Novel chromatin-associated role of Protein Kinase C family members in regulating inducible genes and microRNAs during epithelial to mesenchymal transition

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Declaration Statement

This thesis conforms to the Australian National University guidelines and regulations. The work contained within has not been submitted for the purpose of obtaining any other degree at this, or other universities. This thesis is author's own work and has not been published or written by another person except where due references has been stated in the text. All the experimental data presented in this thesis has been performed and analyzed by the author, unless otherwise acknowledged. Dr. Ben Quah (JCSMR, ANU, Canberra) assisted with the initial FACS analysis.

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The Australian National University

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**List of acronyms and abbreviations**

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<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>µM</td>
<td>Micro molar</td>
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<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>3' UTR</td>
<td>3' untranscribed region</td>
</tr>
<tr>
<td>Ac</td>
<td>Acetylated</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<td>Adenosine monophosphate-activated protein kinase</td>
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<td>ANU</td>
<td>Australian National University</td>
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<td>AOL</td>
<td>Amine oxidase-like</td>
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<td>American Type Culture Collection</td>
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<td>ATF3</td>
<td>Activating transcription factor 3</td>
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<td>B, B</td>
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<td>B lymphocyte-induced maturation protein-1</td>
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<td>Big mitogen-activated protein kinase 1</td>
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<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BRF</td>
<td>Biomolecular Resource Facility</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CaMK</td>
<td>Calcium/calmodulin-dependent protein kinase</td>
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<td>REST corepressor</td>
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<td>Cyclin-dependent kinase 1</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<td>CpGIs</td>
<td>CpG islands</td>
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<td>Conventional or classic PKCs</td>
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<td>Cancer stem cell-like</td>
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<td>CSCs</td>
<td>Cancer Stem like Cells</td>
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<tr>
<td>CREB</td>
<td>cAMP response element-binding</td>
</tr>
<tr>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>CtBP</td>
<td>C-terminal binding protein</td>
</tr>
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<td>Cyanine-3</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenyl-indole</td>
</tr>
<tr>
<td>DDW</td>
<td>Distilled deionised water</td>
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<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNMT</td>
<td>DNA methyltransferase enzymes</td>
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<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
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<td>D-PBS</td>
<td>Dulbecco's Phosphate Buffered Saline</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>E8</td>
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<td>E-cadherin</td>
<td>Epithelial cadherin</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
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<td>ER</td>
<td>Estrogen receptors</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>Definition</td>
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</tr>
<tr>
<td>ESA</td>
<td>Epithelial surface antigen</td>
</tr>
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<td>ETaR</td>
<td>Endothelin-A receptor</td>
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<td>EZH2</td>
<td>Enhancer of zeste homolog 2</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>FAM</td>
<td>6-amino-floresceine</td>
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<td>FCS</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
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<td>FITC</td>
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<td><em>green fluorescent protein</em></td>
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<td>G-protein coupled receptor</td>
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<td>Guanosine-5'-triphosphatase</td>
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<td>Hairy/ enhancer of split</td>
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<td>Histone H2A</td>
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<td>Histone acetyltransferase</td>
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<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)</td>
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<td>HIF-α</td>
<td>Hypoxia-inducible factor 1, alpha</td>
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<td>HMLE</td>
<td>Mammary luminal epithelial</td>
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<td>HMT</td>
<td>Histone methyltransferase</td>
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<tr>
<td>hr</td>
<td>Hour(s)</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
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<td>ILK</td>
<td>Integrin-linked kinase</td>
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<td>IP3</td>
<td>Inositol trisphosphate</td>
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<td>JCSMR</td>
<td>John Curtin School of Medical Research</td>
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<td>Jumonji C</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase (pairs)</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton (s)</td>
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<td>MAO</td>
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<td>MAPK</td>
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<td>MCRF</td>
<td>Microscopy and Cytometry Resource Facility</td>
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<td>MET</td>
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<td>MFs</td>
<td>Microfilaments</td>
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<td>MicroRNA</td>
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<td>Micro liter</td>
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<td>MLL1</td>
<td>Mixed lineage leukemia 1</td>
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<td>MMP-2</td>
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<td>mRNA</td>
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<td>MW</td>
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<tr>
<td>MUC1</td>
<td>Mucin 1, cell surface associated</td>
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<td>N-</td>
<td>Amino terminal</td>
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<td>NCoR</td>
<td>Nuclear receptor corepressor</td>
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<td>NCSC</td>
<td>Non-cancer stem cell</td>
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</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>Pit1</td>
<td>Pituitary transcription factor 1</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<td>PLC</td>
<td>Phospholipase C</td>
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<td>PMA</td>
<td>Phorbol 12-Myristate 13-acetate</td>
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<tr>
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<td>RNA polymerase II</td>
</tr>
<tr>
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<td>miRNA precursors</td>
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<tr>
<td>pri-miRNA</td>
<td>Primary miRNA transcripts</td>
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<tr>
<td>PRK1</td>
<td>protein kinase C (PKC)-related kinase 1</td>
</tr>
<tr>
<td>PSN</td>
<td>Penicillin, streptomycin and neomycin antibiotic cocktail</td>
</tr>
<tr>
<td>Pt</td>
<td>Platelets</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real time polymerase chain reaction</td>
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<td>Ras</td>
<td>Renin-angiotensin system</td>
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Rev
Reverse
Rhob
Ras homolog gene family, member B
Rho
Ras homolog gene family
RNA
Ribonucleic acid
ROS
Reactive oxygen species
rpm
Revolutions per minute
RPMI
Roswell Park Memorial Institute
RT
Reverse transcriptase
RTKs
Receptor tyrosine kinases
SDS
Sodium dodecyl sulfate
SE
Standard error
sec
Second(s)
Ser
Serine
Set7/9
Set domain containing (lysine methyltransferase) 7/9
SF/HGF
Scatter factor/hepatocyte growth factor
SSC
Side scatter
SIK1
Salt-inducible kinases 1
siRNA
Small interfering RNA
SIRT1
Silent mating type information regulator 2 homolog 1
Spermidine
N-(3-Aminopropyl)-1,4-butanediamine trihydrochloride
Spermine
N,N'-Bis(3-aminopropyl)-1,4-diaminobutane
Src
Sarcoma
ST
Stimulated
SWIRM
Swi3, Rsc8, and Moira
T
T cell
TAK1
TGFβ-activated kinase-1
TAP
Tandem affinity purification
TBLR1
Transducer β-Like-Related Protein 1
TE
Tris-EDTA buffer
TF
Transcription factor
TGF-β
Transforming growth factor-β
TGFβR
TGFβ receptor
<table>
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<th>Full Form</th>
</tr>
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<tr>
<td>TI</td>
<td>Total input</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor- α</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>Unit(s)</td>
</tr>
<tr>
<td>UPAR</td>
<td>Urokinase-type plasminogen activator receptor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
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<tr>
<td>v/v</td>
<td>Volume per volume</td>
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<tr>
<td>w/v</td>
<td>Weight per volume</td>
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<tr>
<td>Wnt</td>
<td>Wingless-type</td>
</tr>
<tr>
<td>WntR</td>
<td>Wnt receptor</td>
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<tr>
<td>x g</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>YB1</td>
<td>Y-box binding protein</td>
</tr>
<tr>
<td>Zeb1</td>
<td>Zinc finger E-box binding homeobox 1</td>
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<td>Zonula occludens-1</td>
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Abstract

The epithelial to mesenchymal transition (EMT) is a key step in cancer progression and metastasis. A small subpopulation of tumor cells formed thereby, referred to as Cancer Stem Cell (CSC) cells, potentially have a significant role for metastatic tumor initiation and recurrence. The epigenetic regulation underpinning control of gene expression programs in EMT and CSC formation processes are not well understood, largely because an appropriate inducible \textit{in vitro} human model is lacking.

This thesis describes the establishment of a novel human \textit{in vitro} rapidly inducible breast cancer EMT model amenable to detailed analysis of epigenetic processes. The human breast cancer epithelial cell line, MCF-7 was stimulated with the PKC pathway inducer, PMA. This results in the emergence of a significant cancer stem cell-like subpopulation amidst almost complete mesenchymal conversion. The epithelial cells, CSC-like and non-CSC (NCSC) mesenchymal subpopulations were shown to be distinct in their transcriptional programs and microRNA profiles.

The novel role of PKC isoforms as chromatin-associated enzymes in EMT and CSC formation processes has been indentified for the first time in this thesis. Chromatin immuno-precipitation (ChIP) assays revealed that non-phosphorylated PKC-\(\theta\) predominated in the epithelial state across the promoter of uPAR, whilst phosphorylated PKC-\(\theta\) predominated in the mesenchymal state of MCF-7 cells. In contrast, PKC-\(\beta\) recruitment on the uPAR promoter predominated in mesenchymal state with little enrichment detected in the epithelial state. Similarly, phosphorylated H3T6, which is a surrogate for active PKC-\(\beta\) was highly recruited in the mesenchymal state in comparison to epithelial state. Interestingly, inactive forms of PKC-\(\theta\) and
PKC-β form complexes with LSD1 and Zeb1 on the chromatin template in epithelial cells. However, in mesenchymal cells, active PKC-θ switches its association to the active mark Pol II instead of LSD1 and Zeb1. Importantly, the data suggest that although phosphorylated PKC-θ is required for PKC-β recruitment in the mesenchymal state, the two PKC isozymes, PKC-θ and PKC-β play opposing roles in regulating transcription of inducible EMT genes.

For the first time it has been shown that PKC-θ and PKC-β tether to chromatin on the promoters of microRNA 200 family members during the process of the epithelial to mesenchymal transition. Not only does Pol II co-exist with PKC-θ in the mesenchymal state on the miR 200c promoter but, also, epigenetic tags such as LSD1 and Zeb-1 co-exist with Pol II in mesenchymal state, suggesting that “Pol II capturing” occurs in a PKC-θ containing repressive complex. Additionally, it was shown that PKC activity is important in controlling EMT and CSC formation by regulating EMT associated inducible genes and microRNAs.

Collectively, the data presented in this thesis suggest that PKC isozymes might have “dual role” consisting of signal transduction role and chromatin associated role in controlling EMT and CSC formation processes. Furthermore, a previously undescribed layer of chromatin-tethered enzymes with interconnected function regulates transcription of EMT-associated inducible genes and microRNAs, include PKC isozymes, histone demethylases and DNA methyltransferases.
Publications and presentations resulting from this work

Journal Publications


- He, Yi; Sutcliffe, Elissa; Bunting, Karen; Li, Jasmine; Goodall, Katharine; Poon, Ivan; Hulett, Mark; Freeman, Craig; Zafar, Anjum; McInnes, Russell; Taya, Toshiki; Parish, Christopher; Rao, Sudha. Nuclear Heparanase Modulates H3 Methylation at Actively Transcribed Genes via the Interplay with Key Chromatin Modifying Enzymes. Journal of Immunology and Cell Biology. (Submitted, under review 2011).


Conference Presentations


**Fellowships and Awards:**

• Recipient of Ruth Gani Memorial Travelling Fellowship for Human genetics, 2011

• Recipient of Australian Society for Biochemistry and Molecular Biology (ASAMB) Travel bursary, Combio2011.

• Recipient of Travel award TEMTIA-V, 2011.

• Recipient of Vice Chancellor’s Travel fellowship, 2010.

• Recipient of Endeavour Postgraduate Research Fellowship for PhD, 2008.
Chapter One

Introduction (General)

Although it is well established that the onset of most of the human cancers involves metastasis, the biochemical and genetic factors responsible for metastasis itself are only partly identified (Sporn, 1996). To date, at least four inter-related processes have been recognized in metastasis, which are: (1) activation of the epithelial-mesenchymal transition (EMT) within the tumour cells, (2) remodeling of the extracellular matrix (ECM), (3) migration of the tumour cells to specific secondary sites and (4) induction of neo-angiogenesis at the secondary tumour site.

This chapter provides an inclusive overview of the epithelial to mesenchymal transition (EMT) and its role in cancer invasion and metastasis. This literature review also addresses the relationship of EMT with Cancer Stem like Cells (CSCs) formation, epigenetic changes and involvement of histone modifying enzymes during the process of both EMT and CSC. Finally, the rationale of the research project is described and the aims of the project are introduced.
Chapter 1: Introduction

1.1 Epithelial to Mesenchymal Transition (EMT) and its role in cancer

Epithelial to mesenchymal transition is a normal process during embryonic development (Hay, 1995; Perez-Pomares and Munoz-Chapuli, 2002; Thiery and Sleeman, 2006) and a pathological feature in tumorigenesis (Huber et al., 2005; Mani et al., 2008). EMT is a sequence of events in which cell-cell and extracellular matrix (ECM) contacts are changed to let loose epithelial cells from the surrounding tissue. The cytoskeleton is restructured to confer the ability to move through a three dimensional ECM and a new transcriptional program is induced to retain the mesenchymal phenotype (Radisky, 2005).

1.1.1 Definition of EMT

In mammals, two key cell types are epithelial and mesenchymal cells. Main characteristics of the epithelial cells are: (i) interactions among cells are cohesive, which facilitate the formation of continuous cell layers; (ii) three membrane domains are present: apical, lateral and basal; (iii) tight junctions are present between apical and lateral domains; (iv) existence of apicobasal polarized distribution of the various organelles and cytoskeleton components; and (v) there is lack of mobility of individual epithelial cells with respect to their local environment (Larue and Bellacosa, 2005).

Mesenchymal cells differ from epithelial cells as they have: (i) loose or no interactions among the cells, so there is no continuous layer of cells; (ii) there is not any clear apical and lateral membranes; (iii) apicobasal polarized distribution of organelles and cytoskeleton does not exist; and (iv) these are motile cells with invasive properties. Certain cells change from epithelial to mesenchymal status by a well coordinated process defined as epithelial to mesenchymal transition (EMT). In some cases, EMT could be reversible and cells undertake the
reciprocal mesenchymal to epithelial transition (MET). Figure 1 show a schematic view of structural features associated with epithelial and mesenchymal cells during EMT and MET (Boyer et al., 2000; Larue and Bellacosa, 2005). Schematic representation of the changes in cell morphology and cell-cell contact induced by EMT has been shown in Figure 1.1.

1.1.2 EMT in development

Most adult tissues and organs arise from a series of interconversions between epithelium and mesenchyme during early development in mammals and the mesoderm generated by EMTs develops into multiple tissue types leading to the generation of key epithelial organs, such as the kidney and ovary, via METs (Davies, 1996). Additionally, several other EMT conversions occur later during embryonic development. For example, formation of neural crest cells from the neural tube on embryonic day 8(E8), formation of the atrial and ventricular mesenchymal septa from the endothelium during heart development on E8; formation of the sclerotome from somites on E9; formation of coronary vessel progenitor cells from the epicardium around E10-11; formation of palate mesenchymal cells from the oral epithelium on E13.5; and formation of mesenchymal cells during regression of mullerian tract on E15 (Larue and Bellacosa, 2005). Schematic representation of the role of EMT in development that gives rise to various organs is shown in Figure 1.2. The transition occurring during development involves mesenchymal cells which acquire a morphology that is appropriate for migration in an extracellular environment and settlement in areas that are involved in organ formation, which involves interactions between epithelial and mesenchymal transition. Mesenchymal cells then later participate in formation of epithelial organs through mesenchymal-epithelial transition (MET) (Thiery, 2002). In summary, it appears that during development, EMT is tightly regulated and also requires discontinuing the EMT process in order to form epithelial organs by the process of MET.
Figure 1.1  EMT induces major changes in cell morphology and cell–cell contacts.

A schematic drawing of EMT highlighting the changes in cell shape and loss of intercellular connections including desmosomes, gap junctions and tight junctions. Figure adopted from Boyer et al., 2000.
Figure 1.2  Primary EMT give rise to progenitors of many organs and tissues.

(A) Epiblast cells that internalize at gastrulation give rise to different mesodermal and endodermal populations from which a variety of cell types form. Embryogenic cells undergoing EMT are shown in green. Pt, platelets; B, T and NK, lymphocytes; G, granulocytes; M, macrophages. (B) In turn, the neural crest delaminates from the dorsal neural tube and will generate neurons of the peripheral nervous system, glial and satellite cells, pigment cells, odontoblasts, and craniofacial cartilage, as well as other cell types. Figure adopted from Acloque et al., 2009.
1.1.3 EMT and cancer

As described in section 1.1, during embryogenesis and homeostasis, EMT plays critical roles (Shook and Keller, 2003). Nevertheless, if deregulated, it can disrupt normal epithelial homeostasis and can contribute to pathogenesis such as fibrosis and cancer metastasis. EMT increases the motility and invasiveness of cancer cells during tumorigenesis, and malignant transformation may be associated with distinct and specific signaling pathways promoting EMT (Boyer et al., 2000). Various EMT processes occurring during cancer progression, closely resemble to those occurring in normal development. However, it appears that important differences exist between normal and physiopathological EMT. Normal development and pathological processes principally differ as cellular and molecular events occur in highly controlled and spatio-temporally regulated during development, however during cancerous transformation the events follow a stochastic and time-independent order, or some events may be skipped (Boyer et al., 2000). In addition, it is clear now that the molecular program leading to EMT during tumour progression is characterized by augmentation of only some features of complete EMT in development (Boyer et al., 2000). A schematic representation of EMT in cancer metastasis has been shown in Figure 1.3.

1.1.4 EMT in other diseases

It has been recognized that EMT also occurs in response to epithelial stress or injury in many adult tissues such as kidney and eye (Kalluri and Neilson, 2003; Liu, 2004; Saika et al., 2004). Injury to lens epithelial cells and retinal pigment epithelial cells, results in EMT which leads fibrosis (Hales et al., 1994; Saika et al., 2004; Zuk and Hay, 1994). Several animal models and diseased human kidney biopsies have been confirmed for renal fibrosis (Iwano et al., 2002; Rastaldi et al., 2002; Strutz et al., 1995; Zeisberg and Kalluri, 2004). There is accumulating evidence to show that EMT could be source of myofibroblasts in vivo. Iwano et al. in their study showed that genetically tagged proximal
Figure 1.3  Sites of EMT and MET in the emergence and progression of carcinoma.

Normal epithelia lined by a basement membrane can proliferate locally to give rise to an adenoma. Further transformation by epigenetic changes and genetic alterations leads to a carcinoma in situ, still outlined by an intact basement membrane. Further alterations can induce local dissemination of carcinoma cells, possibly through an epithelial-mesenchymal transition (EMT), and the basement membrane becomes fragmented. The cells can intravasate into lymph or blood vessels, allowing their passive transport to distant organs. At secondary sites, solitary carcinoma cells can extravasate and either remain solitary (micrometastasis) or they can form a new carcinoma through a mesenchymal-epithelial transition (MET). Figure adopted from Thiery, 2002.
tubular epithelial cells gave rise to up to 36% of interstitial fibroblasts via EMT following unilateral-urethral obstruction, which is a model of acute renal injury (Iwano et al., 2002). Exposure to environmental stresses such as hypoxia (Manotham et al., 2004), reactive oxygen species (Radisky et al., 2005; Rhyu et al., 2005), exposure to advanced glycation end products (Oldfield et al., 2001), and treatment with a variety of cytokines and growth factors, also result in EMT.

1.1.5 Characteristics of EMT/Signaling of EMT

Epithelial cells convert into mesenchymal cells by the process of EMT. The term EMT describes a series of events during which epithelial cells lose many of their epithelial characteristics and obtain properties that are typical of mesenchymal cells. This EMT process requires complex changes in cell architecture and behavior, which has been shown schematically in Figure 1.4. It has been demonstrated that a complex network of extracellular activators trigger EMT and extensive crosstalk occurs between the signaling pathways that activate and repress EMT genes. EMT-inducing signaling pathways have many common endpoints, including downregulation of E-cadherin and induced expression of EMT associated genes (Savagner, 2001; Thiery, 2002, 2003; Thiery and Sleeman, 2006). Schematic representation of the signaling pathways that are activated by regulators of EMT and their crosstalk has been shown in Figure 1.5. During the transition process of epithelial to mesenchymal the “EMT proteome” emerges, that reflects a fundamental change in the proteins gained, maintained or lost (Kalluri and Neilson, 2003). Various studies suggest that proteins gained or maintained during this process are Snail1, Slug, Zeb1, Zeb2, Rhob, TGF-β, FSP-1, MMP-2, MMP-9, Vimentin, Fibronectin, HIF-α, Twist, Collagen type I and III, while protein whose expression becomes attenuated are E-cadherin, β-catenin, desmplakin, MUC-1, ZO-1, uPAR and cytokeratin-18 (Carver et al., 2001; Gregory et al., 2008; Ip and Gridley, 2002; Lester et al., 2007; Radisky et al., 2005; Ramaswamy et al., 2003; Zavadil et al., 2001). Many studies have focused mainly on E-cadherin or Snail1 expression and therefore more comprehensive studies on EMT process need to be carried out (Kalluri and Neilson, 2003).
Figure 1.4  The cycle of epithelial-cell plasticity.

The diagram shows the cycle of events during which epithelial cells are transformed into mesenchymal cells and vice versa. The different stages during EMT (epithelial–mesenchymal transition) and the reverse process MET (mesenchymal–epithelial transition) are regulated by effectors of EMT and MET, which influence each other. Important events during the progression of EMT and MET, including the regulation of the tight junctions and the adherent junctions, are indicated. A number of markers have been identified that are characteristic of either epithelial or mesenchymal cells and these markers are listed in BOX 1 and BOX 2. E-cadherin, epithelial cadherin; ECM, extracellular matrix; FGFR2, fibroblast-growth-factor receptor-2; FSP1, fibroblast-specific protein-1; MFs, microfilaments. Figure adopted from Thiery and Sleeman, 2006.
Figure 1.5  Overview of the molecular networks that regulate EMT.

A selection of the signaling pathways that are activated by regulators of EMT and a limited representation of their crosstalk is illustrated. Activation of receptor tyrosine kinases (RTKs) is known to induce EMT in several epithelial cell types and in vivo, but it is now clear that the EMT process often requires co-activation of integrin receptors. The role of transforming growth factor-β (TGF-β) signaling in EMT is established for a limited number of normal and transformed cell lines, whereas in vivo data has indicated a mutual regulation of the TGF-β and NOTCH pathways during EMT. There is now increasing evidence that other signaling pathways could have an important role in EMT, including G-protein-coupled receptors. Matrix metalloproteinases (MMPs) can also trigger EMT through as-yet-undefined receptors. ETαR, endothelin-A receptor; FAK, focal adhesion kinase; GSK3β, glycogen-synthase kinase-3β; H/E(Spl), hairy/ enhancer of split; ILK, integrin-linked kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PAR6, partitioning-defective protein-6; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase-B; ROS, reactive oxygen species; TAK1, TGF-β-activated kinase-1; TGFβR, TGF-β receptor; WntR, Wnt receptor. Figure adopted from Thiery and Sleeman, 2006.
EMT is triggered by interplay of extracellular signaling, including components of extracellular matrix (ECM), such as collagen and hyaluronic acid (Zoltan-Jones et al., 2003), as well as soluble growth factors, such as members of TGF-β and fibroblast growth factor (FGF) families, epidermal growth factor (EGF) and scatter factor/hepatocyte growth factor (SF/HGF) (Zavadil and Bottinger, 2005). These ligands trigger activation of other intracellular effector molecules, such as small GTPase family members- Ras, Rho and Rac- and members of the Src tyrosine-kinase family. These effectors then initiate disassembly of junctional complexes and also the changes in cytoskeletal organization. At the same time, activation of signaling pathways also lead to transcriptional activation of EMT regulators such as snail or SNAIL1 (Barrallo-Gimeno and Nieto, 2005) and slug or SNAIL2, which regulate the alteration in gene expression program that underlie EMT.

E-cadherin represents one of the best characterized epithelial marks and is a central target of many transcriptional regulators as it gets repressed upon activation of EMT transcriptional regulators. Down-regulation of EMT results in the loss of E-cadherin-dependent intercellular epithelial junctional complexes, and E-cadherin mediated sequestration of β-catenin in the cytoplasm is inhibited. This results in nuclear translocation of β-catenin, which feeds into the Wnt signaling pathway by transcriptional activation of LEF/TCF4 (lymphoid-enhancer-binding factor/T-cell-factor-4) (Kim et al., 2002).

