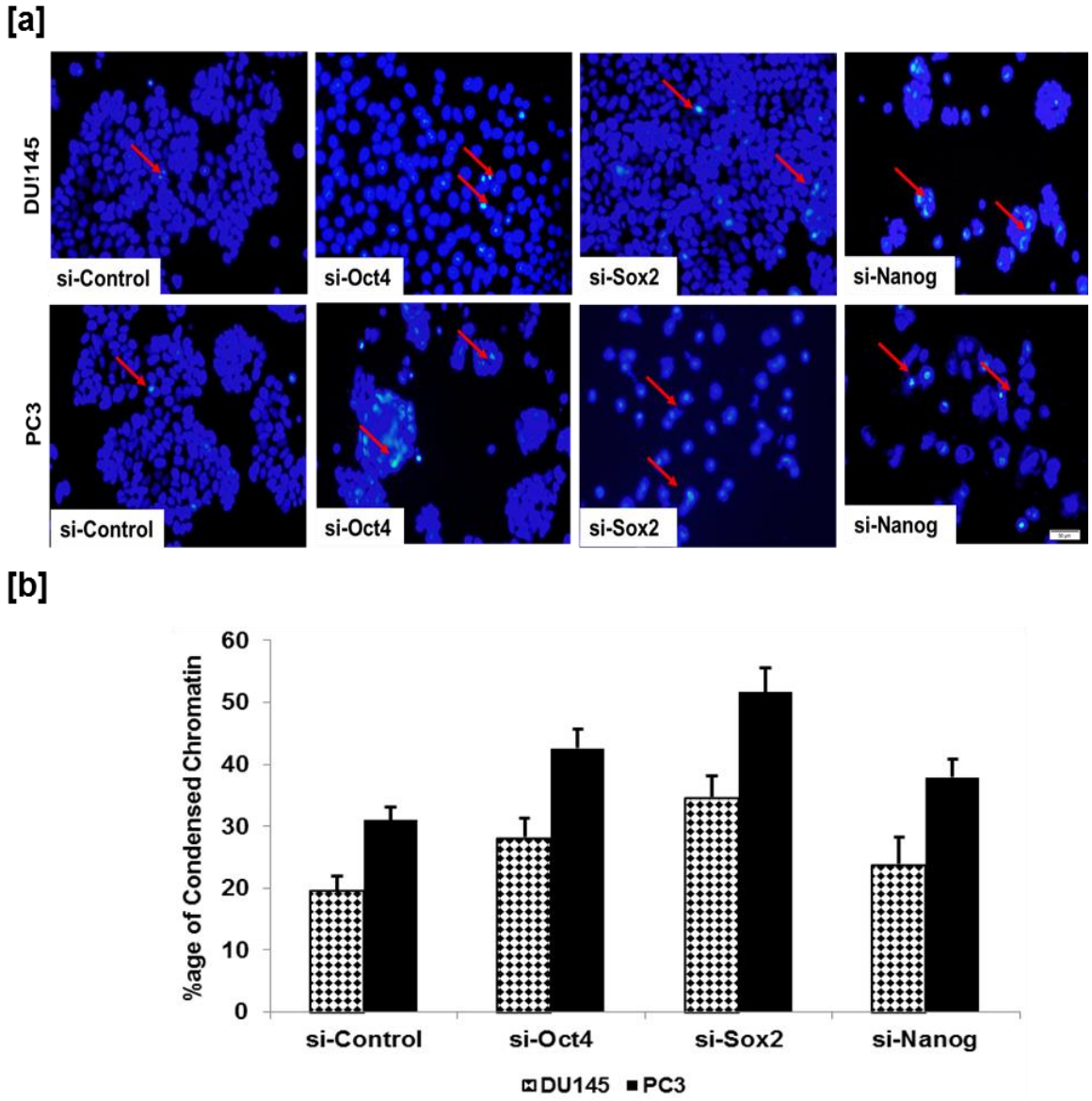


Figure 4.3: Chromatin condensation analysis in prostate cancer cell lines after treatment with si-Oct4, si-Sox2 and si-Nanog. 30 nM of si-RNA against Oct4, Sox2, Nanog were used for 24 h. [a] Representative images of Hoechst 33342 stained nuclei in prostate cancer cell lines (DU145 and PC3) after treatment with si-RNA against Oct4, Sox2 and Nanog and control si-RNA. Magnification 20X, Scale bar 20 µm. [b] Graphical representation of percentage of condensed nuclei per field (n=3, mean ± S.D, $P < 0.05$).

4.3.3 Silencing of Oct4, Sox2 and Nanog diminishes the rate of cell migration in both breast and prostate cancer cell lines:

The ability of cancer cells to migrate from one site to another site and result in tumorigenic growth is considered one of the most detrimental effects of cancer. In order to investigate how Oct4, Sox2 and Nanog influence the migratory behavior in breast and prostate cancer cells, wound healing assay was done. After treatment with the respective si-RNAs, it was observed that upon silencing of oct4, Sox2 and Nanog, there is decrease in number of migrating cells in comparison to si-control treated cells in both breast and prostate cancer. In breast cancer cell lines, the percentage of invaded cells after treatment in MCF7 was 29.64%, 8.915%, 11.56% and 17.86% and in MDA-MB-231 was 33.45%, 13.56%. 12.45% and 23.51% for si-control, si-Oct4, si-Sox2 and si-Nanog treated cells respectively (Fig. 4.4a, 4.4b). In case of prostate cancer cell lines, the percentage of invaded cells were 31.45%, 18.94%, 14.67% and 23% in DU145 and 45.67%, 15.34%, 17% and 27.89% in PC3 cells after treatment with si-control, si-Oct4, si-Sox2 and si-Nanog treated cells respectively (Fig. 4.5a, 4.5b). In case of breast cancer, Oct4 knockdown resulted in lowest migration while Sox2 was more effective in prostate cancer. Nanog showed comparatively lesser effect on cell migration potential in comparison to Oct4 and Sox2. Thus, these pluripotency factors help to mediate migration and invasion of cancer cells most probably by encouraging epithelial to mesenchymal transition.

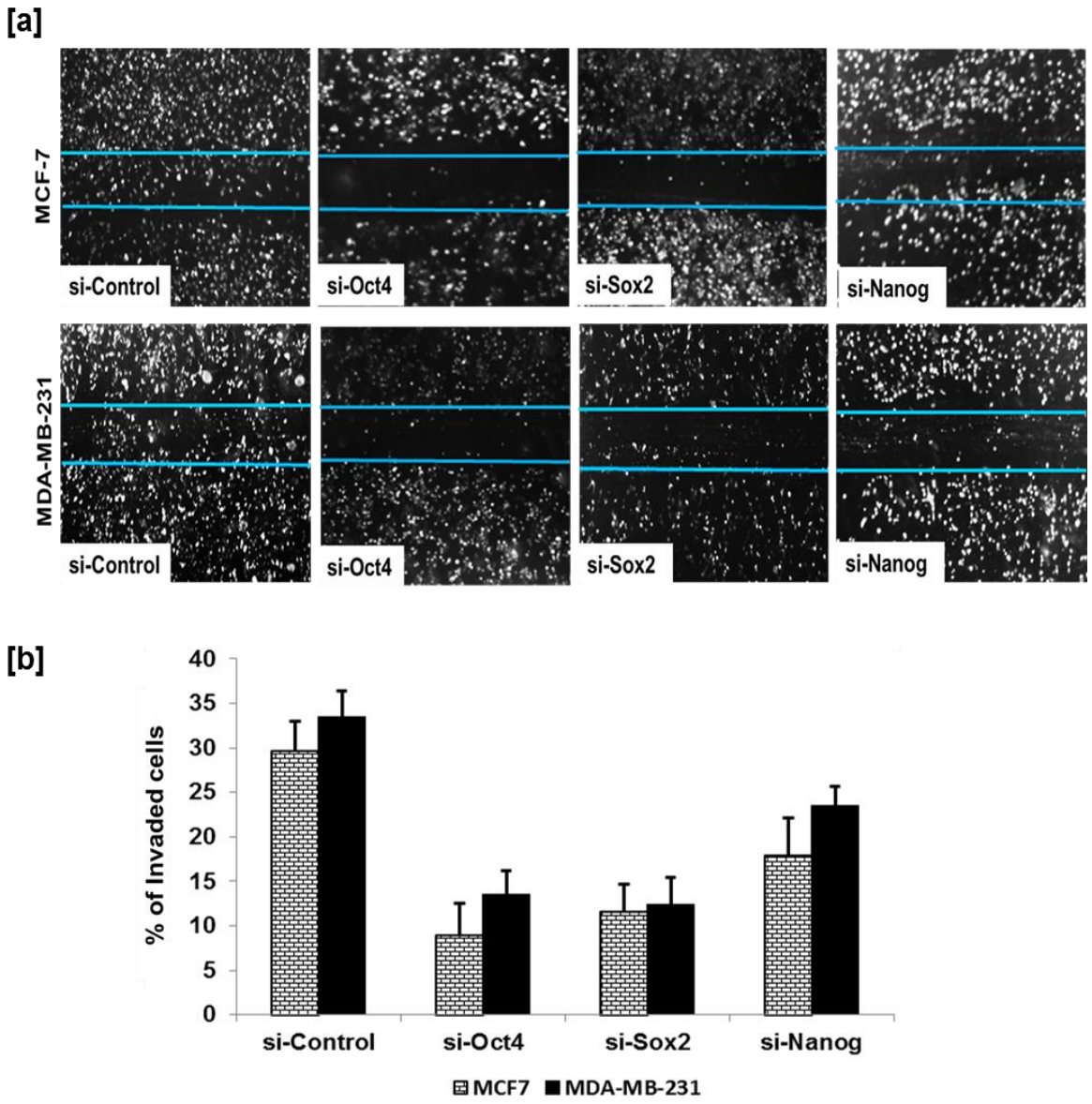


Figure 4.4: Wound healing assay in breast cancer cell lines after knockdown of Oct4, Sox2 and Nanog. 30 nM of si-RNA against Oct4, Sox2, Nanog were used for 24 h. [a] Representative images of migrating cells in breast cancer cell lines (MCF7 and MDA-MB-231) after traetment with si-RNA against Oct4, Sox2 and Nanog and control si-RNA. Magnification 20X, Scale bar 20 μm. [b] Graphical representation of percentage of invaded cells per field (n=3, mean ± S.D, $P < 0.05$).

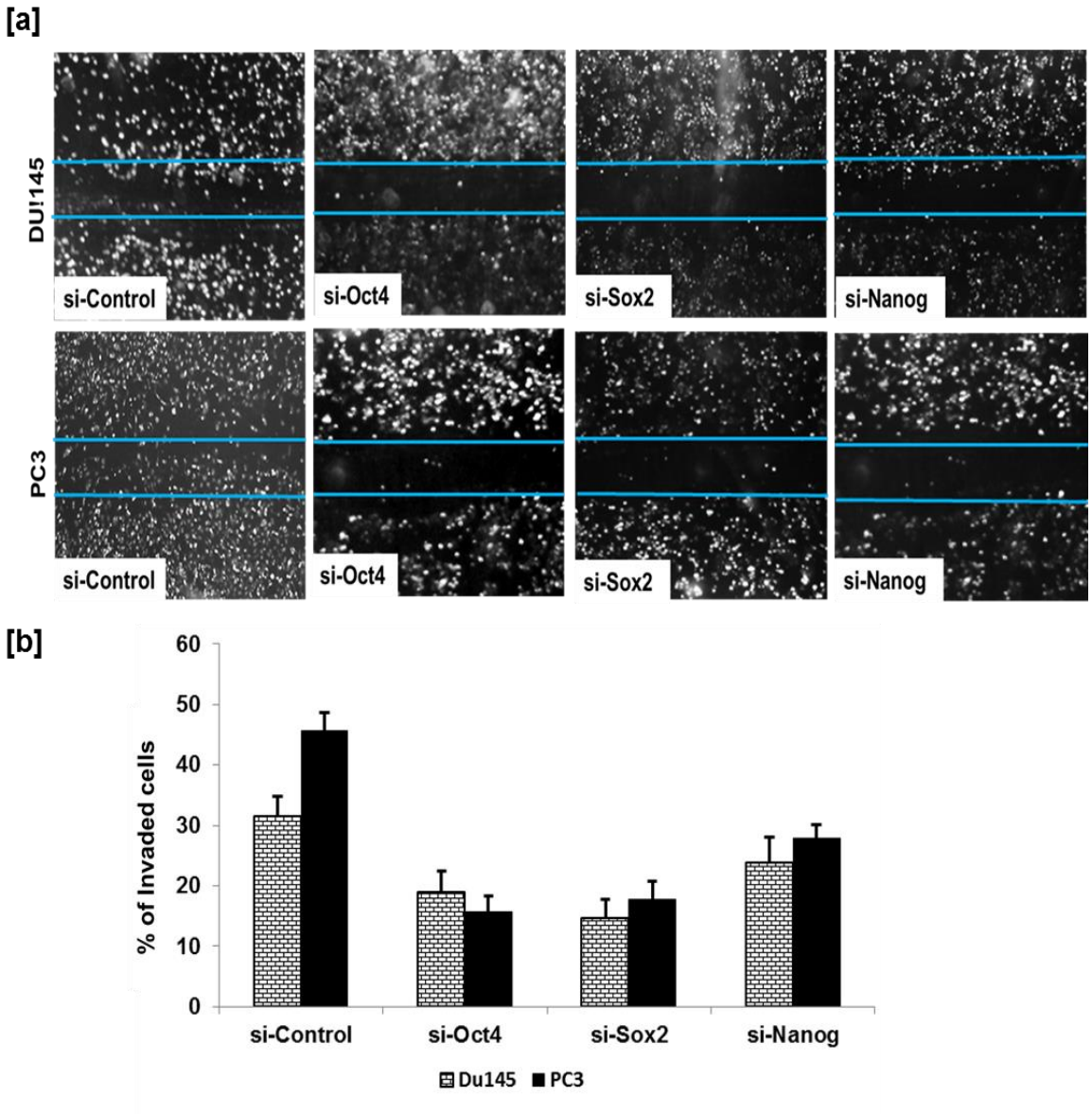


Figure 4.5: Wound healing assay in prostate cancer cell lines after knockdown of Oct4, Sox2 and Nanog. 30 nM of si-RNA against Oct4, Sox2, Nanog were used for 24 h. [a] Representative images of migrating cells in prostate cancer cell lines (DU145 and PC3) after treatment with si-RNA against Oct4, Sox2 and Nanog and control si-RNA. Magnification 20X, Scale bar 20 μm. [b] Graphical representation of percentage of invaded cells per field (n=3, mean ± S.D, $P < 0.05$).

4.3.4 Silencing of Oct4, Sox2 and Nanog inhibits cell growth and colony forming ability in both breast and prostate cancer cell lines:

Colony forming or clonogenic assay was conducted to examine the ability of cancer cells to grow and produce colonies. Oct4, Sox2 and Nanog are known to support self-renewal in embryonic and pluripotent cells. Also, in malignant phenotype they are known to induce clonal growth, self-renewal and proliferation of cancer stem cells. In order to assess whether Oct4, Sox2 and Nanog affect clonal growth in cancer cells, treatment with

si-RNA directed against Oct4, Sox2 and Nanog was done and it was observed that number of colonies formed drastically reduced in comparison to si-control cells. The number of surviving colonies after treatment with si-Oct4, si-Sox2 and si-Nanog in case of MCF7 cells were 39.48%, 19.86% and 45.55% and in case of MDA-MB-231 cells were 27.46%, 36.57% and 59.12% respectively in comparison to 85.31% and 93.12% control si-RNA treated cells (Fig. 4.6a, 4.6b). While Oct4 and Sox2 down-regulation greatly reduced colony forming ability, in comparison when Nanog is silenced, cells showed less effect.

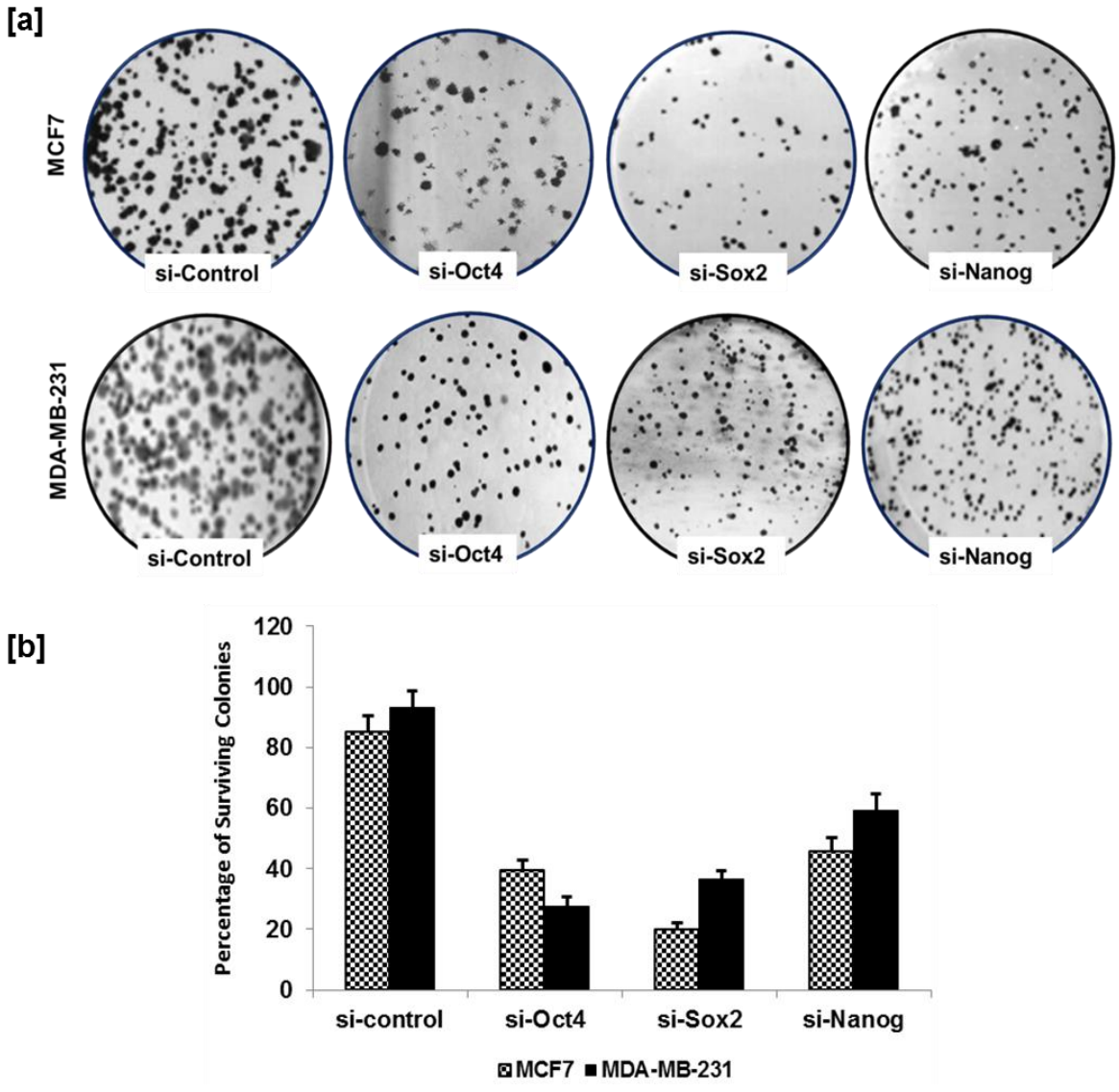


Figure 4.6: Clonogenic assay in breast cancer cell lines after treatment with si-RNA against Oct4, Sox2 and Nanog. 30 nM of si-RNA against Oct4, Sox2, Nanog were used for 24 h. **[**a] Representive images of colonies formed after treatment with si-RNA directed against Oct4, Sox2 and Nanog in breast cancer cell lines (MCF7 and MDA-MB-231). [b] Graphical representation of number of colonies formed (n=3, mean ± S.D, $P < 0.05$).

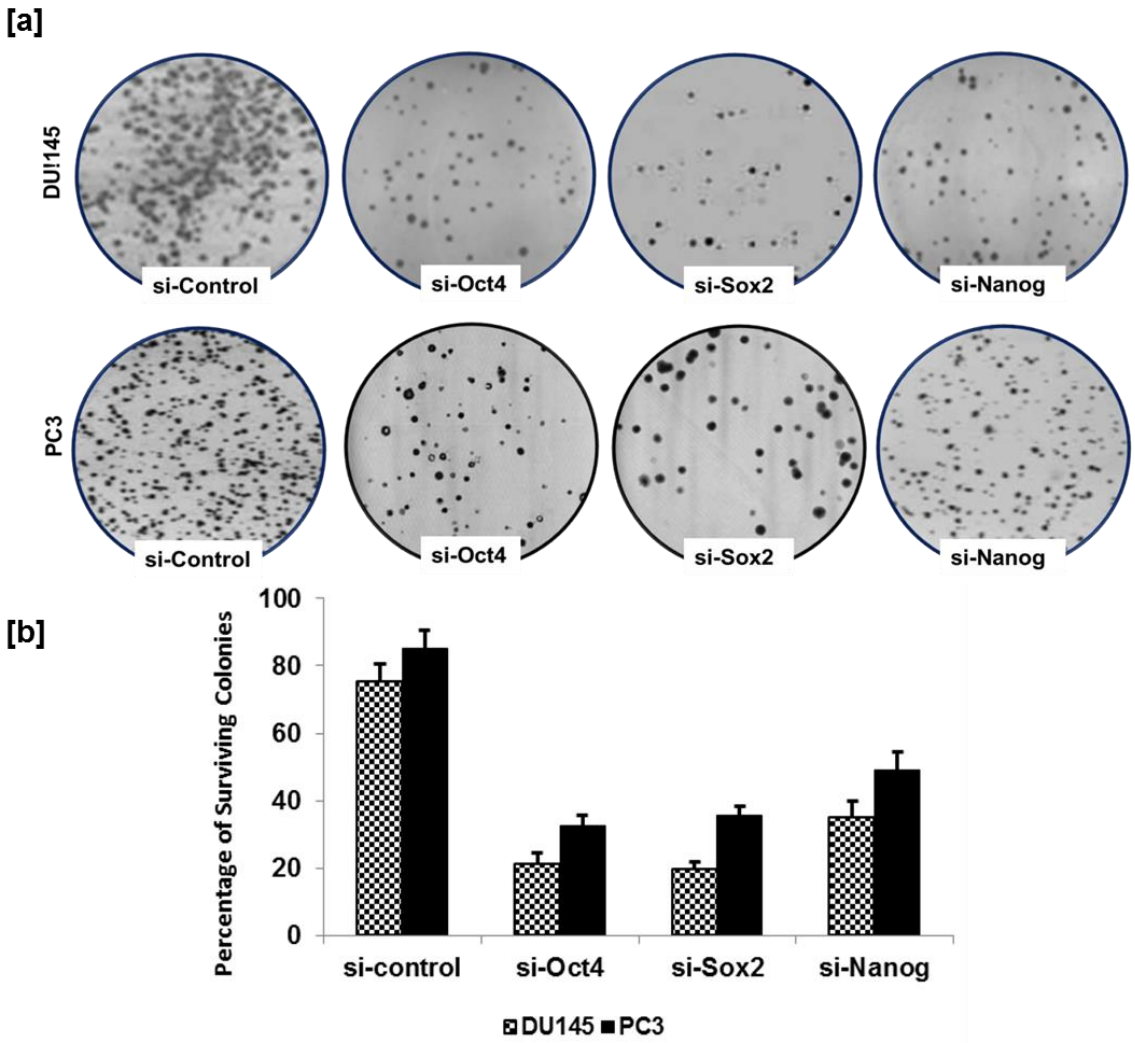


Figure 4.7: Clonogenic assay in prostate cancer cell lines after treatment with si-RNA against Oct4, Sox2 and Nanog. 30 nM of si-RNA against Oct4, Sox2, Nanog were used for 24 h. [a] Representive images of colonies formed after treatment with si-RNA directed against Oct4, Sox2 and Nanog in breast cancer cell lines (DU145 and PC3). [b] Graphical representation of number of colonies formed (n=3, mean ± S.D, $P < 0.05$).

In prostate cancer cell lines, number of surviving colonies drastically reduced in case of cells transfected with si-RNAs. The number of surviving colonies after treatment with si-Oct4, si-Sox2 and si-Nanog in case of DU145 cells were 21.36%, 19.56% and 35.26% and in case of PC3 cells were 32.56%, 35.46% and 48.97% respectively in comparison to 75.32% and 85.23% control si-RNA treated cells (Fig. 4.7a, 4.7b). The above results indicate that in both breast and prostate cancer cell lines, silencing of Oct4, Sox2 and Nanog resulted in decrease in percentage of surviving colonies indicating a decrease in the cell survival ability of breast and prostate cancer cells. The number of colonies in case of primary cell lines like MCF7 and DU145 is lesser

in comparison to metastatic cells implying that these factors might promote cell survival in early stage cancer.

4.3.5 Silencing of Oct4, Sox2 and Nanog causes cell cycle arrest and promotes apoptotic cell death in both breast and prostate cancer cell lines:

Cell cycle analysis was performed in order to assess the role of Oct4, Sox2 and Nanog in cell cycle distribution. In general, upon silencing of these factors, cell cycle was arrested at G_0/G_1 phase indicating that Oct4, Sox2 and Nanog affected cell cycle machinery and promoted unchecked cell division in cancer. In breast cancer cell lines, the distribution of G_0/G_1 in MCF7 cell line after treatment with si-Oct4, si-Sox2 and si-Nanog were 66.78%, 59.56%, 54.21% respectively in comparison to 52.67% in si-control treated cells. Similarly, for MDA-MB-231 cells, percentage of G_0/G_1 phase population was 56.78%, 49.13%, 52.29% respectively for si-Oct4, si-Sox2 and si-Nanog with respect to si-control (32.31%) (Fig. 4.8). The drastic increase in G_0/G_1 cells indicated that Oct4, Sox2 and Nanog are instrumental in dysregulating cell cycle and allowing unchecked cell division.

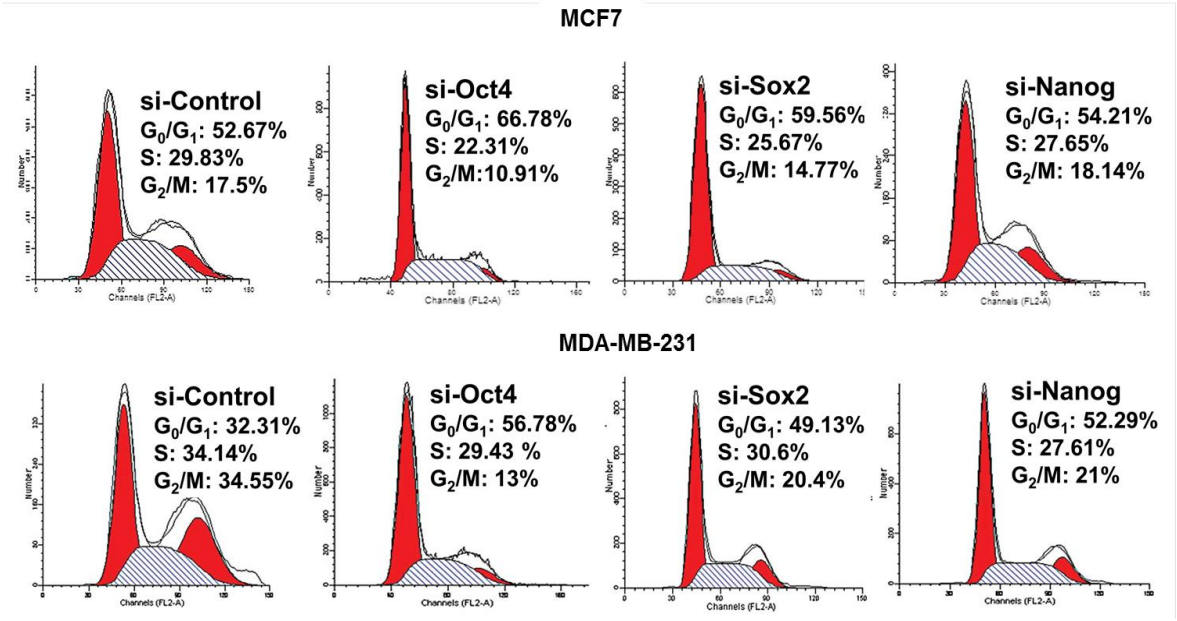


Figure 4.8: Cell cycle analysis in breast cancer cell lines after treatment with si-RNA against Oct4, Sox2 and Nanog. 30 nM of si-RNA against Oct4, Sox2, Nanog were used for 24 h. Representative FACS images showing distribution of different cell populations after treatment with si-RNA against Oct4, Sox2 and Nanog.

In prostate cancer cell lines also, there is increase in G_0/G_1 arrested cells, but it is observed that Oct4 and Sox2 are more effective in inhibiting cell cycle than Nanog as

percentage of G_0/G_1 cells is 52.28% and 56.15% for Oct4 and Sox2 and 46.69 for Nanog knockdown cells in DU145. In PC3, percentage of G_0/G_1 cells after knockdown of Oct4, Sox2 and Nanog is 68.53%, 64.72% and 51.83% respectively (Fig. 4.9).

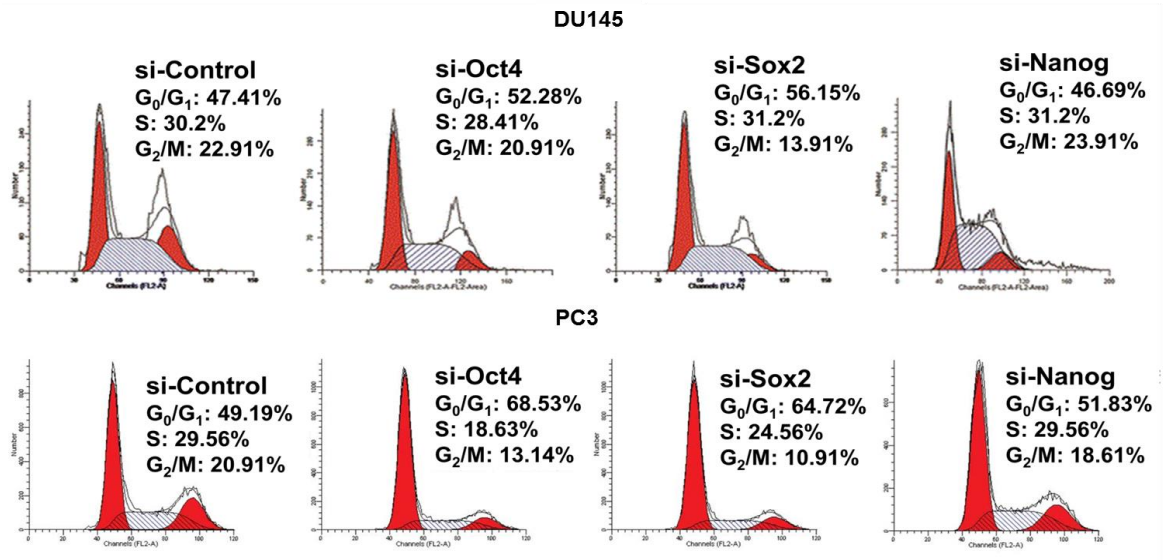


Figure 4.9: Cell cycle analysis in prostate cancer cell lines after treatment with si-RNA against Oct4, Sox2 and Nanog. 30 nM of si-RNA against Oct4, Sox2, Nanog were used for 24 h. Representative FACS images showing distribution of different cell populations after treatment with si-RNA against Oct4, Sox2 and Nanog.

The apoptotic cell population was examined and it was found that in comparison to control si-RNA treated cells, Oct4 and Sox2 silencing increased the apoptotic cell population in breast and prostate cancer cell lines indicating that Oct4, Sox2 and Nanog affect apoptotis (Fig. 4.10a, 4.10b).

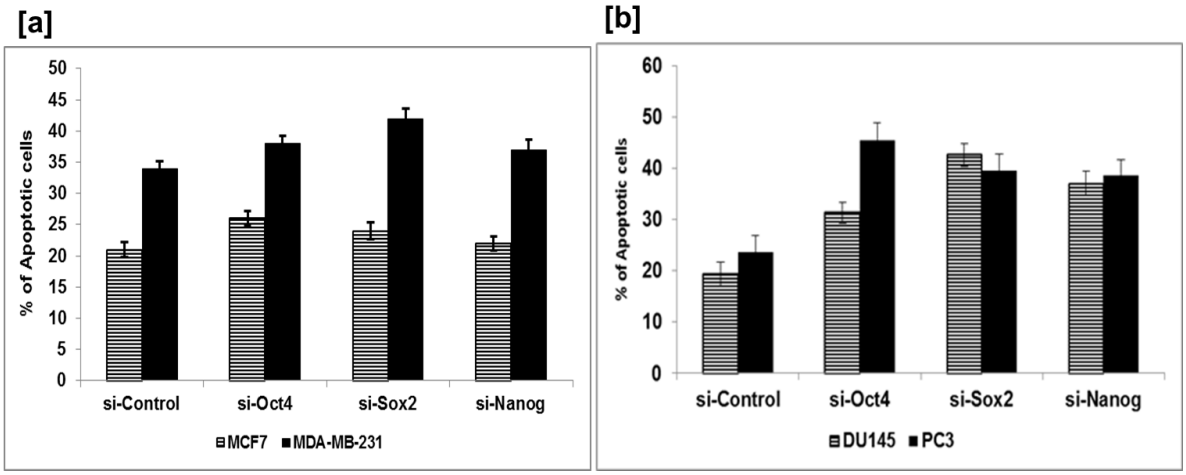


Figure 4.10: Analysis of apoptotic cell death in breast and prostate cancer cell lines. Graphical representation of percentage of apoptotic cell population in [a] breast cancer cell lines (MCF7 and MDA-MB-231) and [b] prostate cancer cell lines (DU145 and PC3) after traetment with si-RNA against Oct4, Sox2 and Nanog and control si-RNA (n=3, mean ± S.D, $P < 0.05$).

4.4 Discussion:

As is evident from the first objective, Oct4, Sox2 and Nanog are over-expressed in both breast and prostate cancer. Therefore, the role of these oncogenic transcription factors in promoting inherent tumorigenicity in breast and prostate cancer was evaluated using si-RNAs directed against them. Although, Oct4, Sox2 and Nanog have always been accredited for contributing towards maintenance of CSC population and promoting stem cell phenotype, we have established that in addition to their role in maintenance of stem cell population, Oct4, Sox2 and Nanog are functionally relevant in tumor bulk in breast and prostate cancer cells.

Colorimetric MTT assay was performed for assessing the rate of proliferation after silencing of Oct4, Sox2 and Nanog in breast and prostate cancer cell lines. It was observed that cell proliferation in Oct4, Sox2 and Nanog silenced cells were lower in comparison to cells transfected with control si-RNA in both breast and prostate cancer. In case of breast cancer, silencing of Nanog resulted in slowest proliferation of cells whereas in case prostate cancer, Oct4 knockdown drastically reduced cell proliferation with much greater effect on PC3 cells (Fig. 4.1). Chromatin condensation analysis using Hoechst stain was performed to study the cytotoxic effect of knockdown of Oct4, Sox2 and Nanog. The blue-fluorescent DNA-binding Hoechst 33342 dye can detect DNA damage such as condensed nuclei induced by apoptosis. It brightly stains the highly condensed, dense chromatin of apoptotic cells in comparison to the chromatin of non-apoptotic cells. Therefore, increased number of condensed nuclei indicates higher DNA damage and apoptotic rate. Upon knockdown of Oct4, Sox2 and Nanog, percentage of condensed chromatin in both breast and prostate cancer cell lines dramatically increased indicating that these oncogenic factors have anti-apoptotic activity in tumor niche. In breast cancer, percentage of condensed nuclei was highest for Sox2 knockdown MDA-MB-231 cells whereas in prostate cancer, cells transfected with si-Nanog showed highest apoptotic nuclei in comparison to cells transfected with control si-RNA (Fig. 4.2, 4.3).

EMT is considered to be a pro-oncogenic process in which primary cancer cells become detached from their site of origin and travel to distant sites to promote oncogenic differentiation of other organs and tissues. Cancer metastasis is one of characteristic hallmarks of malignant progress, and is considered to be influenced by stemness promoting factors that recapitulate embryonic potential in neoplastic cells (Tsai et al.,

2014; Han et al., 2013). Scratch or wound-healing assay was conducted to study the role of Oct4, Sox2 and Nanog in promoting cell migration and motility. For the first time, we have shown that Oct4, Sox2 and Nanog can also support metastatic behavior in non-stem cancer cells. It was observed that after treatment with respective si-RNAs, the percentage of invading or migrating cells significantly decreased in both breast and prostate cell lines indicating that Oct4, Sox2 and Nanog encourage metastatic and invasive behaviour in tumor cells and help in acquiring more aggressive phenotype (Fig. 4.4, 4.5). Clonogenic assay was performed to examine the impact of knockdown of Oct4, Sox2 and Nanog on colony forming ability in breast and prostate cancer cells. It was observed that after gene knockdown, number of colonies formed in comparison to si-control treated cells is significantly lower. In case of breast cancer, si-Sox2 treated cells exhibited the least number of colonies whereas for prostate cancer, knockdown of Oct4 severely decreased the growth of prostate cancer cells (Fig. 4.6, 4.7).

Finally, the impact of Oct4, Sox2 and Nanog over-expression over cell cycle progression and apoptosis was deliberated by performing FACS analysis. Cells transfected with si-RNAs against Oct4, Sox2 and Nanog displayed G_1 phase growth arrest and increase in apoptotic cell population implying that these transcription factors mediate cell cycle progression from G_1 to S phase and help in tumorigenic progression. In case of breast cancer, highest percentage of apoptotic population was observed in Sox2 silenced cells whereas knockdown of Oct4 in prostate cancer cell lines greatly increased apoptotic death (Fig. 4.8, 4.9, 4.10). It has already been evidently proven that Oct4, Sox2 and Nanog influence CSCs properties by confering advantageous properties like increased cellular proliferation and tumor growth, evasion of apoptotic death and improved metastatic and invasive potential. The above-mentioned findings however strengthen our hypothesis that Oct4, Sox2 and Nanog are equally responsible for facilitating oncogenic properties in bulk of breast and prostate cancer cells. In other words, over-expression of oncogenic transcription factors Oct4, Sox2 and Nanog enhance the inherent tumorigenicity in breast and prostate cancer by increasing metastatic behavior, improving survival and proliferation capacity, as well as deregulating cell cycle machinery.

Chapter 5

To elucidate the epigenetic modifications regulating pluripotency inducing TFs in breast and prostate cancer

5.1 Introduction:

The molecular signatures underlying neoplastic transformation are now regarded to be of epigenetic origin with the notion that aberrant alterations in the epigenomic organization are mostly responsible for cancer initiation, maintenance and progression (Hadjimichael et al., 2015). With the epigenetic progenitor model of cancer taking center-stage in the field of oncology, it is generally acknowledged that abnormal epigenetic gene silencing events are superior surrogates to mutational inactivation/silencing during tumorigenic conversion. Early epigenetic variations in the stem/progenitor cell population disrupt the fine balance between cell proliferation and cell division, thus pre-disposing them towards genetic mutations (Klimczak, 2015). As mutational instability affects the phenotypic characteristics, enhancing genetic and epigenetic plasticity and heterogeneity in tumor cell enables them to acquire stemness properties such as invasion, metastasis and drug resistance (Feinberg et al., 2006; Feinberg et al., 2016). This deregulation is possible due to disruption of pluripotency related transcriptional network in stem cells during early cancer development. We hypothesize that the pluripotency transcriptome maintained by nuclear transcription factors Oct4, Sox2 and Nanog is epigenetically regulated during embryonic pluripotency and differentiation. Hence, it is imperative that alterations in the stem cell pluripotency circuitry via epigenetic aberrations are functionally responsible for augmenting the evolution of tumor into potentially aggressive phenotype.

The major players involved in epigenetic deregulation during cancer are DNA methylation and histone modifications. Global loss of methylation was the first epigenetic change to be characterized in human cancers and is one of the major mechanisms responsible for genomic instability (Eden et al., 2003). Epigenetic gene silencing mediated by promoter DNA hypermethylation is another important contributor to malignant transformation. Silencing of crucial tumor suppressor genes, cell cycle

regulatory and DNA repair genes, genes involved in detoxification of carcinogen induced cytotoxicity as well as important signaling molecule results in inhibition of cellular response against carcinogenic insults and paves the way for neoplastic distress (Scaffidi and Misteli, 2010; Kanwal and Gupta, 2012) Similarly, histone modifications mediate tumorigenic potential by providing accessible chromatin environment that facilitates transcriptional activation of oncogenes. These changes affect the overall epigenomic landscape around the pluripotency maintaining transcriptome which in turn allows for transcriptional plasticity responsible for acquisition of stem cell features (Muller et al., 2016). In fact, epigenetic reprogramming of pluripotency inducing transcription factors is now considered as a potential mechanism of tumorigenic adaption of cancer cells.

It has already been discussed how epigenetic regulation modulates the expression and activity of Oct4, Sox2 and Nanog during embryonic development (Christophersen and Helin, 2010). Oct4 gene locus is generally maintained in an active state by the help of activating histone marks such as H3K9Ac, H3K14Ac and H3K4me2/me3; however during differentiation, Oct4 is silenced transiently by repressive histone marks such as H3K9me3 followed by DNA methylation mediated permanent silencing (Deshpande et al., 2009; Athanasiadou et al., 2010). Similarly, Sox2 and Nanog expression during maintainenance of pluripotency and initiation of lineage-speciation and commitment is subjected to extensive epigenetic modifications. The promoter of Nanog is activated by histone acetyltransferases and methyltransferases such as WDR5 and repressed by EZH2 through enrichment of H3K27me3 marks (Baltus et al., 2009; Li et al., 2012). Although epigenetic regulation of Oct4, Sox2 and Nanog is relatively known and established during embryonic development, similar regulatory mechanism(s) responsible for Oct4, Sox2 and Nanog expression in cancer remains poorly understood. As the importance of Oct4, Sox2 and Nanog was acknowledged during generation of induced pluripotency, it was discovered that ESC-like signature mediated by Oct4, Sox2 and Nanog is also prevalent in cancer. Hence, it was assumed that an epigenetic regulatory system is functionally responsible for over-expression of Oct4, Sox2 and Nanog in human cancers and that this system affects the overall activity of these TFs in mediating tumorigenesis (Savarese et al., 2009).

In this light, the present objective has been designed to investigate various epigenetic modifications regulating Oct4, Sox2 and Nanog expression and function in breast and prostate cancer. The role of epigenetic modifications were studied by

employing epigenetic modulators/inhibitors against prominent epigenetic modifying enzymes, for example 5-aza-2'-deoxycytidine (AZA) against DNMTs, Trichostatin A (TSA) against HDACs and 3-Deazaneplanocin A (DZNeP) against EZH2. The effect of ectopic inhibition of the following epigenetic enzymes on the promoter methylation and histone mark enrichment was analyzed via MS-PCR and ChIP assays so as to obtain a clear picture regarding the methylation status and histone marks enrichment that arbitrates aberrant expression of Oct4, Sox2 and Nanog in breast and prostate cancer.

5.2 Materials and Methods:

5.2.1 Cell culture:

Human breast carcinoma cell lines MCF7 and MDA-MB-231 and human prostate cancer cell lines PC3 and DU145 were obtained from National Centre for Cell Science (NCCS), Pune. MCF7 was cultured and maintained in Minimum Essential Medium (MEM, Himedia-AL047A), MDA-MB-231 in Leibovitz's medium (L-15, Himedia-AL011S), PC3 was cultured in Nutrient Mixture F-12 Ham medium (F-12, Himedia-AL106S) whereas DU145 in Dulbecco's Modified Eagle's Medium (DMEM, Himedia-AL007A) supplemented with 10% (v/v) fetal bovine serum (FBS, Himedia-RM1112) and 100 IU/mL penicillin and 0.1 mg/mL streptomycin (Himedia-A002A) in a humified atmosphere of 5% CO_2 at 37° C. The cells were harvested by trypsinization (Trypsin, Sigma-T4049) and the number of living cells was calculated by trypan blue (Himedia-TC193) staining (0.2% v/v) using hemocytometer before seeding for future experiments.

5.2.3 *In vitro* treatment of epigenetic modulators:

In order to determine IC_{30} concentration of AZA (Sigma-A3656), TSA (Sigma-T8552) and DZNeP (Santa Cruz Biotechnology-sc-397045), 5 X 10^3 cells per well were seeded in 96-well microtiter plates. After reaching 70% confluency, growth medium was replaced with respective experimental media containing epigenetic modulators at different concentrations supplemented with 5% FBS. AZA and DZNeP treatment was done with 1, 5, 10, 15, 20, 25 μM concentrations whereas for TSA, 10, 50, 100, 150, 200, 250 nM concentrations were used in all four cell lines. Control cells were treated with di-methyl sulphoxide (DMSO, Himedia-TC185) only.

5.2.4 Cell viability assay by colometric MTT assay:

The effect of the epigenetic modulators on cell viability was assessed by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT, Himedia-TC191) assay, using our standard protocol (Kar et al., 2014). Cells were incubated for 24 and 72 h after treatment with TSA, AZA and DZNeP respectively. Drug-treated cells were trypsinized and washed twice with phosphate buffered saline (PBS) prior to use. Working concentration of 0.8 mg/mL MTT solution was prepared from stock (5 mg/mL PBS, pH 7.2). To each well, 100 µL working MTT solution was added. After incubation in dark for 4 h at 37° C, 100 µL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm and results were expressed as the mean of three replicates as a percentage of control (taken as 100%). The extent of cytotoxicity was defined as the relative reduction in optical density (OD) of treated cells in comparison to control cells, which indicated the amount of viable cells in relation to control (100%). The cell viability was graphically represented and IC_{30} value of drugs was calculated accordingly to decide the optimum dosage for further studies.

5.2.5 Relative mRNA expression analysis by real-time PCR after treatment with epigenetic modulators:

After treatment with sub-lethal dosages of drugs for effective time period, total cellular RNA from each of the four cell lines was extracted with TRI Reagent (Sigma- T9424) according to the manufacturer's instructions. cDNA was prepared from 1 µg of total RNA by using RevertAid^{TM} First Strand cDNA Synthesis Kit (Thermo Scientific-K1622) followed by quantitative real-time PCR (RT-PCR) using Maxima SYBR Green/ROX qPCR Master Mix (2X, Thermo Scientific-K0221). The mRNA level was normalized to house-keeping gene GAPDH used as an endogenous control and calculations were done using $2^{-\Delta\Delta Ct}$ method, as described earlier (Schmittgen and Livak, 2008). The primer sequences are provided in Table 5.1.

Table 5.1: List of sequence and product length of PCR primers used

Gene	Primer Sequence			Product Length (bp)
Real-Time Primers				
Oct4	F	5’- AGCAAAACCCGGAGGAGT-3’		114
	R	5’- CCACATCGGCCTGTGTATATC-3’		
Sox2	F	5’- GGAAATGGAGGGGTGCAAAAGAGG-3’		150
	R	5’- TTGCGTGAGTGTGGATGGGATTGGTG-3’		
Nanog	F	5’- TCCTCCTCTTCCTCTATACTAAC-3’		112
	R	5’- CCCACAATCACAGGCATAG-3’		
GAPDH	F	5’- TGTTGCCATCAATGACCCCTTC -3’		204
	R	5’- CTCCACGACGTACTCAGCGC-3’		
Methyl-Specific PCR Primers				
Oct4	M	F	5’-CGGGATATTTGGTTTCGGATTTC-3’	209
		R	5’-CCCACAAAACTCATACGACGA-3’	
	U	F	5’-TGGGATATTTGGTTTTGGATTTT-3’	210
		R	5’-CCCCACAAAACTCATACAACAAA-3’	
Sox2	M	F	5’-TTTTTTTTATGTAAAATTCGGTAGC-3’	122
		R	5’-AATAAACAACCATCCATATAACGAA-3’	
	U	F	5’-TTTTTTATGTAAAATTTGGTAGTGA-3’	122
		R	5’-AATAAACAACCATCCATATAACAAA-3’	
ChIP PCR Primers				
Oct4	F	5’- CTTCCACAGACACCATTGCC-3’		240
	R	5’- GAGAAGGCGAAATCCGAAGC-3’		
Sox2	F	5’- CAAAGGTTTCTCAGTGGCTGG-3’		190
	R	5’-GGGTTTCTAGCGACCAATCAG-3’		

5.2.6 Western Blotting:

2 X 10^5 cells of each cell line were seeded and incubated till 80% confluency. Cells were harvested and total cellular protein was extracted using lysis buffer (RIPA Buffer, Sigma-R01278) supplemented with protease inhibitor cocktail (PIC, Sigma-P8340). Bradford method was used to quantify the protein concentration and equal amount of cell lysate was loaded and separated in 10% SDS-PAGE and subsequently transferred onto PVDF membrane (Millipore,USA-ISEQ00010). Protein containing membranes were blocked in 3% bovine serum albumin (BSA, Himedia-5RMI05) in PBS containing 0.1% Tween 20

(Himedia-MB067; PBST), followed by overnight incubation with primary antibodies such as rabbit polyclonal anti-Oct4 (Abcam-ab19857), rabbit polyclonal anti-Sox2 (Abcam-ab59776) and rabbit polyclonal anti-Nanog (Abcam-ab21624), then incubated with appropriate HRP conjugated secondary antibody (Goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology-sc-2004) for 2 h at 37° C in dark. The membranes were thoroughly washed with PBST buffer and were developed by Supersignal^{TM} West Femto Chemiluminescent substrate (ThermoScientific-34095). β-Actin (Primary-Mouse IgG_1 (Monoclonal),Santa Cruz Biotechnology-sc-47778 and Secondary-Goat Anti-Mouse IgG-HRP, Santa Cruz Biotechnology-sc-2005) protein levels were used as control for equal protein loading. Relative protein expression was analyzed from the blots obtained using ImageJ quantification software.