Snail1 has been shown to have a central role in regulation of EMT and different phosphorylation events regulate snail1 stability, subcellular localization and function. Other EMT regulating pathways such as AKT/PKB (protein kinase B), Wnt and Hedgehog pathway work through inhibiting glycogen-synthase kinase-3β (GSK3β) (Grile et al., 2003; Polakis, 2000), which phosphorylate a number of transcription factors including p53, Myc and nuclear factor of activated T cells (NFAT) (Beals et al., 1997). Phosphorylation of two serine residues on Snail1 occurs by virtue of GSK3β and one of these serine phosphorylation targets Snail1 for ubiquitination and subsequent degradation, while the other
promotes nuclear transportation (Zhou et al., 2004). Inhibition of GSK3β results in enhanced cellular levels of Snail1 along with concomitant downregulation of E-cadherin (Zhou et al., 2004). Activators of EMT such as EGF also signal to Snail1 through p21-activated kinase-1 (PAK1) (Yang et al., 2005). PAK1 could also phosphorylate Snail1 on a serine residue which is different from the residue phosphorylated by GSK3β. This results in accumulation of Snail1 in the nucleus followed by Snail1-mediated transcriptional repression of target genes.

During the EMT process, both regulations of integrin-mediated contacts as well as the separation of cell-cell contacts takes place and extensive crosstalk between integrin signaling and pathways that regulate EMT has been observed. For example, the TGF-β signaling through p38 MAPK that is required for EMT is dependent on signaling by β1 integrin (Bhowmick et al., 2001). It is the crosstalk between integrins and E-cadherin that co-ordinates the switch from cadherin to integrin-mediated adhesions during EMT (Gimond et al., 1999; Li et al., 2003; Oloumi et al., 2004; Yang et al., 2005).

1.1.6 Models to study EMT

Classic molecular and cell-biology techniques have helped in understanding some of the molecular mechanisms underlying EMT. However, EMT is a very dynamic process that involves many overlapping regulatory pathways as well as intra-and intercellular events, and therefore interdisciplinary approaches are required to understand the complex regulation of EMT.

Several in vitro models of EMT have been described that have involved utilization of human mammary epithelial cells or human breast cancer cell lines (Kokkinos et al., 2007). Recent studies suggest that thousands of genes may contribute to breast cancer pathophysiologies when deregulated by genomic or epigenomic events. There have been studies to define a “system” to appraise the functional contributions of these genes to breast cancer subsets (Neve et al., 2006). In this study, the recurrent genomic and transcriptional characteristics of 51 breast cancer cell lines were found to mirror those of 145 primary breast tumours, although some significant differences were
documented. The cell lines that comprise the system also exhibit the substantial genomic, transcriptional, and biological heterogeneity found in primary tumours (Neve et al., 2006).

A larger transcript analysis has been done by gene array technology on breast cancer cell lines (Blick et al., 2008). This analysis revealed a strong difference between the more epithelial (also called Luminal cells) cells such as MCF-7, T47D and more mesenchymal lines (also called Basal cells) such as Hs578T, BT-549 (Ross et al., 2000) and MDA-MB-435 (Rae et al., 2007) based on their transcriptome. Using the expression of most widely-used markers of EMT (Vimentin, E-cadherin, fibronectin) (Neve et al., 2006), Blick et al., categorized human breast cancer cell line into three subgroups-(i) luminal or epithelial, (ii) basal A (between epithelial and mesenchymal) and (iii) basal B or more mesenchymal (Blick et al., 2008). Gene expression levels for common EMT markers in 51 breast cancer cell lines and their categorization of luminal (epithelial) or basal (mesenchymal) cell type have been shown in Figure 1.6.

Studies by Zajchowski et al. established an association between mesenchymal status and invasiveness (Zajchowski et al., 2001). A mesenchymal gene signature dominated by 24 genes has also been indicated as invasiveness prediction tool of human breast cancer cell lines, whereas high levels of cytokeratins 18 and 19 and plakoglobin, amongst other epithelial markers, predicted non-invasiveness. Other studies have also categorised gene array data into invasive/mesenchymal-like/basal breast cancer cell lines and Luminal/epithelial properties (Lacroix and Leclercq, 2004; Lombaerts et al., 2006).

Epithelial breast cancer cell lines, showing luminal or epithelial phenotype seem to be prone for EMT changes in the presence of appropriate inducers and may obtain a mesenchymal phenotype. This ability of epithelial cells to transform has been exploited for establishing in vitro models of EMT. For example, MCF-7 cells are one of the well accepted model of estrogen receptor (ER)-positive, epithelial
Figure 1.6  Gene expression levels for common EMT markers in 51 breast cancer cell lines. Median centered mRNA expression levels for (a) Vimentin, (b) E-Cadherin, (c) N-Cadherin and (d) fibronectin are shown on a log2 scale. The 51 cell lines are organized by subclass defined in Neve et al., 2006 (Neve et al., 2006). For genes represented by multiple probesets on the arrays, the probeset with the greatest standard deviation across samples was selected. Figure adopted from Blick et al., 2008.
or "luminal" type breast cancer that have been shown to undergo EMT changes in response to oestrogen (Planas-Silva and Waltz, 2007) and the cell adhesion molecule, L1 (Shtutman et al., 2006).

Apart from epithelial or luminal breast cancer cells, basal-B cells, showing phenotype between epithelial and mesenchymal transition have also been shown to be exploited for studying EMT. For example, MDA-MB-468 cells (categorized as basal-B) have been shown to undergo EMT induced by hypoxia, where expression of urokinase-type plasminogen activator receptor (uPAR) and snail were increased. Some reports have compared epithelial cells with mesenchymal cells to demonstrate EMT changes (Gregory et al., 2008). Other EMT models using epithelial cancer cells include a well characterized dog cell line model utilizing MDCK cells, where Pez overexpression transforms normal epithelial morphology of MDCK cells into a mesenchymal phenotype by inducing TGF-β pathway (Gregory et al., 2008; Wyatt et al., 2007). Although TGF-β induced MDCK model remains an invaluable model of EMT but there are many caveats of this model which include (i) difficulty in transferring the findings from dog to human and (ii) the initial events triggering EMT cannot be studied as TGF-β leads to a delayed induction of EMT. Therefore it appears that there is an immediate need for developing a human in vitro model where EMT could be rapidly induced. There is an existing TGF-β induced human mammary luminal epithelial (HMLE) cell model to study EMT but in this model rapid EMT induction does not take place either (Mani et al., 2008).

1.2 Cancer Stem Cells or cancer stem-like cells (CSC)

To give rise to the outgrowth of metastatic tumour in new organ environment, cancer cells have to overcome various types of stresses that may lead to cell death such as loss of adhesion, nutrient depletion, and hypoxia (Bao et al., 2004). Current evidences support the fact that
overwhelming majority of cells that shed from the primary tumour and disseminate to distant secondary sites lack the capacity to self-renew and the ability to form macroscopic metastasis in the new microenvironment. However, a small subset of tumour cells that have stem-like properties can potentially self-renew. This subset, referred to as Cancer Stem Cells (CSCs), known also as "tumour-initiating-cells" or tumorigenic cells, differentiate into heterogeneous populations of cancer cells (Brabletz et al., 2005; Jordan et al., 2006; Li et al., 2007c; Reya et al., 2001), and seed new tumour in a xenotransplant system (Gupta et al., 2009a; Mani et al., 2008; Ouyang et al., 2010). Studies so far show that CSCs normally have features associated with mesenchymal cells and also play a critical role in tumour initiation, growth, metastasis and therapeutic resistance (Bao et al., 2006; Gupta et al., 2009a; Mani et al., 2008).

1.2.1 The cancer stem cell-debate: Myth or reality?

There are studies that have described stem cells in normal tissues, which are capable of renewing themselves through asymmetrical division and at the same time producing progenitor cells whose descendants may eventually differentiate and carry out tissue specific functions (Reya et al., 2001). The concept of stem cells have resulted in emergence of the cancer stem cell hypothesis more recently (Brabletz et al., 2005; Jordan et al., 2006; Li et al., 2007c; Reya et al., 2001), which suggests that cancers are derived from a stem cells compartment in a multi-step process involving the accumulation of mutations in a variety of oncogenes and tumour suppressors (Cho and Vogelstein, 1992; Fearon and Vogelstein, 1990).

There are numerous evidences to support cancer stem cell hypothesis. Firstly, epithelial stem cells are long-term inhabitants of exposed epithelial tissues; they are exclusively susceptible to the accumulation of oncogenic lesions (Miller et al., 2005) and this imitates their favored sites for carcinogenesis. Secondly, these epithelial stem cells share many features with carcinoma cells,
including immortality, absence of contact inhibition and self-renewal. Thirdly, some reports also suggest that the signaling pathways that regulate self-renewal in normal stem cells, become deregulated in cancer stem cells and this is the reason for uncontrolled expansion, abnormal differentiation and formation of tumour with heterogeneous phenotypes (Al-Hajj et al., 2004). Therefore, for conceptual purposes, cancer could be considered as an abnormal organ where growth is determined by atypical sub-populations of the cancer stem cells and differentiated non-tumorigenic cancer cells depending upon their location in the tumour (Al-Hajj et al., 2004; Brabletz et al., 2005; Cho and Vogelstein, 1992; Fearon and Vogelstein, 1990; Hamburger and Salmon, 1977; Locke et al., 2005; Reya et al., 2001).

Several reports have suggested that 25% of the cancer cells within tumour have the properties of CSC (Kelly et al., 2007; Quintana et al., 2008). These findings have lead to controversies whether CSCs exist only as rare subpopulations within tumours and also have raised questions about the general applicability of the CSC model and the very existence of CSCs. Some of the dispute surrounding CSC model seems to start from the confusion regarding the definition of CSC and therefore objections against use of the term CSC. Normal stem cells are usually oligo-or multipotent cells but unlike them, it is unclear whether CSCs can give rise to multiple differentiated cell types or not. However it should be noted here that most essential aspects of stem cell model are capacity for self-renewal, tissue regeneration and giving rise to non-stem cells. In other words, there is nothing intrinsic to the dynamics of normal stem cells that limits the use of the term ‘stem cell’ to define the cells which are oligo-potent (Gupta et al., 2009a). A second key controversy to the CSC model is that it is unclear if normal cellular precursors of the CSCs are in fact, bona fide stem cells. However, it has been established that the characters used to define CSCs are independent of the knowledge about their cellular origins within normal
tissues. Therefore, the CSC model must be judged on the basis of experimental characterizations of the cancer cell populations (Gupta et al., 2009a).

1.2.2 Molecular and cellular characteristics of cancer stem cells

CSC demonstration in cancer cell populations is usually based on the ability to seed tumour at limiting dilutions in vivo. There are certain in vitro properties of CSCs. First, for solid tumors and cancer cell lines, the repertoire of cell surface markers currently used to identify human cancer stem cells includes CD44, CD133, epithelial surface antigen (ESA), and CD24, either singly or in combination. Specifically, the CD44⁺ phenotype is correlated positively with colon, breast, prostate, and pancreatic cancer initiator cells (Al-Hajj et al., 2003; Li et al., 2007b; Maitland and Collins, 2005; O'Brien et al., 2007). Second, CSC-enriched populations have the ability to form spherical colonies in suspension cultures, named tumour mammospheres or tumorspheres (Dontu et al., 2003). Third, CSC-enriched populations show enhanced resistance to chemotherapeutic agents (Bao et al., 2006; Dean et al., 2005; Woodward et al., 2007) and ionizing radiation (Diehn and Clarke, 2006; Woodward et al., 2007).

In breast cancer stem cells, the characteristic surface markers that have been described so far include CD44<sup>high</sup>/CD24<sup>low</sup>, Lin⁻ and ESA (Al-Hajj et al., 2003; Sheridan et al., 2006). More recently, high aldehyde dehydrogenase 1 (ALDH1), which is not a surface marker has been identified as being characteristic marker for breast cancer stem cells (Ginestier et al., 2007; Neumeister et al., 2010; Neumeister and Rimm, 2010).

1.2.3 EMT and cancer stem cells

Since, the cancer stem cell hypothesis does not stipulate the cell of origin for particular cancer, there could be two logical hypotheses, first-tumour may originate from transformation of the normal adult tissue stem cells or second-tumour originate from more differentiated progenitors
that have acquired self-renewal capabilities (McDermott and Wicha, 2010). Importantly, evidence connecting EMT to the emergence of stem cells has been reported by two groups (Mani et al., 2008; Morel et al., 2008). These studies show that induction of EMT by ectopic expression of Snail1, twist or TGF-β treatment (Mani et al., 2008) or activation of Ras-MAPK pathway (Morel et al., 2008) not only results in enhanced migratory and invasive potential of breast cancer epithelial cells, but also significantly enhances their self-renewal, tumour initiating capabilities and expression of breast cancer stem cell markers. Additionally, chronic over-expression of the homeobox protein Six1 in the mouse mammary gland generated highly aggressive tumour with an EMT phenotype, stem cell features and activated Wnt signaling, providing evidence for emergence of cells with combined EMT/CSC phenotypes in vivo (McCoy et al., 2009). Schematic depiction of how EMT process is linked with cancer stem cell formation is shown in Figure 1.7.

More recently, it has been shown that induction of EMT in transformed HMLER breast cancer cells by knock-down of E-cadherin expression displayed an increased population of CD44$^{\text{high}}$/CD24$^{\text{low}}$ cells, and enhanced mammosphere-forming ability compared to their epithelial phenotypic cells (Gupta et al., 2009b). More importantly, they found that cells undergoing EMT demonstrated an increased drug resistance associated with CSCs signatures (Gupta et al., 2009b). These reports strongly suggest that the induction of EMT could generate stem-like cells; however, the molecular mechanisms responsible for such processes are not fully understood.
Figure 1.7 Epithelial–mesenchymal transition and stem cell traits in breast cancer progression.

Breast tumors may originate from the transformation of normal adult tissue stem cells or from more differentiated progenitors that have acquired self-renewal capabilities (left panel). Moreover, a subset of resident mammary gland stem cells (MaSCs, in blue) exhibit epithelial–mesenchymal transition (EMT) features a priori. The EMT features of metaplastic and claudin-low breast tumors may thus signify either that they derive from cells that have undergone EMT or that they originate from deregulated expansion of a pre-existing stem cell pool that expresses EMT-associated markers. Additionally, the induction of sporadic EMT within a tumour bestows migratory and invasive potential coupled with self-renewal capabilities to cancer cells, generating cancer stem cells (CSCs) (right panel). Following extravasation and upon encountering an altered local microenvironment, CSCs (in red) may at least partially revert to an epithelial phenotype (mesenchymal–epithelial transition (MET)) to allow adhesion and proliferation at distal sites. As sporadic EMT and MET are triggered by extracellular stimuli and microenvironment factors, this model provides a plausible explanation for the de novo generation of CSCs from differentiated tumour cells and suggests that passage through EMT and MET is an alternative and/or additional driving force in breast tumorigenesis. Figure adopted from May et al., 2011.
1.3 Epigenetics

Epigenetics is defined as heritable changes in gene activity and expression that do not involve any alteration in DNA sequence (Bird, 2007; Goldberg et al., 2007). One of the best examples of an epigenetic alteration is the differentiation process in which cells carrying identical DNA differentiate into different cell types. Genomic imprinting, which results in monoallelic expression or X chromosome inactivation in female mammalian cells, is also referred to the as an epigenetic phenomena. Epigenetic alterations are preserved during the cell division (Jaenisch and Bird, 2003). Therefore, epigenetic modulation of expression works as a link between genotype and phenotype (Bernstein et al., 2007; Jaenisch and Bird, 2003; Reik, 2007). Different epigenetic phenomena are associated principally due to the fact that DNA exists as chromatin, a close complex with histones (also histone variants) and other chromatin related proteins such as chromatin remodelling proteins. Epigenetic information is mainly stored as chemical modifications to cytosine bases and to the histone proteins. These chemical modifications control chromatin structure and accessibility of DNA. Recently, small non-coding RNAs have been shown to have a major role in the targeting of chromatin-modifying effectors to specific loci on chromatin. In the past decade, epigenetic processes have been increasingly recognized as major contributing factors in human diseases (Esteller, 2008; Feinberg and Tycko, 2004; Ozanne and Constancia, 2007) and consequently, also as therapeutic targets for many diseases (Fiskus et al., 2009; Ganesan et al., 2009; Karberg, 2009). For didactic purposes, epigenetic modifications are grouped into four major categories: DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs (Portela and Esteller, 2010). Also there is an emerging fifth category that includes chromatin bound enzymes such as signalling kinases. This includes Protein Kinase C family members, which are described in section 1.4 of
this thesis. In the current section, chromatin structure and epigenetic modifications have been
described in detail with a focus on their role in transcriptional regulation.

1.3.1 Eukaryotic chromatin structure

In eukaryotic cells genomic DNA is packed along with histones into protein/DNA complexes
called chromatin. The nucleosome is the basic unit of chromatin and a nucleosome is
composed of ~147 base pairs of DNA wrapped around an octamer of the four core histones
(H2A, H2B, H3, and H4) (Figure 1.8). These core histones are tightly packed in globular regions
and have amino-terminal tails that extend outwards from the globular region, making them
accessible to histone modifying enzymes (Luger et al., 1997). Another histone protein, termed
a linker histone H1, interacts with the DNA between nucleosomes. This linker histone is
important for the compaction of chromatin into higher-order structures that comprise
chromosomes. In a non-dividing cell, chromatin is classified into two functional states:
euchromatin or heterochromatin. Euchromatin, which accounts for a less than 4 % of the
genome is the form where DNA is accessible and in an open conformation due to the relaxed
state of nucleosome arrangement. Euchromatin contains genes in both active and inactive
transcriptional states (Koch et al., 2007). Some of these genes are ubiquitously expressed
(housekeeping genes); which others are either developmentally regulated or follow stress-
induced regulation in response to environmental cues. Conversely, heterochromatin
constitutes genomic DNA that exists in a highly condensed packaged unit and, therefore, it
becomes inaccessible to transcription factors or chromatin-associated proteins (Huang et al.,
2004; Jenuwein and Allis, 2001; Talbert and Henikoff, 2006). Heterochromatin mainly consists
of non-coding genes, repetitive sequences and the repressed genes associated with
morphogenesis or differentiation (imprinting or X chromosome inactivation) (Feinberg and
Figure 1.8  **Higher order structuring of chromatin and structure of nucleosome.**

DNA compaction within the interphase nucleus (depicted at left) occurs through a hierarchy of histone-dependent interactions that can be subdivided into primary, secondary, and tertiary levels of structure. Strings of nucleosomes compose the primary structural unit. Formation of 30-nm fibers through histone tail-mediated nucleosome-nucleosome interactions provides a secondary level of compaction, whereas tail-mediated association of individual fibers produces tertiary structures (such as chromonema fibers). Figure adopted from Horn and Peterson, 2002.
Tycko, 2004; Reik, 2007). Heterochromatin also plays a critical role in regulating chromosomal stability and the prevention of mutations and translocations (Huang et al., 2004; Muegge, 2005).

1.3.2 DNA methylation

In mammalian cells, DNA methylation occurs at the 5’ position of the cytosine ring within CpG dinucleotides via addition of a methyl group to create a 5-methylcytosine (m5C) (Figure 1.2). A family of DNA methyltransferase enzymes (DNMTs) has been shown to have role in DNA methylation and its maintenance (Bestor, 2000; Chen and Li, 2004). DNMT3a and DNMT3b function primarily as “de novo” methyltransferases, targeting unmethylated CpGs to initiate methylation. The process of de novo methylation has been reported to occur in early embryonic stem cells and also as a central mechanism of regulation in cancer cells (Okano et al., 1999). In contrast, ubiquitously expressed DNMT1 mainly acts as a “maintenance” methyltransferase and has specificity for hemi-methylated CpG dinucleotide motifs. DNMT1 recognizes hemi-methylated CpGs after DNA replication and copies DNA methylation patterns to a newly synthesized DNA strand based on the DNA methylation pattern in the complementary template strand. This makes it an important element in the inheritance of DNA methylation patterns during DNA replication (Groth et al., 2007; Li et al., 1992).

CpGs are found to cluster in regions termed CpG islands (CpGIs). CpGIs are characterized by more than 50% (G+C) and CpG content, spanning at least 200 bases. On a genome wide scale, methylated DNA is enriched at non-coding regions (e.g., centromeric heterochromatin) and interspersed at repetitive elements (transposons), consequently linked to transcriptional silencing and formation of heterochromatin (Feinberg and Tycko, 2004). However, in euchromatin, CpGIs are found at 60% of the 5’ends of many genes. CpG methylation at the gene promoter-associated regions is considered as a critical factor for the control of gene silencing (Huang et al., 2004; Muegge, 2005). The 5’ regions of genes involved in imprinting, X chromosome inactivation, and tissue-specific
differentiation are hypermethylated, whereas the 5' regions of most housekeeping genes and many regulated genes are frequently unmethylated, which leaves them accessible to transcription factors and chromatin-associated proteins (Jones and Baylin, 2007; Laird, 2003).

1.3.3 Histone modifications

Histones are key players in the epigenetic process and all histones are subject to post-transcriptional modifications. Several types of post-translational modifications occur in the N-terminal histone tails, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP ribosylation (Kouzarides, 2007; Ruthenburg et al., 2007). Site-specific combinations of histone modifications, which have been termed “histone code”, have important roles in biological functions, such as transcriptional activation/repression, DNA repair (Huertas et al., 2009), DNA replication, alternative splicing (Luco et al.), histone deposition, mitosis/meiosis, chromatin condensation (Kouzarides, 2007) and X inactivation (Peterson and Laniel, 2004). In contrast to DNA methylation, which is relatively stable, histone modifications are much more dynamic and respond to hormonal signals, environmental factors or drug treatment (Jones and Martienssen, 2005).

1.3.4 Two key histone modifications, acetylation and methylation

It has been over four decades since the importance of histone acetylation and methylation was discovered and shown to have a role on gene regulation (Allfrey et al., 1964). Although the significance of most of histone modifications are yet to be fully understood, lysine acetylation and methylation are considered to be major modulatory marks for transcriptional activation or repression (Kouzarides, 2007). Schematic depiction of the principal histone modifications is presented in Figure 1.9.
Figure 1.9  Histone modifications.

All the histones are subject to post-transcriptional modifications, which mainly occur in histone tails. The main post-transcriptional modifications are depicted in this figure: acetylation (blue), methylation (red), phosphorylation (yellow) and ubiquitination (green). The number in grey under each amino acid represents its position in the sequence. Figure adopted from Portela and Esteller, 2010.
In 1996, the first nuclear histone acetyltransferase (HAT) Gca5 was identified which had previously been characterized as a transcriptional co-activator protein. Subsequently, a variety of other transcriptional co-activators, such as CREB binding protein (CBP)/p300 were found to have intrinsic HAT activity, and many co-repressors, such as Rpd3, were found to have histone deacetylase (HDAC) activity (Peterson and Laniel, 2004). Acetylation of lysine residues at the N-terminus of histone tails is connected with transcriptional activation by directly affecting chromatin structure (Feinberg and Tycko, 2004). Acetylation abolishes positive charges of the lysine residues and reduces the affinity between histones and the negatively charged DNA-phospho diester backbone, thereby opening the condensed chromatin structure to allow transcriptional machinery easier access to promoter regions. Thus, the effect of histone acetylation relies primarily on the number of lysines modified, which is termed a cumulative effect (Li et al., 2007a). The known acetylation sites and HAT/HDAC enzymes are summarized in Figure 1.10.