5.2.7 Immunocytochemistry:

Immunocytochemistry was performed as per our previous protocol with some modifications. In brief, MCF-7, MDA-MB-231, PC3 and DU145 cells were grown on glass coverslips and treated with AZA (10 μM), TSA (150nm, 100 nM) and DZNeP (10 μM) for 24 and 72 h respectively. The treated cells were fixed by ice cold methanol (Himedia-AS058) and permeabilized by 0.25% triton X-100 (Himedia-RM845) in PBS. Cells were incubated with 1% BSA in PBST for 30 mins to block nonspecific binding of the antibodies. The endogenous peroxidase activity was blocked by incubating in 5% hydrogen peroxide (H_2O_2-Himedia) in methanol for 20 mins followed by incubation in primary antibodies for Oct4 (Abcam-ab19857), Sox2 (Abcam-ab59776) and Nanog (Abcam-ab21624) overnight at 4° C. The cells were then washed in PBS and incubated with HRP-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology-sc-2004) for 1 h followed by another wash. Finally, reactions were visualized by incubation with 3, 3′-Diaminobenzidine tetrahydrochloride (DAB, substrate and chromogen, Sigma-D3939) and counterstained with Mayer's hematoxylin (Thermo Scientific-38803). For negative control, cells were incubated overnight with dilution buffer (no primary antibody).

5.2.8 Bi-sulphite conversion and MS-PCR:

Promoter region of Oct4 and Sox2 were retrieved by http://www.genomatix.de/ software. Retrieved promoters were analyzed by UCSC genome browser. ChIP specific primers were designed by Perl primer software. Meth-primers and bisulfite primers were designed

using meth-primer designing tool (http://www.urogene.org/methprimer/). Genomic DNA was isolated from treated cells by phenol-chloroform method. 2µg of genomic DNA was converted by bisulfite treatment using EpiTect® Bisulfite Kit (Qiagen-59104) according to the manufacturer's instructions. Bisulfite-treated DNA was then used as template in PCR reactions for PCR analysis. Promoter regions of Oct4 and Sox2 were analyzed by bisulfite genomic DNA sequencing with specific primers.

5.2.9 Chromatin immunoprecipitation analysis:

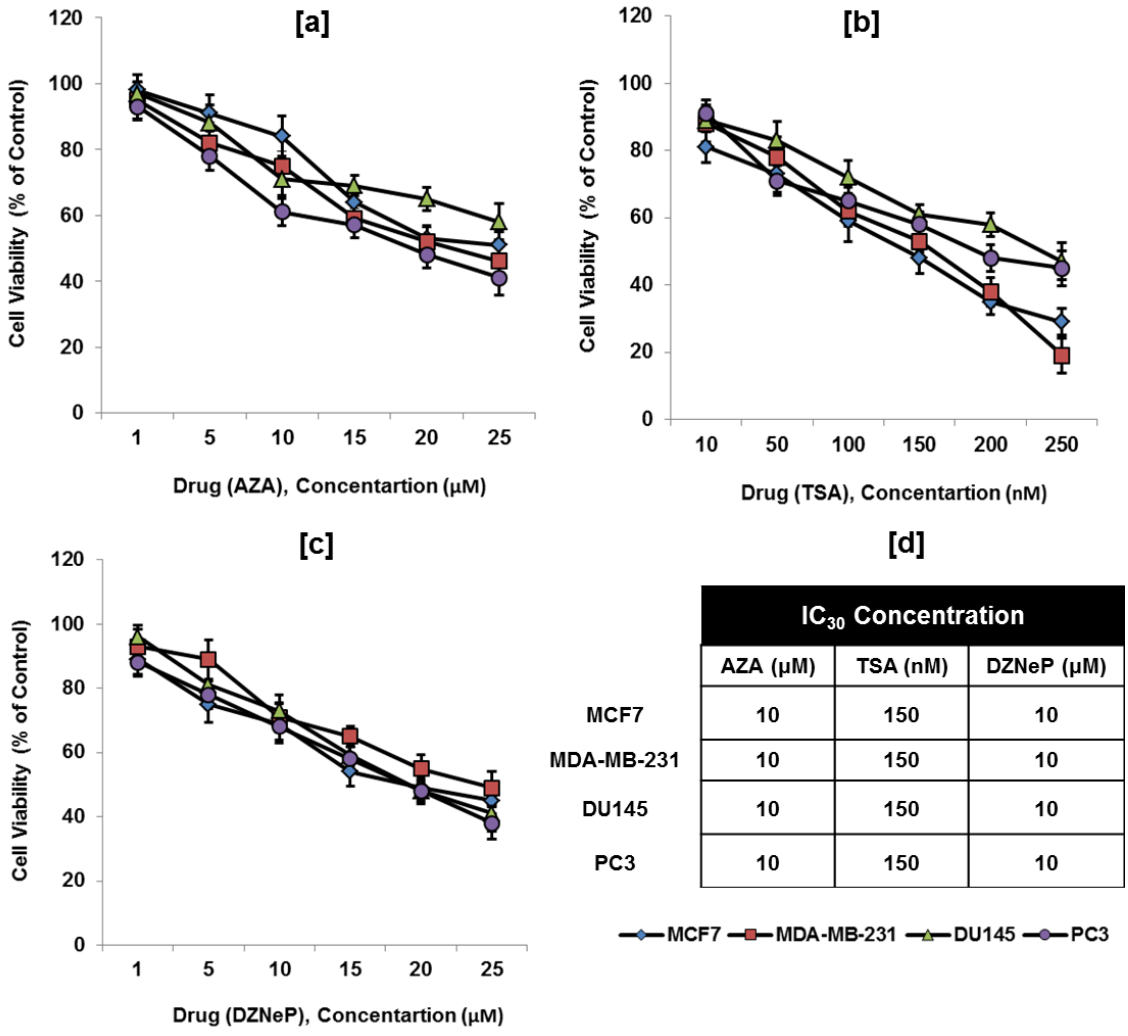
ChIP assay was performed using primary antibodies – Rabbit anti-H3K4me3 (Invitrogen-49-1005), Rabbit anti-H3K9me3 (Invitrogen-49-1008), Rabbit anti-H3K9acS10p (Invitrogen-49-1011) and rabbit anti-H3K27me3 antibody (Abcam-mAbcam6002) by employing Imprint® Chromatin Immunoprecipitation Kit (Sigma-CHP1) according to the manufacturer's instructions. Conventional and real-time PCR techniques were used to analyze ChIP DNA. For RT-PCR (Realplex4 Eppendorf) analysis, Maxima SYBR Green/ROX qPCR Master Mix (2X, Thermo Scientific-K0221) was used. 2µL of sonicated DNA was used for PCR analysis with the following PCR conditions ; 95° C for 2 min, followed by 40 cycles of 95° C for 20s alternating with 55° C for 30s annealing and 72° C extension for 30s. Anti-mouse IgG precipitated DNA was used as template for negative control. Nonspecific antibody (mouse IgG) precipitated DNA was used for normalization. Default input fraction is 1% which is a dilution factor of 100 or 6.644 cycles (i.e. log2 of 100). % Input is calculated as $2^{(-\Delta Ct\ [\text{normalized ChIP}])}$ where ΔCt [normalized ChIP] = (Ct [ChIP] - (Ct [Input] - Log2 (Input Dilution Factor))). Fold of enrichment is calculated as $2^{(-\Delta\Delta Ct\ ChIP)}$.

5.3 Results:

5.3.1 Epigenetic modulators affect cell viability and hinder growth in breast and prostate cancer cell lines in a dose-dependent manner:

The effect of three different epigenetic modulators (AZA, TSA and DZNeP) on cell viability after treatment for 24h (TSA) and 72h (AZA, DZNeP) was assessed by colorimetric MTT assay in both breast and prostate cancer cell lines. It is observed that cell viability of treated cells gradually decreased with increase in concentration of modulators in comparison to untreated control cells indicating dose-dependent behaviour (Fig. 4.1). The IC_{30} concentration (concentration of drug at which 70% of cells remain

viable) of each drug were almost similar; IC_{30} value of AZA for MCF7, MDA-MB-231, DU145, PC3 was 10 µM, IC_{30} value of TSA was found to be 150 nM for MCF7 and MDA-MB-231 and 100 nM for DU145 and whereas IC_{30} value of DZNeP was observed to be 10 µM for all the four cell lines (Fig. 4.1). The IC_{30} value was considered as the effective drug concentration for further experiments. The inhibitory effect of epigenetic modulators on cancer cell growth is suggestive of the fact that epigenetic changes induced by treatment with AZA, TSA and DZNeP affect growth and proliferation by regulating Oct4, Sox2 and Nanog expression.



[d]

	IC_{30} Concentration		
	AZA (µM)	TSA (nM)	DZNeP (µM)
MCF7	10	150	10
MDA-MB-231	10	150	10
DU145	10	150	10
PC3	10	150	10

Figure 5.1: Cell viability assay to determine IC_{30} concentration after treatment with epigenetic modulators. [a] AZA, [b] TSA and [c] DZNeP treatment in breast (MCF7 and MDA-MB-231) and prostate (DU145 and PC3) cell lines, [d)] IC_{30} value corresponding to different cell lines for different drugs (n=3, mean±S.D; $P < 0.05$).

5.3.2 Expression profiling of Oct4, Sox2 and Nanog gene expression after treatment with epigenetic drugs AZA, TSA and DZNeP:

Expression of Oct4 after treatment with AZA, TSA and DZNeP:

All the four cell lines were treated with AZA, TSA and DZNeP to observe the changes in Oct4 expression level and attempts were made to correlate Oct4 expression with effect of epigenetic modulators. After AZA treatment, Oct4 mRNA expression increased by 1.67 folds and 3.85 folds, in MCF7 and MDA-MB-231 cells (Fig. 5.2) and by 2.36 and 2.57 folds in DU145 and PC3 respectively (Fig. 5.3). Increased Oct4 expression was observed after TSA treatment individually in all four cell lines. Folds of increase in Oct4 mRNA level were 4.03 in MCF7 and 11.3 in MDA-MB-231 (Fig. 5.2), 12.2 folds in DU145 and 24.35 folds in PC3 respectively (Fig. 5.3). After DZNeP treatment, Oct4 expression increased by1.39 folds in MCF7 and 2.54 folds in MDA-MB-231 (Fig. 5.2), 3.21 folds in DU145 and 3.45 folds in PC3 respectively (Fig. 5.3).

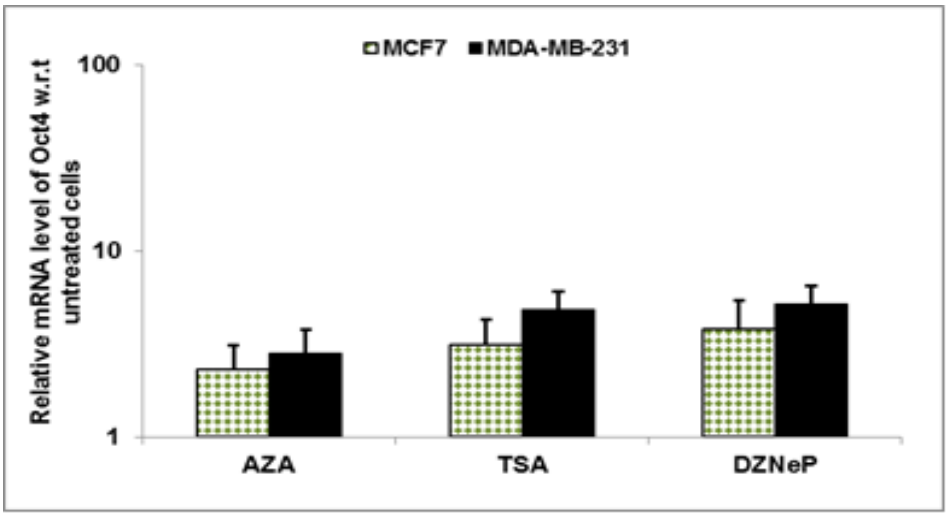


Figure 5.2: RT-PCR analysis of Oct4 expression in MCF7 and MDA-MB-231. AZA, TSA and DZNeP treatment increased Oct4 mRNA level in both cell lines. Oct4 mRNA level was measured by reverse transcription–PCR. The Oct4 level in DMSO treated cells for AZA, TSA and DZNeP treated cells was normalized to 1 (n=3, mean±S.D., $P < 0.05$).

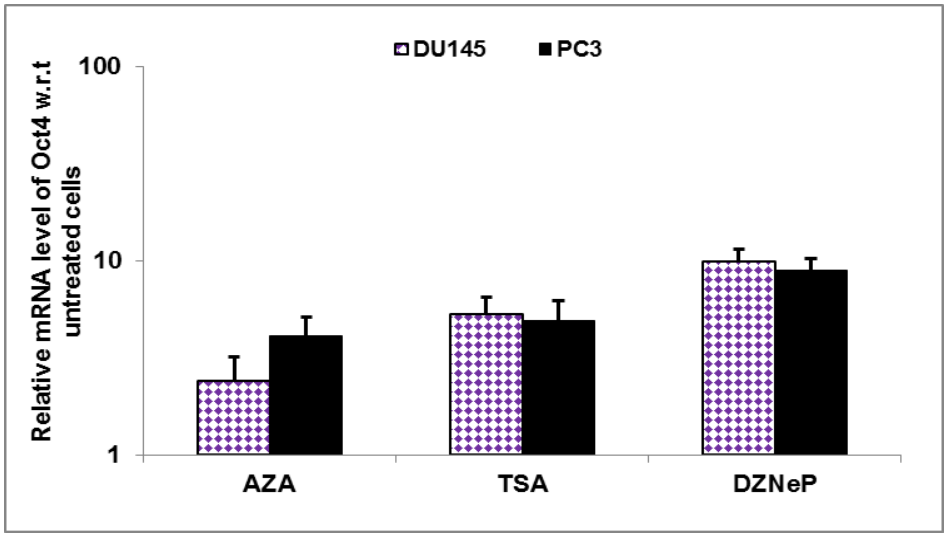


Figure 5.3: RT-PCR analysis of Oct4 expression in DU145 and PC3. AZA, TSA and DZNeP treatment increased Oct4 mRNA level in both cell lines. Oct4 mRNA level was measured by reverse transcription–PCR. The Oct4 level in DMSO treated cells for AZA, TSA and DZNeP treated cells was normalized to 1 (n=3, mean±S.D., $P < 0.05$).

Expression of Sox2 after treatment with AZA, TSA and DZNeP:

After AZA treatment, Sox2 mRNA level increased by 2.34 folds in MCF7, 3.5 folds in MDA-MB-231 (Fig. 5.4), 6.7 folds in DU145 and 5.78 folds in PC3 (Fig. 5.5). In case of treatment with TSA, there was 4.8 folds increase for MCF7 and 3.9 folds increase in case of MDA-MB-231 (Fig. 5.4), 5.4 folds for DU145 and 4.65 folds for PC3 (Fig. 5.5). After DZNeP treatment, the increase in Sox2 expression for MCF7, MDA-MB-231, DU145 and PC3 are 7.8, 6.9, 6.5 and 5.8 folds respectively (Fig. 5.4, 5.5).

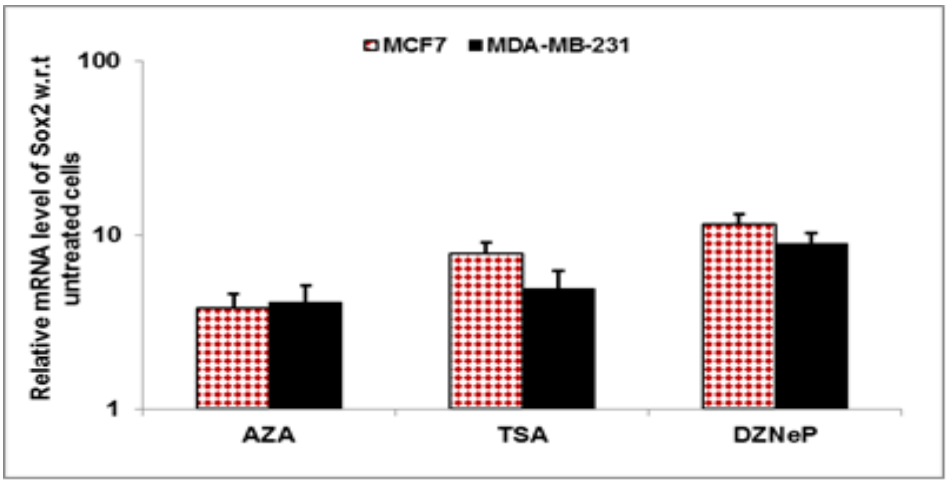


Figure 5.4: RT-PCR analysis of Sox2 expression in MCF7 and MDA-MB-231. AZA, TSA and DZNeP treatment increased Sox2 mRNA level in both cell lines. Sox2 mRNA level was measured by reverse transcription–PCR. The Sox2 level in DMSO treated cells for AZA, TSA and DZNeP treated cells was normalized to 1 (n=3, mean±S.D., $P < 0.05$).

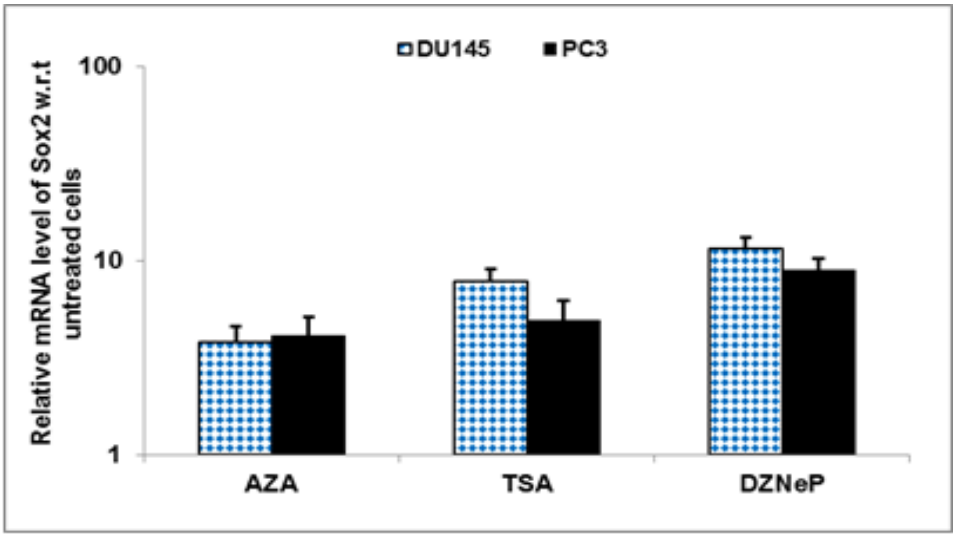


Figure 5.5: RT-PCR analysis of Sox2 expression in DU145 and PC3. AZA, TSA and DZNeP treatment increased Sox2 mRNA level in both cell lines. Sox2 mRNA level was measured by reverse transcription–PCR. The Sox2 level in DMSO treated cells for AZA, TSA and DZNeP treated cells was normalized to 1 (n=3, mean±S.D., $P < 0.05$).

Expression of Nanog after treatment with AZA, TSA and DZNeP:

Similar to Oct4 and Sox2, there is increase in expression of Nanog but the increase is not as significant as in case of Oct4 and Sox2 for all the epigenetic modulators (Fig. 5.6, 5.7). The effect of all the above drugs on the transcript level expression shows that epigenetic regulation works at transcriptional level of gene regulation.

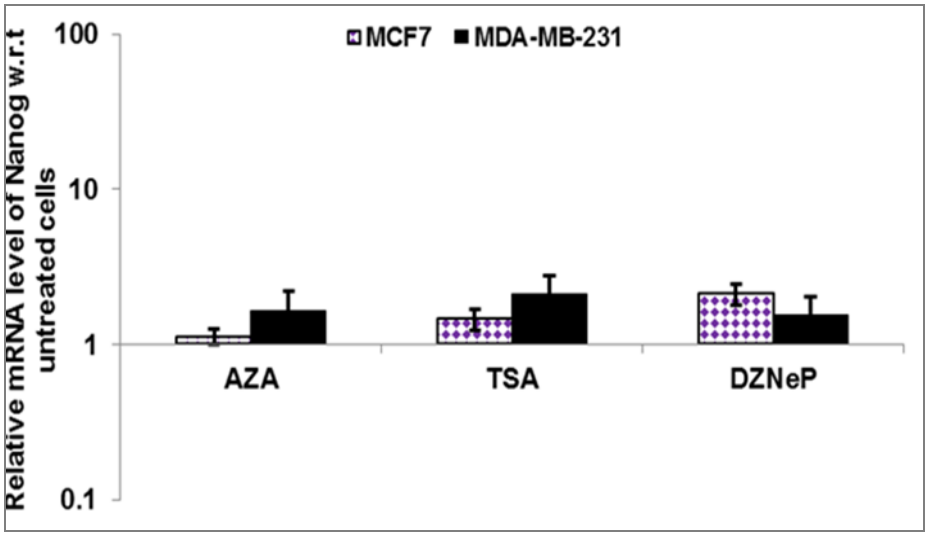


Figure 5.6: RT-PCR analysis of Nanog expression in MCF7 and MDA-MB-231. AZA, TSA and DZNeP treatment increased Sox2 mRNA level in both cell lines. Sox2 mRNA level was measured by reverse transcription–PCR. The Sox2 level in DMSO treated cells for AZA, TSA and DZNeP treated cells was normalized to 1 (n=3, mean±S.D., $P < 0.05$).

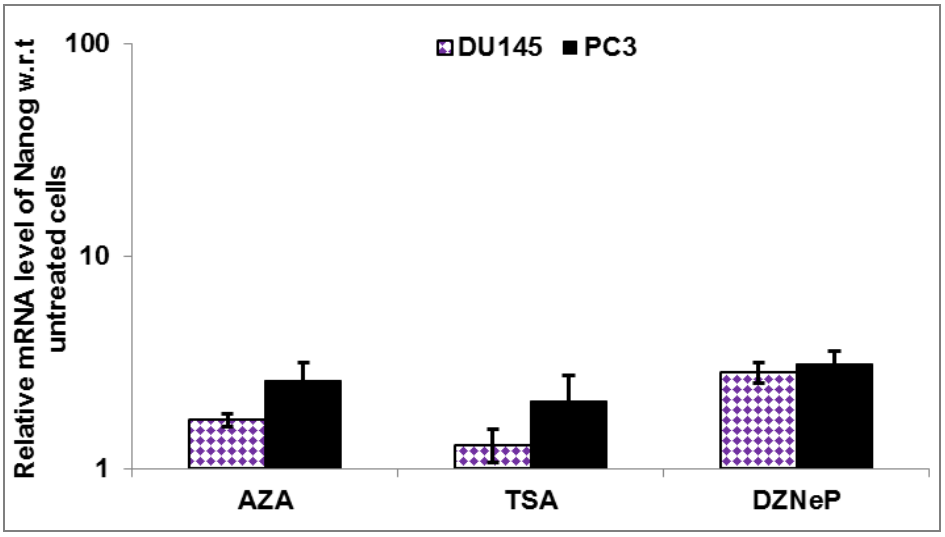


Figure 5.7: RT-PCR analysis of Nanog expression in DU145 and PC3. AZA, TSA and DZNeP treatment increased Nanog mRNA level in both cell lines. Nanog mRNA level was measured by reverse transcription–PCR. The Nanog level in DMSO treated cells for AZA, TSA and DZNeP treated cells was normalized to 1 (n=3, mean±S.D., $P < 0.05$).

5.3.3 Expression profiling of Oct4 and Sox2 protein expression after treatment with epigenetic modulators AZA, TSA and DZNeP:

Similar to mRNA expression, protein expression of Oct4 and Sox2 was found to be highest in breast and prostate cancer respectively. In breast cancer cell lines MCF7 and MDA-MB-231, Oct4 expression was analyzed as it showed the highest expression at the transcript level. In MCF7 cells, Oct4 expression was highest after DZNeP tretament, but in MDA-MB-231 cells, Oct4 expression was highest after TSA treatment (Fig. 5.8a). In case of prostate cancer cell lines, Oct4 expression was highest after TSA treatment in both DU145 and PC3 cell lines (Fig. 5.8b). The expression at protein level corraborated with mRNA level indicating that histone modifications play an important role in regulating the expression of pluripotency inducing transcription factors.

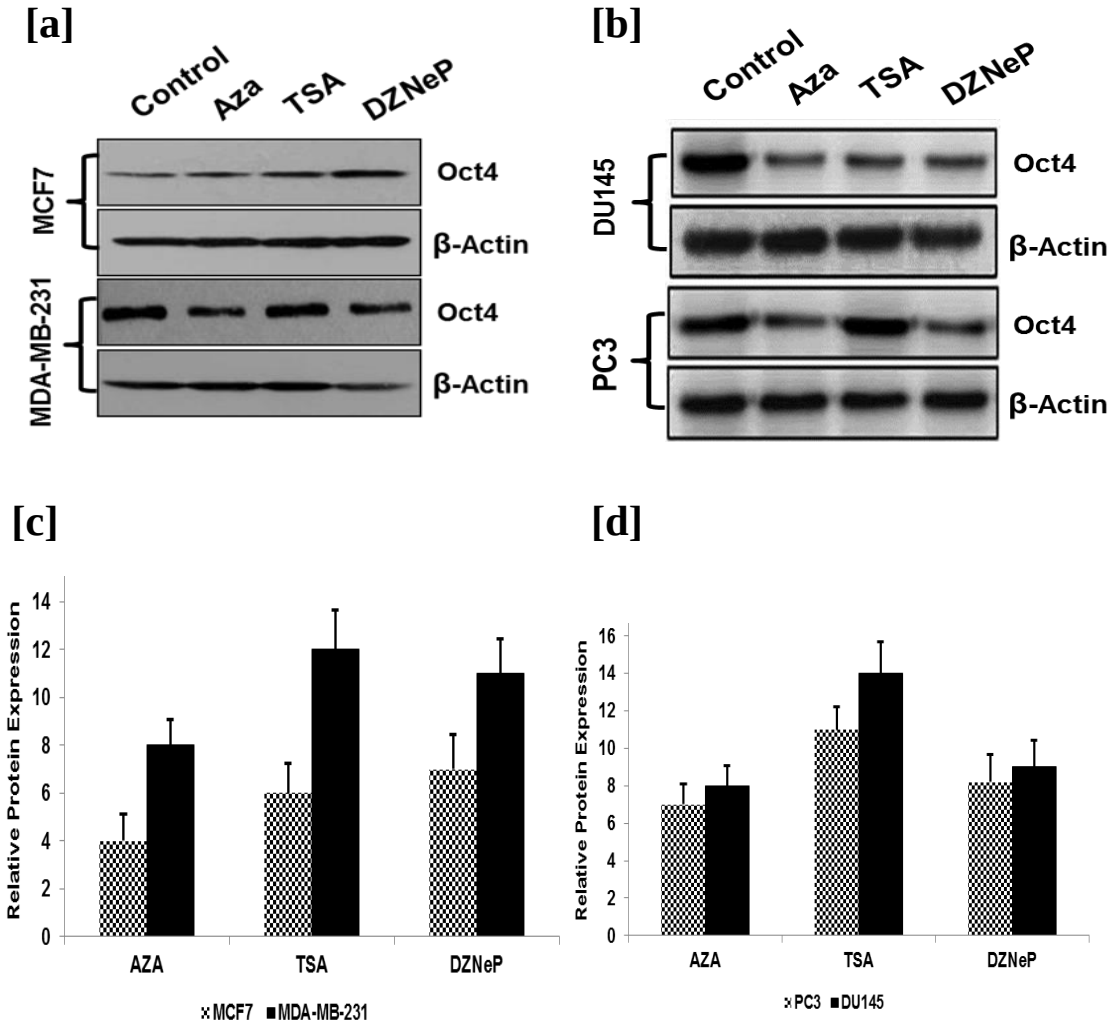


Figure 5.8: Western blot analysis of Oct4 expression in breast and prostate cancer cell lines. **P**rotein expression and quantification was measured by western blot in [a] breast (MCF7 and MDA-MB-231) and [b] prostate (DU145 and PC3) cell lines with antibody specific for Oct4 after treatment with epigenetic modulators. Graphical representation of relative expression level of Oct4 protein in [c] breast and [d] prostate cancer cell lines after treatment with epigenetic modulators.

Similarly, Sox2 was found to be expressed higher than Oct4 and Nanog; hence, Sox2 protein expression was analyzed after treatment with AZA, TSA and DZNeP. There is increase in protein expression of Sox2 in each of the four cell lines (Fig. 5.9a, 5.9b). However, DZNeP treated cells showed highest expression (Fig. 5.9c, 5.9d). The findings are suggestive of the fact that epigenetic inhibitors can affect the corresponding epigenetic enzyme and therefore affect its regulatory function over pluripotency inducing transcription factors and other important genes which might have role to play in cancer.

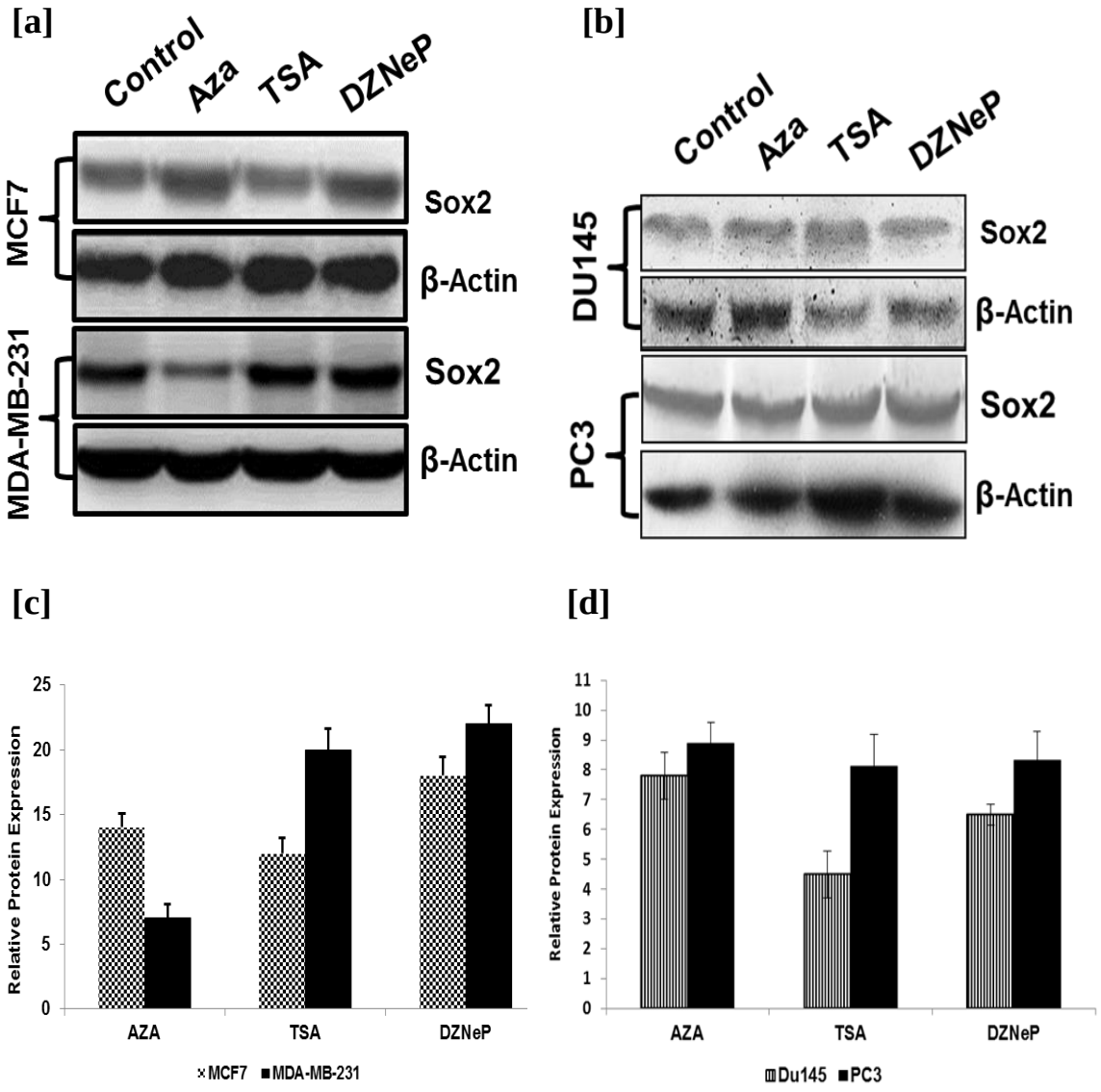


Figure 5.9: Western blot analysis of Sox2 expression in breast and prostate cancer cell lines. **P**rotein expression and quantification was measured by western blot in (a) breast (MCF7 and MDA-MB-231) and (b) prostate (DU145 and PC3) cell lines with antibody specific for Sox2 after treatment with epigenetic modulators. Graphical representation of relative expression level of Sox2 protein in (c) breast and (d) prostate cancer cell lines after treatment with epigenetic modulators.

5.3.4 Analysis of promoter DNA methylation of Oct4 and Sox2 in breast and prostate cancer cell lines:

In order to understand the effect of promoter DNA methylation of Oct4 and Sox2 expression, *in silico* analysis of methylation in Oct4 and Sox2 promoters was conducted. Promoter regions of Oct4 and Sox2 were retrieved using http://www.genomatix.de software followed by analysis in the UCSC genome browser (Fig. 5.10a, 5.11a).

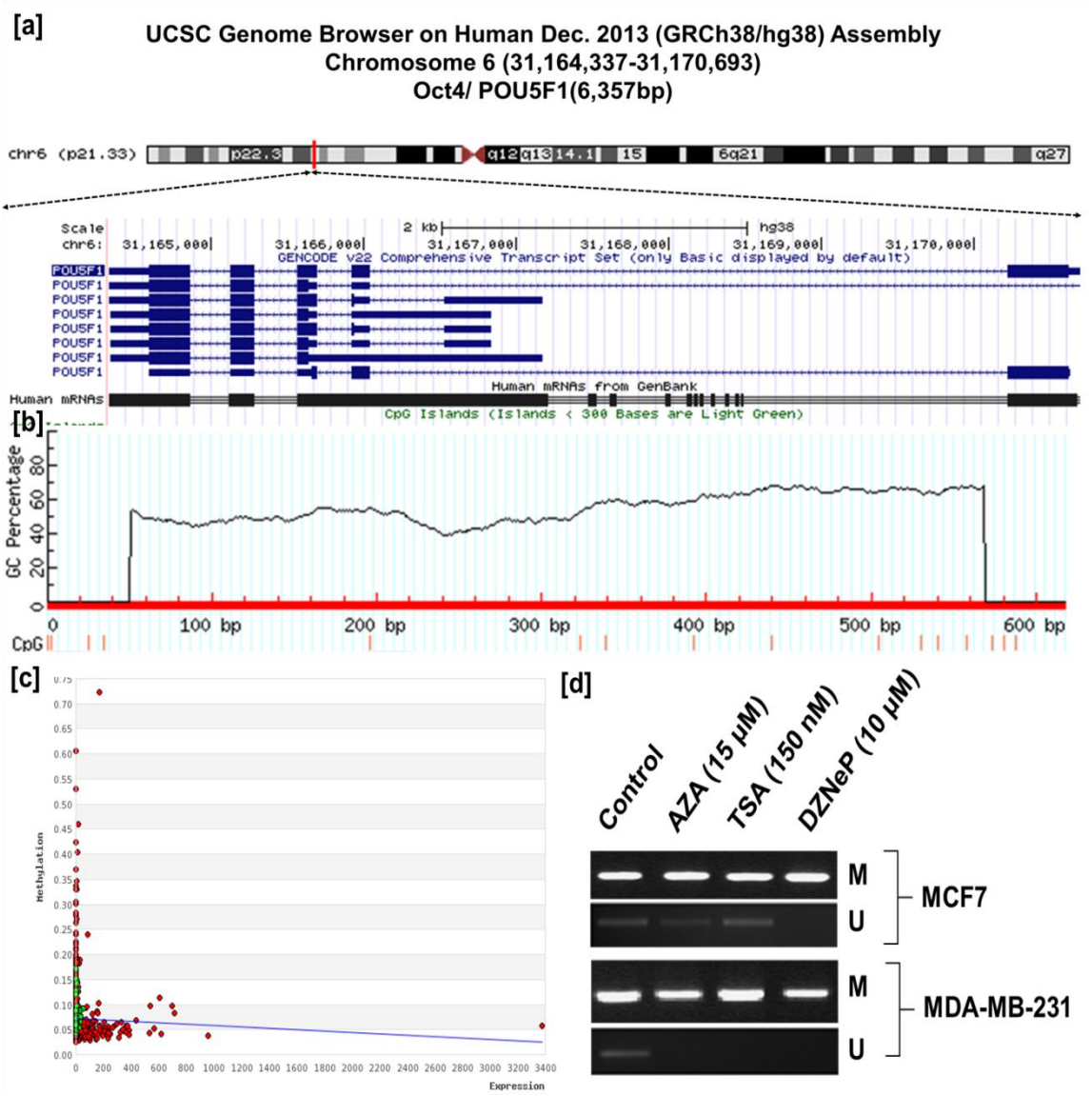


Figure 5.10: Promoter DNA methylation analysis of Oct4 promoter in breast cancer cell lines MCF7 and MDA-MB-231 [a] UCSC genome browser view of Oct4 chromosomal location, [b] The CpG island promoter distribution of Oct4, [c] Analysis of co-relation between promoter DNA methylation and mRNA expression of Oct4 in breast cancer, [d] Methylation-specific PCR of Oct4 promoter in MCF7 and MDA-MB-231. U, results with primers specific for unmethylated sequence. M, results with primers specific for methylated sequence.

It was observed that while Oct4 promoter has no CpG islands (Fig. 5.10b), Sox2 promoter contains CpG island composed of around 270 bps (Chr3: 181,711,970-181,713,540; 1571 bp) with 70% CG percentage (Fig. 5.11b). In order to analyze the influence of promoter methylation on expression of Oct4 and Sox2, relative co-relation between these two parameters was analyzed by methHC software. In both cases, promoter methylation was negatively co-related with mRNA expression indicating that over-expression of Oct4 and Sox2 in breast and prostate cancer is independent of promoter

DNA methylation (Fig. 5.10c, 5.11c). In order to validate the above findings experimentally, methyl specific PCR was done to establish the methylation pattern in Sox2 promoter region in prostate cancer. No significant changes in methylation pattern was detected after treatment with epigenetic inhibitors indicating that promoter DNA methylation doesn't affect the over-expression of Oct4 and Sox2 in breast and prostate cancer respectively (Fig. 5.10d, 5.11d).

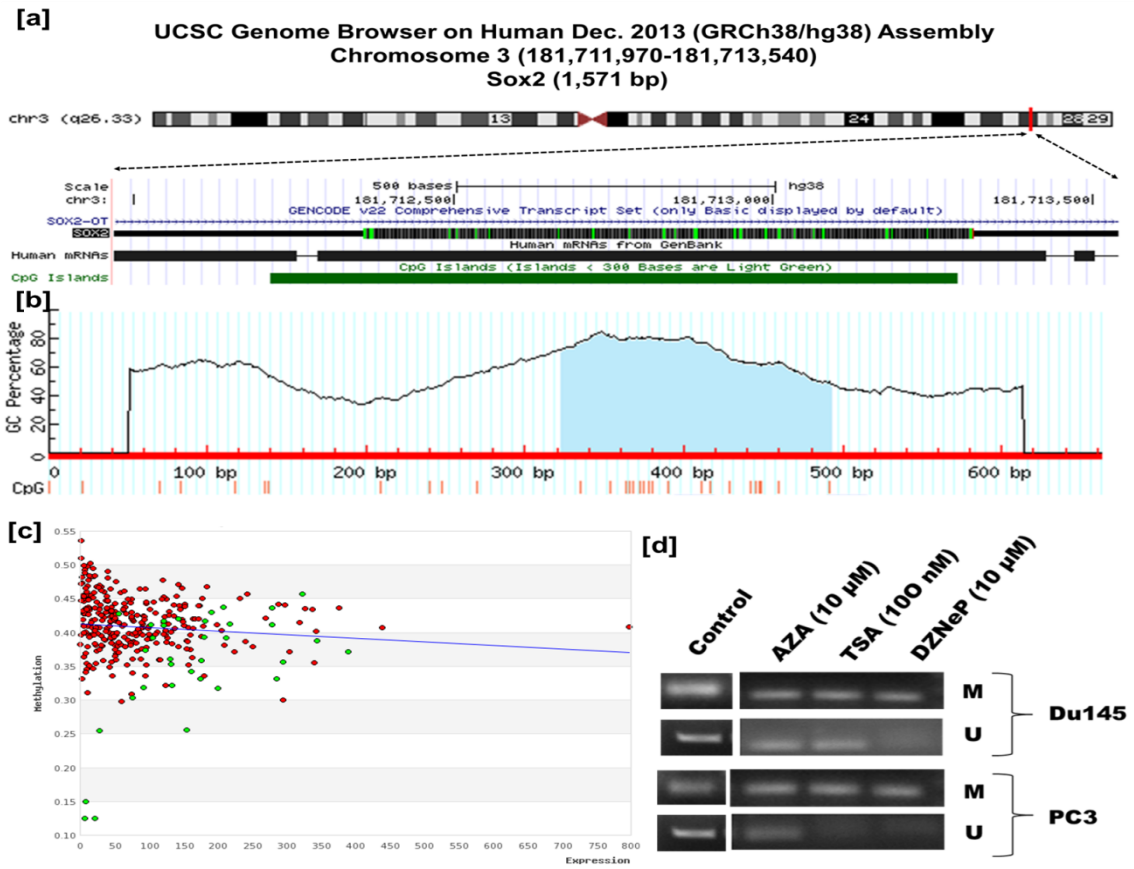


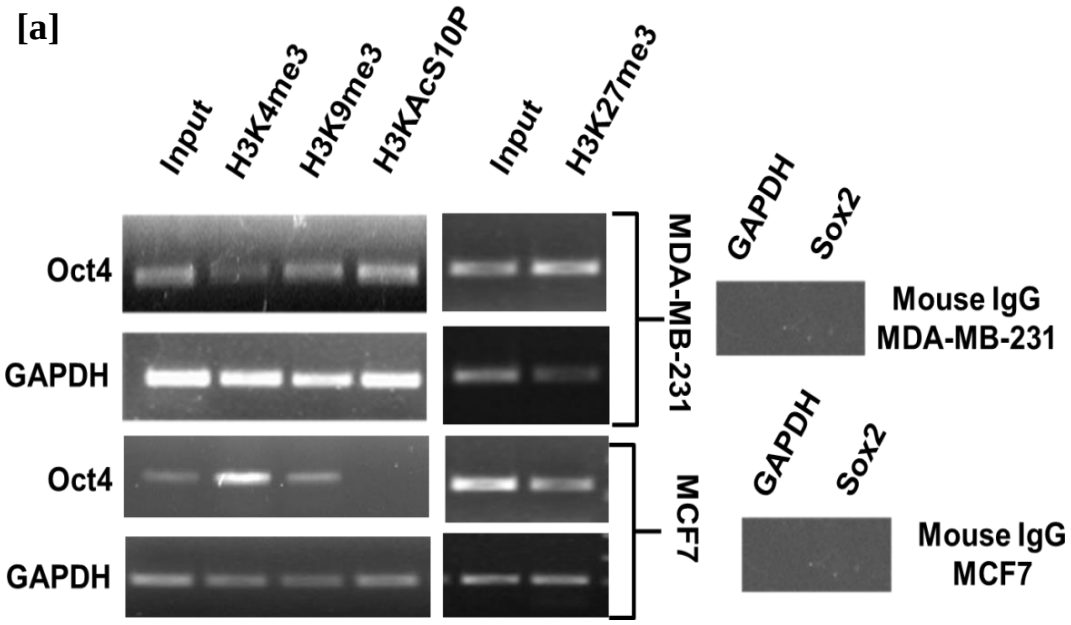
Figure 5.11: Promoter DNA methylation analysis of Sox2 promoter in prostate cancer cell lines DU145 and PC3. [a] UCSC genome browser view of Sox2 chromosomal location. [b] The CpG island promoter distribution of Sox2. [c] Analysis of co-relation between promoter DNA methylation and mRNA expression of Sox2 in prostate cancer. [d] Methylation-specific PCR in DU145 and PC3. U, results with primers specific for unmethylated sequence. M, results with primers specific for methylated sequence.

5.3.5 Analysis of gene specific histone modifications of Oct4 and Sox2 promoters in breast and prostate cancer cell lines:

Detection of H3K4me3, H3K9me3, H3K27me3, H3K9AcS10p level in Oct4 promoter in breast cancer cell lines:

After methylation analysis, it has been observed that DNA methylation has no significant effect on Oct4 expression in breast cancer cell lines. So epigenetic marks other than

promoter DNA methylation must be responsible for regulating Oct4 expression. To investigate whether histone modifications are involved in the regulation of Oct4 expression, PCR analysis was performed in breast cancer cell lines MCF7 and MDA-MB-231 to identify the enrichment of H3K4me3 and H3K9acS10p (active histone marks) as well as H3K9me3 and H3K27me3 (repressive histone marks) in the Oct4 promoter region. The region amplified by ChIP-primers was similar to the amplified region which was analyzed by bisulphite sequencing. The presence of H3K4me3, H3K9me3, H3K27me3 and H3K9acS10p in the CGI promoter region of Oct4 is evident from PCR analysis (Fig. 5.12a). This was followed by RT-PCR analysis which revealed that promoter region of Oct4 gene has high levels of H3K4me3, H3K9acS10p and low levels of H3K9me3 and H3K27me3. In MCF7, H3K4me3, H3K9acS10p, H3K9me3, H3K27me3 occupancy was 1.67, 0.59, 0.36 and 0.18 and in MDA-MB-231, occupancy was 5.23, 1.93, 0.67 and 0.34 respectively. Fold of H3K4me3 and H3K9acS10p enrichment were higher in MDA-MB-231 than in MCF7 (Fig. 5.12b).