While histone acetylation is positively associated with actively transcribed genes (Roh et al., 2005) histone methylation either activates or represses transcription, depending upon the site and degree (mono-, di-, and trimethylation) of the modifications (Ruthenburg et al., 2007). In contrast to acetylation, histone methylations are regulated with immense specificity. One histone methyltransferase (HMT) modifies one single lysine on a single histone (Kouzarides, 2007). Six lysine (K) residues on histone H3 and H4 (H3K4, H3K9, H3K27, H3K36, H3K79 and H4K20) are subjected to mono-, di- and tri-methylation. Importantly each methylation state represents a specific epigenetic mark with a precise biological meaning and well defined chromatin localization (Margueron et al., 2005). H3K4, H3K36 and H3K79 are implicated in activation of transcription, whereas H3K9, H3K27 and H4K20 are connected to transcriptional repression.
<table>
<thead>
<tr>
<th>Enzymes that Modify Histones</th>
<th>Acetyltransferase</th>
<th>Residues Modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAT1</td>
<td>H4 (K5, K12)</td>
<td></td>
</tr>
<tr>
<td>CBP/P300</td>
<td>H3 (K14, K18) H4 (K5, K8)</td>
<td>H2A (K9) H2B (K12, K15)</td>
</tr>
<tr>
<td>PCAF/GCN5</td>
<td>H3 (K9, K14, K18)</td>
<td></td>
</tr>
<tr>
<td>Tip60</td>
<td>H4 (K5, K8, K12, K16)</td>
<td>H3 K14</td>
</tr>
<tr>
<td>HB01 (ScEsa1, SpMST1)</td>
<td>H4 (K5, K8, K12)</td>
<td></td>
</tr>
<tr>
<td>ScSAS3</td>
<td>H3 (K14, K23)</td>
<td></td>
</tr>
<tr>
<td>ScSAS2 (SpMST2)</td>
<td>H4 K16</td>
<td></td>
</tr>
<tr>
<td>ScRtTR109</td>
<td>H3 K56</td>
<td></td>
</tr>
</tbody>
</table>

| Decetylases               | SirT2 (ScSir2)   | H4 K16           |

| Lysine Methytransferase   | Suv39H1          | H3K9             |
|                          | Suv39H2          | H3K9             |
|                          | G9a              | H3K9             |
|                          | ESET/SETD81      | H3K9             |
|                          | EuHMTag/SLP      | H3K9             |
|                          | Cll8             | H3K9             |
|                          | SpCid4           | H3K9             |
|                          | Mll1             | H3K4             |
|                          | Mll2             | H3K4             |
|                          | Mll3             | H3K4             |
|                          | Mll4             | H3K4             |
|                          | Mll5             | H3K4             |
|                          | Set1a            | H3K4             |
|                          | Set1b            | H3K4             |
|                          | Ash1             | H3K4             |
|                          | Sc/Spo SET1      | H3K4             |
|                          | Set2 (Sc/Spo SET2) | H3K36       |
|                          | Nsd1             | H3K36             |
|                          | Symd2            | H3K36             |
|                          | Dtt1             | H3K79             |
|                          | Sc/Spo DOT1      | H3K79             |
|                          | Pr-SET 7/8       | H4K20             |
|                          | Suv34 20H1       | H4K20             |
|                          | Suv34 20H2       | H4K20             |
|                          | SpSet9           | H4K20             |
|                          | Ezh2             | H3K27             |
|                          | Riz1             | H3K9             |

| Lysine Demethytransferase | LSD1/BHC110      | H3K4             |
|                          | JhdM1a           | H3K36             |
|                          | JhdM1b           | H3K36             |
|                          | JhdM2a           | H3K9             |
|                          | JhdM2b           | H3K9             |
|                          | JmjD2A/JhdM3A    | H3K9, H3K36       |
|                          | JmjD2B           | H3K9             |
|                          | JmjD2C/Gasc1     | H3K9, H3K36       |
|                          | JmjD2D           | H3K9             |

| Arginine Methytransferase | CARM1            | H3 (R2, R17, R26) |
|                          | Prmt4            | H4R3             |
|                          | Prmt5            | H3R8, H4R3       |

| Serine/Threonine Kinases | Haspin           | H3T3             |
|                         | MsK1             | H3S28             |
|                         | MsK2             | H3S28             |
|                         | Ckii             | H4S1             |
|                         | Mst1             | H2B514             |

| Ubiquitases             | Bmi/Ring1A       | H2A1K119         |
|                         | Rnf20/Rnf40      | H2B1K120         |

| Proline Isomerases      | ScFpr4           | H3P30, H3P38     |

Only enzymes with specificity for one or a few sites have been included, along with the sites they modify. Human and yeast enzymes are shown. The yeast enzymes are distinguished by a prefix: Sc (Saccharomyces cerevisiae) or Sp (Saccharomyces pombe). Enzymes that fall within the same family are grouped.

Figure 1.10  List of enzymes modifying histones and residues modified by them.

Figure adopted and modified from Kouzarides, 2007.
Histone methylation had been thought of as an irreversible epigenetic mark until the discovery of the first lysine specific histone demethylase LSD1 (also known as AOF2 and KDM1) in 2004 (Shi et al., 2004). Following the identification of LSD1, another family of more than 30 histone demethylases which were structurally different from LSD1 were described, all of which sharing a motif designated the Jumonji C (JmJC) domain (Klose et al., 2006a) and also revealing a substrate specificity. Recently, another protein LSD2 (or AOF1) related to LSD1 has been reported to have a demethylase activity (Yang et al., 2010c). Discovery of these enzymes spawned a new era in perception of chromatin dynamics. Further understanding of the regulation of these enzymes could provide significant insight into fundamental mechanisms of many biological processes and human diseases. Currently known site-specific HMTs and histone lysine demethylases (KDMs) are listed in Figure 1.10.

1.3.5 Understanding elucidation of histone modifications

Histones may be modified at different sites concurrently. The core histones structuring the nucleosome can each have several modifications; therefore it gives rise to cross-talk among the different marks. Communication between histone modifications may occur at the same site (Wang et al., 2008), in the same histone tail (Duan et al., 2008) and among different histone tails (Nakanishi et al., 2009). Histone modification cross talk is schematically exemplified in Figure 1.12.

Global genome-analyses have discovered that distribution of histones and histone modifications correlates with transcriptional state and a schematic depiction of this has been presented as Figure 1.11. Activated gene regions are enriched in active histone markers such as methylation at H3K4, H3K36, or H3K79 and global acetylation at core histone (Edmunds et al., 2008; Heintzman et al., 2007; Koch et al., 2007; Krivtsov et al., 2008; Steger et al., 2008). “Tri or dimethylation” at histone H3K4 and H3/H4ac are enriched around the transcriptional start sites, while methylation marks H3K36 and H3K79 are mainly distributed downstream of the activated regions of gene. Besides that H3K36me3
at the 3' end of active genes has been shown to have a role in the suppression of inappropriate
initiation from cryptic start sites within the coding region (Kouzarides, 2007). Additionally, histone
H2A has been demonstrated to be replaced with the histone variant H2A.Z around the transcriptional
start sites. In contrast, high levels of repressive histone markers such as H3K27me2 and H3K9me2/3
have been reported to be enriched around the transcriptional start sites of genes that are not
expressed or are expressed at low levels.

Histone modifications occur in a combinatorial manner. A series of coordinated chromatin
modifications take place during the transition of a naive chromatin template to active euchromatin or
establishment of repressive heterochromatin. As shown in Figure 1.12, combinations of active marks
are progressively induced while simultaneously counteracting repressive modifications (Goldberg et
al., 2007). For example, histone lysine methylation has been linked to DNA methylation and is thus
implicated in gene silencing. The major outcome after the establishment of the “histone code” at a
given locus is the interpretation of this epigenetic information by the transcriptional machinery to
bring biological consequences. Some “effector” proteins have been reported that can recognize these
specific histone modifications and bind to the modified histone tails (Goldberg et al., 2007).

1.3.6 Lysine specific demethylase-1 (LSD1)

Methylation marks were originally thought to be static but since the discovery of LSD1, it has been
established that methylation marks are dynamic and regulated by both histone methyltransferases
and histone demethylases. LSD1 is a histone demethylase which catalyzes the demethylation reaction
of mono- and dimethylated histone H3 lysine 4 (Shi et al., 2004). LSD1 is highly conserved in
organisms ranging from Schizosaccharomyces pombe to human. There are three major domains in
LSD1: an N-terminal SWIRM (Swi3p/Rsc8p/Moira) domain, a C-terminal AOL (amine oxidase-like)
domain, and a central protruding Tower domain (Aravind and Iyer, 2002).
Figure 1.11 Genome-wide distribution patterns of histone modifications from a transcription perspective.

The distribution of histones and their modifications are mapped on an arbitrary gene relative to its promoter (5' IGR), ORF, and 3' IGR. The curves represent the patterns that are determined via genome-wide approaches. The squares indicate that the data are based on only a few case studies. With the exception on K9 and K27 methylation, most of the data are based on yeast genes. Figure adopted from Li et al., 2007a.
Figure 1.12  Histone modification cross-talks.

Histone modifications either positively or negatively affect other modifications. A positive effect is indicated by an arrowhead and a negative effect is indicated by a flat head. Figure adopted from Bannister and Kouzarides, 2010.
The C-terminal catalytic domain is homologous to amine oxidases of the flavin adenine dinucleotide (FAD)-dependent enzyme family including mono- and poly-aminoxidase. The N-terminal SWIRM domain seems to be important for chromatin binding (Anand and Marmorstein, 2007). The Tower domain, inserted into the AOL domain, forms a long helix-turn-helix structure and serves as a platform for binding of LSD1 partner proteins such as co-repressor element silencing factor, CoREST (Lee et al., 2005). LSD1 activity is very specific for only on mono- and dimethylated H3K4 through a flavin-dependent mechanism (Forneris et al., 2005; Shi et al., 2004) and, importantly, it cannot demethylate trimethylated lysine residues (Forneris et al., 2005). The catalytic activity of LSD1 could be reduced by the presence of other activation markers such as Lys hyperacetylation or Ser 10 phosphorylation on H3 (Forneris et al., 2005). This study suggests that other enzymes, including histone deacetylases, arginine demethylases and serine phosphatases might be operating before LSD1 activity commences. Therefore, it appeared that LSD1-mediated H3K4 demethylation could be a final stage in the epigenetic process associated with gene repression (Forneris et al., 2008).

1.3.7 LSD1 association with gene regulation

Originally LSD1 was recognized as a component of transcriptional repressor complexes comprising transcriptional co-repressor protein (CoREST) and HDAC1/2 in non-neuronal cells and neuronal precursors (Dallman et al., 2004; Saleque et al., 2007). LSD1-CoREST-HDAC complex also has a role in silencing mature B-cell genes through direct interaction with the transcriptional repressor B lymphocyte-induced maturation protein-1 (Blimp-1) (Su et al., 2009). The LSD1-CoREST-HDAC core also inhibits cell proliferation by repressing the PTEN gene through forming a complex with the constitutive transrepressor TLX (Yokoyama et al., 2008). LSD1 also directly interact with p53 to confer p53-mediated
transcriptional repression, such as the repression of the alpha-fetoprotein (AFP), while the well known p53 target gene p21 is actively transcribed without recruitment of LSD1. It suggests that LSD1 is targeted to chromatin by p53 but possibly in a gene specific manner (Tsai et al., 2008). Another significant indication of LSD1 involvement in gene repression is that the DNA methylase regulator DNMT3 recognizes unmethylated histone tails at H3K4 (Ooi et al., 2007). This study suggests role of LSD1 in the arrangement and dissemination of heterochromatin through LSD1-dependent H3K4 demethylation, followed by de novo DNA methylation.

Recently, LSD1 has been shown to have role in transcriptional activation mediated by nuclear androgen receptors (AR) and estrogen receptors (ER) and functions as an H3K9 demethylase (Garcia-Bassets et al., 2007; Metzger et al., 2005). Metzger et al., 2005 demonstrated that, following hormone treatment, AR and LSD1 co-localize on promoters and stimulate only H3K9 demethylation without altering the H3K4 methylation status and promote ligand dependent transcription of AR target genes resulting in enhanced tumour cell growth. They also showed that LSD1 knock-down resulted in decreased activation of AR-responsive promoters.

A recent genome-wide analysis of LSD1 promoter occupancy subsequent to estrogen treatment of MCF-7 cells produced the striking finding about a possible activatory role for LSD1 (Garcia-Bassets et al., 2007). It was shown that LSD1 occupies nearly 20 % of the total assayed promoters and 84 % of these promoters were associated with RNA polymerase II and additionally with activation markers such as dimethyl-H3K4 and acetyl-H3K9 which is suggestive of that LSD1 is widely involved in gene activation rather than repression.
The dual role of LSD1 in gene repression and activation has been confirmed by regulation of growth hormone expression during pituitary development (Wang et al., 2007). This study shows that activation of growth hormone expression during the early phases is regulated though recruitment of a LSD1-containing mixed lineage leukemia 1 (MLL1) coactivator complex on the transcriptional activator pituitary transcription factor 1 (Pit1). Pit1 is later replaced by zinc finger E-box binding homeobox 1 (ZEB1), a transcriptional repressor which recruits a co-repressor complex containing C-terminal binding protein (CtBP), CoREST and LSD1, switching off growth hormone expression. LSD1 has also been shown to function beyond its histone demethylation job. It has been identified that non-histone substrates also exist for LSD1 (Huang et al., 2007a; Wang et al., 2009). Demethylation of p53 at Lys370 site by LSD1 controls tumour suppressor activity because this demethylation is essential for efficient binding of transcriptional co-activator p53-binding protein-1. Through this interaction, LSD1 blocks proapoptotic activity of p53 (Huang et al., 2007a). Very recently, a DNA methyltransferase has also been recognized as a non-histone substrate for LSD1 (Wang et al., 2009). Methylation of DNMT1 leads to its protein degradation. LSD1 directly demethylates and hence stabilizes DNMT1 maintaining global DNA methylation. Thus, LSD1 coordinates not only histone methylation but also DNA methylation to regulate chromatin structure and gene activity (Wang et al., 2009) during mouse embryogenesis. Furthermore, Set7/9 (also known as KMT7) which plays an important role in lysine methylation of histone and non-histone proteins also methylates DNMT1 (Esteve et al., 2009; Pradhan et al., 2009). Lysine specific demethylase (LSD) works as antagonist of Set7/9 methylation. Thus there is a fine-tuning between Set7/9 and LSD interaction with DNMT1 and it has been suggested as a means for epigenetic regulation (Pradhan et al., 2009).
1.3.8 Epigenetic control of transcription in EMT and CSC

During EMT, there is repression of epithelial proteins such as E-cadherin, α and γ-catenins, desmoplakin, zona occludens-1 and cytokeratin-18 (Coughlin, 1999). Whilst some of the mesenchymal markers such as α-smooth muscle actin, fibronectin, vimentin, type I and III collagens, N-cadherin and FSP1 are gained (Arciniegas et al., 2007). A wide array of transcription factors are involved in regulating EMT and, perhaps TGF-β is capable of controlling expression of a significant fraction. At the molecular level, EMT transcriptional reprogramming occurs through transcription factors such as Zeb1/TCF8, Zeb2, Snail1, Zeb2, Slug, E12/E47 FOXC2 and Twist (Kang and Massague, 2004; Thiery et al., 2009; Yang and Weinberg, 2008). Additionally, subsequent activation of factor-specific and overlapping EMT signal-transduction pathways takes place (Blick et al., 2008; Huber et al., 2004). Among these transcription factors, the Twist, Snail1 and Zeb family members are well investigated in both EMT and CSCs. A summary of transcription factors which play a role in EMT is presented in Figure 1.13.

Induction of EMT by ectopic expression of Snail, Twist or TGF-β treatment in HMLE cells results in acquisition of stem cell properties: the ability to self-renew and initiate tumour (Mani et al., 2008; Morel et al., 2008). Twist1 directly stimulates the expression of BMI1, which encodes a polycomb-group protein for maintaining self-renewal through transcriptional repression of p16INK4A-ARF locus. Twist1 and BMI1 synergistically repress expression of both E-cadherin and p16INK4A and therefore cooperate in promoting EMT and tumour initiation (Yang et al., 2010a). Additionally, down-regulation of CD24 by Twist1 also modulates CSC phenotype (Vesuna et al., 2009).
<table>
<thead>
<tr>
<th>EMT transcription factors</th>
<th>Functions in maintenance of stemness of CSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twist</td>
<td>Twist 1 induces HMLEs into mesenchymal-like cells and dedifferentiates HMLEN cells into CSCs via EMT. Constitutively expressing Twist 1 in HMLER cells augments the stem-like cell pool, mammosphere formation, and tumorigenic property in vivo. Twist 1 induces a breast CSC phenotype by downregulating the expression of CD24</td>
</tr>
<tr>
<td>Snail1</td>
<td>Constitutively expressing Snail1 in HMLER cells augments the stem-like cell pool, mammosphere formation, and tumorigenic property in vivo. Snail1 promotes ovarian cancer cells to acquire CSC characteristics</td>
</tr>
<tr>
<td>Slug</td>
<td>Protects hematopoietic progenitor cells from radiation-induced apoptosis in vivo; promotes ovarian cancer cells to acquire CSC characteristics</td>
</tr>
<tr>
<td>ZEB1</td>
<td>ZEB1 is overexpressed in CSCs induced by LBX1 via EMT. ZEB1 promotes tumorigenesis and links activation of EMT and maintenance of stemness of CSCs by repressing stemness-inhibiting miRNAs</td>
</tr>
<tr>
<td>ZEB2</td>
<td>ZEB2 is overexpressed in CSCs induced by Twist 1, TGF-β, or LBX1 via EMT</td>
</tr>
<tr>
<td>Goosecoid</td>
<td>Promotes breast cancer cell motility and metastasis via EMT</td>
</tr>
<tr>
<td>FOXC2</td>
<td>FOXC2 is highly expressed in basal-like breast cancer and confers stem cell properties on epithelial cells</td>
</tr>
<tr>
<td>YB-1</td>
<td>Ectopic expression of YB-1 confers MCF-10A cells various stem cell properties</td>
</tr>
<tr>
<td>LBX1</td>
<td>Promotes cell migration and enlarges CD44\textsuperscript{hi}CD24\textsuperscript{low} cell population and contributes to breast cancer progression via EMT</td>
</tr>
<tr>
<td>Six1</td>
<td>Promotes the expansion of stem/progenitor cell population in the mouse mammary gland and subsequent mammary tumor development via EMT</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>HIF-1α overexpression promotes EMT and metastasis by inducing Twist 1; high levels of HIFs in hypoxic tumor cells may promote cancer cells to acquire properties of CSCs</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>HIF-2α-specific target genes are expressed at significantly higher levels in glioblastoma stem cells in comparison to nonstem cancer cells under hypoxia. HIF-2 is also required for VEGF expression in GSCs, but not nonstem cancer cells. HIF-2α might provide CSCs a growth advantage even without hypoxia in vitro and in vivo</td>
</tr>
</tbody>
</table>
The snail family members Snail1 and Slug are highly conserved zinc-finger transcriptional repressors implicated in embryonic development and tumorigenesis. Both Snail1 and slug are up-regulated by the TGF-β, Wnt, FGF, HGF, GSK3 and ER signaling pathways (Bachelder et al., 2005; Zhou et al., 2004). Snail1 represses transcription of the E-cadherin gene in cultured cells (Batlle et al., 2000; Cano et al., 2000) and also during embryonic development (Carver et al., 2001). TGF-β activates both snail and slug directly through Smad3 (Zavadil et al., 2004).

Zeb1 and Zeb2, two members of the Zeb family, are implicated in malignancy of various human tumours and are important regulators of EMT and they also contribute to drug resistance and stemness (Carver et al., 2001). Interestingly, Zeb1 can promote tumorigenesis and link the activation of EMT with CSC by stemness-inhibiting microRNAs (Wellner et al., 2009). Additionally, a recent study describes cooperation of Snail1 and Twist in regulation of Zeb1 during EMT (Dave et al.).

During EMT, mammalian Y-box binding protein (YB1) has been shown to promote cap-independent translation of Snail1, twist and Zeb2/SIP1 together with the up-regulation of the stem cell markers p63, CD44 and CD10. Simultaneously, YB1 also represses cap-dependent translation of growth-promoting genes and CD24 (Evdokimova et al., 2009). Molecular targets of YB1 in metastatic breast cancer cell lines have also shown to include stem cell associated marker such as CD44 and CD49f as well as c-KIT, BMI1 and members of Wnt and Notch signaling pathways (To et al., 2010).

Ladybird homeobox 1 (LBX1) protein is a newly discovered player in the process of EMT and it transcriptionally targets Zeb1, Zeb2, Snail1 and TGF-β2. Ectopic expression of LBX1 in mammary epithelial cells induces EMT along with increase in mammosphere formation.
and CD44^{high}/CD24^{low} cell proportions (Yu et al., 2009). Therefore both YB1 and LBX1 may function as master regulators of the EMT/stemness program.

Two recent reports have focused the role of the microenvironment in promoting tumour progression by effecting EMT-dependent manifestation of CSC properties. First, adaptive response gene, activating transcription factor 3 (ATF3) might serve to assimilate stromal signals from the tumour microenvironment along with acquisition of combined EMT/CSC phenotype in mammary epithelial cells (Yin et al., 2010). Second, signaling by urokinase-type plasminogen activator receptor (uPAR) also instigates EMT (Lester et al., 2007) and CSC in MDA-MB-468 cells exposed to hypoxia (Jo et al., 2010).

1.3.9 MicroRNAs involved in EMT and CSC

MicroRNAs (miR) are small non-coding RNAs molecules that regulate the translation of targeted messenger RNA (mRNA) variously by inhibiting ribosome function, causing removal of the 5’ cap structure, promoting the deadenylation of the poly (A) tail and even mediating destruction of the target mRNA (Filipowicz et al., 2008). MicroRNAs have the potential either to regulate the expression of many target mRNAs simultaneously or just a few. As a result they collaborate in the control a range of cell functions, including cell proliferation, stem cell maintenance, and differentiation. To date, 706 miRs have been identified in humans and 547 in mice. MiRNAs originate from genes that are transcribed by RNA polymerase II. Nascent primary miRNA transcripts (pri-miRNA) are trimmed into miRNA precursors (pre-miRNA) by Drosha, a member of RNAase III enzyme family, in conjunction with the double-stranded RNA-binding protein Pasha in the nucleus. Pre-miRNAs are then exported from the nucleus in a GTP-dependent fashion by exportin-5, followed by processing by a second RNAse III endonuclease caller Dicer. This
results in mature miRNA (ranging from 18-24 nucleotides in length), which in turn are assembled into a ribonucleoprotein complex known as RNA-induced silencing complex (RISC) that includes Argonaute protein Ago2 (Bartel, 2004; Gregory and Shiekhattar, 2005; Meister et al., 2004). The miR-RISC complex may lead to complementary base-pairing interactions between miRNAs and 3’ untranslated region (3’ UTR) of target mRNAs, often repressing the gene translation or cleaving the target mRNA, depending on the base-pairing features between miR and the target mRNA (Ambros, 2004; Cullen, 2004).

Each vertebrate microRNA (miR) may bind up to 200 gene targets, and each gene may contain multiple binding sites for different miRs, therefore microRNAs could potentially regulate 30% protein coding genes in human genome (Kent and Mendell, 2006; Krek et al., 2005), which highlights importance of microRNAs as global regulators of gene expression. MicroRNAs often target various components of cellular networks or signaling pathways (Cui et al., 2006; Shi et al., 2007). Recent studies have identified critical roles of microRNAs in a large number of biological processes, including development, differentiation, apoptosis, cell cycle progression, cellular proliferation, cancer initiation, and cancer metastasis (Calin and Croce, 2006; Calin et al., 2004; Chen and Rajewsky, 2006; Cummins and Velculescu, 2006; Esquela-Kerscher and Slack, 2006; Tili et al., 2007; Zhang et al., 2007a; Zhang et al., 2007b).

Recently, microRNA mediated processes have been shown to be an important regulator of EMT (Wu and Zhou, 2008) and the discovery of miRNA control has added an additional level of complexity to the molecular networks regulating EMT, metastasis and stemness (Shimono et al., 2009; Wellner et al., 2009). Members of the miR-200 family (miR 200a,
miR 200b, miR 200c and miR 429) are rapidly emerging as new master regulators of
differentiation by directly targeting transcripts encoding Zeb1 and Zeb2/SIP1 and
therefore block repression of E-cadherin and eliciting MET. In addition, Zeb1 also
suppresses members of the miR 200 family, by establishing a reciprocal feedback loop
leading to MET (Gregory et al., 2008; Park et al., 2008). Importantly, miR 200c has been
shown to have role in tumour initiation through EMT, stemness and most recently a role
in the emergence of migrating CSCs at the invasive front of tumour has been also
demonstrated (Shimono et al., 2009; Wellner et al., 2009). Indeed, molecular targets of
miR 200 family include the stem cell-associated factors such as BMI1 and KLF4 (Gregory
et al., 2008; Shimono et al., 2009; Wellner et al., 2009). Akt isoforms have a role in
induction of EMT by regulating expression of miR 200 family in TGF-β treated MCF-10A
cells (Ilipooulos et al., 2009). This same study also described the overall activity of Akt as
controlling stemness by regulating miR 200 family (Ilipooulos et al., 2009). Using an
inducible Src oncogene (ER-Src) model in MCF-10 A, it was reported that miR 200 family
was inhibited during cancer stem cell induction and inhibition of miR 200c showed
increased CSC formation. Interestingly, it was also shown that miR 200b directly targeted
Suz-12, which is a subunit of a polycomb repressor complex (PRC2) (Ilipooulos et al.,
2010). They found that loss of miR 200 during CSC formation could increase Suz12
expression and hence E-cadherin repression. Conversely, ectopic expression of Suz12 in
transformed cells promoted CSC formation (Ilipooulos et al., 2010). Other miRNAs such
as let-7, which targets HMGA2 and HRAS, have also been shown to have role in EMT and
CSC formation (Yu et al., 2007). MiR 34a, a P53 target directly represses CD44 in prostate
cancer and thus has been linked to a key negative regulator of stemness (Liu et al., 2011).
Additionally, miR-9, which is a myc-activated microRNA, has been also shown to regulate
E-cadherin and cancer metastasis (Ma et al., 2010). These reports strongly suggest that miRNAs, especially miR 200 family members, link the EMT phenotype with stem cell signatures and are therefore promising therapeutic targets of master regulators of EMT and stemness.

1.3.10 Chromatin modifiers involved in EMT and CSC

Although the role of many different EMT regulators has been well demonstrated, it is unknown whether chromatin modifiers are also required to coordinate different EMT regulators to mediate EMT.

Besides being a critical regulator of EMT, Snail1 over-expression induces resistance to apoptosis and tumour reoccurrence in breast cancer (Kajita et al., 2004; Moody et al., 2005; Vega et al., 2004). Tandem affinity purification (TAP) combined with mass spectrometry analysis to identify chromatin modifying enzymes interacting with Snail1 revealed that LSD1 is a key chromatin modifying enzyme interacting with Snail1 (Lin et al., 2010b). The SNAG domain of Snail1 and the amine oxidase domain of LSD1 were required for their mutual interaction. Interestingly, it was also demonstrated that the sequence of the SNAG domain is similar to that of the histone H3 tail, and the interaction of Snail1 with LSD1 can be blocked by LSD1 enzymatic inhibitors and a histone H3 peptide (Lin et al., 2010b). They also found that formation of a Snail1-LSD1-CoREST complex was critical for the stability and function of these proteins during invasion and metastasis. Furthermore, it was demonstrated that the SNAG domain of Snail1 was critical for recruiting LSD1 to its target gene promoters (Lin et al., 2010b). Previous studies have shown that Snail1 induces repressive histone modifications at E-cadherin promoter through recruitment of histone deacetylases (HDACs) and H3K27 methyltransferase EZH2.
(Herranz et al., 2008; Peinado et al., 2004). Another study showed that Snail1 physically recruits LSD1 to reduce level of dimethylated H3K4 at its target genes, and that LSD1 is required for Snail1-mediated transcriptional repression (Lin et al., 2010a) during EMT.

Epigenetic modifications during TGF-β mediated EMT were investigated, and although it was found that DNA methylation was unchanged during EMT in AML12 cells, a global reduction in H3 Lys 9 dimethylation (H3K4Me9), an increase in the H3 Lys4 trimethylation (H3K4Me3) and an increase in H3 Lys36 trimethyltylon (H3K36Me3) were observed (McDonald et al., 2011). It was also demonstrated that these changes were largely dependent on LSD1 and that loss of LSD1 function had marked effect in EMT-driven cell migration and chemo-resistance (McDonald et al., 2011). Genome-scale mapping using ChIP on chip analysis showed that chromatin changes were mainly specific to Large Organized Heterochromatin K9 Modifications (LOCKs), which suggested that EMT is characterized by reprogramming of specific chromatin domains across the genome (McDonald et al., 2011).

A very recent report focused on the identification of chromatin modifiers that are regulated by hypoxia to coordinately mediate the transition of EMT marker genes during EMT. This study showed that HIF-α-induced histone deacetylase 3 (HDAC3) is essential for hypoxia induced EMT and metastatic phenotypes. Under hypoxia, HDAC3 interacts with hypoxia induced WDR5, recruits the histone methyltransferase (HMT) complex to increase histone lysine methylation specific HMT activity and activates mesenchymal gene expression (Wu et al., 2011). HDAC3 also works as a corepressor of epithelial gene expression. This study also showed that knockdown of WDR5 abolishes mesenchymal gene activation but not repression of epithelial genes during hypoxia. In summary this
study demonstrated that hypoxia induces a unique set of chromatin modifiers that coordinately regulates EMT though distinct mechanisms (Wu et al., 2011).

1.4 Signal Transduction Kinases: a newly discovered layer of chromatin modifying enzymes

Among the enzymes that modify histones, kinases mainly depend on the activation of specific upstream signaling pathways leading to cascades of protein phosphorylation and regulation of transcription in the nucleus. Recently, significant progress in understanding the roles of this particular type of modification has been made through the elucidation of mechanisms by which gene expression is directly affected through specific kinase dependent phosphorylation of histones (Bungard et al., 2010; Cerutti and Casas-Mollano, 2009; Dawson et al., 2009; Metzger et al., 2010; Perez-Cadahia et al., 2009). Furthermore, there is evidence that some kinases (PKCs, PIM1, IKKa, RsK2, Akt/PKB, MSK1/2, JNK1, PRK1, Chk1, DIK/ZIP, MST1, AMPK and JAK2) that modify histones, also modify non-histone substrates including chromatin remodeling factors and transcription factors (Cha et al., 2005; Fischle et al., 2003; Huang et al., 2007b; Lee et al., 2010; Lehtinen et al., 2006).

There are substantial studies in eukaryotes currently, which indicate that signal transduction kinases show a different function in the cytoplasm than from the nucleus, where they effect transcriptional activity (Anest et al., 2003; Birbach et al., 2002; Bungard et al., 2010; Saccani et al., 2002; Yamamoto et al., 2003). The growing body of evidence of signaling kinases translocating between cytoplasm and nucleus, suggests the existence of a mechanism where extracellular signals generated at plasma membrane result in signals being transferred to nucleus. Indeed one such example is, adenosine monophosphate-activated protein kinase (AMPK), which has been shown to activate
transcription through direct association with chromatin and phosphorylation of histone H2B at serine 36 (Bungard et al., 2010). Apart from that, protein kinase C family members have recently been shown to modify histones (Lee et al., 2010; Metzger et al., 2010; Sutcliffe et al., 2011). Non-histone substrates such as CREB binding protein (CBP) at serine 1382 and 1386 as well as histone H3S10 are phosphorylated by IKKα. Phosphorylation of CBP by IKKα is also accountable for accelerated cell growth by switching the binding preference of CBP from p53 to NF-κB (Huang et al., 2007b). Akt is responsible for phosphorylation of p300 at S1884, which is known to enhance its enzymatic activity (Huang and Chen, 2005). Akt also has a histone modifier, enhancer of zeste homolog 2 (EZH2) as a nonhistone substrate. Phosphorylation of EZH2 at serine 21 by Akt has a negative effect on the methyltransferase activity of EZH2 since it impedes binding of EZh2 to histone H3, which results in deceased H3K27 trimethylation and de-repression of silenced target genes (Cha et al., 2005). Cyclin-dependent kinase 1 (CDK1) has been shown also to phosphorylate EZH2 at multiple sites, inhibiting its enzymatic activity (Chen et al., 2010). Further, phosphorylation of silent mating type information regulator 2 homolog 1 (SIRT1) by JNK1 regulates enzymatic activity of SIRT1 (Nasrin et al., 2009). Kinases without known histone substrate also exist in chromatin apart from the kinases which have been reported to modify histones. For example, salt-inducible kinases 1(SIK1), S6K1, Abl, CaMK and big mitogen-activated protein kinase 1 (BMK1) have been shown to phosphorylate histone modifiers such as class II histone deacetylases (HDACs) and SRC-3 processing weak histone acetyltransferase activity but any direct histone substrates have not been yet reported (Berdeaux et al., 2007; Lai et al., 2010; McKinsey et al., 2000; Oh et al., 2008; Yang et al., 2010b).
1.4.1 Signal Transduction kinase: Protein Kinase C family

The discovery of the link between PKC and cancer, lead several researchers to focus on the role of this signaling pathway. It is now well recognized that there are at least 12 isozymes in this PKC family of serine threonine kinases accounting for 2% of kinases in human (Mackay and Twelves, 2007). PKC isozymes have been shown to have an essential role in cellular signaling transduction involved in cell proliferation, differentiation, apoptosis and angiogenesis (Griner and Kazanietz, 2007). PKC activity dysregulation and its expression have been demonstrated in several malignancies (Assert et al., 1999; Cacace et al., 1996; Frey et al., 1997; Gokmen-Polar et al., 2001; Heit et al., 2001; Koren et al., 2004; O’Brien et al., 1989; Scaglione-Sewell et al., 1998; Wang et al., 1999; Weichert et al., 2003). This has led to therapeutic targeting of the PKC enzymes for treatment of cancer and now there are many PKC inhibitors in the clinical studies (Mizuno et al., 1993; Pajak et al., 2008; Rizvi et al., 2006; Thavasu et al., 1999).

The classification of PKC isoforms is based on structural and activational characteristics (Newton, 1995; Nishizuka, 1992). The PKCs are divided into three subfamilies: conventional or classic PKCs (cPKC), non-classic or novel PKCs (nPKC) and atypical PKCs (aPKC); their binding and activational characteristics are summarized in Figure 1.14 and Figure 1.15. The classic PKC isozymes (cPKC) α, β1, β2 and γ are calcium-sensitive, phospholipid and diacylglycerol-activated kinases. Novel isoforms (nPKC) δ, μ, ε and θ are calcium-insensitive, phospholipid-dependent, and diacylglycerol-dependent. The atypical (aPKC) isoforms: ζ, η and λ/τ are both calcium-insensitive and diacylglycerol-insensitive. Activation of PKC isoforms results in changes in their subcellular location. For example, PKC-α and PKC-ζ translocate from cytosol to the perinuclear membrane on activation (Disatnik et al., 1994). They all comprise a C-terminal serine/threonine protein kinase
domain (AGC class) linked through a variable 'V3' domain to a regulatory domain (Figure 1.14). The latter comprises three functional elements (i) an inhibitory region (pseudosubstrate site), (ii) a C1 domain (one copy or as a tandem repeat-C1A, C1B) and (iii) a C2 or PB1 domain (Roffey et al., 2009).

Here it is important to state that several PKC isozymes have different tissue specific roles (Griner and Kazanietz, 2007). The basis for this disparity is chiefly due to the variety of pathways activated by each isozyme and its distinct sub-cellular localization and access to substrate upon activation (Wang et al., 1999).

Stimulation of tyrosine-kinase receptors and G-protein-couples receptors activates phospholipase C (PLC). Activation of PLC increases the plasma membrane DAG levels which results in re-localization and activation of PKC isozymes (Newton, 2003). Binding of PKC to membrane induces conformational changes that expose the binding sites of the kinase domain. The subsequent downstream events include activation of pathways such as MEK-ERK (Cai et al., 1997; Marshall, 1996), PI3K-Akt pathways (Balendran et al., 2000) and signal transduction from membrane to nucleus. A schematic diagram of PKC isoforms in intracellular signaling pathways has been shown in the Figure 1.16.

All members of the PKC family are activated through allosteric effectors; these comprise lipids, proteins and the combination of the two. Inactivity is determined by the interaction of the regulatory domain with the catalytic domain and this is partly driven by interaction of the inhibitory pseudosubstrate site in the regulatory domain with the substrate binding pocket in the catalytic domain (Pears et al., 1990). However, this model is based on mutational studies and no complete structures yet exist to corroborate this working model.
Figure 1.14  Classification and structural characteristics of PKC isoforms.

Although the catalytic domain of PKC is conserved, the three subgroups have different regulatory domains. The classical PKC isoforms (cPKC) share all typical regulatory features: the autoinhibitory pseudosubstrate motif, two DAG-binding C1 domains (C1a and C1b) and the calcium-binding C2 domain. Novel PKC isoforms (nPKC) lack a calcium-binding motif but contain an extended N-terminal domain that can receive regulatory signals, and they are still regulated by DAG. Finally, the catalytic activity of atypical PKC isoforms (aPKC) is independent of DAG and calcium, and they seem to be regulated mainly through intracellular localization, which is regulated by interaction with regulatory proteins and nuclear localization signals (NLS) and nuclear export signals (NES) in their regulatory domain. Figure adopted from Spitaler and Cantrell, 2004.
Figure 1.17  Schematic representation of PKC activation.

Activation of G-protein coupled receptor (GPCR) or receptor tyrosine kinase (RTK) leads to phospholipase C (PLC) mediated generation of inositol trisphosphate (IP3) and diacylglycerol (DAG) from cell membrane phospholipids. DAG activates and translocates PKC to cell membranes, or sometimes to cytoskeleton or nucleus. IP3 releases calcium from intracellular stores, such as endoplasmic reticulum (ER) which potentiates cPKC activation. Finally, activated PKC phosphorylates its specific substrates on serine/threonine residues. Figure adopted from Koivunen et al., 2006.
Figure 1.16  Schematic of placement of PKC isoforms in intracellular signaling pathways.

Figure adopted from Teicher, 2006.
1.4.2 Protein kinase C family in Cancer progression

The role of PKC family members in carcinogenesis has been recognized for decades. Tumour promoters such as phorbol 12-myristate 13-acetate (PMA) bind to the DAG binding site of PKC with high affinity and promote prolonged activation of PKC and subsequent down regulation of the enzyme (Blumberg et al., 1984; Lu et al., 1998; Sharkey et al., 1984). Overall, the function of PKC in cancer is complex because much of the data indicate that the isozymes subtly regulate many pathways involved in cellular transformation (Mackay and Twelves, 2007). The isozymes most commonly displaying alterations in the expression during cancer progression are α, β and δ, but abnormal expression of other isozymes may also take place (Koivunen et al., 2006).

PKC-α overexpression has been shown in high grade urinary bladder, prostate and endometrial cancers, where as low grade tumour and normal epithelial of the respective organs show significantly lower expression of this enzyme (Fournier et al., 2001; Koren et al., 2004; Koren et al., 2000; Langzam et al., 2001; Martinez-Gimeno et al., 1995; Varga et al., 2004). In contrast, colon, hepatocellular, and basal cell cancers display downregulation of PKC-α expression (Ainsworth et al., 2004; Gokmen-Polar et al., 2001; Kerfoot et al., 2004; Neill et al., 2003; Tsai et al., 2000). Recently, it has been reported that PKC-α activity supports migration of breast cancer cells in vitro and its overexpression correlates to tumour grade, proliferating activity and poor prognosis (Lonne et al., 2010).

Overexpression of PKC-β contributes in several ways to tumour formation, being involved in tumour-host mechanisms such as inflammation (Leitges et al., 1996) and angiogenesis in breast cancer (Sledge and Gokmen-Polar, 2006) and retinal tissue (Suzuma et al., 2002). Overexpression of PKC-βI appears to be an early event in colon cancer development (Gokmen-Polar et al., 2001) and transgenic elevated expression levels of PKC-βII in the
intestine induces hyper-proliferation and invasive phenotype in epithelial cells (Yu et al., 2003; Zhang et al., 2004). It has also been demonstrated that the PKC-β specific inhibitor enzastaurin inhibits the activation of the Akt-GSK3 dependent survival pathway in colon cancers (Graff et al., 2005). In diffuse large B-cell lymphoma patients, PKC-β is one of the most overexpressed genes (Shipp et al., 2002), while loss of PKC-β expression has been reported in melanoma cell lines (Krasagakis et al., 2002).

PKC-γ is mainly expressed in neuronal tissues (Saito and Shirai, 2002) and very little is known about its role in cancers. Transformation of mammary epithelial cells upon PKC-γ expression has been observed, but it is unclear if this leads to breast cancer or not (Mazzoni et al., 2003). PKC-γ has also been shown as a prognostic factor for b-cell lymphomas (Kamimura et al., 2004).

PKC-δ is a ubiquitously expressed PKC isozyme and has been shown to have a role in many cellular processes including proliferation, differentiation and apoptosis. Interestingly, PKC-δ translocates to golgi in response to IFN-γ and ceramide (Kajimoto et al., 2004), while it is transported to the nucleus in response to etoposide and irradiation (Reyland et al., 1999) and to mitochondria in response to UV radiation, PMA and oxidative stress (Majumder et al., 2001). PKC-δ has been shown to have both a positive and negative role in tumour progression (Jackson and Foster, 2004). Down-regulation of PKC-δ with prolonged phorbol-ester treatment in Src-overexpressing fibroblast confers a malignant phenotype, which is suggestive of its tumour suppressive role. However, pro-tumorigenic sonic hedgehog signaling and Wnt signaling are dependent on PKC-δ/ERK pathways (Riobo et al., 2006).

PKC-δ is overexpressed in colon cancers and down-regulated in malignant gliomas, bladder carcinomas and endometrial tumours (Griner and Kazanietz, 2007; Reno et al., 2008). Higher expression of PKC-δ has also been linked with poor prognosis of breast cancer as
well (McKiernan et al., 2008). Furthermore, anti-estrogen resistance in breast cancer cells has been linked with PKC-δ and also to tamoxifen resistance (Nabha et al., 2005). Conversely, PKC-δ activation in prostate cancer helps in promoting extrinsic apoptosis through the release of death receptor ligands (Gonzalez-Guerrico and Kazanietz, 2005). PKC-δ is a potential tumour suppressor gene for human cutaneous squamous carcinomas (Yadav et al., 2010). On the other hand, PKC-δ overexpression occurs in human ductal carcinomas and pancreatic carcinoma cell lines (Mauro et al., 2010). On the contrary, PKC-δ has also been linked to an inhibitory role in cell autophagy, suppressing the catabolic processes in pancreatic cancers (Ozpolat et al., 2007).

Studies on role of PKC-θ, in the in breast carcinoma and the multi-drug resistance has been linked well before the concept of cancer stem cells was even introduced (Budworth et al., 1997). This study showed that both PKC-α as well as PKC-θ play a role in maintenance of multidrug resistance phenotype. Indeed a very old clinical report shows that concordant increase occurs in the expression of PKC-θ and MDR1 genes in leukemia cells from relapsed AML patients (Beck et al., 1996). Furthermore, kinase negative PKC-θ but not constitutively active PKC-θ inhibited capillary tube formation, ring like structure in endothelial angiogenesis assay as well as mitogenesis and motility in endothelial cells (Meller et al., 1998), and also PKC-θ has been linked with cytoskeleton reorganization (Pietromonaco et al., 1998). PKC-θ has also been demonstrated to promote c-rel mediated mammary tumorigenesis in mice and humans by repressing the synthesis of estrogen receptor-α (ER-α) (Belguise and Sonenshein, 2007). PKC-θ is also required for NK cell activation and in vivo control of tumour progression (Aguilo et al., 2009).

Another PKC isozyme, PKC-ε is considered as an oncogene. Overexpression of PKC-ε in NIH 3T3 fibroblasts results in saturation density, facilitating growth in soft agar and induced
tumour formation in nude mice (Mischak et al., 1993). Overexpression of this isoforms has also been shown to have a link with a metastatic phenotype in colonic epithelial cells (Pan et al., 2005; Perletti et al., 1996) and also to squamous cell carcinogenesis (Verma et al., 2006). PKC-ε expression levels were found higher in primary high grade astrocytoma cells (Sharif and Sharif, 1999) and human renal carcinoma (Sharif et al., 2001). PKC-ε is also associated with aggressive and motile phenotype in breast cancer cells (Pan et al., 2005) and head and neck squamous cell carcinoma (Pan et al., 2006). Overexpression of PKC-ε has also been found in prostatic cancers, where it is associated with the conversion from androgen-dependent to androgen-independent state (Wu et al., 2002). It has also been amplified in 28% of thyroid cancers (Knauf et al., 1999).

Taken together, distinctive functions of different isoymes and their coordinated actions have lead to the hypothesis that altered PKC activation balance (increased proportion of cPKCs to nPKCs) is an important factor at least in the aggressiveness of a cancer (Koivunen et al., 2006). Schematic illustration of PKC activation balance theory is shown in Figure 1.17.

1.4.3 A novel role for chromatin associated Protein kinase C family enzymes in EMT

PKC isoymes have been shown to have ability to dwell in nucleus (Martelli et al., 1999), and to phosphorylate histones in vitro (Inoue et al., 1977; Yu et al., 1998). Interestingly, the core activity of PKC-phosphoinositide signaling remains intact at both the plasma membrane and inside the nucleus (Visnjic and Banfic, 2007). Despite the evidence, the mechanism of transcriptional control of mammalian genes by PKCs was unidentified until a recent study (Sutcliffe et al., 2011). In this study, it was demonstrated that PKC-θ, an isozyme of PKC, physically associates with regulatory regions of inducible immune response
Figure 1.17  PKC activation balance theory.

Overall response to PKC activation seems to depend on presence or activity of different isoenzymes in a particular cell. As an example, several cancer initiating factors lead to increased proportional activity of PKC$\alpha/\beta$ compared to PKC$\delta/\theta$. This in turn favors the development of malignant phenotype. Figure adopted from Koivunen et al., 2006.
genes in human T cells. This chromatin associated PKC-θ forms an active nuclear transcription complex as well as binds to promoter regions of microRNAs involved in cytokine regulation. Another recent report demonstrated that PKC-βI leads to phosphorylation of histone H3 at threonine 6 (H3T6) during androgen receptor (AR)-dependent gene activation. PKC-βI mediated phosphorylation then prevents LSD1 from demethylating H3K4 (Metzger et al., 2010). During this process, activation of PKC-βI requires androgen-dependent recruitment of gatekeeper kinase protein kinase C (PKC)-related kinase 1 (PRK1) (Metzger et al., 2008). Interestingly, this study also showed that increased levels of PKC-βI and phosphorylated H3T6 positively correlate with high Gleason scores of prostate carcinomas and also that inhibition of PKC-βI blocks AR-induced tumour cell proliferation in vitro and cancer progression of tumour xenografts in vivo (Metzger et al., 2010).