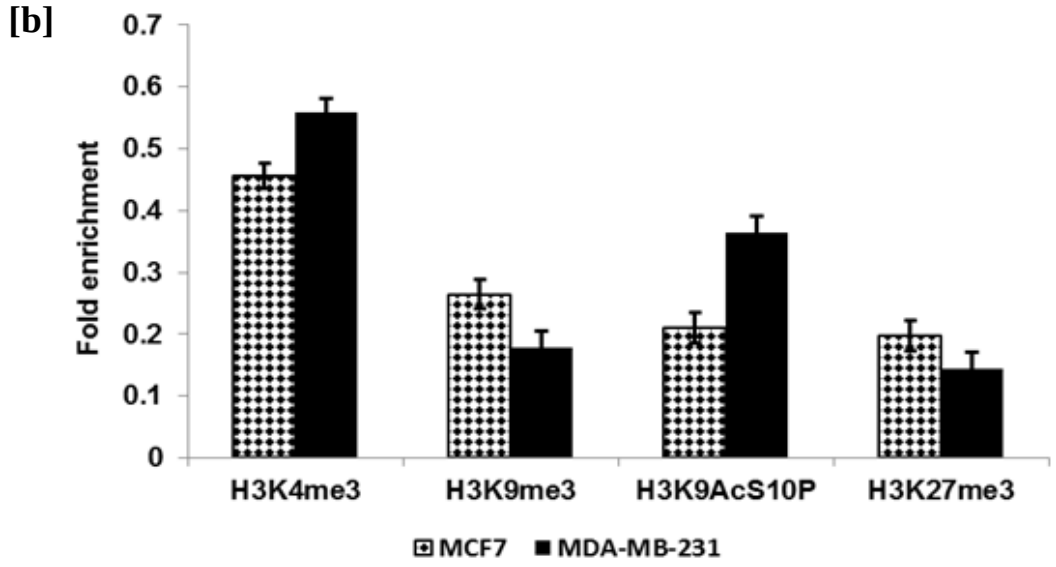


Figure 5.12: H3K4me3, H3K9me3, H3K27me3 and H3K9acS10p occupancy in Oct4 promoter in breast cancer cell lines MCF7 and MDA-MB-231. Histone post-translational modifications in CGI promoter region of Oct4 [a] PCR analysis Oct4 promoter H3K4me3 enrichment and mouse IgG precipitated DNA used as negative control, [b] Graphical representation of RT-PCR analysis of H3K4me3, H3K27me3, H3K9acS10p and H3K9me3 occupancy in Oct4 promoter in MCF7 and MDA-MB-231. The Oct4 level in input was normalized to 1 (n=3, mean± S.D., $P < 0.05$).

Detection of H3K4me3, H3K9me3, H3K27me3, H3K9acS10p level in Sox2 promoter in prostate cancer cell lines:

Since there was no significant change in promoter DNA methylation for regulation of Sox2 gene expression, it was assumed that histone modulations might be involved. Accordingly, ChIP was performed for visualizing histone tail modifications. ChIP of Sox2 gene promoter was executed in PC3 and DU145 to identify the abundance of H3K4me3, H3K9me3, H3K27me3 and H3K9acS10p in the Sox2 promoter. From PCR analysis, presence of higher amount of H3K4me3 in the promoter region of Sox2 was evident (Fig. 5.13a). This was followed by RT-PCR analysis of ChIP-precipitated DNA which revealed that promoter of Sox2 gene has high level of H3K4me3 enrichment in both the cell lines. Relative presence of H3K4me3 was 1.67 and 1.92 folds in DU145 and PC3, respectively. But level of H3K27me3, H3K9me3 and H3K9acS10p occupancy was 0.60, 0.61, 0.89 folds in DU145 and 0.43, 0.64, 0.45 folds in PC3 cells, very much lower than H3K4me3 in both cell lines. The apparent levels of H3K4me3 and H3K9acS10p enrichment in the Sox2 promoter region of untreated cells indicates that perhaps H3K4me3 enrichment is the cause of relatively very high Sox2 expression in prostate cancer cell lines.

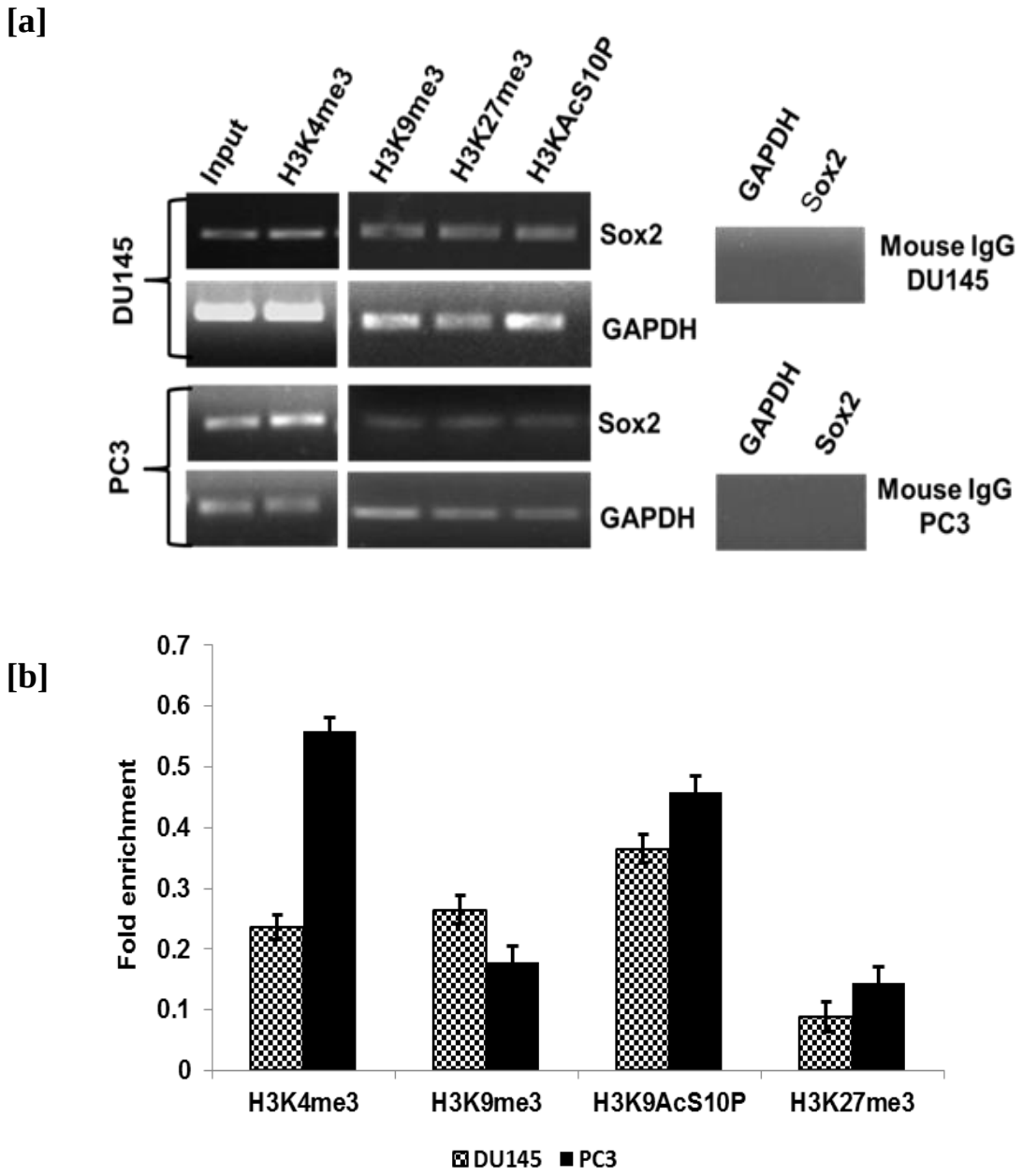


Figure 5.13: H3K4me3, H3K9me3, H3K27me3 and H3K9acS10p occupancy in Sox2 promoter in prostate cancer cell lines DU145 and PC3. Histone post-translational modifications in promoter region of Sox2 [a] qPCR analysis Sox2 promoter H3K4me3 enrichment and mouse IgG precipitated DNA used as negative control. [b] Graphical representation of RT-PCR analysis of H3K4me3, H3K27me3, H3K9acS10p and H3K9me3 occupancy in Sox2 promoter in DU145 and PC3 cells. The Sox2 level in input was normalized to 1 (n=3, mean± S.D, $P < 0.05$).

5.4 Discussion:

In this study, we attempted to investigate the epigenetic regulatory mechanisms controlling Oct4, Sox2 and Nanog expression in breast and prostate cancer. Our investigations on post-operated and FFPE breast and prostate cancer tissues had confirmed over-expression of Oct4, Sox2 and Nanog in metastasis tissue samples in comparison to that in primary cancer tissues. The findings further indicated that enhanced Oct4, Sox2 and Nanog expression during metastasis stage facilitates cancer progression by encouraging pro-tumorigenic attributes and enhancing the inherent tumorigenicity in breast and prostate cancer. Therefore, we attempted to decipher the regulatory mechanisms behind over-expression of these TFs in breast and prostate cancer. Ectopic modulation by epigenetic inhibitors such as AZA, TSA and DZNeP were conducted so as to assess the impact of epigenetic enzymes on the gene and protein level expression of Oct4, Sox2 and Nanog.

After treatment with AZA, TSA and DZNeP, cell viability was seen to decrease in a dose-dependent manner implying that the epigenetic inhibitors affect the cell growth and survival by affecting in turn the epigenetic enzymes (Fig. 5.1). Next, the transcript level expression of Oct4, Sox2 and Nanog was analyzed after treatment which showed that the expression level of Oct4 and Sox2 were differentially up-regulated in the respective breast and prostate cancer cell lines. A sharp rise in expression at mRNA level of Oct4 and Sox2 gene after treatment with AZA, TSA and DZNeP with respect to untreated samples clearly depicts that Oct4 and Sox2 are directly controlled by epigenetic modifications with histone modifications playing a more significant impact on expression level (Fig. 5.2, 5.3, 5.4, 5.5, 5.6, 5.7). To validate whether, gene expression is exemplified at the protein level, Western Blotting analysis and immunofluorescence was performed. A concordant elevation in protein expression of Oct4 and Sox2 was observed in both breast and prostate cancer cell lines corresponding to their transcript level expression (Fig. 5. 8, 5.9).

The differential pattern of over-expression of Oct4 and Sox2 after treatment with AZA, TSA and DZNeP made us ponder regarding the effector modification controlling their expression. Therefore, analysis of promoter DNA methylation in Oct4 and Sox2 was conducted by means of *in silico* and *in vitro* investigations. The promoter regions of Oct4 and Sox2 were obtained using Genomatix software and analyzed by UCSC genome

browser. The Oct4 chromosomal location and promoter size was determined to be Chr 6: 31,164,337-31,170,693; 6357 bp). However, analysis of promoter sequence revealed no significant CpG island in the sequence (Fig. 5.10). Further, negative co-relation between DNA methylation and mRNA expression of Oct4 was observed in methHC analysis. MS-PCR also revealed that promoter DNA methylation is not affecting the mRNA expression of Oct4. Similar analysis was performed for Sox2 and it was found that Sox2 promoter contained CpG island made up around 270 bps (Chr3: 181,711,970-181,713,540; 1571 bp) with high (70%) CG percentage (Fig. 5.11). However, promoter DNA methylation was again found to be negatively co-related with mRNA expression of Sox2, implying that in case of both Oct4 and Sox2, promoter DNA methylation is ineffective and doesn't influence Oct4 and Sox2 over-expression in cancer.

Finally, histone modifications present in the promoters of Oct4 and Sox2 were analyzed by chromatin immunoprecipitation (ChIP) assay. In case of Oct4 promoter, enrichment of H3K4me3, H3K9acS10p enrichment and low level of H3K9me3 and H3K27me3 was observed in breast cancer cell lines. Fold of H3K4me3 and H3K9acS10p enrichment were higher in MDA-MB-231 than in MCF7 implying that over-expression of Oct4 in metastatic advanced stage breast cancer is facilitated by active histone marks (Fig. 5.12). Similar results were also obtained in case of Sox2 promoter analysis in prostate cancer with high levels of H3K4me3 and H3K9acS10p alongside low levels of H3K27me3 and H3K9me3 occupancy in both the cell lines (Fig. 5.13). Thus, it is confirmed that over-expression of Sox2 and Oct4 in breast and prostate cancer is mostly regulated by active histone methylation and acetylation marks such as H3K4me3 and H3K9acS10p in absence of promoter DNA methylation.

Chapter 6

To explore the connection between oncogenic Hedgehog signaling pathway and pluripotency inducing TFs in breast and prostate cancer

6.1 Introduction:

Phenotypic and functional identity of cells during the course of development is an integrated function of extrinsic signaling cues translated by the intrinsic pluripotency inducing transcription factors into diverse cellular features and physiological behavior. In contrast, unrestrained signaling circuits disrupt the homeostatic balance between cell growth and cell death resulting in physiological disorders, degenerative diseases and in many instances, leading to the development of aggressive and metastatic cancers (Patra et al., 2011). Pluripotency inducing factors in concert with developmental signaling pathways are now envisioned as the common instructive cues influencing ESCs identity as well as facilitating CSC phenotype. In case of ESCs, they function as differentiation repressors and guide ESCs into their respective cell fates, whereas in case of CSCs, aberrant expression of transcriptional regulators in concert with constitutive activation of oncogenic signaling pathways encourages malignant transformation (Ben-Porath et al., 2008; Patra et al., 2008; Liu et al., 2013). Henceforth, understanding the delicate functional relationship between pluripotency inducing TFs and cellular signaling pathways is of paramount importance in order to clearly elaborate the molecular mechanisms behind carcinogenesis. In this chapter, the co-operative interaction between developmentally inclined HH signaling and core pluripotency inducing TF, Sox2 in prostate cancer is thoroughly investigated in order to throw light on the nexus between these two developmental manipulators.

Sox2 is a high-mobility group DNA-binding transcription factor which contributes critically towards maintenance of clonogenicity, pluripotency and self-renewal properties of undifferentiated ESCs, regulating cellular identity of adult tissue progenitor cells during developmental transition as well as in adult tissue homeostasis and regeneration

(Liu et al., 2013; Sarkar and Hochedlinger, 2013; Kamachi and Kondoh, 2013). Sox2 is also one of key transcription factors whose ectopic introduction can revert back differentiated somatic cells into ESC like condition known as iPSCs (Takahashi and Yamanaka, 2006). In recent years, it has been increasingly acknowledged that Sox2 is amplified and functionally relevant in various cancer types including glioblastoma, osteosarcoma, lung, esophagus, oral, breast, ovarian, pancreatic and gastric where it promotes oncogenic phenotype via cellular proliferation, contributes towards cancer maintenance by evading apoptotic signals and encourages acquisition of more aggressive tumor properties such as metastasis and lymph node invasion (Chou et al., 2013; Dai et al., 2014; Hutz et al., 2014; Santini et al., 2014). Not surprisingly, Sox2 is also vindicated for causing tumorigenic changes in the prostate; higher expression of Sox2 is observed in prostate cancer tissues with advanced histologic grade in comparison to normal prostate or benign prostate hyperplasias ascertaining the claim that Sox2 encourages more "stem-cell like" tumor phenotype that increases the chemo-resistant property of prostate cancer cells resulting in tumor recurrence. Studies have shown that Sox2 is associated with increased cancer aggressiveness by promoting EMT and stimulating castration-resistance by decreasing the dependence of prostate cancer cells on androgen receptor (AR) signaling for their growth and survival (Lin et al., 2012; Kregel et al., 2013; Russo et al., 2016).

The HH signaling pathway is a central coordinator in embryonic developmental scheme participating in cell proliferation, cell fate determination, patterning and differentiation. It plays a crucial role during organogenesis in many adult tissues by facilitating epithelial-to-mesenchymal transitions, tissue repair and regeneration as well as stem cell maintenance (Barakat et al., 2010; Kar et al., 2012). Given the strategic importance of this HH signaling as a developmental morphogen, it is imperative that inactivation of the pathway results in developmental defects such as holoprosencephaly, spinal cord anomalies and hyper-activation of this pathway is responsible for neoplastic transformation. HH signaling pathway can mediate initiation, maintenance and progression of many different types of cancers through constitutive activation of the pathway via up-regulation of HH ligands and pathway components or by genetic and epigenetic modifications in the pathway (Teglund and Toftgard, 2010; Yang et al., 2010). Deregulation in the pathway promotes tumorigenic potential such as increased metastatic behavior, improved survival capability via up-regulation of anti-apoptotic agents, enhanced proliferative capacity through modulation of the cell cycle machinery and

elevation of tumor invasiveness (Clement et al., 2007; Kasper et al., 2009). With regards to prostate cancer, it is widely known that HH signaling is actively present in the urogenital epithelium from where the prostate derives, mainly promoting ductal budding and extension. HH signaling is relatively quiescent in adult prostate, but is still present and important for regeneration, maintenance and repair of prostate epithelium. Consequently, deregulated HH signaling is implicated in development and progression of prostate cancer to more aggressive and even therapy-resistant state in an autocrine fashion (Datta and Datta, 2006; Chen et al., 2011 (a); Chen et al., 2011 (b); Gonnissen et al., 2013).

A growing body of evidence shows that Sox2 is associated with oncogenic signaling pathways controlling tumor cell physiology by affecting cell fate determination, proliferation, and apoptosis in cancer cells (Weina and Utikal, 2014). It has been already reported that Sox2 endows proliferation and survival advantages on adult lung stem/progenitor cells by enhancing EGFR pathway mediated oncogenic phenotypes (Chou et al., 2013). Similarly, it was also established that Sox2 expression is functionally responsible for sustaining the proliferation of melanoma cells and inducing self-renewal of melanoma initiating cells (MIC) by supporting HH signaling (Santini et al., 2014). Thus, targeting Sox2 and its associated oncogenic signaling partner i.e HH pathway is a novel approach for inhibiting neoplastic growth and turning of the tumor "switch" in prostate cancer. In this note, the present study has been carried out to elucidate in details the connection between oncogenic pluripotency inducing transcription factor Sox2 and HH signaling pathway in androgen independent prostate cancer. We have thoroughly investigated the role of Sox2 and its interaction with HH signaling in prostate cancer cell proliferation and tumorigenicity in two prostate cancer cell lines DU145 and PC3 as well as in human immortalized cell line HaCaT considered as the normal phenotypic counterpart to prostate cancer cell lines. We have shown that silencing of Sox2 leads to a remarkable growth suppression and apoptosis in prostate cancer cells. Similarly, HH signaling pathway is also shown to contribute significantly towards prostate cancer progression. Most importantly, simultaneous Sox2 abrogation and HH impairing greatly reduces the ability of prostate cancer cells to proliferate, migrate and progress towards a more aggressive state, thus providing a novel therapeutic approach for treatment of chemotherapy resistant aggressive prostate cancer.

6.2 Materials and Methods:

6.2.1 *In silico* analysis of Sox2 expression from publicly available databases:

Pre-experimental *in silico* analyses of expression of Sox2 and HH pathway components was done by employing publicly available online bioinformatics tools cBioPortal having links to TGCA database (Cerami et al., 2012; Gao et al., 2013). From patient samples available in this database, cross-cancer histogram for copy number alteration (CNA), oncoprint analysis (indicating amplification, deletion and mutations) and genomic profiles (indicating mRNA up-regulation and down-regulation) of Sox2, SHH, SMO, PTCH and GLI1 were examined for all cancer types and separately for prostate cancer. Further, mutually exclusive relationship between Sox2 and HH pathway components in prostate cancer was also computed. The survival analysis plot individually for Sox2 and together for HH components in prostate cancer was also plotted.

6.2.2 Cell culture:

Human prostate cancer cell lines PC3 and DU145 and immortalized human keratinocytes HaCaT were obtained from National Centre for Cell Science (NCCS), Pune. PC3 was cultured and maintained in Nutrient Mixture F-12 Ham medium (F-12, Himedia-AL106S) whereas DU145 and HaCaT in Dulbecco's Modified Eagle's Medium (DMEM, Himedia-AL007A) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Himedia-RM1112) and 100 IU/mL Penicillin and 0.1 mg/mL streptomycin (Himedia-A002A) in a humified atmosphere of 5% CO_2 at 37° C. The cells were harvested by trypsinization (Trypsin, Sigma-T4049) and the number of living cells was calculated by Trypan blue (Himedia-TC193) staining (0.2% v/v) using hemocytometer before seeding for future experiments.

6.2.3 *In vitro* drug treatment and transfection:

si-RNA against Sox2 (Sox2 si-RNA (h), sc-38408) and control si-RNA (control si-RNA-A, sc-37007) were purchased from Santa Cruz Biotechnology. For determining the optimum concentration of si-RNA that completely abolished gene and protein expression, 5 X 10^5 cells of each of the three cell lines were seeded and after 24 h incubation, were treated with three different concentrations (10, 20, 30 nM) of si-RNA using Lipofectamine 2000 (Invitrogen-11668019) and serum free opti-mem (Gibco-31985-070) according to manufacturer's instructions. Six hours after transfection, opti-mem was

replaced by the respective cell culture mediums and cells were left to incubate for next 24 hours. 30 nM of si-RNA efficiently blocked the expression of Sox2 protein in all the three cell lines. Control si-RNA was also used at the same concentration.

Similarly, for determining the IC_{30} concentration of Oct4-activating compound 1 (OAC1, Santa Cruz Biotechnology-sc-397046) and cyclopamine (Santa Cruz Biotechnology-sc-200929), 5 X 10^3 cells per well were seeded in 96-well microtiter plates and treated with drugs at different concentrations (5, 10, 15, 20, 25, 30 μM) mixed in respective medias supplemented with 10% FBS after reaching 70% confluency. Control cells were treated with di-methyl sulphoxide (DMSO, Himedia-TC185) only. For simultaneous transfection with si-Sox2 and treatment with cyclopamine, first transfection was carried out and after 6 h, cyclopamine at the IC_{30} concentration was administered dissolved in complete media and cells were then incubated for 72 h.

6.2.4 Cell viability and proliferation analysis by colorimetric MTT assay:

The effect of the drugs on cell viability was assessed by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT, Himedia-TC191) assay, using standard protocol (Kar et al., 2014). Briefly, drug-treated cells after incubation for 72 h were washed twice with phosphate buffered saline (PBS). Working concentartion of 0.8 mg/mL MTT solution was prepared from stock (5 mg/mL PBS, pH 7.2). To each well, 100 μL working MTT solution was added. After incubation in dark for 4 h at 37° C, 100 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm and results were expressed as the mean of three replicates as a percentage of control (taken as 100%). The extent of cytotoxicity was defined as the relative reduction in optical density (OD) of treated cells in comparison to control cells, which indicated the amount of viable cells in relation to control (100%). The cell viability was graphically represented and IC_{30} value of drugs was calculated accordingly to decide the optimum dosage for further studies.

For assaying cellular proliferation after treatment with si-RNAs, the above described procedure was followed but with slight variations. Briefly, 2 X 10^3 cells were seeded in 35mm plates and treated with desired concentrations of si-RNAs. After 24, 48, 72 and 96 h incubation, 100 μL MTT solution was added to each well and left in the incubator at 37° C for 4 h in dark. Subsequently, 100 μL of DMSO was added and optical absorbance was measured at 570 nm. Number of extra cells present in comparison to the

number of cells seeded earlier i.e. cell number (plotted in the graph) = Number of cells after 24/48/72/96 h – number of cells originally seeded (Santini et al., 2014).

6.2.5 Relative mRNA expression analysis by real-time PCR:

After treatment with the desired concentrations of si-RNAs for 24 h and drugs for 72 h, total cellular RNA from each of the three cell lines was extracted with TRI Reagent (Sigma-T9429) according to the manufacturer's instructions. cDNA prepared from 1 µg of total RNA prepared using RevertAid^{TM} First Strand cDNA Synthesis Kit (Thermo Scientific-K1622) followed by quantitative RT-PCR using Maxima SYBR Green/ROX qPCR Master Mix (2X, Thermo Scientific-K0221) in the Realplex4 Eppendorf system. The mRNA level was normalized to house-keeping gene GAPDH used as an endogenous control and calculations were done using $2^{-\Delta\Delta Ct}$ method, as described earlier (Schmittgen and Livak, 2008).

6.2.6 Western Blotting:

2 X 10^5 cells of each cell line were seeded, incubated till 80% confluency and treated with desired concentrations of si-RNAs for 24 h and drugs for 72 h. Cells were harvested and total cellular protein was extracted using lysis buffer (RIPA Buffer, Sigma-R01278) supplemented with protease inhibitor cocktail (PIC, Sigma, P8340). Bradford method was used to quantify the protein concentration and equal amount of cell lysate was loaded and separated in 10% SDS-PAGE and subsequently transferred onto PVDF membrane (Millipore, USA-ISEQ00010). Protein containing membranes were blocked in 3% bovine serum albumin (BSA, Himedia-5RMI05) in PBS containing 0.1% Tween 20 (Himedia-MB067) (PBST), followed by overnight incubation with primary antibodies for anti-Sox2 (Abcam-ab59776) and β-Actin (Santa Cruz Biotechnology-sc-47778) then incubated with appropriate HRP conjugated secondary antibody (Goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology, sc-2004, Goat anti-mouse IgG-HRP, Santa Cruz Biotechnology, sc-2005) for 2 h at 37° C in dark. The membranes were thoroughly washed with PBST buffer and were developed by Supersignal^{TM} West Femto Chemiluminescent substrate kit (Thermo Scientific-34095). β-Actin protein levels were used as control for equal protein loading. Relative protein expression was analyzed from the blots obtained using ImageJ quantification software.

6.2.7 Immunohistochemistry:

A total of 33 prostate (BPH=15, malignant=18) FFPE tissue samples from cancer tissue library were selected and analyzed. The tissue embedded in parafilm blocks were cut into 0.5μm sections and subjected to antigen retrieval with tris-EDTA buffer, endogenous peroxidase blocking and rinsed with tris-buffered saline (TBS) containing 0.025% Triton X-100 (TBS-T). After incubation with primary antibody -- rabbit polyclonal anti-Sox2 (Abcam-ab59776) at 4° C for overnight, tissue sections were rinsed with TBS and incubated at room temperature for 1 h with secondary antibody (Goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology, sc-2004). All specimens were developed with 3, 3′-Diaminobenzidine tetrahydrochloride (DAB, Sigma-D3939).

6.2.8 Immunocytochemistry:

Immunocytochemistry was performed as per our previous protocol with some modifications (Patra et al., 2001; Patra et al., 2002; Patra et al., 2009; Patra et al., 2011). In brief, HaCaT, DU145 and PC3 cells were grown on glass coverslips and treated with desired concentrations of si-RNAs for 24 h and drugs for 72 h. The treated cells were fixed in ice cold methanol (Himedia, AS058) and permeabilized by 0.25% triton X-100 (Himedia-RM845) in PBS. Cells were incubated with 1% BSA in PBST for 30 min to block non-specific binding of antibodies. The endogenous peroxidase activity was blocked by incubating in 5% hydrogen peroxide (H_2O_2, Himedia) in methanol for 20 min followed by overnight incubation in primary antibodies for Sox2 (Abcam, ab59776) and β-Actin (Santa Cruz Biotechnology-sc-47778) at 4° C. The cells were then washed in PBS and incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology-sc-2004, sc-2005) for 1 h followed by another PBST wash. Finally, cells were visualized by incubation with 3, 3-Diaminobenzidine tetrahydrochloride (DAB, substrate and chromogen, Sigma-D3939) and counterstained with Mayer's hematoxylin (Sigma-38803). For negative control, cells were incubated overnight with dilution buffer (no primary antibody).

6.2.9 Chromatin condensation analysis:

2 X 10^4 cells per well of each cell line were seeded in 6-well plates separately, and then treated with desired concentrations of si-RNAs for 24 h and drugs for 72 h. Cells were then stained with Hoechst 3342 (1 μg/mL, Invitrogen-H1399) dissolved in serum-free

media followed by incubation for 10 min at 37° C. Finally, images were taken under UV filter using Epi-fluorescent Microscope (Olympus IX71, excitation wavelength of 355 to 366 nm, emission wavelength of 465 to 480 nm) at 20X magnification. Nuclei with condensed chromatin were counted amongst total number of nuclei in the field, and the percentage of condensed nuclei was calculated and plotted graphically (Kar et al., 2014).

6.2.10 Wound healing or scratch assay:

2 X 10^5 cells per well were seeded in 6-well plate and incubated at 37° C until a fully confluent and adherent monolayer is formed. Scratch is then made using sterile 200 µL micropipette tip. Cells were washed thoroughly with PBS to remove the debris and smoothen the edge of the scratch. Treatment with the desired concentrations of si-RNAs for 24 h and drugs for 72 h was done. Images were taken using phase contrast microscopy at 0 h (immediately after treatment) and 72 h.

6.2.11 Cell cycle analysis by FACS:

HaCaT, DU145 and PC3 cells after treatment with desired concentrations of si-RNAs for 24 h and drugs for 72 h were incubated in their respective media with 10% FBS for 24 h. The cells were then trypsinized, collected by centrifugation (500 × g for 5 min at 4° C), washed twice with PBS and then fixed in 90% ice-cold methanol. After incubation at -20° C for 1 h, cells were centrifuged and resuspended in PBS followed by treatment with RNaseA (500 U/mL, Fermentas-EN0531) to digest residual RNAs and stained with propidium iodide (PI, 10 µg/mL, Sigma-81845). Samples were incubated for 30 min at 4° C and cell cycle analysis was performed with a Becton-Dickinson fluorescence-activated cell sorter (FACS). The percentage of apoptotic cells was graphically represented.

6.2.12 Statistical analysis:

Three independent experiments were done to calculate the P value and validate the results. All data are presented as means ± standard deviation (SD). Statistical analysis was performed using the Student's t-test. Values of $P < 0.05$ were considered as significant values.

6.3 Results:

6.3.1 Sox2 is highly amplified in cancer and this amplification is co-related to its biological function:

Pre-experimental *in silico* analyses were undertaken to have a comprehensive overview of Sox2, SHH, PTCH, SMO and GLI1 expression and function in all cancer types, particularly in prostate cancer. Using the publicly available cBioportal database, we observed the copy number alteration including amplification, deletion and mutations in almost all major types of cancer. All the five genes were subjected to wide-spread alterations with highest amplification for Sox2 in lung cancer (55%), for SHH in ovarian cancer (11%), for PTCH in sarcoma (2.5%), for SMO in ovarian cancer (6.4%) and for GLI in case of glioblastoma (8.4%) (Fig. 6.1a). Next, copy number alteration of all the five genes was done in prostate cancer which showed that Sox2 is amplified in 8%, SHH in 6%, PTCH in 7%, SMO in 10% and GLI in 5% of all prostate cancer patients with simultaneous mRNA up-regulation for all genes (Fig. 6.1b). Next, mutual exclusivity between Sox2 and all the components of HH pathway were computed and it was found that the alterations are mutually exclusive, however alterations in Sox2 and GLI1 were found to be co-current (p-value: 0.199, Log odds ratio 1.748). Survival (both with disease and disease-free) study for cases with alterations in Sox2 did not yield any significant result (Fig. 6.1c, 6.1d). However, survival analysis for cases with alterations in SHH, PTCH, SMO and GLI when done together showed significant values of 0.00158 (Fig. 6.1e). All the above results agree that both Sox2 and components of HH pathway are over-expressed and actively participate in mediating prostate cancer.

Table 6.1: Mutual exclusivity and Co-occurrence in prostate adenocarcinoma patients

Prostate adenocarcinoma (n= 333)				**Alterations in 20 (6.0%) cases**
Gene 1	**Gene 2**	**p-value**	**Log odds ratio**	**Association**
Sox2	GLI1	0.199	1.748	Tendency towards co-occurrence
Sox2	PTCH	0.801	<-3	Tendency towards mutual exclusivity
Sox2	SMO	0.863	<-3	Tendency towards mutual exclusivity
Sox2	SHH	0.929	<-3	Tendency towards mutual exclusivity

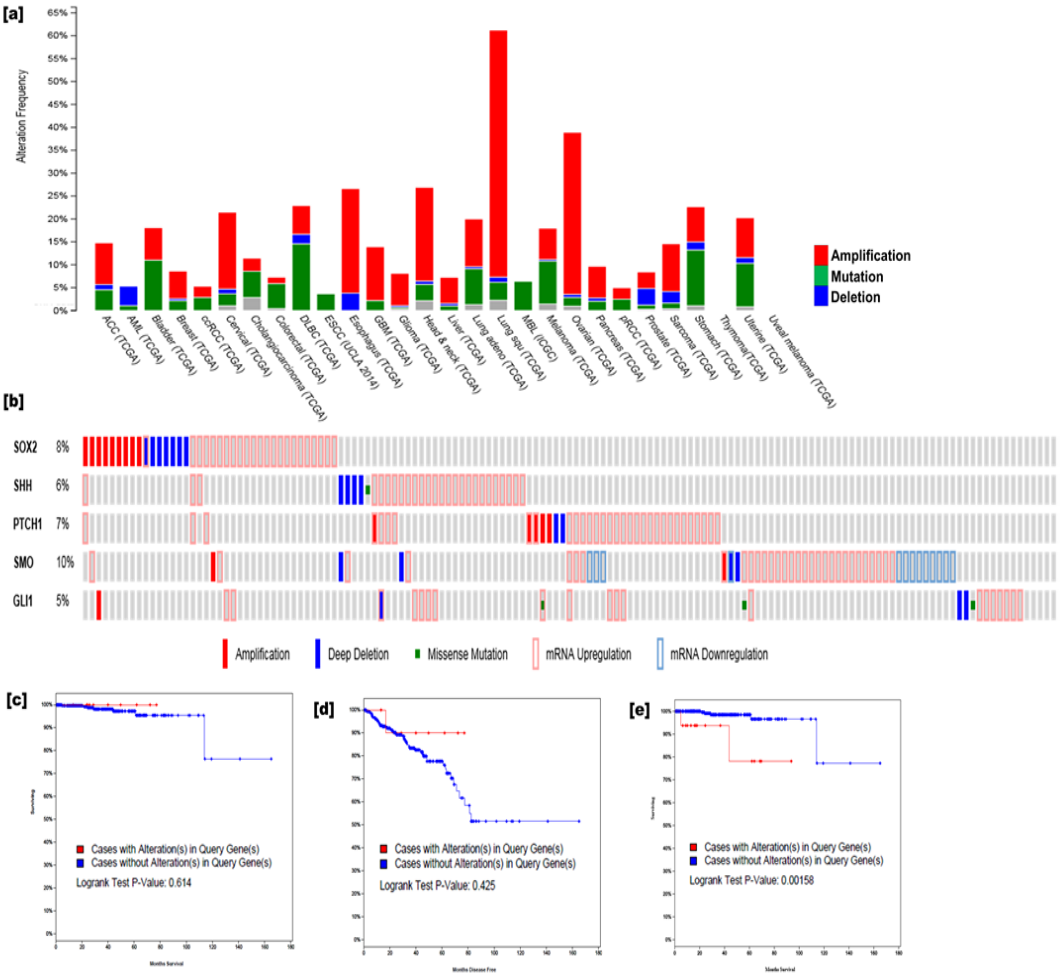


Figure 6.1: Pre-experimental *in silico* analysis of Sox2, SHH, PTCH, SMO and GLI1 using cBioportal. [a] Histogram showing cross-cancer summary of copy number alterations for Sox2, SHH, PTCH, SMO and GLI1 (28 studies/5 genes) depicted wide-spread alterations in all the 5 genes in almost all cancer types. [b] Oncoprint analysis in prostate cancer showed that Sox2 was amplified in 8%, SHH in 6%, PTCH in 7%, SMO in 10% and GLI in 5% of all patient tissue samples. [c] Survival plot with disease in case of alteration in Sox2 depicted no significant difference. [d] Survival plot for disease-free state with alteration in Sox2 was not significant. [e] Survival plot with disease in case of alteration in all the HH components showed significant value (P= 0.00158).

6.3.2 Sox2 expression in prostate cancer cells is indicative of its role in prostate tumorigenesis:

As is evident from the above mentioned *in silico* data analysis, Sox2 as well as SHH, PTCH, SMO, GLI were found to be amplified and showed up-regulation at the mRNA level in prostate cancer in prostate cancer. To validate the above data in cell lines, we employed qRT-PCR to examine the expression of all the 5 genes in two human prostate cancer cell lines-DU145 and PC3 taking human immortalized cell line HaCaT as the control. Our results agree with the databases and showed that in comparison to HaCaT

cells, Sox2, SHH, PTCH, SMO and GLI are over-expressed in both androgen independent prostate cancer cell lines, with PC3 showing a relatively higher level of expression of all the 5 genes (Fig. 6.2a). Protein expression of Sox2 further augmented the data obtained from RT-PCR with PC3 cells exhibiting higher Sox2 protein expression in comparison to HaCaT and DU145 cells (Fig. 6.2b, 6.2c). Immunohistochemistry done from a cohort of 33 prostate tissues comprising both benign and malignant samples showed that higher expression of Sox2 protein is observed in malignant prostate tissues, further strengthening our hypothesis that expression of Sox2 is tissue-stage specific and is involved in progression of cancer stages and mediating aggressive cancer phenotype (Fig. 6.2d). Immunocytochemical detection provided evidence for higher Sox2 expression in prostate cancer cells in comparison to HaCaT cells and also highlighted the nuclear localization of Sox2 (Fig. 6.2e).

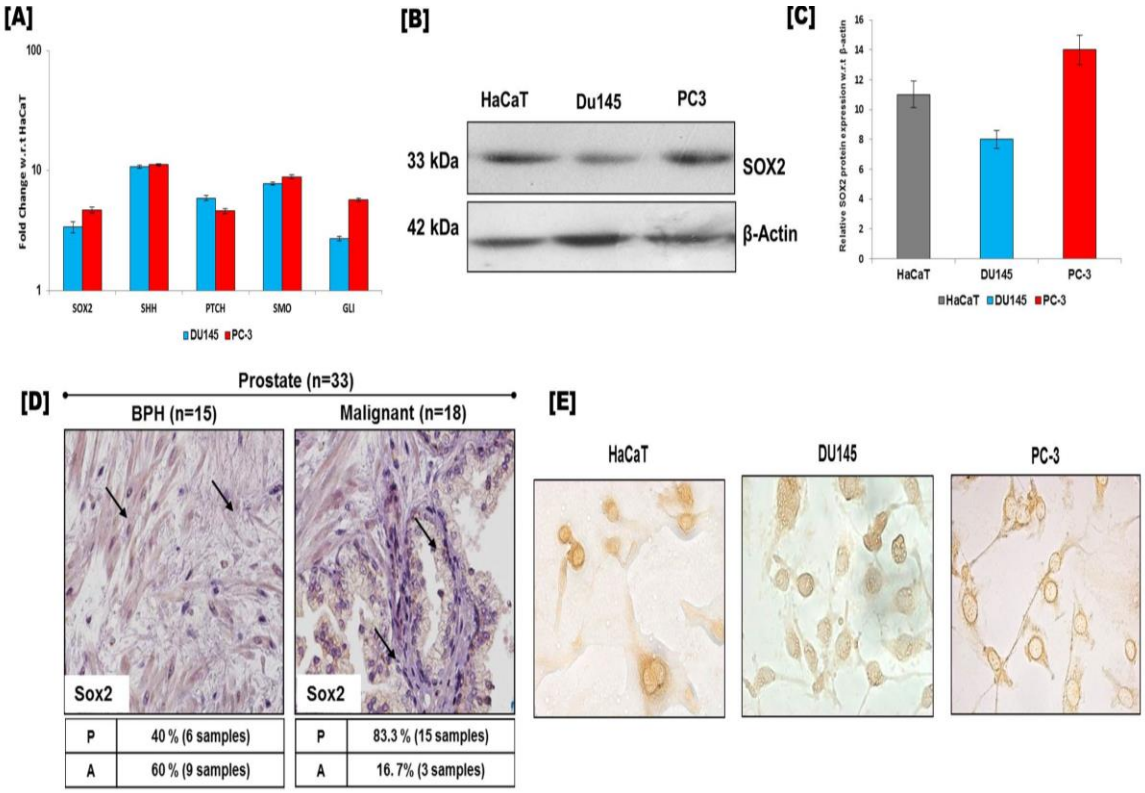


Figure 6.2: Sox2 is over-expressed in both the androgen independent prostate cancer cell lines. [a] qRT-PCR analysis for relative expression of Sox2, SHH, PTCH, SMO and GLI1 at the transcript level in DU145 and PC3 cells showed that all 5 genes are over-expressed in prostate cancer cell lines in comparison to HaCaT cells. [b] Western blot analysis showed that Sox2 protein expression is highest in case of PC3 cells in comparison to HaCaT and DU145. [c] Graphical representation of Sox2 protein expression after quantification using ImageJ software showed highest expression in case of PC3 cells. [d] Immunocytochemical staining of Sox2 protein in three cell lines showed nuclear localization of Sox2. Error bars indicate SD (n=3, mean± S.D, $P < 0.05$).

6.3.3 Silencing of Sox2 hinders cell proliferation and induces apoptotic cell death:

To assess the functional impact of Sox2 in prostate cancer, HaCaT, DU145 and PC3 cell lines were stably transfected with 30 nm of Sox2 si-RNA. As shown, all the three cell lines showed lower levels of Sox2 protein expression in comparison to cells treated with control si-RNA (Fig. 6.3a). From colorimetric MTT assay for assessing the rate of proliferation after silencing of Sox2, it was observed that cell proliferation in Sox2 silenced HaCaT, DU145 and PC3 cells was lower in comparison to cells transfected with control si-RNA with PC3 cells showing the slowest rate of cell proliferation (Fig. 6.3b). To evaluate the effect of Sox2 knockdown on cell cycle distribution, flow cytometric analysis was done. In case of treatment with si-control, percentage of apoptotic cells was found to be 18% for HaCaT, 21% in DU145 and 16% in PC3 cells. But after treatment with si-Sox2, the percentage of apoptotic cells increased to 24% in HaCaT, 25% in DU145 and 27% for PC3 cells in comparison to control cells (Fig. 6.3c). Chromatin condensation analysis using Hoechst stain was performed to study the cytotoxic effect of Sox2 knockdown. The morphological changes induced by apoptosis such as packaged/condensed nuclei can be detected by blue-fluorescent Hoechst 33342 dye which brightly stains the highly condensed, dense chromatin of apoptotic cells in comparison to the chromatin of non-apoptotic cells. Sox2 knockdown cells show higher chromatin condensation as is evident from 34%, 28% and 42% condensed cells in HaCaT, DU145 and PC3 respectively with respect to 21%, 13% and 24% condensed cells in control si-RNA transfected cells (Fig. 6.3d, 6.3e). Scratch assay was performed to evaluate the wound healing capacity of the Sox2 silenced cells and it was observed that after Sox2 si-RNA treatment, migration of cells drastically reduced. While cells transfected with control si-RNA had 31%, 29% and 45% migrated cells respectively for HaCaT, DU145, and PC3 cells, Sox2 silenced cells had lower migration as 22%, 11% and 31% respectively (Fig. 6.3f, 6.3g). Altogether, these results indicate that interference with Sox2 function impedes cell and metastatic growth by reducing cell proliferation, stimulating apoptosis and constraining invasive ability of prostate cancer cells.

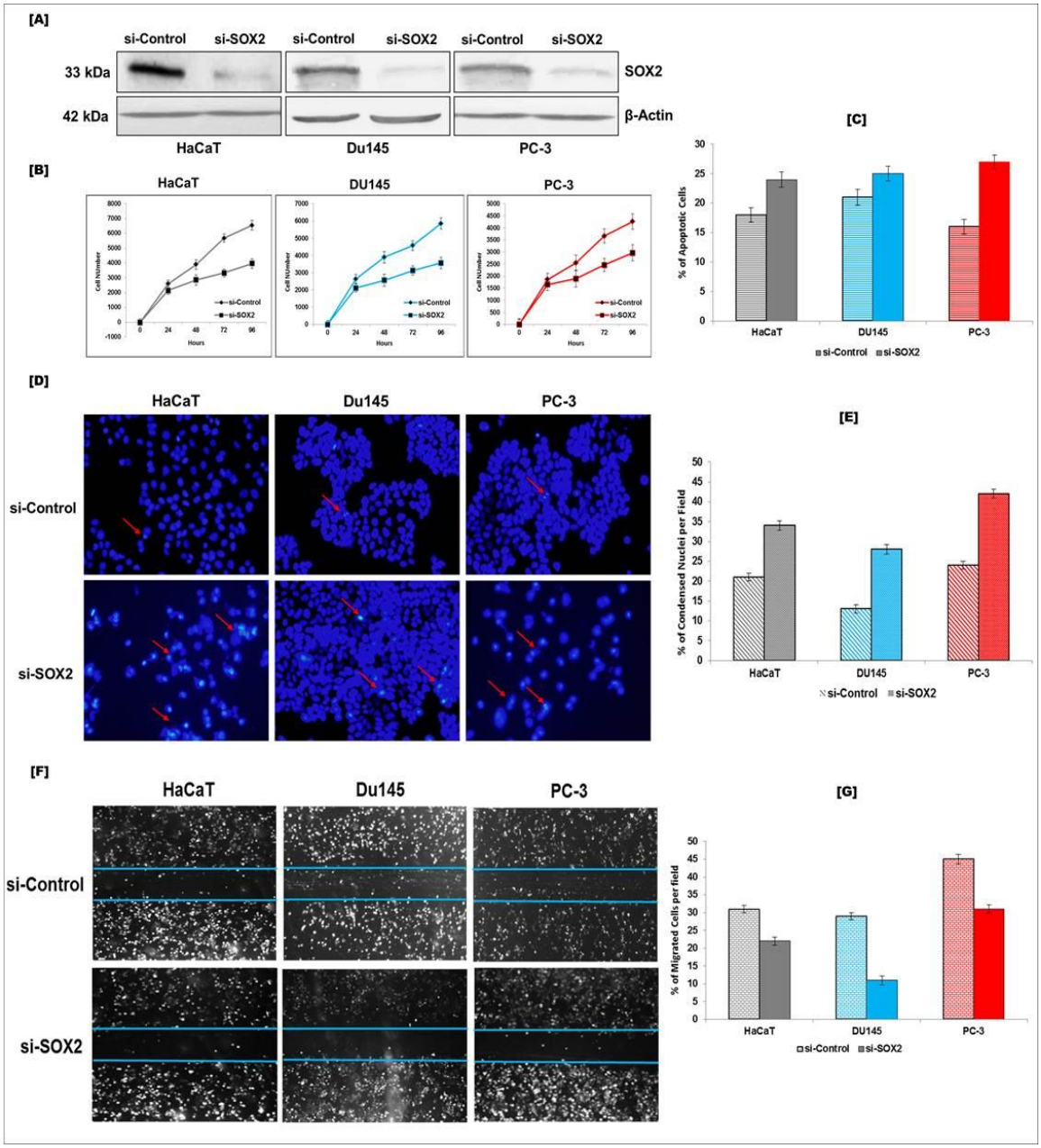


Figure 6.3: Silencing of Sox2 affects tumorigenic properties of prostate cancer. [a] Western blot analysis showed that Sox2 protein expression was completely abolished after treatment with 30 nM si-RNA against Sox2. [b] Graphical representation of cell proliferation in HaCaT, DU145 and PC3 after treatment with si-RNA against Sox2 and control si-RNA for 96 h showed that PC3 cells were highly restricted in proliferating ability. [c] Graphical representation of percentage of apoptotic cell population showed increase in apoptotic cells for si-Sox2 treated cells w.r.t control si-RNA treated cells. [d] Chromatin condensation analysis after treatment with si-RNA against Sox2 and control si-RNA. Magnification 20X. [e] Graphical representation of percentage of condensed nuclei per field showing increase in number of condensed nuclei. [f] Scratch assay after treatment with si-RNA against Sox2 and control si-RNA showed decrease in number of migrating cells. Magnification 20X. [g] Graphical representation of percentage of invaded cells per field. Error bars indicate SD (n=3, mean± S.D, $P < 0.05$).

6.3.4 Over-expression of Sox2 boosts cell proliferation and facilitates apoptotic escape:

In our study, for the first time, over-expression of Sox2 was achieved via use of a chemical activator called Oct4-activating compound 1 (OAC1). OAC1 is known to increase transcription of Oct4-Nanog-Sox2 triad, enhance and accelerate efficiency of iPSC reprogramming methods. Through MTT assay, IC_{30} concentration of OAC1 was determined to be 15 μM for DU145, PC3 and 10 μM for HaCaT cells and henceforth used for future experiments (Fig. 6.4a). The relative mRNA expression of all the three core transcription factors Oct4, Sox2 and Nanog was evaluated by real time PCR and results indicated over-expression of all the three factors in comparison to the housekeeping genes GAPDH (Fig. 6.4b). The protein expression of Sox2 was studied by Western Blotting in all three cell lines after treatment with OAC1 which showed elevated expression in comparison to control untreated cells, with PC3 again exhibiting higher protein expression in comparison to the other cell lines (Fig. 6.4c). The percentage of apoptotic cells in control cells were 14.4%, 13% and 18.5% and after OAC1 treatment were 11%, 10% and 12% for HaCaT, DU145, PC3 respectively. This decrease is accompanied by increase in S and G_2 phase population, implying that higher expression of Sox2 could promote G_1 to S phase transition of the cell cycle and evasion of apoptosis (Fig. 6.4d). The number of condensed chromatin was not significantly high as was the case after si-Sox2 treatment, which may further prove that over-expression of Sox2, facilitates apoptotic escape (Fig. 6.4e, 6.4f). Even the migration properties of prostate cancer cells were boosted after treatment with OAC1 showing 12%, 19% and 31% for HaCaT, DU145, PC3 respectively (Fig. 6.4g, 6.4h). All the above results implicate Sox2 as a mediator of malignant transformation and validate Sox2 as an essential player during acquisition of stem cell properties.