It is evident that different PKC family members target different sites on histone H3, for example, PKC-α and PKC-β phosphorylate H3T6, whereas PKC-δ phosphorylates H3T45 respectively. Recently, orphan nuclear receptor RORα phosphorylation turned out to be a non-histone substrate phosphorylated by PKC-α in the nucleus. Phosphorylation of RORα results in the inhibition of canonical Wnt/β-catenin target genes in colon cancer (Lee et al., 2010). Another PKC isoform, PKC-δ has a non-histone substrate, Transducer β-Like-Related Protein 1 (TBLR1), in addition to histone H3T45. TBLR1 phosphorylation at S123 by PKC-δ is required for overcoming C-terminal binding protein (CtBP)-and nuclear receptor corepressor (NCoR)-dependent transcriptional repression checkpoint (Perissi et al., 2008). Considering the large number of PKC family members and their importance as key signal transduction kinases, the discovery of many more histone and nuclear non-histone targets is possible in the near future.
1.5 Scope of this investigation

Epigenetic mechanisms including DNA and histone modifications result in silencing of genes without changing the coding sequence of the gene. Even though these events are heritable, they are potentially reversible, thus opening up new avenues for therapeutic intervention. The importance of epigenetic changes in human cancer is only now being recognized in the medical community and an emerging area of therapeutic research is of epigomic—drugs (epi-drugs). In recent years, several epi-drugs such as HDAC inhibitors and DNMT inhibitors have reached various phases of clinical trials and are showing promising therapeutic outcomes. Current knowledge suggests agents that intervene in the epigenetic process by "turning back on" silenced genes may represent a significant advancement in treating many forms of cancer. A key step in cancer progression and metastasis is the epithelial to mesenchymal transition (EMT). Recent studies suggest that only a small subpopulation of these mesenchymal tumour cells referred to as Cancer Stem-like cells (CSC) in fact contribute to metastatic tumour initiation and recurrence. To date, the molecular mechanisms, in particular, the epigenetic signatures underpinning CSC formation, EMT and the relationship between these two processes are not clearly defined. One of the major limitations in this emerging field has been the lack of in vitro human inducible models of CSCs. The investigations reported in this thesis focus on the epigenetic mechanisms that control the induced gene transcription program during the process of EMT and CSC formation.

1.5.1 Specific aims

The principal objective of this work has been to understand the contribution of epigenetic regulation with the focus on the newly discovered category of chromatin associated enzymes, namely PKCs that may play role in regulation of gene expression programs during epithelial to
mesenchymal transition. Thus the primary aims of the research were following (the schematic representation of the objectives of this thesis is shown in Figure 1.18):

1. To establish a human in vitro inducible model of the epithelial to mesenchymal transition and cancer stem cell processes suitable for epigenetic analysis (chapter three).

2. To investigate the role of chromatin associated PKCs in regulating the epithelial to mesenchymal transition and formation of CSC (chapter four).

3. To understand how various epigenetic enzymes cooperate in regulating inducible gene transcription program and microRNAs involved in the epithelial to mesenchymal transition and formation of CSC (chapter five).
To date, epigenetic mechanisms involved in EMT are poorly defined

Specifically:

• What epigenetic signatures are laid down on genes involved in EMT/CSC formation process?
• Which of these epigenetic signatures are essential for the EMT/CSC formation process?
• Do distinct transcriptional programs separate CSC from NCSC populations?

Figure 1.18  Schematic illustrations of the objectives of the investigation reported in understanding the epigenetic regulation involved in epithelial to mesenchymal transition.

Epigenetic signatures underlying the epithelial to mesenchymal transition (EMT) and cancer stem cell (CSC) formation processes are unknown. It is unclear what epigenetic marks are present in epithelial cells and how EMT signal introduce mesenchymal marks. Epithelial cells are shown as green tightly packed clusters of cobblestone-shaped cells. Mesenchymal cells are depicted as blue elongated sickle shaped cells with lose cell-cell contact. It is also unknown that how these mesenchymal cells acquire self renewal properties to become cancer stem-like cells, hence this will also be included as one of the objective of the project. Additionally, role of a signal transduction kinase, Protein Kinase C (PKC) family will be investigated in regulating these epigenetic signatures during the process of EMT and CSC formation.
Chapter two

Materials and methods

This chapter provides in depth description of the materials and methods used for all the experimental procedures presented in this research thesis. In addition, this chapter also outlines the detailed analysis methodologies for Flow cytometric experiments and transfections. Besides that, Oligonucleotide primer sequences used in this research project and calculation methods for analysis of both cDNA and genomic DNA samples using Real-Time PCR, are also described in detail. All the procedures contain sufficient details for experimental replication. Details (suppliers and catalogue numbers) of the reagents used are specified in Appendix.
Chapter 2: Materials and methods

2.1 General Reagents

2.1.1 Chemicals

All the reagents used were either classified molecular biology or analytical grade.

2.1.2 Cell lines from the American Type Culture Collection (ATCC)

The adherent human mammary adenocarcinoma cell lines including MCF-7 (ATCC® number HTB-22), MDA-MB-231(ATCC® number HTB-26), MDA-MB-468(ATCC® number HTB-132), human adherent mammary ductal carcinoma T-47D (ATCC® number HTB-133) and human cervix carcinoma including HeLa (ATCC® number CCL-2) were obtained from ATCC (VA, USA). Stocks were stored at -196°C in 5 x 10⁶ cells/ml aliquots in either RPMI or DMEM Complete medium (section 2.1.3) containing 45% heat inactivated foetal calf serum (FCS; Sigma-Aldrich, St. Louis, MO) and 9% dimethyl sulfoxide (DMSO) solution (Cambridge Isotope Laboratories, Inc., Andover, MA). Cell stocks were thawed in complete media, RPMI or DMEM, and checked for mycoplasma contamination (as described in section 2.2.3). Cell lines were frozen as stocks described in cryotubes (NUNC, Roskilde, Denmark) at -70°C overnight and removed to long term storage in liquid nitrogen.

2.1.3 Media, buffers, and solutions

All media, buffers and solutions were obtained either from JCSMR Media/Wash-up Facility, ANU, Canberra, Australia or purchased from Gibco (Invitrogen Corporation, NY). RPMI-1640 (Gibco #11875-093) or Dulbecco’s Modified Eagle Medium (DMEM) (1X, liquid, low glucose, Gibco #12320-32) complete cell culture media were freshly prepared for tissue culture according to experimental demand by supplementing RPMI/DMEM plus HEPES with 10% heat inactivated FCS, 0.1% PSN antibiotics (section 2.1.4) and 2mM L-glutamine.
2.1.4 Antibiotics

Antibiotic stocks were dissolved in DDW filtered through 0.22 μm filters (Millipore, NSW, Australia) and prepared by the JCSMR Media Facility, ANU, Canberra, Australia. Penicillin, streptomycin and neomycin (PSN) antibiotics (1000 x stock): 30.07 g/L Penicillin G Sodium (MP Biomedicals, LLC), 50 g/L Streptomycin Sulphate (Sigma-Aldrich, St. Louis, MO) and 50g/L Neomycin Sulphate (Sigma-Aldrich, St. Louis, MO). PSN was added to all RPMI/DMEM Complete medium (section 2.1.3) except where otherwise stated.

2.1.5 Oligonucleotides

Primer/probe sets for gene expression analysis were purchased online from Taqman® Gene expression Assays (Applied Biosystems, Foster City, CA). Human Taqman® probe sets used for quantitative cDNA real-time PCR included laminin-5, Fibronectin, Integrin-β, snail-1, uPAR, E-cadherin, vimentin, MMP-1, Zeb1, CD44, CD24, LSD1, PKC-θ and cyclophilin A. All primer sequences used for quantitative real-time PCR analysis of transcript are listed in Table 2.1.

All genomic DNA oligonucleotides were purchased online as Guaranteed Oligos from EasyOligoes Australia (Sigma-Aldrich, St. Louis, MO) as 100μM stocks. Primer concentrations were optimized to achieve similar amplification efficiencies between different primer sets for the same gene. All oligonucleotide primer sequences used for quantitative real-time PCR are listed in Table 2.2.

2.1.6 Antibodies and conjugates

All antibodies for this study were purchased from commercial sources. Details of all commercially purchased antibodies are listed in Table 2.3.

2.1.7 Kits

100mM dNTP set (4x25μmol) kit (Astral scientific Pty. Ltd., NSW, Australia)
Platinum® Taqman DNA Polymerase (Invitrogen, Carlsbad, CA)
QIAmp® Blood Mini Kit (Qiagen, Valencia, CA)
Superscript™ III RNaseH-Reverse Transcriptase kit (Invitrogen, Carlsbad, CA)
PowerSYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA)
Taqman® universal PCR Master Mix (Applied Biosystems, Foster City, CA)
Taqman® MicroRNA Reverse Transcription kit

2.1.8 Enzymes and markers

Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Mannheim, Germany)
DNase I, RNase-free (Roche Diagnostics, Mannheim, Germany)
Proteinase K (solution), RNA grade (Invitrogen, Carlsbad, CA)
Superscript™ III RNaseH- Reverse Transcriptase (Invitrogen, Carlsbad, CA)

2.2 Cell Culture

2.2.1 Mammalian cell-lines

The adherent human mammary adenocarcinoma MCF-7 cells lines were grown in DMEM Complete media (section 2.1.3). After thawing the cells were kept in DMEM Complete media in a sterile 75cm² flask for 2 days before first splitting. Once confluent (after 2 days), cells were washed with 10 ml pre-warmed D-PBS (Gibco-BRL, Gaitherburg, MA) before adding 1ml of 0.05% trypsin-EDTA (1X) (Gibco-BRL, Gaitherburg, MA) on washed cells. Cells were then incubated for 3 minutes at 37°C in order to detach the cells, followed by addition of another 10 ml DMEM media. Cells were then centrifuged at 300 x g for 10 minutes prior to re-suspension in 1 ml of fresh complete DMEM media and counted subsequently by Vi-CELL-XR counter (section 2.2.2) before splitting at desired density of cells. Cells were passaged subsequently every 2-3 days depending on experimental demands and were sub-cultured when reached 80% confluences. For most of the experiments, 4x10⁴ cells/well of a 24 well plate, 4x10⁵ cells/ 75cm² flask or 4x10⁶ cells/375cm² flask were seeded one day before the experiment unless otherwise stated. MDA-MB-231All cells were grown in a humidified atmosphere of 5% CO₂/O₂ and incubated at 37°C in a Hepa-Filtered Infrared (IR) Incubator (Forma Scientific Inc., Materietta, OH).

All other cell lines used were grown and passaged in same way except using RPMI-1640 Complete media.
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FORWARD PRIMER
REVERSE PRIMER
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| uPAR     | OLIGO
FORWARD PRIMER
REVERSE PRIMER
Seq
GGGAAGCAAGCAAGGGTTA
GTTTTCAGGGATAGACTGG |
| Zeb1     | OLIGO
FORWARD PRIMER
REVERSE PRIMER
Seq
GCAGCCCAGCCTATATAAGGA
GACGCATTTATCGCCCCCTCT |
| Laminin-5| OLIGO
FORWARD PRIMER
REVERSE PRIMER
Seq
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CTCCGCTTAAAGGAACATCA |
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FORWARD PRIMER
REVERSE PRIMER
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CAAGATGTGCGCAAGGAGA |
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FORWARD PRIMER
REVERSE PRIMER
Seq
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ACGGCGCTCAGGAAAGGAT |
| miR-200b-429#2  | OLIGO
FORWARD PRIMER
REVERSE PRIMER
Seq
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GACGGGCTTAAATGAGTGT |
| miR-200c-141 | OLIGO
FORWARD PRIMER
REVERSE PRIMER
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2.2.2 Cell viability and density counts

Cell viability was determined by using Vi-CELL-XR Counter (Beckman Coulter Ireland Inc., Galway, Ireland). Commercially available Vi-CELL Counter reagent packs were purchased from Beckman Coulter Ireland Inc., Galway, Ireland. A 50μl aliquot of re-suspended cells (in 1 ml media) was diluted 1:10, in 450μl media and loaded in the Vi-CELL counter cup to perform cell count and viability check. Cell viability counts were constantly >98% unless otherwise stated.

2.2.3 Mycoplasma detection

Prior to freezing and after thawing cells were always checked for mycoplasma contamination and only mycoplasma-free cells were used for all the experiments. Mycoplasma detection was performed with MycoAlert Q Mycoplasma detection kit (Lonza, ME USA).

2.2.4 Stimulation conditions

MCF-7 cells were seeded one day before stimulation at a set density according to the experimental demand (section 2.2.6). Potential inducers of EMT were tested including Phorbol 12-Myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO), TNF-α/TNFsf1A (R&D systems, Minneapolis, MN), IL-6 (R&D systems), EGF-α (R&D systems, Minneapolis, MN) and TGF β (R&D systems, Minneapolis, MN) at various concentrations and incubation length as specified in Table 2.4.

2.2.5 PKC inhibition conditions

The PKC inhibitors used, their stock concentration and supplier information are described in Table 2.5. Corresponding control samples with equivalent concentration of dissolving media or vehicle, usually DMSO (unless otherwise stated) was also included for each experiment.
2.2.6 Epithelial to mesenchymal transition (EMT) assay

EMT assays were performed in 24 well plates (Costar, Corning Inc., Corning, NY, USA). For this assay, usually MCF-7 cells were seeded at $4 \times 10^4$/well/500μl media one day before the experiment unless otherwise specified. Cells were stimulated with various EMT stimuli prepared in warm media the following day and monitored for EMT changes under the microscope specified in section 2.4.2. The percentage of EMT was generally calculated based on phenotype counting 100 cells per field under the microscope. All the potential EMT stimuli used in this thesis are described in Table 2.4 and they were prepared and stored as per supplier specification.

2.2.7 Wound healing/Scratch assay

Wound healing assays were performed in 24 well plates. Cells were seeded at $8 \times 10^4$/well/500μl DMEM media 36 hours before the start of assay or until cell monolayer was grown. Usually monolayer was scratched with a 1 ml sterile pipette tip held vertically to create a wound in the middle of the well, except for the transfection experiments where cells were first transfected and then scratched. The monolayer was then washed 3 times with PBS (500μl) to remove the scratched cells before adding 500μl complete DMEM media. Next, monolayer was stimulated with potential EMT stimulus according to the experimental protocol. The cell monolayer was monitored for the healing of the wound and pictures were taken at different time intervals. The method for taking pictures and microscope used are described under the section 2.4.2. All the wound healing assays were performed in duplicate wells and the entire procedure was repeated at least twice.
### Table 2.4  Details of potential EMT stimuli

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<th>EMT Inducer</th>
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<th>Stock concentration</th>
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<td>PMA</td>
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<td>R&amp;D systems (210-TA/CF)</td>
<td>100 μg/ml in PBS</td>
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<tr>
<td>Recombinant human EGF</td>
<td>R&amp;D systems (236-EG)</td>
<td>10 μg/ml in 10mM Acetic acid with 0.1% BSA</td>
<td>50 ng/ml for 2 hour to 60 hour</td>
</tr>
<tr>
<td>Recombinant human IL-6</td>
<td>R&amp;D systems (206-IL)</td>
<td>10 μg/ml in PBS</td>
<td>50 ng/ml for 2 hour to 60 hour</td>
</tr>
<tr>
<td>Recombinant human TGF-β</td>
<td>R&amp;D systems (240/b-CF)</td>
<td>10 μg/ml in PBS</td>
<td>2.5 ng/ml for 2 hour to 60 hour</td>
</tr>
</tbody>
</table>

### Table 2.5  PKC Inhibitor information

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Supplier &amp; Catalogue number</th>
<th>Stock concentration</th>
<th>Final concentration &amp; Pre-incubation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bisindolylmaleimide-I</td>
<td>Calbiochem (203293)</td>
<td>2.4 mM in DMSO</td>
<td>1 μM for 1 hour</td>
</tr>
<tr>
<td>Go6976</td>
<td>Calbiochem (365253)</td>
<td>1.3 mM in DMSO</td>
<td>1 μM for 1 hour</td>
</tr>
</tbody>
</table>
2.2.8 Mammosphere assay

The following mammosphere culture media components were purchased from StemCell Technologies Inc., BC, Canada: Mammocult Basal medium (Human) (catalogue number-05621), Mammocult Proliferation Supplements (Human) (catalogue number-05622), heparin (catalogue number-07904) and hydrocortisone (catalogue number-07904). Hydrocortisone powder was freshly dissolved into mammocult basal medium to get $10^{-4}$ M solution on the day of experiment. 50 ml of Mammocult complete media was then prepared by addition of 45 ml Mammocult basal medium, 5 ml mammocult proliferation supplements, 100μl of the 0.2% heparin stock, 500μl of $10^{-4}$ M stock of hydrocortisone and 50μl of the PNS. Mammocult complete media was either used on same day or stored for not more than 7 days at 4°C.

MCF-7 cells were grown in a 175 cm$^2$ cell culture flask and harvested using a cell scraper (Zellschaber, Switzerland) or FACS sorted as specified in section 2.3.2. Importantly trypsin treatment was never used for harvesting cells as it interferes with the mammosphere assay. Harvested cells were then re-suspended in 1 ml mammocult complete media and centrifuged at 500 x g for 3 minutes at 20°C. The cell pellet was then re-suspended in 1 ml mammocult complete media before counting the cells on the ViCELL counter. Cell dilutions were then prepared to stain 40,000 cells/2 ml and 2 ml of cells were seeded in the 6 well-ultra low adherent, flat bottom plates (Costar, Corning Inc, Corning, NY, USA). Cells were either stimulated or not treated according to the experimental protocol and incubated at 37°C, under 5% CO$_2$ for 7 days in a Hepa-Filtered Infrared (IR) Incubator (Forma Scientific Inc., Materietta, OH). Mammospheres larger than 60 μm were counted per well on day 7 and pictures were taken with an Olympus
microscope (section 2.4.2). All the mammosphere assays were performed in duplicate wells and the entire procedure was repeated at least twice.

2.3 Immunofluorescence

2.3.1 Intracellular staining

Cells from each well were harvested by treatment with trypsin and followed by two washes with PBS. The washed cells then re-suspended in 1000μl of freshly prepared paraformaldehyde (PFA) (2% in PBS) (pH 7-8) (Analytical Grade, UNIVAR, Seattle, WA), incubated for 15 minutes at room temperature in a 15 ml falcon tube. After fixing the cells with PFA, cells were washed twice with PBS by centrifuging at 300 x g and re-suspended in 250μl of PBS for storage at 4°C (not more than 2 weeks) or used immediately. In case of immediate use, 100μl of the fixed cells were placed on Poly-L-lysine (Sigma-Aldrich, St. Louis, MO) pre-treated coverslips and spun down on cytopsin 2 (Shandon, England) at 300 x g for 3 minutes to facilitate sticking of cells on the coverslip. Each coverslip was then transferred into a single well of a 24 well plate (Corning Incorporated Costar®, Corning, NY) containing 200μl PBS to prevent the cell drying. Coverslips were twice washed with PBS and then incubated for 15 minutes at room temperature with 1% solution of Triton X-100 (Sigma-Aldrich, St. Louis, MO) diluted in PBS. Wells were washed with the PBS twice before addition of 1% Bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) in PBS (blocking solution) and incubated for 2 hours at room temperature. Primary antibodies (1:100 or 2 μg/ml) were diluted in blocking solution prior to addition to the cells (on coverslips in 24 well plates) and incubated at 4°C overnight (with lid of the plates be closed to prevent evaporation). The primary antibodies used for intracellular staining was anti-laminin-5 (details in Table 2.3). Next
day excess primary antibody was removed by washing 3 times with 200μl of blocking solution for 5 minutes at room temperature. Conjugated secondary antibodies (Donkey anti-rabbit IgG) tagged with FITC (details in Table 2.3) were then diluted 1:100 in blocking solution and incubated with the cells at 37°C for 30 minutes. Again, the cells were washed 3 times with the blocking solution for the removal of excessive secondary antibody. Samples were then stained with 100ng/ml of DAPI (Sigma-Aldrich, St. Louis, MO) in 200μl of PBS for 5 minutes. Afterwards, the cells were washed twice in blocking solution, rinsed in PBS and kept in PBS before mounting the coverslip on the glass slide (Livingstone, NSW, Australia) with help of 5 μl of mounting media-vectashield (Vector Laboratories, Burlingame, CA). The outer boundary of the coverslip then sealed with nail-polish, slides were then left overnight to dry at 4°C in dark and either microscopic analysis was performed or slides were stored in slide archive box at 4°C.

2.3.2 Fluorescence-activated cell sorting (FACS)

Cells from each well/flask were harvested by means of trypsin treatment followed by two washes with PBS. Washed cells then re-suspended in an antibody cocktail consisting anti-CD44-APC, anti-CD24-PE antibodies (details in Table 2.3) and Hoechst 33258 dye (final dilution of 1:1000) (Invitrogen, Carlsbad, CA) in 1% FCS-PBS solution. Cells re-suspended in antibody cocktail were incubated for 20 minutes at 4°C. Cells were next washed twice with PBS and re-suspended in 1% FCS-PBS solution (volume based on cell number) and kept on ice until analyzed by FACS. Forward scatter (FSC) and side scatter parameters were selected with FITC fluorochrome excited by 488 nm argonion laser, PE fluorochrome excited by 488 nm and Hoechst fluorochrome excited by 350 nm helium-cadmium UV laser.
Flow Cytometry data was produced using either BD FACS LSR Flow Cytometer (Becton Dickinson Biosciences) or BD FACS Aria™ II Flow Cytometer (Becton Dickinson Biosciences) and analyzed using the data acquisition software CellQuest Pro (Becton Dickinson Biosciences) and FlowJo (Tree Star Inc., Ashland, OR) software at the MCRF facility, JCSMR, ANU, Canberra, Australia. Single colour controls were used to set compensation parameters. Isotype controls were used for all corresponding primary antibodies in each experiment.

2.3.3 CD44 and CD24 staining optimization in MCF-7 cells

The FACS gating strategy used in this thesis is adopted and modified from the pioneer breast cancer stem cell publications (Al-Hajj et al., 2003) which sorted cells based on the CD44\textsuperscript{high} and CD24\textsuperscript{low} expression. Expression of CD44\textsuperscript{high} and CD24\textsuperscript{low} has been shown to be associated with human breast cancer stem cells (Al-Hajj et al., 2003; Sleeman et al., 2006; Mani. et al., 2008). To confirm that anti-CD44-APC and anti-CD24-PE antibodies were specific, isotype control antibodies were used. The isotype (negative controls) used for anti-CD24-PE was PE Mouse-Anti-human IgG2aK and for anti-CD44-APC was APC-Mouse-Anti-human IgG2bK. First, all the cells were stained with Hoechst 33258 to monitor cell viability (Figure 2.1 A). In addition cells were stained with varying concentrations of either APC or PE isotypes controls as indicated in Figure 2.1 B and C. Figure 2.1 A shows a representative FACS plot of the gating strategy used to exclude dead cells (Hoechst negative) for analysis. Hoechst 33258 stain was always integrated in the gating strategy to ensure that no dead cells were included in the analysis, leading to sorting of only live cells. Figure 2.1 B & C show a representative FACS histogram plot of PE-isotype control and APC-isotype control, which showed no binding of the isotype antibody control, which confirms.
Figure 2.1  Specific staining of anti-CD24-PE and anti-CD44-APC on live MCF-7 cells for FACS analysis.

(A) FACS plot showing FSC fluorescence against Hoechst 33258 fluorescence. Gate was made on live cells which are Hoechst negative cells. (B) FACS histogram plot showing APC-anti-CD24 antibody binding relative to APC isotype control (APC-Mouse-anti-human IgG2kb). All the cells were stimulated with PMA (0.65 ng/ml for 60 hours) and stained with Hoechst 33258 for 20 minutes on ice. (C) FACS histogram plot showing PE-anti-CD44 antibody binding relative to PE Isotype control (PE-Mouse-anti-human IgG2kb). All the cells were stimulated with PMA (0.65 ng/ml for 60 hours) and stained with Hoechst 33258 for 20 minutes on ice.
the binding specificity of anti-CD24-PE (1:100 dilution) and anti-CD44-APC (1:100 dilution) antibodies to cells relative to controls. All the cells used for this experiment were stimulated with PMA (0.65ng/ml) for 24 hours before staining and staining was done for 20 minutes on ice. All the experiments were done at least twice before any conclusions were drawn (only representative FACS plot are shown in this thesis).

2.4 Microscopy

2.4.1 Fluorescence microscopy

Cells were stained as outlined either in section 2.3.1 or 2.3.2 and mounted on coverslips as described in section 2.3.1. Stained cells were viewed under oil immersion at x 100 magnification using Olympus Fluorescence 1X71 microscope (Olympus, Tokyo, Japan) or 60x magnification or Leica confocal microscope (Leica microsystems). Images on Olympus Fluorescence 1X71 microscope were captures using DPController camera software version 1.2.1.108 (2002 Olympus optical Co., LTD) and images on Leica confocal microscope were captured using Leica application suite, 2.0.0 program. Images were analysed using Photoshop CS3 (Adobe Systems Inv., San Jose, CA). GFP/FAM vector transfected wells or flasks were viewed under Olympus Fluorescence 1X71 microscope using FITC excitation filter 406-495 nM filter (WIB).