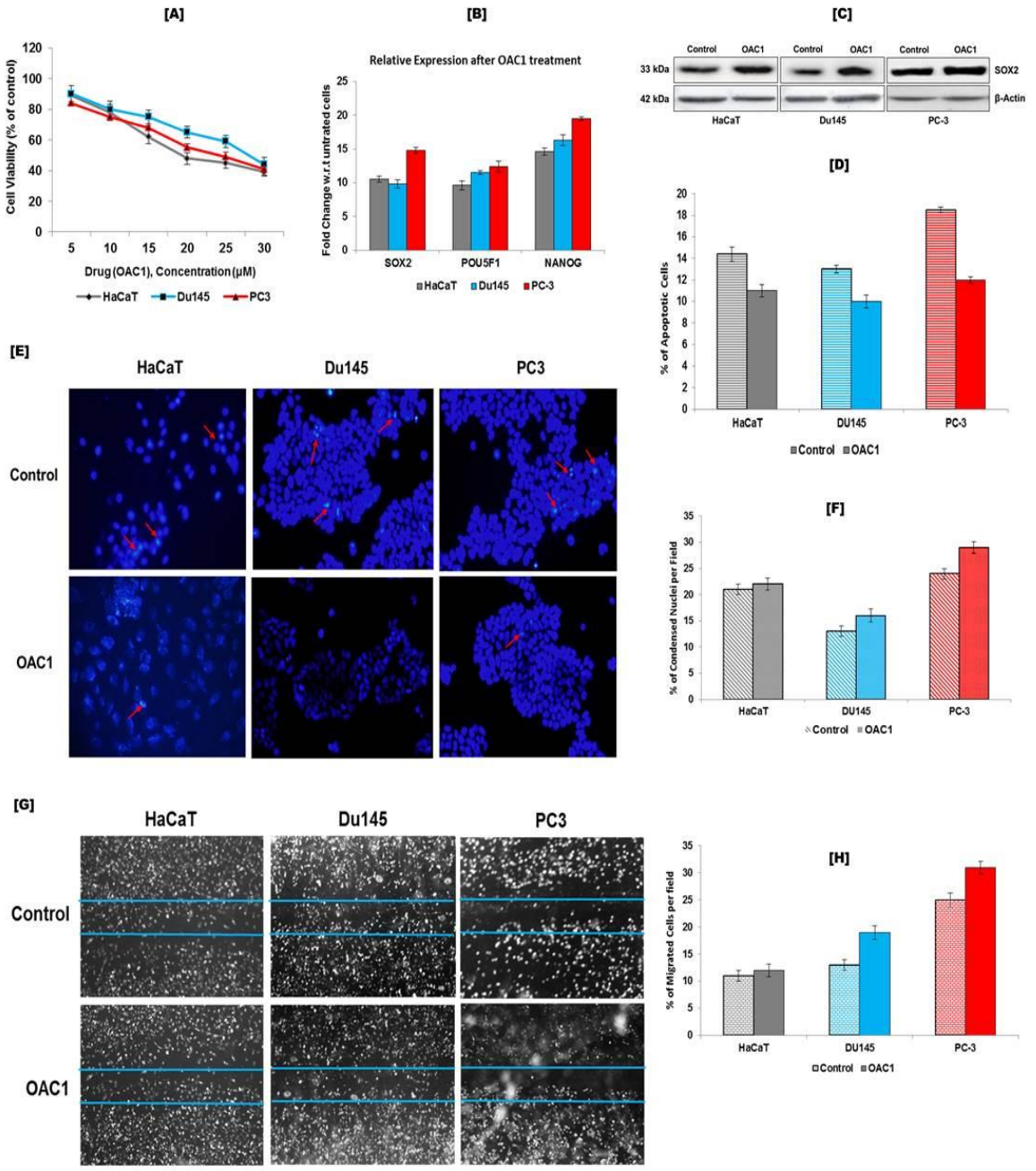


Figure 6.4: Over-expression of Sox2 induced by chemical activator OAC1. [a] Colorimetric MTT assay determined the IC_{30} concentration of OAC1 to be 15 μM for DU145, PC3 and 10 μM for HaCaT cells. [b] Relative gene expression of pluripotency inducing transcription factors-Oct4, Sox2 and Nanog after treatment with OAC1 showed elevated expression in all the three cell lines. [c] Western blot analysis showed that after OAC1 treatment, Sox2 protein expression increased in all cell lines with PC3 showing highest expression. [d] Graphical representation of percentage of apoptotic cell population showed decrease in apoptotic cells for OAC1 treated DU145 and PC3 cells. [e] Chromatin condensation analysis after treatment with OAC1 showed no significant condensed nuclei. Magnification 20X. [f] Graphical representation of percentage of condensed chromatin after treatment with OAC1. [g] Scratch assay in HaCaT, DU145, PC3 after treatment with OAC1 showed increase in cell migration. Magnifications 20X. [h] Graphical representation of percentage of invaded cells per field after treatment with OAC1. Error bars indicate SD (n=3, mean± S.D, $P < 0.05$).

6.3.5 Consequences of inactivating HH signaling pathway in prostate cancer:

Constitutively active HH signaling pathway is known to promote malignant growth in prostate tissues; hence the role of HH signaling pathway was investigated by down-regulating SMO using cyclopamine and subsequently inhibiting the pathway. MTT assay was performed to decide the optimum concentration of drug to be used and IC_{30} value of 15 μM for 72 h was determined as the ideal concentration for subsequent treatments (Fig. 6.5a). From the cBioportal, it was evident that alterations in Sox2 and GLI1 pathway were co-occurent, hence in order to the gene expression of HH pathway components – SHH, PTCH, SMO and GLI was examined along with Sox2 after cyclopamine treatment. It was seen that transcript level expression of all the HH pathway components in cyclopamine treated DU145 and PC3 cells was reduced in comparison to their expression in HaCaT cells observed earlier (Fig. 6.5b). In agreement with the above results, Sox2 protein expression was also greatly reduced in all the three cell lines further highlighting the connection between Sox2 and HH pathway (Fig. 6.5c). After FACS analysis, we observed that percentage of apoptotic cells is higher for drug treated HaCaT, DU145 and PC3 cells with 17%, 23% and 29% apoptotic cells in comparison to untreated cells with 8.3%, 15.2% and 19% apoptotic cells (Fig. 6.5d). Upon treatment with cyclopamine, it was observed that chromatin condensation increases considerably. Percentage of condensed chromatin was 26% in case of HaCaT, 32% for DU145 and 36% for PC3 cells; with highest percentage in case of PC3 in comparison to control untreated cells with 18%, 23% and 19% condensed nuclei for HaCaT, DU145 and PC3 respectively (Fig. 6.5e, 6.5f). Similarly, scratch assay provided concomitant results showing that cyclopamine reduced the invasive potential of the cell lines with PC3 cells showing least invasion (Fig. 6.5g, 6.5h). Thus, inactivating HH signaling pathway in prostate cancer cells by using cyclopamine can be considered to be possible mechanism of targeting aggressiveness in advanced prostate cancer cells.

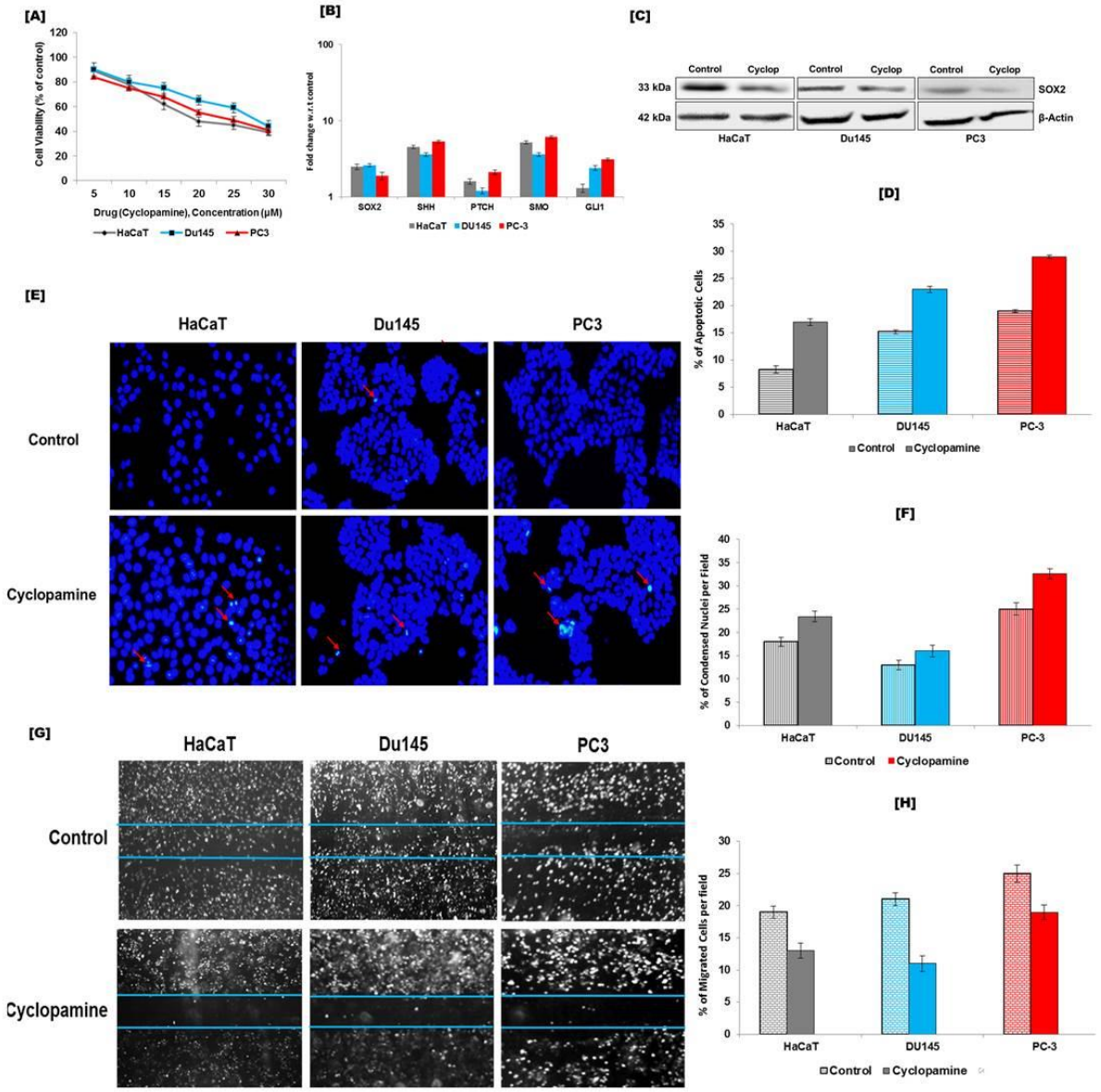


Figure 6.5: Inhibition of HH pathway receptor SMO by using cyclopamine. [a] MTT assay determined the IC_{30} concentration of cyclopamine to be 15 μM for 72 h for all three cell lines. [b] Relative expression analysis showed decrease in gene expression for Sox2, SHH, PTCH, SMO and GLI in all three cell lines. [c] Protein expression of Sox2 after treatment with cyclopamine showed decrease in protein expression with PC3 showing almost complete absence of protein. [d] Graphical representation of percentage of apoptotic cell population after treatment with cyclopamine showed increase in apoptosis. [e] Chromatin condensation analysis after treatment with cyclopamine. Magnification 20X. [f] Graphical representation of percentage of condensed chromatin after treatment with cyclopamine showed DNA damage in all cell lines. [g] Scratch assay after treatment with cyclopamine. Magnification 20X. [h] Graphical representation of percentage of invaded cells per field after treatment with cyclopamine showed decrease in migration and invasion potential. Error bars indicate SD (n=3, mean± S.D, $P < 0.05$).

6.3.6 HH signaling pathway and Sox2 can be targeted concomitantly in prostate cancer:

In order to evaluate the effect of simultaneous inhibition of both Sox2 and HH pathway, we used si-RNA against Sox2 as well as HH pathway inhibitor cyclopamine simultaneously in HaCaT, DU145 and PC3 cell lines. At the transcript level, Sox2 expression was drastically reduced in DU145 and PC3 cells in comparison to HaCaT. After treatment with both cyclopamine and si-Sox2, expression level of Sox2 in HaCaT was 2.4, in DU145 were 3.2 folds, in PC3 were 1.9 folds as compared to 4.5, 5.1 and 3.9 folds change in HaCaT, DU145, and PC3 respectively when treated with si-Sox2 alone (Fig. 6.6a). In all the three cell lines, number of viable cells drastically reduced as compared to that observed when only Sox2 was silenced. PC3 showed the lowest cell proliferation capacity among all the three lines (Fig. 6.6b). These results support the hypothesis that endogenous Sox2 might associate with HH pathway in regulating prostate cancer cell proliferation. Next we investigated the impact of parallel inhibition of Sox2 and HH pathway on prostate cancer properties. From FACS analysis we observed that percentage of apoptotic cells in HaCaT, DU145 and PC3 were 31.4%, 29%, 34.2% after co-treatment which is comparatively higher in comparison to controls—RNA and si-Sox2 treatment done earlier (Fig. 6.6c). Chromatin condensation was also significantly higher with HaCaT, DU145 and PC3 showing 22%, 26% and 49% of condensed chromatin (Fig. 6.6d). Identically, invasive ability of the cells was also greatly reduced when subjected to simultaneous treatment with both the agents (Fig. 6.6e). Results obtained from FACS, chromatin condensation and scratch assays further strengthened the hypothesis that Sox2 and HH pathway work together in promoting tumorigenic nature. In order to understand how Sox2 and HH work at the molecular level, we analyzed the gene expression of EMT gene such as E-cadherin and apoptotic markers like Bcl-2 and Bax. At the transcript level, E-cadherin gene expression was significantly down-regulated in comparison to only si-Sox2 treated cells. Change in expression of E-cadherin in HaCaT was 2.8 folds, 3.5 folds in DU145 and 1.9 folds in PC3 which were in agreement with the reduction in percentage of migrating cells shown earlier (Fig. 6.6f). Similarly the expression of pro-apoptotic agent Bax was enhanced, while anti-apoptotic marker Bcl-2 in both DU145 and PC3 was relatively down-regulated in comparison to HaCaT (Fig. 6.6g). In case of Bax, the change in expression was 4.4 folds, 3.6 folds and 5.1 folds for HaCaT, DU145 and PC3 respectively and for Bcl-2; expression was changed by 2.4, 1.7 and 2.3 folds for HaCaT,

DU145 and PC3 with respect to house-keeping gene GAPDH (Fig. 6.6h). This data further substantiates our claim that both Sox2 and HH pathway integrate at the molecular level to promote cancer aggressiveness. Also, targeting Sox2 and HH pathway simultaneously will be more beneficial as a therapeutic option in comparison to differential treatment using HH inhibitors or gene knock-down of Sox2 individually.

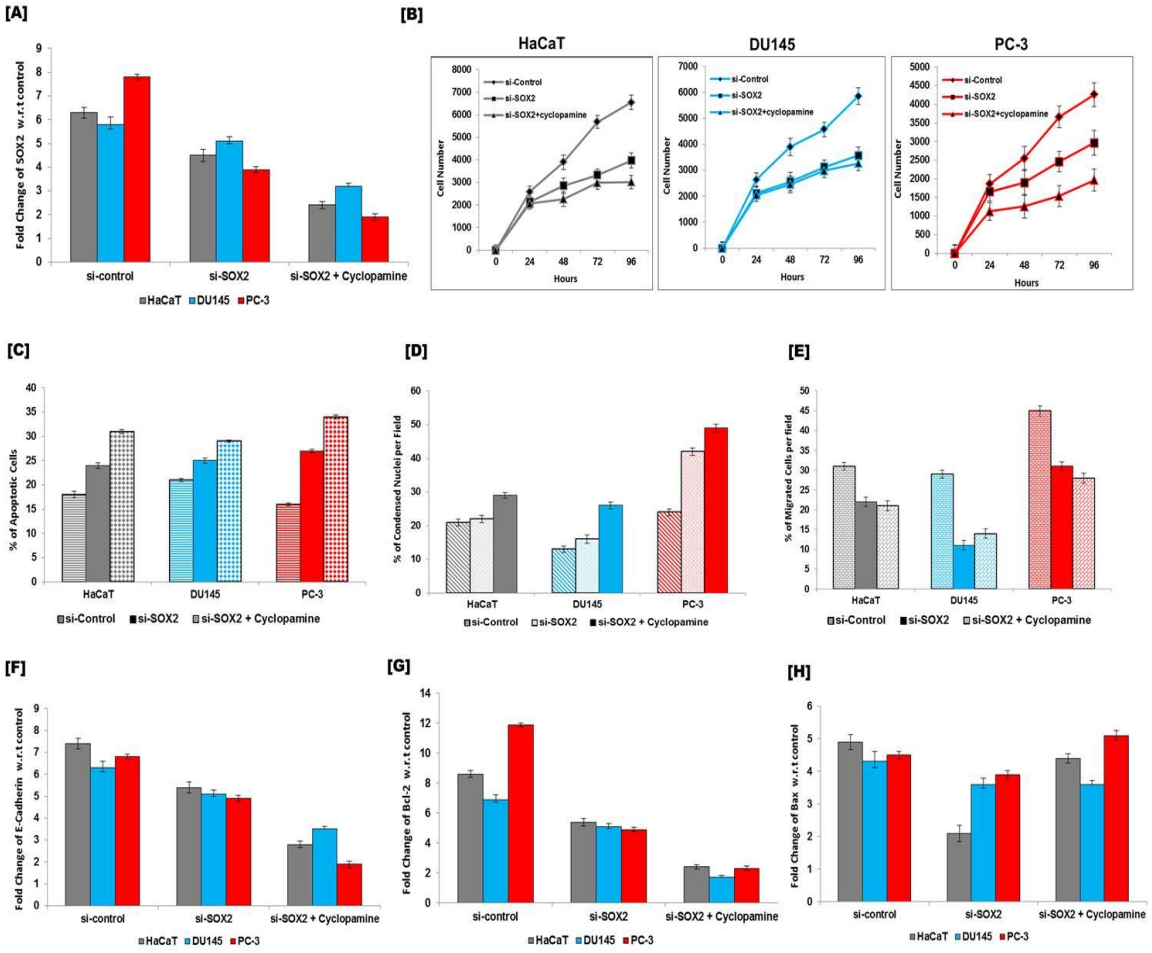


Figure 6.6: Co-treatment using si-RNA against Sox2 and cyclopamine against HH receptor. [a] Relative gene expression of Sox2 after si-control, si-Sox2 and si-Sox2+Cyclopamine treatment showed down-regulation of Sox2 gene expression. Error bars indicate SD (n=3). [b] Cell proliferation in HaCaT, DU145 and PC3 after treatment with si-RNA against Sox2 and cyclopamine showed drastic decrease in cell number in PC3. [c] Graphical representation of percentage of apoptotic cells showed huge increase after co-treatment. [d] Graphical representation of percentage of condensed chromatin showed increase in chromatin condensation. [e] Graphical representation of percentage of invaded cells per field showed reduction in migration potential in all three cell lines. [f] Relative gene expression of E-cadherin was decreased in PC3 and DU145 in comparison to HaCaT. [g] Relative gene expression of Bcl-2 also showed down-regulation. (h) Relative gene expression of Bax showed increase in expression w.r.t house-keeping gene GAPDH. Error bars indicate SD (n=3, mean± S.D, $P < 0.05$).

6.4 Discussion:

Prostate cancer is a widespread malignancy considered to be second major cause of cancer-related deaths in men. Tumor heterogeneity in prostate cancer is profound; during early neoplastic changes, tumor growth is dependent upon AR signaling; however, at the advanced stages, cancer cells become androgen independent and consequently become resistant to chemo-therapy. Androgen deprivation therapy however effective in the initial prognosis, fails to prevent post-therapeutic recurrence. Lack of proper insight into the molecular mechanisms behind advanced prostate cancer largely impinges on the therapeutic strategies currently employed. As more and more studies are emphasizing on the role of pluripotency factors and signaling pathways in facilitating aggressive tumor phenotype, addressing the issue of prostate cancer therapy in light of the above factors could serve as an effective treatment policy. In this study, we have described for the first time an important connection between pluripotency inducing transcription factors and cellular signaling pathways in prostate cancer. Pluripotency transcription factors have always been accredited for contributing towards maintenance of CSC population and promoting stem cell phenotype. However, the major finding of our investigation establishes that in addition to its role in maintenance of stem cell population, Sox2 is functionally relevant in non-stem prostate cancer cells. We have shown that depletion of Sox2 reduces cellular proliferation, progressively restricts metastatic ability, leading to significant increase in apoptotic death. Our study strongly suggests that in prostate cancer, Sox2 is essential for tumorigenic properties and that targeting this transcription factor is a viable therapeutic option. Furthermore, we provide evidence that Sox2 is functionally affiliated to HH signaling in controlling prostate cancer characteristics. Both Sox2 and HH pathway components are amplified in prostate cancer and their co-expression is significantly co-related to aggressive properties of advanced prostate cancer. Down-regulating both Sox2 and HH pathway concomitantly results in significant decrease in metastatic behavior, reduced survival capacity and cell proliferation, reduction in migration and invasiveness as well as greater degree of apoptotic death in androgen independent prostate cancer cells, thus affecting all the major hallmarks of aggressive tumor phenotype. In line with other studies, our findings suggest that HH-Sox2 axis is as much crucial for maintenance and survival of tumor bulk as it is for CSC population in prostate cancer.

Sox2, an important pluripotency inducing transcription factor, is known to be over-expressed in almost all human cancer types, including prostate cancer. In the current study, we demonstrated that Sox2 mRNA and protein were expressed in both of the androgen independent prostate cancer cell lines, with PC3 showing a relatively higher endogenous Sox2 expression (Fig. 6.2). This finding agrees with the previous studies where it was shown that androgen independent cell lines DU145 and PC3 have higher Sox2 expression than androgen dependent LnCaP cell line. This fact signifies that Sox2 might play a vital role during evolution of prostate cancer from androgen dependent to androgen independent mode. Upon silencing Sox2, we observed lower cell proliferation indicating that Sox2 is responsible for encouraging oncogenic growth and proliferation in prostate cancer. Our data also indicated that silencing of Sox2 resulted in apoptotic death and DNA damage in prostate cancer cells (Fig. 6.3). However, the detailed molecular mechanisms by which Sox2 knockdown directs apoptotic induction must be investigated further in order to discern downstream transcriptional targets of Sox2 in apoptotic pathways. DU145 cells were reported to have lower Sox2 gene and protein expression in comparison to PC3. Given the fact that DU145 is moderately and PC3 is highly metastatic, the above-mentioned expression pattern might indicate towards possible contribution of Sox2 during prostate cancer cell invasion. Sox2 knockdown greatly reduced the wound healing ability of prostate cancer cells especially PC3, demonstrating that Sox2 is an important contributor to EMT and encourages metastastic behaviour in prostate cancer cells. We also observed that knockdown of Sox2 expression lead to cell cycle arrest in G_1 phase resulting in more apoptotic population. In contrast, upon chemical activation by OAC1, gene and protein expression of Sox2 greatly increased along with concurrent decrease in apoptotic cell population, reduction in condensed nuclei and increase in percentage of invading cells. In comparison to HaCaT, the above-mentioned changes were more profound in DU145 and PC3 cells indicating that Sox2 over-expression favors tumorigenic properties. All the above data substantiate our claim that silencing of Sox2 is equally effective in diminishing the oncogenic properties in prostate cancer cells as it is in case of prostate cancer stem cells (Fig. 6.4).