2.4.2 Phase-contrast microscopy

Phase contrast microscopy was utilized for EMT and wound healing assays under 10 x or 20 x magnification of Olympus Fluorescence 1X71 microscope. Images were captured and analysed as described in section 2.4.1 except that wound healing assay pictures were also analysed by Image J software (Free software in Public domain developed by NIH).
2.5 Transfection

2.5.1 DNA transfection

Conditions were optimized for DNA transfection in MCF-7 and T-47D cells by using two commercially available transfection agents, FuGENE 6 (Roche Diagnostics, Mannheim, Germany) (the detailed method for FuGENE 6 is described under section 2.5.2) and Lipofectamine LTX (Invitrogen, Carlsbad, CA) (the detailed method for Lipofectamine LTX is described under section 2.5.3) respectively. To achieve the maximum transfection efficiency, initially, both of the transfection reagents were examined at varying ratio of reagent: DNA/oligo (GFP-expression vector for optimization). For all the DNA transfections cells were seeded at $1 \times 10^5$ cells per well in 500 μl of antibiotic-free media 24 hour prior to the commencement of transfection in 24 well plate and transfections were performed as per the manufacture’s guidelines. The dilution medium used for transfections, was OptiMEM® I Reduced-Serum Medium (1X), liquid (Invitrogen, Carlsbad, CA). Transfection percentage was checked after 36-48 hours by FACS as described under section 2.3.2. A typical example of FACS based selection criteria for transfection efficiency of the GFP-tagged vector is shown in Figure 2.2. Florescent microscope was also used for ensuring the transfection of GFP-tagged vector (detailed method of microscopy is described in section 0). Cells were stimulated (stimulation conditions described under section 2.2.4) following 48 hours post-transfection at different time points. Transfection efficiency of various transfection agents have been shown in Figure 2.3 (For T-47D cells), Figure 2.4, Table 2.6 (For HeLa cells) and Figure 2.5, Table 2.7 (For MCF-7). A detailed selection criterion for best cell model based on transfection efficiency has been described under section 3.1.
2.5.2 DNA transfection using FuGENE 6 reagent

For a single reaction in 500 µl total volume (per well of 24 well plate), 0.9 µl FuGENE 6 was added into a 1.5 ml eppendorf tube containing 10 µl of OptiMEM® I Reduced-Serum Medium, mixed well and incubated for 5 minutes at room temperature. After 5 minutes, either 0.4 µg or 0.3 µg GFP tagged DNA (volume of DNA was calculated depending upon the concentration of specific DNA oligonucleotide used) was added into the tube to get a 2.25:1 or 3:1 ratio of FuGENE 6 respectively. DNA and the complex were then incubated for 45 minutes at room temperature. After 45 minutes, 20 µl of the FuGENE-DNA complex was added on top of the cells drop wise and mixed by swirling the plate and plates were incubated at 37°C for 36-48 hours.

2.5.3 DNA transfection using Lipofectamine LTX and plus reagent

For a single reaction in 500 µl total volume (per well of 24 well plate), either 250 ng or 500 ng of GFP-tagged DNA was added in tubes containing 100 µl of OptiMEM® I Reduced-Serum Medium and mixed gently. 1 µl of Plus reagent per 1 µg of DNA was then added to these tubes and incubated for 10 minutes at room temperature. This step was followed by addition of 1.25 µl Lipofectamine LTX per reaction, followed by incubation of tubes for 25 minutes at room temperature. After 25 minutes of incubation, 100 µl of different LTX-DNA complexes were added on top of the cells drop wise and mixed by swirling the plate and plates were incubated at 37°C for 36-48 hours.
Figure 2.2 Transfection efficiency of FuGENE 6 using Hoechst dye and GFP tagged vector.

(A) FACS plot showing percent live T-47D cells after transfection with FuGENE 6 as a transfection reagent and Hoechst 33258 for dead cells detection. (B) FACS plot showing percent transfected cells among the live T-47D cells. (C) FACS plot showing percent live MCF-7 cells after transfection with FuGENE 6 as a transfection reagent and Hoechst 33258 for dead cells detection. (D) FACS plot showing percent transfected cells among the live MCF-7 cells.
Figure 2.3  Transfection efficiency of various transfection reagents in T-47D cells.

Live T47D cells after 48 hours post-transfection with following transfection reagent and GFP-tagged vector ratio: (A) 3 μl FuGENE 6 and 1 μg DNA, (B) 2.25 μl FuGENE 6 and 1 μg DNA, (C) 1.25 μl LTX and 250ng DNA with Plus reagent and (D) 1.25 μl LTX and 500ng DNA with Plus reagent.
Figure 2.4  Transfection efficiency of various transfection reagents in HeLa cells.

Live HeLa cells after 48 hours post-transfection with following transfection reagent and GFP-tagged vector ratio: (A) 3 μl FuGENE 6 and 1 μg DNA, (B) 2.25 μl FuGENE 6 and 1 μg DNA, (C) 1.25 μl LTX and 250ng DNA with Plus reagent and (D) 1.25 μl LTX and 500ng DNA with Plus reagent. (Relative results upon FACS analysis shown in Table 2.6)

Table 2.6  FACS analysis of HeLa cells followed by treatment of various transfection reagents.

<table>
<thead>
<tr>
<th>No.</th>
<th>Transfection reagent</th>
<th>% Live HeLa cells using Hoechst dye</th>
<th>GFP vector's transfection % of live HeLa cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FuGENE 6 2.25 μl:1 μg DNA</td>
<td>53</td>
<td>74</td>
</tr>
<tr>
<td>2</td>
<td>FuGENE 6 3 μl:1 μg DNA</td>
<td>48</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>LTX 1.25 μl: 250 μg DNA with Plus reagent</td>
<td>49</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>LTX 1.25 μl: 500 ng with Plus reagent</td>
<td>48</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>88</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 2.5 Transfection efficiency of various transfection reagents in MCF-7 cells.

Live MCF-7 cells after 48 hours post-transfection with following transfection reagent and GFP-tagged vector ratio: (A) 3 μl of FuGENE 6 and 1 μg DNA, (B) 2.25 μl of FuGENE 6 and 1 μg DNA, (C) 1.25 μl LTX reagent and 250 ng DNA with Plus reagent and (D) 1.25 μl LTX reagent and 500 ng DNA with Plus reagent, (Relative results upon FACS analysis shown in Table 2.7).

Table 2.7 FACS analysis of MCF-7 cells followed by treatment of various transfection reagents.

<table>
<thead>
<tr>
<th>Number</th>
<th>Transfection reagent</th>
<th>% Live MCF-7 cells using Hoechst dye</th>
<th>Transfection % of live MCF-7 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FuGENE 6 2.25:1</td>
<td>85</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>FuGENE 6 3:1</td>
<td>60</td>
<td>81</td>
</tr>
<tr>
<td>3</td>
<td>LTX 1.25 μl: 250 μg DNA with Plus reagent</td>
<td>56</td>
<td>75</td>
</tr>
<tr>
<td>4</td>
<td>LTX 1.25 μl: 500 ng with Plus reagent</td>
<td>45</td>
<td>73</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>85</td>
<td>0</td>
</tr>
</tbody>
</table>
2.5.4 SiRNA Transfection

Lyophilized silencer® FAM™ labeled negative control siRNA (Mock) (AM4620); validated PKC-β siRNA (s11095) assays were purchased from Ambion, Applied Biosystems, Foster City, CA and validated PKC-θ siRNA (sc-36252) was purchased from Santacruz Biotechnologies, California. The specificity of these siRNAs has been previously published (Sutcliffe et al., 2011). Forward transfections with 10nM siRNA were performed in MCF-7 cells by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Forward transfection methods were performed in 24 well plates (4 x 10⁴ cells) for EMT assays and for transcript analysis, while for Chromatin Immuno-Precipitation (ChIP) assays where large quantities of cells were required, transfections were performed in 25Cm² flasks (4 x 10⁵ cells).

Briefly, for setting up forward transfection reactions in 24 well plates, 4 x 10⁴ MCF-7 cells per well were seeded in 500 µl of antibiotic free media 24 hours prior to transfection. 250 µl DEPC-water was added in the 20 nmol lyophilized siRNA stock to get a 20 µM concentration stock. To achieve a final siRNA concentration of 10 nM (for one reaction in total volume of 500 µl media in one well of 24 well plate), 3 µl of 20 µM siRNA stock was further diluted in 50 µl Opti-MEM® I and incubated for 5 minutes at room temperature. This step was immediately followed by a further dilution of Lipofectamine by adding 1 µl Lipofectamine (for one reaction) in 50 µl Opti-MEM® I, followed by 5 minutes incubation at room temperature. 50 µl of diluted siRNA and 50 µl of diluted Lipofectamine solutions were then mixed together to get 100 µl of the siRNA-lipofectamine complex. This complex was subsequently incubated in the dark, at room temperature for 20 minutes. The resultant siRNA-lipofectamine complex was carefully pipette onto the surface of the cells and mixed by gently rocking the plate
back and forth. Plates were incubated for 48-72 hours and transfection efficiency was checked by flow cytometry (Figure 2.6 A) (refer to section 2.3.2 for detailed method of flow cytometry analysis). The knockdown was checked with three methods: (1) transcript analysis on gene of interest was carried out to confirm the knockdown of specific gene, for example, LSD1 knockdown by LSD1 directed siRNA is shown in Figure 2.6 B using LSD1 oligonucleotide (described in Table 2.1) for real-time PCR from Taqman. (2) at the ChIP level (please refer to ChIP results for specific genes) (3) optimization experiments were carried out for each siRNA to confirm maximal transfection efficiency (70-80%) in MCF-7 (Figure 2.6 A and B). Therefore, the results presented in this thesis are reproducible. Each mock (control) and gene specific siRNA knockdown experiment was performed three independent times and only one representative experiment is shown in the this section.
Figure 2.6 Transfection efficiency of FAM labeled siRNA control (mock) with Lipofectamine 2000 using forward transfection strategy

(A) FACS plot showing transfection efficiency of silencer® FAM™ labeled negative control siRNA. Forward transfection of MCF-7 cells with silencer® FAM™ labeled negative control siRNA was performed or cells were treated without control siRNA, transfected cells were incubated for 48-72 hr and FACS analysis was performed for FAM positive cells.

(B) LSD1 knockdown using forward siRNA strategy decreases the transcription LSD1 in both non-stimulated and PMA-stimulated MCF-7 cells. MCF-7 cells were transfected with either 10nM Mock siRNA (silencer® FAM™ labeled negative control siRNA) or LSD1 siRNA followed by either Non-stimulation (NS) or 60 hr of PMA stimulation (0.65 ng/ml) after 48 hr of transfection. Taqman® real time PCR for LSD1 was performed on cDNA synthesized from total RNA isolated from cells. Threshold cycle (Ct) values generated for each time points were converted to arbitrary copy number and normalized to Cyclophilin A reference levels. mRNA levels are expressed as arbitrary copies normalized to Cyclophilin A reference levels. Data represent mean ± standard error (SE) from three independent experiments.
2.5.5 MicroRNA transfection

FAM labeled control pre-miRNA (001093) assays, pre-miR 200b (PM 10492), pre-miR 200c (PM 11714) or pre-miR 200c* (PM 12741) (Applied Biosystems, Foster City, CA) were used for forward transfection of microRNAs. MicroRNA transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) by a similar method described for siRNA transfection (as described in section 2.5.4).

2.6 Molecular Biology Techniques

For RNA isolation procedure (section 2.6.1), all the pipettes, tube holders and gloves were always pre-treated with RNase Zap (Ambion, VIC, Australia). RNase, DNase, pyrogen free, sterile microtubes (Axygen Scientific, Inc., Union City) and Pre-sterile aerosol resistant tips and fresh bench coat were routinely used for all the molecular biology work described in this section.

2.6.1 Total RNA isolation

For most of the experiments, total RNA was extracted from $5 \times 10^5$ to $1 \times 10^6$ MCF-7 cells unless otherwise specified. Cells were first thawed in 1 ml of Trizol® Reagent (Invitrogen, Carlsbad, CA) for 5 minutes at room temperature to inactivate RNases, followed by trituration to dislodge the cell pellet. The dissociated cells suspended in Tizol (1 ml) were transferred into a 1.5 ml eppendorf tube for 5 minutes for homogenisation. RNA was then extracted by addition of 200μl chloroform and mixing was done vigorously before centrifuging the samples at 8,000 x g for 30 minutes at 4°C. The aqueous layer was then collected into a fresh 1.5 ml eppendorf tube and an equal volume of isopropanol was added to the aqueous layer and mixed gently. After 5 minutes at room temperature, samples were either snap frozen on dry ice and stored at -70°C overnight or until the isolation procedure could be re-commenced. After thawing the
samples quickly, the samples were again centrifuged at 8,000 x g for 30 minutes at 4°C to precipitate the RNA. To remove all traces of isopropanol, next the RNA pellets were washed with 1 ml of ice cold 80% ethanol (Analytical UNIVAR, Seattle, WA) before centrifuging at 3,600 x g for 10 minutes at 4°C. All ethanol then was removed and pellets were allowed to air dry for 5 minutes. RNA samples were then solubilised by re-suspending them in 50 µl of nuclease-free DEPC (Diethylpyrocarbonate treated) water (Ambion, VIC, Australia). Next, 2 µl of the sample was taken out to measure RNA quality and quantity on Nano-drop® Spectrophotometer ND 1000 (Nanodrop Technologies, Inc., Wilmington, DE, USA) using ND-1000 V3.30 software. All the RNA samples were found pure as they had A_{260}/A_{280} ratio of 1.9-2.1 and this ratio provides an estimate of RNA purity with respect to contaminants such as proteins that absorb in the UV spectrum.

2.6.2 First strand cDNA synthesis

Superscript TM® III kit (Invitrogen, Carlsbad, CA) was used for cDNA synthesis. Master mix-1 was prepared by adding 1 µl of 5 µM oligo (dT) and 1 µl of 100mM dNTPs and mixed by flicking. Mastermix-2 was prepared by adding 2 µl of RT buffer, 2 µl of 3mM mgCl₂, 2 µl of DTT mix, 1 µl of RNase out, 1 µl of superscript III and mixed by flicking. For 1 µg RNA, 2 µl of the master mix-1 was added to the samples, mixed and incubated at 65°C for 5 minutes and then samples were placed on ice to stop the reaction. This step was followed by addition of 10 µl of the master mix-2 per sample and incubation of samples at 50°C for 50 minutes. The reaction was then stopped by incubating samples at 85°C for 5 minutes followed by placing the samples on the ice for 2 minutes. Finally 1 µl of RNaseH was added to each sample and samples were incubated at 37°C for 20 minutes. All the samples were either snap frozen on dry ice or used immediately for quantitative Real-Time PCR analysis.
2.6.3 Quantitative Real-Time PCR (qRT-PCR) analysis

TaqMan® Gene expression Assays (Applied Biosystems, Foster City, CA) were used to perform qRT-PCR on an ABI PRISM 7900 HT fast Real-Time PCR sequence detector (PerkinElmer/PE, Applied Biosystems, Foster City, CA) using the FAM probe channel. A total reaction volume of 10μl was used with cDNA diluted at 1:20 with DEPC water for the PCR, as detailed in the manufacture's guidelines (PerkinElmer/PE, Applied Biosystems, protocol PN 4333458). For all genomic DNA, Power SYBR Green real-time PCR (PerkinElmer/PE, Applied Biosystems, Foster City, CA) reactions were performed and the ChIP samples were diluted at 1:5. Each PCR was performed in duplicate wells using thermocycler conditions as follows: stage 1- 50°C for 2 minutes for 1 cycle; stage 2- 95°C for 10 minutes for 1 cycle; stage 3- 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. For all the primers sets, no template controls were always included to test for PCR amplification of any contaminating DNA within the PCR mix. Dissociation curves were performed for each primer set to confirm amplification of a single product using the following PCR conditions: stage 1- 95°C for 15 seconds; stage 2- 60°C for 20 seconds; with a minimum ramp speed to reach stage 3- 95°C for 15 seconds. PCR reactions were performed using Optical PCR 384 well reaction plates (Applied Biosystems, Foster City, CA).

2.6.4 Data analysis of cDNA experiments

All the threshold cycle (Ct) values from the PCR amplification plots were converted to arbitrary copy number using the formula $100000/2^{(Ct-17)}$ in Microsoft excel spread sheet, where a $C_t$ value of 17 was set to $10^5$ copies and assuming that each cycle increase equated to a 2 fold increase in input DNA. All the primers were checked against an amplicon standard curve to show that above formula produced results that were similar to results obtained with amplification standard curve method. Cyclophilin A primer (section 2.1.5) PCR reactions were
performed concurrently for each experiment to normalize for differences in RNA input and cDNA synthesis. All experiments were performed in duplicate.

2.6.5 cDNA synthesis for MicroRNA

The TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) was used to convert total RNA into cDNA for microRNA analysis with specific TaqMan® miRNA primers assays. Reagents of TaqMan® MicroRNA Reverse Transcription Kit were allowed thawing completely on ice before preparing RT master mix by adding following components per 15μl reaction volume; 0.15 μl dNTP mix (100mM), 1 μl Multiscribe™ RT enzyme (50U/μl), 1.5 μl 10 x RT Buffer, 0.19 μl RNase Inhibitor (20 U/μl) and 4.16 μl Nuclease free water to get a total volume of 7 μl. All the components were mixed gently, centrifuged briefly and then this RT master mix was placed on ice until the RT reaction plate was prepared. RT reaction plate was then prepared; by first combining total RNA (1-10 ng per 15 μl reaction) with the RT master mix in a ratio of 5 μl RNA: 7 μl RT master mix. MicroRNA RT primers were then thawed on ice and tubes to be used for RT reaction were labeled with appropriate numbers. Next, 12 μl of the RT master mix containing total RNA was dispensed into each tube before adding 3 μl of 5x RT primers to the appropriate tubes to bring the total volume 15 μl per tube. All the tubes were then mixed gently, centrifuged briefly and incubated on ice for 5 minutes or until ready to load on thermal cycler for reverse transcription at following thermal cycles at 15 μl reaction volume – Hold for 16°C for 30 minutes for 1 cycle; Hold for 42°C for 30 minutes for cycle 2; Hold for 85°C for 5 minutes for cycle 3 and 4°C for ∞ for cycle 4. After completion of the run, samples were either saved at -20°C or used immediately for qPCR.
2.6.6 qPCR amplification for MicroRNA cDNA

qPCR reaction was performed by preparing the 20 μl qPCR reaction mix by addition of the following components into appropriate tubes: 1 μl of TaqMan® small RNA Assay (20x); 1.33 μl of product from RT reaction; 10 μl of TaqMan® Universal PCR master Mix II (2 X, no UNG) and 7.67 μl nuclease free water. Components were then mixed gently and centrifuged briefly. All the qPCR reactions were performed in triplicate. 20 μl of the complete qPCR reaction mix (including assay and RT product) were then transferred into each of three wells of a 384-well plate. Plate was then sealed, centrifuged briefly and PCR amplification was performed as described in section 2.5.2.

2.7 Chromatin assays

2.7.1 Chromatin immunoprecipitation (ChIP) assay

Between 1-5 x 10^6 Cells were harvested following various treatments according to the experimental requirement and re-suspended in 10 ml DMEM completer media at room temperature after counting then at Vi-CELL counter. Cells were then cross linked with freshly prepared 1% paraformaldehyde (PFA) (Analytical UNIVAR, Seattle, WA) for 10 minutes at room temperature with continuous but slow rotations on rotary wheel. Next, the reaction was quenched by the addition of 2M glycine solution (AnalaR, Merck, Darmstadt, Germany) to a get a final concentration of 125mM and mixed further for 10 minutes at room temperature on the rotary wheel. Cells were then washed three times with 10 ml ice cold PBS and the cell pellet was either snap frozen on dry ice or used immediately afterwards. SDS Lysis Buffer (Upstate Biotechnology, Billerica, MA) was prepared by addition of 1 x complete protease inhibitor solution (1 tablet dissolved in 1 ml DEPC water) (Roche Diagnostics, Mannheim, Germany) and cell pellet was then re-suspended in 250 μl of the in the SDS lysis buffer for 10 minutes at room
temperature. Cells were sonicated (10 sec pulses for 2 minutes on 1 liter ice cold water mixed with ice, 70% maximum output) to shear the chromatin to obtain an average DNA fragment size of 250-500 bp using a Cole Palmer Ultrasonic processor (Cole Plamer, Vernon Hills, IL). After the sonication, samples were centrifuged at 10,2000 x g for 5 minutes at room temperature to clear cellular debris and the supernatant was then diluted to 1:10 with ChIP dilution buffer (Upstate Biotechnology, Billerica, MA). Antibodies as per the experiment requirement were aliquoted to the 1.5 ml eppendorf tubes before adding sonicated chromatin from 0.5-1 x 10^6 cells diluted in the dilution buffer and the ChIP mixture was then incubated with antibodies overnight at 4°C on rotary wheel. For all the experiments total genomic DNA without any antibody (named Total Inputs) for each condition was snap frozen and stored at -70°C, also a sample without any antibody (named No antibody) was processed in parallel with the ChIP samples. Next, immune complexes were bound by addition of 60μl of salmon sperm DNA/Protein A agarose beads at 4°C for 1 hour at rotary wheel. Samples were then centrifuged at 2500 x g for 2 minutes at 4°C and the supernatant was discarded before washing beads at 4°C for 5 minutes on a rotary wheel with each of the following washing buffers from Upstate Biotechnology (Billerica, MA) in the same order as described; first wash- 500μl of low salt immune complex wash buffer; second wash- 500μl of high salt immune complex wash buffer; third wash-500μl of LiCl immune complex wash buffer; fourth wash-500μl of low salt immune complex wash buffer and fourth wash- 1 ml TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Protease Inhibitor-complete, 1 x (Roche Diagnostics, Mannheim, Germany) was added to all the wash buffers immediately before use. DNA-protein complexes were then eluted from the beads with 400μl of the freshly made elution buffer (1% (w/v) SDS, 100mM NaHCO₃) for 30 minutes at room temperature on rotary wheel. Samples and total input controls were then incubated to hydrolyse cross links (or reverse-cross link) at 66°C overnight after adding 16 μl of
5M sodium Chloride (Sigma-Aldrich, St. Louis, MO). Next day, samples were treated with 1μl of Protease K solution (20μg/μl) (Invitrogen, Carlsbad, CA) for 1 hour at 45° C. Digested protein was removed from the ChIP samples by addition of equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) saturated with 10mM Tris, pH 8.0, 1 mM EDTA (Sigma-Aldrich, St. Louis, MO), mixing the samples and subsequent centrifugation at 10,200 x g for 20 minutes at room temperature for collection of the aqueous layer. Genomic DNA was precipitated from aqueous fraction by the addition of 2.5 volumes of ice-cold absolute ethanol (Analytical UNIVAR, Seattle, WA), 0.1 volume of 3 M sodium acetate buffer solution pH 5.2 (Sigma-Aldrich, St. Louis, MO) and 25μg of GeneElute™ linear polyacrylamide (Sigma-Aldrich, St. Louis, MO) for at least 24 hours at -20°C. Next, samples were pelleted by centrifugation and washing with 80% ice cold ethanol and pellets were allowed to air dry prior to suspension in 20μl of the DEPC water for real time PCR analysis. The oligonucleotides used for performing real-time PCR on ChIP samples have been listed in Table 2.2. The amount of precipitated target DNA sequence was calculated as described in section 2.7.3. A schematic illustration of the ChIP method is shown in Figure 2.7.