Aberrantly active HH signaling plays crucial role during tumorigenic progression and metastasis of prostate cancer and prostate cancer stem cells. We have used the natural antagonist cyclopamine to inhibit SMO receptor and effectively downregulate HH pathway. After cyclopamine treatment, there is marked reduction in the number of condensed nuclei as well as increase in the apoptotic cell populations. Moreover, data

from the scratch assay also agrees with the above findings implying that HH pathway plays an important role during prostate cancer metastasis and progression (Fig. 6.5). We further provide strength to the claim that HH signaling and Sox2 work together to promote prostate cancer progression by targeting them simultaneously. Gene expression of Sox2 was greatly reduced after co-treatment signifying that Sox2 is a downstream target of HH in prostate cells. Our findings are in sync with other studies; for example, Sox2 is an important contributor in Sonic Hedgehog-associated medulloblastoma. Further, in melanoma initiating cells via chromatin immunoprecipitation (ChIP) assays, it was reported that putative GLI-binding sites are present at about 300 bp upstream to Sox2 transcription start site (TSS), within the core proximal promoter region of Sox2 (−528 and +238 from the TSS). Thus, Sox2 expression at transcriptional level is influenced by HH signaling by the binding of GLI1 and GLI2 to Sox2 promoter. Moreover, HH-GLI and epidermal growth factor receptor (EGFR) signaling synergistically interact to initiate Sox2 and Sox9 expression in basal cell carcinoma and tumor-initiating pancreatic cancer cells. As previously mentioned, Sox2 knock-down resulted in reduction of cell migration, so we decided to investigate the connection between EMT gene and HH-Sox2 axis. The transcript level expression of E-cadherin was greatly reduced in all three cell lines, but most prominently in PC3 indicating that metastatic ability of PC3 cells can be targeted by the above method. It is generally known that HH pathway regulates components of both cell-intrinsic and cell-extrinsic pathways of apoptosis including Bcl-2 and Bax. We have shown that upon treatment with cyclopamine and si-Sox2, the transcript level expression of Bcl-2 was decreased and that of Bax increased, along with an increase in apoptotic cell population. Therefore, HH-Sox2 can be considered as better therapeutic target for addressing androgen-independent aggressive prostate cancer (Fig. 6.6).

Although the probability of selectively targeting Sox2 or other oncogenic transcription factors for tissue-specific treatment options for cancer is challenging and debatable; recently, studies have focused on developing new strategies to inhibit Sox2. In this line, we have for the first time adopted the method of downregulating both Sox2 by gene knockdown and HH pathway via inhibiting its receptor molecule using cyclopamine. Our study firmly establishes Sox2 as a potential therapeutic target in prostate cancer by providing rational evidences of its involvement in prostate cancer. First, we showed that silencing of Sox2 reduces proliferation and induces apoptosis in non-cancer stem cell population of androgen independent prostate cancer cells. Secondly, we demonstrated that

HH signaling pathway knockdown greatly hampers the tumorigenicity of prostate cancer cells. Finally, we have established that HH-Sox2 axis is a better therapeutic target with much higher reduction in cell proliferation, complete inhibition of migration and elevated chromatin condensation than individual treatment policies (Fig. 6.7). Although, this strategy needs to be further validated *in vivo* before it can be safely and effectively applied to patients; co-targeting both transcription factor and oncogenic signaling pathway can be adopted as a novel therapeutic approach for treatment of prostate cancer. As contemporary research in the field of cancer biology is turning towards more unconventional methods for treatment of cancer, co-targeting Sox2 and HH signaling pathway might just be the right arsenal against therapeutically resistant prostate cancer.

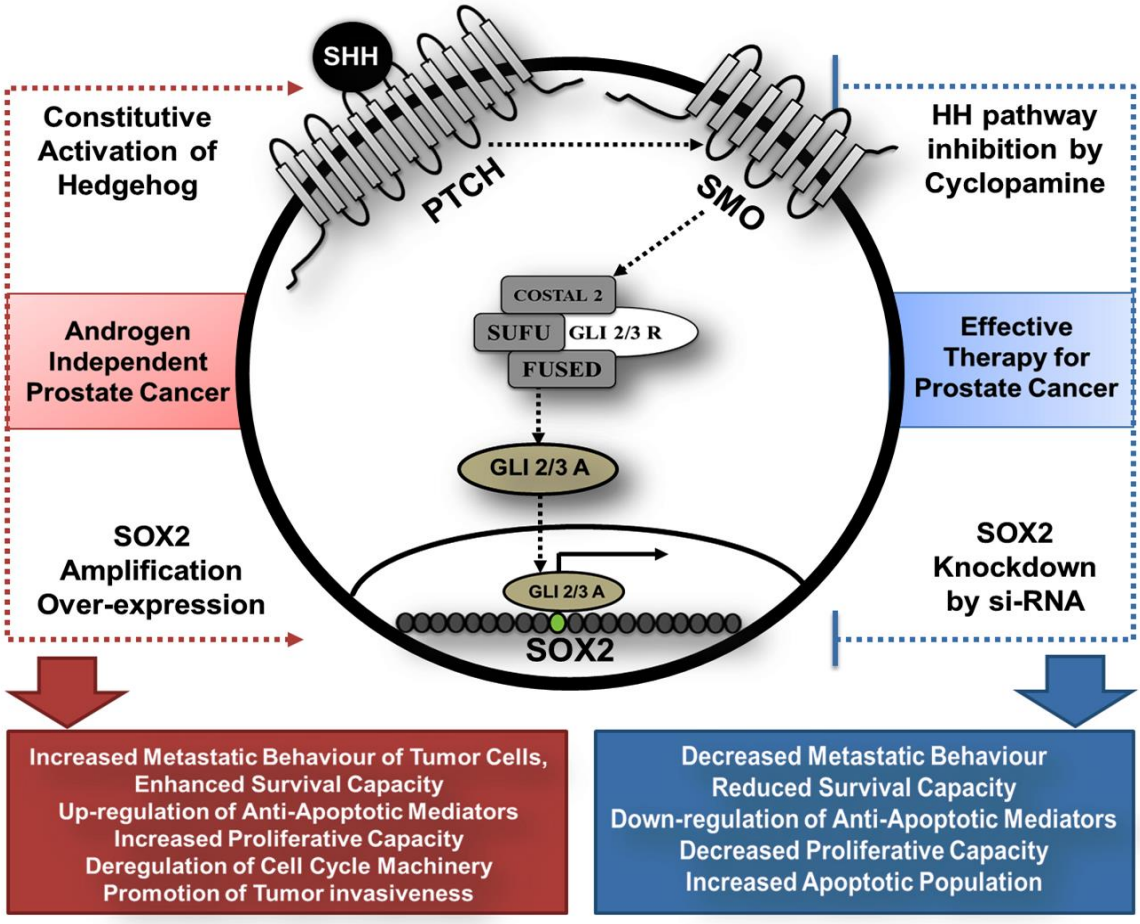


Figure 6.7: Sox2 and HH signaling Pathway can be targeted together for prostate cancer therapy. Sox2 and HH pathway both work synergistically to promote aggressive tumor phenotype in prostate cancer by increasing metastasic behaviour, improving survival and proliferation capacity, as well as deregulating cell cycle machinery. However, by targeting both the factors, tumorigenic potential in androgen independent prostate cancer can reduced to a great extent thus presenting a novel therapeutic approach.

Chapter 7

General Discussion and Conclusions

Efficient gene transcription programs and coordinated regulation of the basal transcriptional machinery underlies smooth homeostatic functioning of cells in any biological system. This system of regulation is jointly undertaken by three important enforcers which are cellular signaling cascades, epigenetic modifiers and chromatin remodeling agents and finally, core group of nuclear transcription factors. These agents synergistically co-operate with each other to form the cornerstone of cellular specialization and functional potential. Given the strategic significance of these factors in maintaining cellular structure, any deregulation or aberrant behavior by any one of the factors can disrupt the delicate molecular stability inside a cell and cause widespread mayhem leading to pathophysiological conditions and disorders. Cancer is one such disorder which gradually destabilizes the entirety of molecular organization and breaks down barriers against de-differentiation. In other words, malignant transformation symbolizes the onset of cellular anarchy exemplified by the aberrant activity of pluripotency transcription factors, epigenetic modifiers and developmentally inclined signaling pathways, all of which work to destroy developmental capacity of a cell.

In recent years, epigenetic definition of neoplasm explains that early, initiating abnormal epigenetic events predispose a group of progenitor cells towards acquiring of oncogenic mutations and plasticity that favors malignant growth. In the abnormally changed progenitor population of pre-malignant cells, signaling pathways which are normally engaged in translating developmental and differentiation specific cues to functional specialization, become constitutively active and provide signals for deregulated growth, proliferation and survival. These signaling pathways with the help of epigenetic modifiers activate inappropriate transcriptional programs that provide crucial advantages to the growing population of neoplasia ready cells. The nuclear transcription factors involved in this act are effectively regulated to trigger expression of genes crucial to maintaining self-renewal, proliferation and survival of cancer cells. Thus, pluripotency inducing transcription factors that generally maintain embryonic and stem cell identity become rogue and facilitate oncogenic reprogramming. The most notable transcription factors involved in this scheme include Oct4, Sox2 and Nanog which together form the

"Holy Trinity" of transcription factors. These three factors are now considered as tumor progenitor genes and thought to mediate tumorigenic reprogramming and evolution from normal cellular state. In the light of above rationale, this work was designed to understand in details the role of pluripotency transcription factors in mediating inherent tumorigenic potential under the influence of epigenetic modifications and signaling pathways. The aim of the project was to establish whether Oct4, Sox2 and Nanog are oncogenic in nature and elucidate the epigenetic effectors that guide them in their role. Firstly, the expression profile of Oct4, Sox2 and Nanog in breast and prostate cancer was established. Secondly, by gene knockdown experiments, the direct involvement of Oct4, Sox2 and Nanog in pro-tumorigenic characters such as cell proliferation, EMT and metastatic invasion, anti-apoptotic nature was examined. Next, epigenetic modifications especially DNA methylation and histone modifications regulating the expression and function of these factors were investigated. Finally, the interaction of HH signaling and pluripotency inducing transcription factors was studied in order to fully visualize the upstream regulatory control over these factors.

In the first step, expression of Oct4, Sox2 and Nanog in different stages of clinical breast and prostate tissue samples was studied. With the help of publicly available database cBioportal which is linked to TCGA database, we learnt that Oct4, Sox2 and Nanog are amplified in breast and prostate adenocarcinomas. In order to validate in silico data, we examined protein expression of Oct4, Sox2 and Nanog in FFPE tissues and found that all the three factors are over-expressed in stage-specific manner, as metastatic samples exhibited higher protein expression in comparison to primary stage tissues. To further confirm this result, we analyzed relative mRNA and protein expression of Oct4, Sox2 and Nanog in breast and prostate cancer cell lines. It was observed that Oct4, Sox2 and Nanog were over-expressed in both breast and prostate cancer cell lines in comparison to non-tumorigenic control used. The results clearly established that Oct4, Sox2 and Nanog are over-expressed in breast and prostate cancer and can be considered as oncogenic markers that can facilitate malignant transformation by facilitating inherent tumorigenic properties in breast and prostate tumor cells.

Next, we analyzed the involvement of Oct4, Sox2 and Nanog by conducting a series of gene knockdown experiments using si-RNA against Oct4, Sox2 and Nanog. Tumorigenic properties such as cell proliferation, colony formation, migration and invasion as well as cell cycle deregulation were examined after gene silencing. It was

observed that cell proliferation was drastically reduced in cells treated with si-Oct4, si-Sox2 and si-Nanog with respect to control si-RNA cells indicating that Oct4, Sox2 and Nanog promote cancer cell proliferation and that by preventing their expression, tumor growth can be checked. Secondly, chromatin condensation analysis revealed that after gene silencing of Oct4, Sox2 and Nanog, percentage of condensed nuclei drastically increased and apoptotic DNA damage was greatly enhanced. This suggests that Oct4, Sox2 and Nanog helped in protecting cells against apoptotic death and that down-regulating these factors can reset cellular death in tumor cells. Next, migration and colony forming ability of breast and prostate cancer cells were investigated and results indicated that Oct4, Sox2 and Nanog are responsible for promoting metastatic behavior in tumor cells. Finally cell cycle analysis was done after si-RNA treatment and it was observed that upon inhibition of Oct4, Sox2 and Nanog, G_0/G_1 arrest is induced and apoptotic cell population greatly increased. All of the above findings substantiated the claim that over-expression of Oct4, Sox2 and Nanog in breast and prostate cancer enhanced tumorigenic characteristics such as increased cellular proliferation and cell survival, augmented apoptotic cell population and enhanced metastatic invasive properties. This is for the first time that the role of Oct4, Sox2 and Nanog in maintenance and survival of tumor bulk as opposed to CSC population was investigated. The results further emphasized another fundamental finding that Oct4, Sox2 and Nanog are as actively involved in mediating oncogenic features in non-stem cancer cells as they are known to do in cancer stem cells and tumor-initiating cells.

After establishing that Oct4, Sox2 and Nanog are over-expressed and facilitate malignancy in breast and prostate cancer, we investigated the various epigenetic mechanisms responsible for controlling their behavior. Breast and prostate cancer cells were treated with well-known epigenetic inhibitors AZA (which acts against DNMT1), TSA (which act against HDACs) and DZNeP (which acts against EZH2) and transcript and protein level expression of Oct4, Sox2 and Nanog was analyzed. After treatment with these modulators, mRNA and protein levels of Oct4, Sox2 and Nanog were found to be up-regulated in comparison to untreated cells which suggested that epigenetic modifications play a crucial role in regulating these factors. Promoter DNA methylation was analyzed by bisulphite conversion followed by methyl specific PCR. The promoter of Sox2 was found to be unmethylated and hence DNA methylation induced silencing was found to be lacking. Next, histone enrichment profile containing H3K4me3, H3K9me3

H3K27me3 and H3K9AcS10p in the promoter of Sox2 and Oct4 was analyzed. It was observed that both the promoters were enriched with active histone marks H3K4me3 and H3K9AcS10p and repressive marks like H3K9me3 and H3K27me3 were very low. This proved that the over-expression of Oct4 and Sox2 in breast and prostate cancer was facilitated by active histone modifications like H3K4me3 and H3K9AcS10p in the absence of promoter DNA methylation.

Finally, to investigate the interaction between signaling pathways and pluripotency factors we performed experiments to understand the link between Sox2 and HH pathway in prostate cancer. We provide strong evidence implying that Sox2 is functionally associated with HH signaling in controlling prostate cancer characteristics. Both Sox2 and HH pathway components are over-expressed in prostate cancer and their co-expression is significantly co-related to aggressive properties of advanced prostate cancer. Down-regulating both Sox2 and HH pathway concomitantly results in significant decrease in metastatic behavior, reduced survival capacity and cell proliferation, reduction in migration and invasiveness as well as greater degree of apoptotic death in androgen independent prostate cancer cells, thus affecting all the major hallmarks of aggressive tumor phenotype. In line with other studies, our findings suggest that HH-Sox2 axis is as much crucial for maintenance and survival of tumor bulk as it is for CSC population in prostate cancer. From all the above results and findings, we have clearly explained the role of epigenetic modifications and signaling pathways in influencing pluripotency transcription factors as they mediate tumorigenic activities in case of breast and prostate cancer. In the contemporary field of clinical oncology, novel strategies of targeting cancer cells are in demand. Our project provides one such fresh prospective on therapeutic targeting of malignancy by simultaneously acting against all the three enforcers of malignant phenotype i.e. transcription factors, epigenetic agents and signaling pathways. Based on our findings from the above mentioned objectives, we have tried to formulate a novel strategy and have proposed the following model wherein co-targeting pluripotency inducing TF such as Sox2, epigenetic modifiers and signaling molecules can effectively counteract and restrict oncogenic transformation in solid tumors (Fig. 7.1).

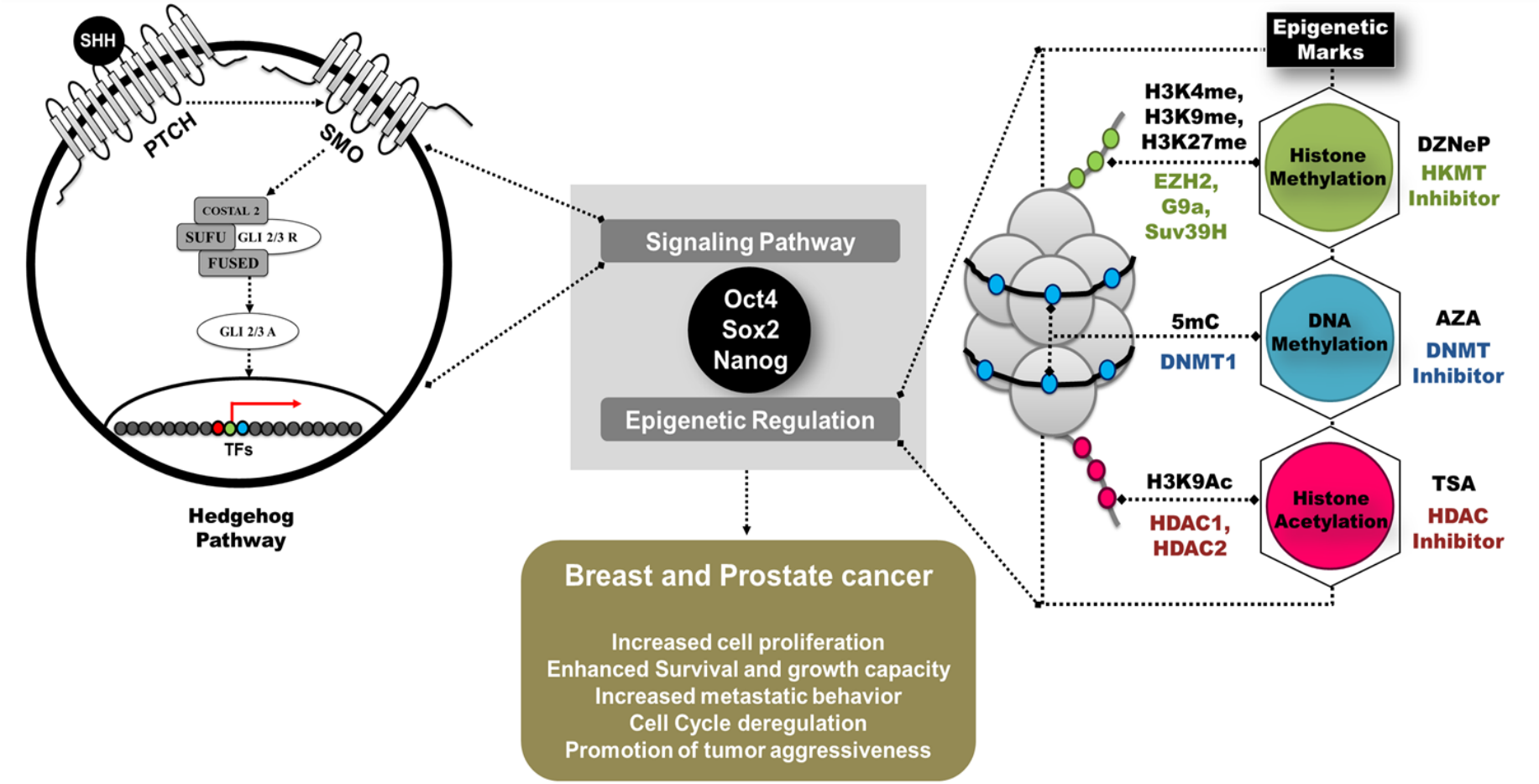


Figure 7.1: A schematic representation of the mechanism by which HH signaling pathway and epigenetic modifications influence pluripotency inducing transcription factors-Oct4, Sox2 and Nanog in breast and prostate cancer.

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List of Publications:

Peer-reviewed Publications

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- **Kar S,** Sengupta D, Deb M, Rath SK, Pradhan N. **(2017)**
  SOX2 function and Hedgehog Signaling Pathway are co-conspirators in promoting Androgen Independent Prostate Cancer
  Biochim Biophys Acta., 1863(1), 253-265

- **Kar S,** Sengupta D, Deb M, Shilpi A, Parbin S, Rath SK, Pradhan N, Rakshit M, Patra SK. **(2014)**
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- **Kar S**, Parbin S, Deb M, Shilpi A, Sengupta D, Rath SK, Rakshit M, Patra A, Patra SK. **(2013)**
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Publications as co-author:

- Patra SK, Sengupta D, Deb M, **Kar S**, Kausar C. **(2017)**
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- Parbin S, Shilpi A, **Kar S**, Pradhan N, Sengupta D, Deb M, Rath SK, Patra SK. **(2016)**
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- Deb M, Sengupta D, Rath SK, **Kar S**, Parbin S, Shilpi A, Pradhan N, Bhutia SK, Roy S, Patra SK. **(2015)**
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- Deb M, **Kar S**, Sengupta D, Shilpi A, Parbin S, Rath SK, Londhe VA, Patra SK. **(2014)**
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- Sengupta D, Deb M, **Kar S,** Shilpi A, Parbin S, Mallick B, Patra SK. **(2014)**
  Epigenetic microRNA Regulation of Multiple Chromatin Functions: A Perspective in Cancer
  Epigenetic Diagnosis & Therapy, DOI: 10.2174/2214083201666131227200815

Conference Proceedings

- **Kar S,** Rath SK, Sengupta D, Deb M, Pradhan N, Parbin S, Shilpi A, Patra SK. **(2015)**
  Exploring the epigenetic profile of SOX2 in prostate cancer cells
  In: Cell Symposium: Stem Cell Epigenetics, Sitges, Spain, 20nd – 22nd September, 2015

- **Kar S,** Deb M, Sengupta D, Shilpi A, Parbin S, Pradhan N, Rath SK, Patra SK. **(2013)**
  MBD proteins as prognostic biomarkers for epigenetic cancer therapy
  In: 100th Indian Science Congress Session, Kolkata, India, 3rd - 7th January, 2013

- **Kar S,** Deb M, Sengupta D, Shilpi A, Patra SK. **(2011)**
  DNA methylation as a prognostic marker: Diagnostic and therapeutic implications in cancer research
  In: 1st Global Cancer Genomics Consortium TMC Symposium, ACTREC, Mumbai, India, 10th - 12th November, 2011

- Patra SK, Deb M, Sengupta D, **Kar S**, Shilpi A. **(2012)**
  Reversible methylation at DNA-cytosine-5-carbon by DNA methyltransferase and possible mechanism of inhibition by 5- aza-2'-deoxycytidine
  In: Biochemical Society Annual Symposium, Cambridge, UK, 10th - 12th January, 2012

- Sengupta D, Deb M, **Kar S,** Shilpi A, Parbin S, Patra SK. **(2012)**
  Expression of DNMT1 and its modulation by epigenetic modifiers in breast cancer cells
  In: International Seminar on Emerging Trends In Cell & Molecular Biology, Jadavpur University, Kolkata, India, 14th December, 2012

- Deb M, Sengupta D, **Kar S,** Shilpi A, Parbin S, Patra SK. **(2012)**
  Clusterin expression and its epigenetic control switch in human breast cancer
  In: 2nd Global Cancer Genomics Consortium TMC Symposium, ACTREC, Mumbai, India, 19th – 21st November, 2012

- Patra SK, Deb M, Sengupta D, **Kar S**, Shilpi A. **(2012)**
  Molecular mechanisms of DNA-methylation during development, ageing and cancer: DNA-methyltransferase and DNA demethylase in action
  In: National Seminar on Current Trends in Chemistry–VI (NSCTC-VI), Kalyani University, West Bengal, India, 2nd March, 2012

Newsletters

- **Kar S,** Patra SK. **(2014)**
  Dynamics of DNA Demethylation and Epigenetic Reprogramming in Stem Cells
  ISSRF (Indian Society for the Study of Reproduction and Fertility) Newsletter, September, 2014

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Cell Culture	:	Culture and maintenance of cancer cell lines, Gene knockdown studies by using anti-sense oligonucleotides and si-RNA, , Cell cycle analysis by FACS, Cell viability Assays, Cell Proliferation and Cytotoxicity assays, Soft agar assays, Wound healing assays, cell study by Fluorescence and Confocal Microscopy, SP characterization, Cancer Stem cell culture for generating CSCs etc.
Biochemistry	:	Quantitative and qualitative estimation of carbohydrates, proteins, nuclei acids, lipids, chromatography techniques, enzyme assays.
Bio-Informatics	:	Meta-analysis of data sets from publicly available databases such as TCGA, Oncomine, cBioportal, KM plotter, GOBO, methHC, USCS genome browser, ENCODE database, Galaxy, Software packages such as Schrödinger, Swissprot, Modeller, Hex, Autodock

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