2.7.2 Sequential ChIP

Primary ChIP of was performed as described above in section 2.7.1 until the TE buffer washing step. Thus, immunoprecipitates from the primary ChIP were dissolved in 60μl elution buffer containing 10mM DTT (Superscript™ III RNaseH-Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) in DEPC water and incubated for 1 hour in a 37° C water bath. Tubes were flicked every 15 minutes during this incubation period. Next, samples were diluted with 1:40 of ChIP dilution buffer before taking 400μl of the samples to be frozen at -70°C to use as a total genomic input for secondary ChIP. The second antibody was added to these samples and immunoprecipitation was carried out again as the ChIP protocol (as defined in section 2.7.1.)
except that immune complexes were bound to 60μl of the salmon sperm DNA/Protein A agarose beads for 2 hour at 4°C. Sequential ChIP samples were then eluted, the cross-linking was reversed and the genomic DNA was precipitated as method described in section 2.7.1 above. Sequential ChIP analysis was carried out using the ChIP enrichment ratio method described in section 2.7.3 and then expressed as a fold change with respect to the non-stimulated samples. A schematic illustration of the sequential ChIP method is shown in Figure 2.8.

### 2.7.3 Data analysis for ChIP experiments

All ChIP assays were carried out in the presence of a non-antibody control as well as an isotype specific control antibody. The negative control is a non-antibody control and the enrichment values from these are routinely low and are included as background subtractions in the calculations of ChIP enrichment ratio. C\textsubscript{T} values from the real time PCR amplification plots were first converted to arbitrary copy number using formula the 100000/2^{(C\textsubscript{T}-17)} (section 2.6.4). Sample data was next normalized to the corresponding total input arbitrary copy number. Fold change above the no antibody control was then calculated to get ChIP enrichment ratio. ChIP enrichment ratio values were multiplied by a factor of 10 except the ChIP enrichment ratio values for histone modifications. This method of analysis was adopted from Pokholok et al. who established that the ChIP enrichment ratio presented on the linear scale better emphasizes signal versus noise in the display of ChIP-on-ChIP data over a logarithmic scale (Pokholok et al., 2006). Consequently, all the ChIP data in this thesis is presented on a linear scale. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. In some cases fold change was calculated with respect to the non-stimulated sample, which was set as 1. Statistical significance was determined by two-tailed Paired-t test using GaraphPad Prism 5.03 for Windows.
All the chip samples were always normalized with the Total input (TI) of each corresponding sample. If the recovery of the ChIP samples was low in the stimulated cells and CSC subset, then the arbitrary copies achieved in the real-time PCR analysis would be low but this was not the case and arbitrary copies from the samples were in same Ct range as the others. Since the "ChIP enrichment ratio" method has been used for analysis of the, the recoveries of the ChIP DNA across the different samples did not affect the analysis and interpretation of the data. This ChIP analysis method by using "ChIP enrichment ratio" is considered as the best method for calculating ChIP data (Pokholok et al., 2006). Therefore, the results were not likely to be effected due to poor ChIP recoveries.

For sequential ChIP analysis, the genomic DNA recovered from the sequential ChIP experiments was quantified by SYBR Green real-time PCR using primers specific for the promoter regions of the uPAR or miR 200c. The $C_t$ values from the PCR amplification plots were converted to arbitrary copy number using the formula $100000/2^{\Delta (C_t-17)}$ (section 2.6.4). The no antibody control was subtracted from the data for each sample, which were then normalised to the corresponding total input (TI-1) that was taken prior to the first immunoprecipitation. Data generated were then normalised to their respective 2nd total immunoprecipitation input (TI-2). Finally, fold change was then calculated with respect to the non-stimulated sample, which was set as 1. These values were then used to prepare the sequential ChIP plots shown in Figures. This method of sequential ChIP analysis has been adopted from Sutcliffe et al., 2009. Statistical significance was determined by two-tailed Paired-t test using GaraphPad Prism 5.03 for Windows. This method of sequential ChIP analysis has been adopted from Sutcliffe et al., 2009.
Figure 2.7  Schematic depiction of ChIP assay.

Proteins are cross linked to DNA with formaldehyde before cells lysis. Chromatin is then fragmented by sonication and a protein-specific antibody is added. This allows purification of specific protein-DNA complexes. Cross-links are reversed to isolate and purify DNA and the read-out is performed by real-time PCR. The control for the ChIP procedure omits the antibody addition step. Cross-linked DNA could also be used for ChIP on ChIP and ChIP sequencing analysis. Adapted and modified from (Massie and Mills, 2008).
Figure 2.8  Schematic representation of Sequential ChIP assay.

DNA is first cross linked with protein using formaldehyde before cell lysis. Chromatin is then fragmented by sonication. Chromatin is immune-precipitated with first antibody, followed by a wash. Next, immuno-precipitation of chromatin is carried out with second antibody, followed again by a wash. Chromatin is then eluted and, ultimately, cross-linking is reversed to obtain purified DNA. The read-out is performed via real-time PCR.
Chapter three

Establishing an inducible *in vitro* model of the human epithelial to mesenchymal transition for epigenetic analysis
Chapter 3: Establishing an inducible *in vitro* model of the human epithelial to mesenchymal transition for epigenetic analysis

**Introduction**

The epithelial to mesenchymal transition (EMT) is an extreme example of cell plasticity and is characterized by the concomitant loss of epithelial cell characteristics and gain of mesenchymal cell properties (Kalluri, 2009; Thiery et al., 2009). There is very little known about the role of epigenetic regulation in EMT and, to date, an inducible *in vitro* human EMT model suitable for detailed chromatin analysis has not been available; although TGF-β induced dog and human breast cancer models (Gregory et al., 2008; Mani et al., 2008) have been described but these models are not ideal for studying epigenetic regulation in the EMT process.

The key criteria for establishing a human *in vitro* model of EMT for epigenetic analysis include: (i) choice of a human cell line that is epithelial in origin (ii) in which high proportion of the cells are transfectable with minimal cell death in order to determine effect of EMT reagents and effect of protein knockdown by siRNA based strategies (iii) and in which EMT is inducible, leading to the rapid induction of the EMT associated signature genes. This chapter describes the establishment and characterization of a novel human *in vitro* model of the epithelial to mesenchymal transition for epigenetic analysis. Firstly, the selection of a suitable epithelial cell line and EMT inducing agent have been described, followed by optimization of the concentration of the ideal EMT stimuli to obtain maximum EMT and minimum cell death. EMT was initially measured by morphological changes and then the wound healing assay was used to confirm EMT-like morphological changes for
development of an inducible in vitro model. Secondly, transcript analysis of the EMT expression programs was carried out by quantitative real-time PCR to validate EMT-like morphology at the molecular level. Thirdly, antibody staining for one of the EMT-related proteins (laminin-5) was undertaken to confirm that transcript analysis (of laminin-5) correlated with findings at the protein level.

There is evidence that cells undergoing EMT may also acquire some stem cell properties (Mani et al., 2008; Morel et al., 2008) and that neoplastic epithelial cells undergoing EMT might represent the fraction that have been termed as cancer 'stem' cells (Gupta et al., 2009b). These cancer stem cells have been shown to be responsible for tumour initiation (Berx et al., 2007; Dean, 2005; Dean et al., 2005), reoccurrence of tumour and resistance to chemotherapy (Creighton et al., 2009). Only a few studies have been undertaken to characterize these cells epigenetically and a major impediment in this field so far has been lack of an inducible human in vitro model. Breast cancer stem cells are defined by a CD44\textsuperscript{high}/CD24\textsuperscript{low} phenotype as described by (Al-Hajj et al., 2003). After establishing the in vitro inducible model of EMT, it was assessed if this novel EMT model system also produced a CD44\textsuperscript{high}/CD24\textsuperscript{low} subpopulation in the induced EMT population. The presence of a CD44\textsuperscript{high}/CD24\textsuperscript{low} subpopulation was confirmed by FACS analysis and the ability of those cells to form mammospheres (a property associated with mammary epithelial stem cells) was investigated. Finally, the targeted transcript analysis was carried out to determine the expression of signature genes for EMT and CSC populations.
3.1 MCF-7 cells are a readily transfectable epithelial cell line ideal for detailed epigenetic analysis of induced EMT

Based on the classification of human epithelial (or luminal) and mesenchymal (or basal) cells by Blick et al. (refer to section 1.1.6), three epithelial cell lines; HeLa, T-47D and MCF-7 were chosen to determine their suitability for transfection with a GFP-expression plasmid mediated by a lipid based reagent, FuGENE 6. The FuGENE 6 reagent had been found to be the most optimal reagent for transfection of these epithelial cell types (HeLa, T-47D and MCF-7) as described earlier in this thesis (please refer to section 2.5.1 for detailed optimization).

Figure 3.1 shows the transfection efficiency of the FuGENE 6 reagent into three cell lines; MCF-7, HeLa and T-47D. The results showed that under these conditions MCF-7 cells displayed maximal transfection (approximately 80%) with the GFP-expression plasmid with greatest live cell percentage (approximately 85%) (Figure 3.1 A). In comparison, HeLa cells showed approximately 74% transfection with 54% live cells (Figure 3.1 B) while T-47D cells displayed essentially no transfection (0%) (Figure 3.1 C). Therefore, MCF-7 cells were chosen as the most suitable cell line for the study of induced EMT.

3.2 EMT-like morphological changes induced by PMA and other EMT agents in MCF-7 cells

MCF-7 cells were treated with various EMT promoting stimuli for 0, 12, 48 and 60 hours and the resultant EMT changes were monitored subsequently by phase-contrast microscopy followed by capturing images. Cells undergoing EMT were identified accordingly using microscopy for characteristic morphological changes such as alterations in shape of cell, gaining arms for motility, reduced compactness of the cell colonies, lose of
cell to cell and cell to extracellular matrix interactions like shown in the Figure 3.2 F. The various EMT stimuli used included exposure to TNF-α, EGF, IL-6, TGF-β and PMA (phorbol 12-myristate 13-acetate). The PKC inducer (Hwang et al.; Moolten et al., 1981), PMA, was also included as one of the stimuli because PKC signaling has been implicated previously in the EMT process (Dissanayake et al., 2007; Serova et al., 2010; Wu et al., 2006). The concentrations and incubation time for various stimuli are described in section 2.2.4. For this experiment, PMA was used at 20 ng/ml concentration as this concentration has been shown to induce gene expression in other systems such as T cell biology (Sutcliffe et al., 2011; Sutcliffe et al., 2009).

The representative images presented in Figure 3.2 show the effect of PMA and physiological EMT stimuli on morphology of MCF-7 cells after 60 hours of stimulation. Strikingly, PMA induced maximal EMT-like morphological changes in MCF-7 cells with approximately 95% cells converting to mesenchymal phenotype (Figure 3.2 F). In comparison, other stimuli such as TNF-α (Figure 3.2 B) and EGF (Figure 3.2 C) showed only 7-10% conversion, whilst TGF-β (Figure 3.2 E) and IL-6 (Figure 3.2 D) demonstrated minimal EMT-like morphological changes (2-5%).
Figure 3.1  

MCF-7 cells display maximum transfection efficiency using FuGENE 6 reagent

Transfection of the GFP-expression vector (1 μg) using 2.25 μl of FuGENE 6 (in 1: 2.25 ratio) was carried out in following epithelial cell lines, (A) MCF-7 cells, (B) HeLa cells and (C) T-47D cells. Images were subsequently captured using 10 x magnification of Olympus 17X1 microscope. Representative images are shown with % live cells and % transfection efficiency indicated below the images.
Figure 3.2  PMA stimulation results in maximal EMT in MCF-7 cells

MCF-7 cells were either (A) non-stimulated (NS) or stimulated with (B) TNF-α (20 ng/ml), (C) EGF (50 ng/ml), (D) IL-6 (50 ng/ml), (E) TGF-β (2.5 ng/ml) or (F) PMA (20 ng/ml) for 60 hr. Phase contrast images of live MCF-7 cells were captured subsequently with Olympus 17X1 microscope using 10 x magnification.
Figure 3.3  Graphical representation of EMT-like morphological changes generated in Figure 3.2.

MCF-7 cells were either non-stimulated (NS) or stimulated with TNF-α (20 ng/ml), EGF (50 ng/ml), IL-6 (50 ng/ml), TGF-β (2.5 ng/ml) or PMA (20ng/ml) for 60 hr. Three or more phase contrast images were captured for every stimulus using Olympus 17X1 microscope. At least 200 cells were counted in every image and average percentage (%) of the cells undergoing EMT was calculated and subsequently a graph was plotted. Results are shown as average ± standard error from two independent experiments.
Proportion of MCF-7 cells undergoing EMT in response to various EMT treatments has also been shown graphically in Figure 3.3, where it is very clear that PMA is the best EMT inducer. Besides EMT-like morphological changes, the compactness of cell colonies was also taken into consideration to assess the effect of EMT stimuli. As shown in Figure 3.2 A, non-stimulated cells showed tightly packed clusters of cobblestone-shaped cells, while PMA stimulation caused a clear morphological change, with cells acquiring an irregular but elongated shape and loss of cell-cell contact (Figure 3.2 F) in comparison to other EMT stimuli in MCF-7 cells. In summary, PMA induces greatest degree of EMT-like morphological changes in MCF-7 cells within 60 hours of stimulation.

3.3 Optimization of PMA concentration for maximum EMT and minimum cell death

Results from the previous sections provided the information that PMA was the ideal inducer of EMT in MCF-7 cells, so optimization of the PMA concentration was carried out to obtain maximal EMT and minimal cell death. Serial dilution of 20 ng/ml of PMA was done in warm DMEM media to get dilutions as 20 ng/ml, 2 ng/ml, 1 ng/ml, 0.65 ng/ml, 0.5 ng/ml, 0.33 ng/ml, 0.29 ng/ml, 0.25 ng/ml, 0.22 ng/ml and 0.2 ng/ml. Cells were stimulated with all above mentioned dilutions in duplicate wells, subsequently monitored by phase-contrast microscopy and images were captured following 0, 4, 18 and 48 hours of PMA treatment. Cells undergoing EMT were counted and a graph was plotted after 48 hr post-stimulation (Figure 3.4). Percentage of cell death caused at each dilution was also measured using Vi-CELL-XR-cell counter after 48 hour of stimulation and a graph was plotted (Figure 3.4). Treatment of MCF-7 cells with 0.65 ng/ml PMA yielded maximum percent EMT along with minimal observable cell death (Figure 3.4). PMA at 0.65 ng/ml concentration resulted in little change in cell viability and cell number after the stimulation. In comparison the dilutions 20 ng/ml, 2 ng/ml or 1 ng/ml also attained maximum EMT but at the same time resulted in high percentage of cell death after 48 hours of stimulation. While 0.65 ng/ml concentration of PMA was better
MCF-7 cells were either non-stimulated (NS) or stimulated for 48 hr with PMA ranging in concentration between 0.02 ng/ml to 20 ng/ml. For each dilution, three phase contrast images were taken with Olympus 17X1 microscope and at least 200 cells were counted in each image before calculating the average of the cells undergoing either percent EMT (as measured by morphology) or percent cell death (as measured by Vi-CELL-XR cell counter).
at inducing higher EMT in comparison to lower concentrations of PMA (dilutions ranging from 0.5 ng/ml to 0.2 ng/ml), it resulted in minimal cell death. Consequently, PMA concentration of 0.65 ng/ml concentration was chosen as the optimal PMA concentration to induce EMT in this thesis.

3.4 PMA increases migration ability of cells in vitro

PMA was compared for its ability to promote wound healing with physiological stimuli such as TNF-α by scratching a wound in monolayer of MCF-7 cells and images were captured at 0 hour and 18 hour. Both 0 hr and 18 hr images were than overlaid to observe the difference in migration (refer to section 2.2.7 for detailed wound healing assay). The wound healing assay revealed that after 18 hours of treatment, wound was completely closed with PMA stimulus (Figure 3.5 A) but TNF-α did not close the wound (Figure 3.5 B) and in fact showed similar wound closing properties to non-stimulated cells (Figure 3.5 C).

3.5 Transcript analysis of PMA induced EMT

The mRNA expression of a cohort of EMT marker genes (namely: laminin-5, uPAR, Zeb1, E-cadherin and Vimentin) was explored following induction of EMT in MCF-7 cells with either PMA or TNF-α as stimulus. A time course study was carried out at 0, 4, 24 and 48 hours intervals to capture the inducible nature of genes by both the agents (Figure 3.6).

As shown in Figure 3.6, among all the genes tested, PMA resulted in much greater induction levels of the genes compared to TNF-α as early as 4 hours post-stimulation. For example, laminin-5 although demonstrated similar kinetics of expression, at 24 hours post-stimulation, it showed 20 fold induction by PMA treatment compared to TNF-α, which lead approximately 5 fold induction (Figure 3.6 A). Expression of the uPAR gene showed a similar trend because PMA treatment resulted in 9 fold up-regulation of this gene whilst TNF-α only lead to 2 fold induction at 24 hours post-stimulation (Figure 3.6 B). After 24 hours of stimulation, Zeb1 was 2.5 fold induced upon PMA treatment but in comparison
Figure 3.5  PMA stimulation leads more effective wound healing than TNF-α

MCF-7 cells were stimulated for 18 hr with (A) PMA (0.65 ng/ml), (B) TNF-α (20 ng/ml) or (C) left untreated, non-stimulated (NS). Phase contrast images of wound healing assay were subsequently captured by Olympus 17X1 using 10 x magnification at time points, 0 hr (red line) and 18 hr (green line). An overlaying (red and green line together in one image) of the two images was done to show wound healing ability of the treatment.
Figure 3.6 (I)

(A) PMA treatment

Laminin-5

FC (relative to NS)

Stimulation time (hr)

TNF-α treatment

Laminin-5

FC (relative to NS)

Stimulation time (hr)

(B) PMA treatment

uPAR

FC (relative to NS)

Stimulation time (hr)

TNF-α treatment

uPAR

FC (relative to NS)

Stimulation time (hr)

(C) PMA treatment

Zeb1

FC (relative to NS)

Stimulation time (hr)

TNF-α treatment

Zeb1

FC (relative to NS)

Stimulation time (hr)
Figure 3.6 (I) and (II) Transcription of EMT marker genes induced in MCF-7 cells following treatment with PMA

cDNA was synthesized from total RNA isolated from PMA (0.65 ng/ml) or TNF-α (10 ng/ml) stimulated MCF-7 cells, for the time points indicated. Taqman® real time PCR was subsequently performed for (A) Laminin-5, (B) uPAR, (C) Zeb1 (D) E-cadherin and (E) Vimentin. Threshold cycle (Ct) values were converted to arbitrary copy number, normalised to cyclophilin A and were expressed as fold change (FC) relative to the non-stimulated sample. Data represent the mean ± standard error (SE) of three independent experiments.
TNF-α did not upregulate expression of Zeb1 (Figure 3.6 C). The classic epithelial marker gene E-cadherin showed similar kinetics and demonstrated decreased mRNA expression following both PMA and TNF-α treatment (Figure 3.6 D) at 24 hour-post stimulation, however, the classical mesenchymal marker vimentin was not very inducible by either PMA or TNF-α in MCF-7 cells (Figure 3.6 E). Expression of microRNA 200 family members; microRNA 200b and 200c, was also measured. It was observed that both miR 200b and miR 200c were highly expressed in non-stimulated cells while their expression was greatly reduced in PMA-stimulated cells (Figure 3.7 A & B).

**3.6 Protein expression of laminin-5 in PMA-induced EMT**

The extracellular protein laminin-5 has been demonstrated to have role in EMT (Nakaya et al., 2008; Xu et al., 2003) and cancer (Giannelli et al., 2005). Intracellular immunofluorescence staining of non-stimulated and stimulated MCF-7 cells was performed with laminin-5 antibody (refer to section 2.3.1 for detailed method of intracellular staining). Images were taken by confocal microscope to observe the difference in the staining patterns between two treatments. Figure 3.8 shows that in non-stimulated cells laminin-5 is confined to a small area and displays minimal staining (Green fluorescence) as the cells themselves are highly compact and cell to cell interactions are tight (demonstrated by closely compacted DAPI stained blue nuclei in the Figure 3.8 A). On the other hand PMA stimulated cells not only cover more area and gain arm-like projections but also show increased laminin-5 staining (Figure 3.8 B). It is clear in the images that upon PMA stimulation laminin-5 covers more area as the size of cell expands and localization of laminin-5 is visible in specific areas (line-like packets) of cytoplasm.
Figure 3.7  
PMA stimulation results in decreased expression of microRNA 200 family members

cDNA levels for (A) miR 200b, (B) miR 200c were measured by Taqman® microRNA real-time analysis from non-stimulated (NS) and PMA stimulated cells (ST) (0.65 ng/ml for 60 hr). Threshold cycle (Ct) values generated for each time points were converted to arbitrary copy number and normalized to RNU6B reference levels. MicroRNA levels are expressed in arbitrary copy numbers. Data represent the mean ± standard error (SE) of three independent experiments.

Figure 3.8  

Figure 3.8  Laminin-5 shows differential intracellular staining patterns between non-stimulated and stimulated MCF-7 cells

MCF-7 cells were either (A) non-stimulated (NS) or (B) stimulated with PMA (ST) (0.65 ng/ml for 60 hr) and were subsequently stained with anti-laminin-5 antibody (green colour) and DAPI stain (nuclear stain) (blue colour) respectively. Confocal microscopic images of MCF-7 were captured using Leica microscope at 60 x magnification.
3.7 PMA induces CD44\textsuperscript{high}/CD24\textsuperscript{low} CSC-like subpopulation

Using the optimized antibody staining conditions, an assessment was made whether stimulation of MCF-7 cells with PMA could induce a CSC-like (CD44\textsuperscript{high} and CD24\textsuperscript{low}) subpopulation (refer to section 2.3.3 for detailed staining method for CD44\textsuperscript{high} and CD24\textsuperscript{low}). The flow cytometry gating strategy used was to first gate on PMA-induced CSC-like population followed by copying this gate on non-stimulated cells. Figure 3.10 showed that after PMA stimulation, a CD44\textsuperscript{high} and CD24\textsuperscript{low} (CSC-like) sub-population (approximately 10%) emerged. This latter observation could not be due to non-specific antibody binding as isotype control antibodies did not produce this sub-population (Figure 3.9).

Additionally, PMA stimulation routinely generated four sub-populations namely: (i) CD44\textsuperscript{high}/CD24\textsuperscript{low} (CSC-like), (ii) CD44\textsuperscript{low}/CD24\textsuperscript{low}, (iii) CD44\textsuperscript{low}/CD24\textsuperscript{high} and (iv) CD44\textsuperscript{high}/CD24\textsuperscript{high}, which were sorted using flow cytometry (Figure 3.10 A) followed by transcript analysis. Transcript analysis showed that CD44 transcript level was higher in CD44\textsuperscript{high}/CD24\textsuperscript{low} subpopulation in comparison to other cell types (Figure 3.10 C). In contrast, CD24 transcript of CD44\textsuperscript{high}/CD24\textsuperscript{low} sub-population remained as low as non-stimulated (NS) cells (Figure 3.10 C). Therefore, CD44 high/CD24 low sub-population was named as CSC-like and rest of the other three populations jointly were called as non-cancer stem cells (NCSCs) so that further epigenetic analysis was made possible to identify if these two major sub-populations differentiate in epigenetic properties.

Transcript analysis also showed that mRNA profile of Laminin-5 and uPAR (genes other than CD24) were also distinct between 4 sub-populations, specifically, both Laminin-5 and uPAR showed higher mRNA expression in CD44\textsuperscript{high}/CD24\textsuperscript{low} sub-populations than
CD44^{high}/CD24^{high} sub-population. Therefore, CD44^{high}/CD24^{low} sub-populations are different from CD44^{high}/CD24^{high} sub-population.

For detailed epigenetic analysis, two populations; CSC-like (CD44^{high} and CD24^{low}) cells and NCSC cells (rest of EMT population) were sorted using BD FACS Aria™ II sorter. The gating strategy for this experiment is shown as a representative FACS plot in Figure 3.10. Interestingly, using this gating strategy, 5.959 ± 3.633 percent CSC-like cells were recovered upon stimulation with PMA (0.65ng/ml for 60 hours) in comparison to 0.181 ± 0.158 percent CSC-like cells from non-stimulated cells (Figure 3.11). PMA stimulation results in formation of CSC-like cells rather than killing the cells with higher CD24 phenotype because there was only slight change in cell viability and cell number after PMA stimulation (0.65 ng/ml) (figure 3.4); therefore there could not be a selection of CSC-like cells simply due to the preferential death of non-CSCs.

3.8 Confirmation of CD44^{high}/CD24^{low} subpopulation by mammosphere assay

The Mammosphere assay was used to confirm presence of cancer stem-like cells (refer to section 2.2.8 for detailed method of mammosphere assay). It is an in vitro cultivation system that allows for propagation of human mammary epithelial cells in an undifferentiated state, based on their ability to proliferate in suspension, as non-adherent mammospheres (Dontu et al., 2003). It is clear from Figure 3.12 (representative pictures) and Figure 3.13 that PMA treatment of MCF-7 cells leads to more mammosphere (1329 ± 59 mammospheres/40,000 cells) formation in comparison to non-stimulation (631 ± 14.5 mammospheres/40,000 cells) or TNF-α stimulation (171 ± 4.5 mammospheres/40,000 cells). These results confirmed the induction of a CSC-like subpopulation by PMA treatment in MCF-7 cells.
Figure 3.9  
FACS plot showing specific staining of MCF-7 cells with APC-anti-CD44 antibody and PE-anti-CD24 antibody.

MCF-7 cells were either stimulated (ST) with PMA (0.65 ng/ml for 60 hr) or non-stimulated (NS) and subsequently staining of the cells for FACS analysis was carried out. Cells were either (i) stimulated (ST)-stained with PE isotype control antibody alone (red colour population), (ii) stimulated (ST)-stained with APC-anti-CD44 antibody and PE-anti-CD24 antibody (green colour population) or (iii) non-stimulated (NS)-stained with APC-anti-CD44 antibody and PE-anti-CD24 antibody (blue colour population).
Figure 3.10

FACS gating strategies for sorting of PMA-induced $\text{CD}44^{\text{high}}/\text{CD}24^{\text{low}}$-cancer stem-like cell (CSC) sub-population

MCF-7 cells were either left untreated, non-stimulated (NS) or stimulated (ST) with PMA (0.65 ng/ml) for 60 hr. Cells were subsequently stained with Hoechst, APC-anti-CD44 and PE-anti-CD24 cocktail prior to FACS sorting. Cancer stem-like cell (CSC) population was defined by $\text{CD}44^{\text{high}}/\text{CD}24^{\text{low}}$ stain. Gates for CSC-like and NCSC sub-populations were first made on PMA stimulated populations and these gates were copied to non-stimulated population to confirm that the CSC-like population was below 0.1% in non-stimulated cells. Representative FACS plot of 10 independent experiments has been shown for highlighting the gating strategy.

Figure 3.11

Figure 3.11

PMA Stimulation results in a high percentage of $\text{CD}44^{\text{high}}/\text{CD}24^{\text{low}}$ or CSC-like sub-population in vitro

MCF-7 cells were either left untreated, non-stimulated (NS) or stimulated (ST) with PMA (0.65 ng/ml) for 60 hr. FACS analysis was carried out using gating strategies described in Figure 3.10 and subsequently, the mean % CSC-like subpopulation was plotted (Error bars are standard errors) from ten independent experiments.
Figure 3.10 B  FACS gating strategies for sorting of PMA-induced CD44\textsuperscript{low}/CD24\textsuperscript{high}, CD44\textsuperscript{low}/CD24\textsuperscript{low}, CD44\textsuperscript{high}/CD24\textsuperscript{high} and CD44\textsuperscript{high}/CD24\textsuperscript{low}\textsubscript{sub-populations.}

MCF-7 cells were either left untreated, non-stimulated (NS) or stimulated (ST) with PMA (0.65 ng/ml) for 60 hr. Cells were subsequently stained with Hoechst, APC-anti-CD44 and PE-anti-CD24 cocktail prior to FACS sorting. Populations were defined by high or low CD44 and CD24 stain and sorted as CD44\textsuperscript{low}/CD24\textsuperscript{high}, CD44\textsuperscript{low}/CD24\textsuperscript{low}, CD44\textsuperscript{high}/CD24\textsuperscript{high} and CD44\textsuperscript{high}/CD24\textsuperscript{low}. Representative FACS plot of two independent experiments has been shown for highlighting the gating strategy.
Figure 3.10 B  PMA-induced CD44<sup>high</sup>/CD24<sup>low</sup> sub-population shows higher CD44 and lower CD24 transcript profile.

Transcript analysis was carried out on MCF-7 cells, either non-stimulated (NS) or PMA stimulated (0.65 ng/ml for 60 hr) and FACS sorted CD44<sup>low</sup>/CD24<sup>high</sup>, CD44<sup>low</sup>/CD24<sup>low</sup>, CD44<sup>high</sup>/CD24<sup>high</sup> and CD44<sup>high</sup>/CD24<sup>low</sup> sub-population for genes (A) CD44 and (B) CD24, (C) Laminin-5 and (D) uPAR. Taqman® real time PCR was performed on cDNA synthesized from total RNA isolated from above said four populations. Threshold cycle (Ct) values were converted to arbitrary copy number, normalised to cyclophilin A and were expressed as fold change (FC) relative to the non-stimulated sample. Data represent the mean of fold change ± standard error (SE) of two independent experiments.
Figure 3.12  
PMA leads to the generation of greater number of mammospheres in comparison to TNF-α stimulation in MCF-7 cells

Mammosphere assay was performed with 4 x 10^4 MCF-7 cells/well in ultra low attachment 6 well plates. Cells were either (A) non-stimulated (NS), or stimulated with (B) PMA (0.65 ng/ml) or (C) TNF-α (10 ng/ml). Images were captured using phase contrast microscopy for mammosphere assay after 6 days of assay commencement.

Figure 3.13

![Graph showing mammosphere generation](image)

Figure 3.13  
Graphical representation of Figure 3.12 showing greater number of mammosphere generation by PMA in comparison to TNF-α stimulation in MCF-7 cells

Mammosphere assay was performed with 4 x 10^4 MCF-7 cells/well in ultra low attachment 6 well plates. Cells were either (i) non-stimulated (NS), or stimulated with (ii) PMA (0.65 ng/ml) or (iii) TNF-α (10 ng/ml). Only mammospheres larger than 60 μm were counted after 6 days of treatment. Experiment was performed in duplicate and mammospheres in each well were counted for average. Data represent the average ± SE of two independent experiments.
3.9 CSC-like and Non-CSC subpopulations show distinct transcript profile

To investigate the transcript profile of CD44<sup>high</sup>/CD24<sup>low</sup>-CSC-like and non-CSC (NCSC) sub-populations, these sub-populations were sorted using FACS sorter, 60 hours post-PMA treatment (as described in section 3.7 above) and RNA was extracted from non-stimulated, CSC-like and NCSC cells for subsequent transcript analysis. As shown in the Figure 3.14. CD44 transcript level was 52 fold higher in CSC-like subpopulation and it was 15 fold higher in NCSC sub-population in comparison to non-stimulated cells (Figure 3.14 A). At the same time, CD24 transcript expression in CSC-like population remained as low as non-stimulated cells, while in the NCSC population CD24 level was 6.4 fold higher than non-stimulated cells. (Figure 3.14 B) Transcript level of other EMT related gene such as laminin-5 and uPAR were respectively 300 and 37 fold higher in CSC-like subpopulation and 86 and 10 fold higher in NCSC than non-stimulated cells (Figure 3.14 C and D). Whereas, transcript level of genes such as fibronectin and integrin-β did not change much between CSC-like and NCSC sub-populations (Figure 3.14 E and F).

Expression level of the miR 200 family members; miR 200b and miR 200c was also measured (Figure 3.15). Consistent with previous studies, both miR 200b and miR 200c expression level were very low in CSC-like cells in comparison to non-stimulated cells (Figure 3.15 A and B). Cumulatively, these results suggest that PMA induced model for studying EMT, CSC-like and NCSC cells is most suitable. In addition, it was also established that CSC-like and NCSC sub-populations have distinct transcript profiles.
Figure 3.14  PMA induced CSC-like sub-populations have distinct transcriptional profile

Transcript analysis was carried out on MCF-7 cells, either non-stimulated (NS), or PMA stimulated (0.65 ng/ml for 60 hr) and FACS sorted sub-population-cancer stem-like cells (CSC-like) and non-cancer stem like cells (NCSC), for genes (A) CD44, (B) CD24, (C) laminin-5, (D) uPAR, (E) Fibronectin, (F) Integrin-β. Taqman® real time PCR was performed on cDNA synthesized from total RNA isolated from above said three populations. Threshold cycle (Ct) values generated for each time points were converted to arbitrary copy number and normalized to Cyclophilin A reference levels. mRNA levels are expressed in arbitrary copy numbers and fold change in comparison to non-stimulated cells is shown above the error bars. Data represent the mean ± standard error (SE) of three independent experiments.
Figure 3.15

**Figure 3.15**  PMA induced CSC-like and NCSC sub-populations results in reduced expression of miR200 family members.

MicroRNA cDNA levels for (A) miR 200b and (B) miR 200c were measured by TaqMan® microRNA real-time analysis from either MCF-7 cells left untreated, non-stimulated (NS), or PMA stimulated (0.65 ng/ml for 60 hr) FACS sorted sub-populations-cancer stem-like cells (CSC-like) and non-cancer stem like cells (NCSC). Threshold cycle (Ct) values generated for each time points were converted to arbitrary copy number and normalized to RNU6B reference levels. MicroRNA levels are expressed in arbitrary copy and data represent the mean ± standard error (SE) of three independent experiments.
Discussion

Human MCF-7 cells are a well-accepted model of estrogen-receptor-positive, luminal type breast cancer that has been shown to undergo EMT changes in response to oestrogen (Planas-Silva and Waltz, 2007). Based on the data presented in this chapter, it appears that PMA induces rapid EMT in majority of the MCF-7 cells (90-95%), producing an ideal basis for epigenetic analysis of cells undergoing this process since chromatin based assays require large cell numbers and also the background from non-EMT cells would be low.

In MCF-7 cells, PMA induces EMT in a high proportion of cells in comparison to other physiological EMT stimuli such as TNF-α and TGF-β, which was confirmed by morphological changes (Figure 3.1) and wound healing assay (Figure 3.4). PMA stimulation of MCF-7 cells decreased the expression of E-cadherin but failed to induce vimentin, however it induced other EMT characteristic genes such as laminin-5, uPAR and Zeb1 (Figure 3.5). The fact that different cell types have a different ability to express genes should be taken into consideration because there was not much difference in vimentin transcript expression after PMA stimulation until 48 hours. Ma et al., also showed that in SUM149 human breast carcinoma cells, miR 9 (a microRNA regulating E-cadherin and metastasis) down-regulated E-cadherin expression by 50% but failed to induce vimentin, other mesenchymal markers and fibroblastic cell morphology, while the same system in HMLE cells resulted in 70% decrease in E-cadherin and five-fold increase in the mesenchymal marker vimentin (Ma et al., 2010). Recently, He et al., also demonstrated that PMA induces EMT in ARCaP prostate cancer cell lines (He et al., 2010). In their study, PMA reduced expression of E-cadherin and CK-18 and concurrently PMA induced expression of vimentin.
PMA rapidly (within 4-24 hours) induces EMT in MCF-7 cells (Figure 3.6), and therefore, it is a very good model to study the initial events triggering EMT. Additionally, because it is possible to easily manipulate this system using pharmacological inhibitors, gene mutants and siRNA strategies, there is opportunity to investigate the process of EMT at the molecular level. The stability of pharmacological inhibitors, transiently expressed mutants or gene knockdown in the *in vitro* systems is not much, hence to have a system where rapid EMT is occurring and manipulations are achievable, is very powerful. Rapid induction of EMT results in a system which undergoes substantial changes in gene transcriptional program prior to and after cell division also provides a system in which epigenetic mechanisms that occurred either prior to or after cell division could also be dissected. There is an existing inducible model of EMT by TGF-β in human mammary epithelial cells (HMLEs) (Mani et al., 2008) but it takes 12-22 days for induction of EMT and therefore this system is not easily manipulated to study the initial events of EMT.

Mani and colleagues demonstrated that inducing EMT in immortalized human mammary epithelial cells (HMLEs), either by treating cells with TGF-β or by over-expressing the key transcription factors that control EMT (i.e., Snail and Twist), almost all the cells acquired a CD44\textsuperscript{high}/CD24\textsuperscript{low} profile phenotype (Mani et al., 2008). However in this model it was not possible to study non-cancer stem like cells because all the cells converted to CSC-like cells. Another inducible model using IL-6 and tamoxifen in MCF-10 cells showed that CSC and NCSCs are related but that different cell types arose from a transformed common cell precursor (Iliopoulos et al., 2011). That study also demonstrated that IL-6 was sufficient to convert NCSCs into CSCs. Results presented in this chapter demonstrated the establishment of a novel inducible human *in vitro* model for EMT by stimulating MCF-7 cells using PMA. This model should provide a useful means to study
epigenetic signatures involved in EMT and simultaneously provides a model to study formation of CSC-like and NCSC subpopulations (Figure 3.10). To our knowledge, this is the first human inducible model where both CSC and NCSC subpopulations could be studied in controlled in vitro conditions.

During the optimization of the MCF-PMA-EMT model following measures were taken to avoid preferential shift of CSC percentages: (i) Percentage of cell death caused by PMA stimulation was measured using Vi-CELL-XR-cell counter after stimulation and without stimulation. Because, there was little change in cell viability and cell number after the PMA stimulation (0.65 ng/ml) (Figure 3.4); therefore there could not be a selection of CSC-like cells simply due to the preferential death of non-CSCs. (ii) during flow cytometry analysis MCF-7 cells were always stained with a cocktail of APC-anti-CD44, PE-anti-CD24 along with the Hoechst 33258, specifically to monitor cell viability. A flow cytometry gating strategy was always included in analysis to exclude dead cells (Hoechst negative cells) (Figure 2.1 A). No dead cells were included in the analysis leading to sorting of only live cells, therefore there is no preferential selection of any cell types to shift the CSC-like cell percentages.

This PMA induced MCF-7 model suggested that the CSC-like and NCSC subpopulations are distinct in their transcript profile as there are genes such as laminin-5 and uPAR which were highly expressed in CSC-like subpopulations in comparison to NCSC and non-stimulated epithelial cells (Figure 3.14 C and D), while other EMT genes such as integrin-β were higher in NCSC populations (Figure 3.14 F). Expression of miR 200c family members goes down in both CSC-like and NCSC sub-populations in comparison to non-stimulated populations (Figure 3.15). This result is in accordance with another study which
demonstrated recently that miR 200 family gets inhibited during CSC formation (Iliopoulos et al., 2010).

Further studies combining markers of CSCs and EMT should provide insight into the possibility that CSCs have been generated by EMT occurring within the breast tissue and if this occurrence is predictive of metastasis. With the aim of eradicating breast cancer, there is a great interest and excitement in the possibility of identifying and treating the subpopulation of cancer stem cells that fuel tumour growth. However, there remains a need to determine whether the CD44\textsuperscript{high}/CD24\textsuperscript{low} cells are true tumorigenic cells across all the various breast cancer subtypes, or whether these are exclusive to more basal tumour types. Providentially, the field is budding with the identification of new potential markers, such as protein C receptor (Abraham et al., 2005), which may permit further enrichment and identification of therapeutic targets for treatment of breast cancer. In this succession, this PMA induced model of EMT/CSC will be proven to have new insights into novel potential markers as well as a quick screen to identify new cancer therapies.

Taken together, the results presented in this chapter provide evidence that PMA induces EMT in MCF-7 cells and that from this easily manipulated \textit{in vitro} EMT model, large cell number of cancer stem like cells and non-cancer stem like cells could be isolated for subsequent epigenetic analysis. Furthermore, this model provides the opportunity to study the role of chromatin-associated PKCs during EMT, which will be described in next chapter.
Chapter Four

Chromatin-associated PKC family members involved in phorbol ester induced epithelial to mesenchymal transition and formation of cancer stem cell-like subpopulation
Chapter 4: Chromatin-associated PKC family members involved in phorbol ester induced epithelial to mesenchymal transition and formation of cancer stem cell-like subpopulation

Introduction

Cellular signal transduction pathways modify gene expression programs in response to changes in the extracellular stimuli. These signaling pathways are often initiated by cascades of protein phosphorylation that conclude in regulation of nuclear transcription. The activation of signal transduction pathways results ultimately in phosphorylation of transcription factors (Hill and Treisman, 1995; Karin and Hunter, 1995), histones (Clayton and Mahadevan, 2003), chromatin-modifying complexes and the transcription machinery itself (Edmunds and Mahadevan, 2004; Yang et al., 2003). Until recently, the central kinases in these pathways were not thought to be associated directly with modulating transcription itself. However, pioneer studies in yeast have demonstrated that signal transduction kinases translocate to the nucleus, associate with chromatin and regulate gene expression (Pascual-Ahuir et al., 2006; Pokholok et al., 2006). These chromatin-associated kinases play a role as part of the integral structure of the transcriptional machinery as well as an enzymatic part in phosphorylating their target proteins (Bungard et al., 2010; de Nadal and Posas, 2010; Edmunds and Mahadevan, 2006).

Protein Kinase C (PKC) family members have long been known for their involvement in the signaling pathways that transduce the carcinogenic effects of phorbol esters (Castagna et al., 1982; Kikkawa et al., 1983). Moreover, various PKC isozymes have been implicated also in the process of the EMT and metastasis (Dissanayake et al., 2007; Koivunen et al., 2006).
To determine the involvement of PKC-mediated pathways in the PMA-induced model of EMT and CSC formation the PKC inhibitor Bisindolylmaleimide-I (Martiny-Baron et al., 1993) and Go6976 were used to challenge EMT induction by PMA as judged by cellular morphological changes and at the transcript level. The effects of these PKC inhibitors on the emergence of the CSC-like subpopulation were determined using a combination of FACS analysis and the mammosphere assay. Bisindolylmaleimide-I is a derivative of bisindolylmaleimide (2-methyl-1H-indol-3-yl-BIM-1), which works through inhibiting phosphorylation (Amos et al., 2005; Roberts et al., 2004) in an ATP-competitive manner, where the bound inhibitor adopts a non-planar conformation in the ATP-binding site, with the kinase domain being arrested in an intermediate open conformation (Grodsky et al., 2006; Toullec et al., 1991). Initially bisindolylmaleimide was introduced as a PKC-β II inhibitor and it provided a means to obtain the first structural description of a conventional PKC kinase domain (Grodsky et al., 2006), although subsequently other derivatives of bisindolylmaleimide have been shown to potently inhibit both PKC-βI and PKC-βII isozymes and, additionally inhibit PKC-α and PKC-γ isozymes (Zhang et al., 2003). Bisindolylmaleimide derivatives have been demonstrated to inhibit cell migration (Sliva et al., 2002); EMT (He et al., 2010) and some are in pre-clinical studies as potential anti-cancer therapeutics (Ohtsuka and Zhou, 2002; Pajak et al., 2008).

In this chapter, chromatin association of two PKC isozymes namely, PKC-θ and PKC-β was explored in the context of both epithelial to mesenchymal transition and cancer stem cell formation processes. In previous studies, chromatin-associated PKC-θ and PKC-β have been demonstrated to have a role in T cell biology (Sutcliffe et al., 2011) and prostate cancer respectively (Metzger et al., 2010). Using ChIP assays, the effect of bisindolylmaleimide-I on the chromatin recruitment PKC-θ, PKC-β and key EMT-related protein regulators such as the transcription factor Zeb1 (Sanchez-Tillo et al.; Spadaerna et al., 2008; Wellner et al., 2009) and chromatin demethylase enzyme LSD1 (Lin et al., 2010a; Lin et al., 2010b) was examined. The dependence upon histone modifications such as H3K4Me2 and H3K4Me1 were also screened in these studies because LSD1 causes demethylation
of H3K4Me2 into H3K4Me1 (Forneris et al., 2005; Shi et al., 2004). Sequential ChIP assays (as described in section 2.7.2) were carried out subsequently to identify the co-occupants of chromatin on selected EMT-associated gene promoters with PKCs during PMA-induced EMT. Both PKC-θ and PKC-β isozymes have been shown to play role in various types of cancer progression (Aguilo et al., 2009; Belguise and Sonenshein, 2007; Gokmen-Polar et al., 2010; Ou et al., 2008), so specific roles for both of these isozymes were investigated by knockdown of these proteins using siRNA strategies (described in section 2.5.4) followed by morphological, FACS, transcript and ChIP analysis.

4.1 PKC activity is essential for PMA-induced EMT

MCF-7 cells were pre-treated either with the broad spectrum PKC inhibitor, bisindolylmaleimide-I (1 μM) or cPKC-specific inhibitor Go6976 (1 μM) for one hour or they were incubated with vehicle alone, followed by PMA stimulation (0.65 ng/ml) for 60 hours and ultimately screened for EMT-like morphological changes. Consistent with the results presented in the preceding chapter (Figure 3.4 F), PMA stimulation without any inhibitor (vehicle alone) treatment, resulted in EMT-like morphological changes in the majority of cells (Figure 4.1 A). Pre-incubation with bisindolylmaleimide-I (1 μM) abolished the EMT induced by PMA (Figure 4.1 B) in MCF-7 cells. In contrast, pre-incubation with Go6976 (1 μM) did not prevent PMA-induced EMT-like morphological changes (Figure 4.1 C). The effects of both bisindolylmaleimide-I and Go6976 on transcript levels of EMT/ CSC related genes in the PMA-induced EMT model of MCF-7 cells were also determined. The real-time PCR analysis was consistent with results described above as bisindolylmaleimide-I (1 μM) abrogated the PMA-induced increase in transcription level of laminin-5, CD44 and uPAR genes (Figure 4.2 A). In contrast, consistent with the observation of morphological changes, transcript analysis showed that pre-treatment with Go6976 (1 μM) resulted in increased mRNA expression of PMA-induced EMT/CSC genes such as laminin-5, CD44 and uPAR genes (Figure 4.2 B).
Figure 4.1

The broad spectrum PKC inhibitor, Bisindolylmaleimide-1 inhibits PMA-induced EMT in MCF-7 cells but the cPKC-specific inhibitor, Go6976 increases PMA-induced EMT.

Phase contrast microscopy images of non-stimulated (NS) and PMA (0.65 ng/μl for 60 hr) stimulated (ST) MCF-7 cells were captured either (A) without pre-treatment of PKC specific inhibitor, (B) with 1 hr pre-treatment of Bisindolylmaleimide (1 μM) before PMA stimulation or (C) with 1 hr pre-treatment of Go6976 (1 μM) treatment prior to PMA stimulation.
Figure 4.2

(A)

![Graph showing fold change for Bisindolylmaleimide-I on Laminin-5, uPAR, and CD44 with treatment (ST) and non-treatment (NS).](image)

(B)

![Graph showing fold change for Go6976 on Laminin-5, uPAR, and CD44 with treatment (ST) and non-treatment (NS).](image)

Figure 4.2  Bisindolylmaleimide-I treatment inhibits PMA-induced transcription of key EMT/CSC genes whilst Go6976 treatment increases PMA-induced transcription of key EMT/CSC genes in MCF-7 cells

MCF-7 cells were either left untreated, non-stimulated (NS) or pre-treated with either (A) bisindolylmaleimide-I (1 μM), or (B) Go6976 (1 μM) for 1 hour prior to PMA (0.65 ng/μl for 60 hr) stimulation (ST). Taqman® real time PCR analysis for EMT/CSC genes-laminin-5, uPAR and CD44 was performed on cDNA synthesized from total RNA. Threshold cycle (Ct) values generated for each time point were converted to arbitrary copy number and normalized to Cyclophilin A reference levels. mRNA levels are expressed in fold change in comparison to non-stimulated cells. Data represent the mean ± standard error (SE) of two independent experiments.
4.2 The role of PKC activity in the formation of the CSC-like sub-population induced by PMA

MCF-7 cells were subjected to 1 hour pre-incubation of either bisindolylmaleimide-I (1 µM) (a broad spectrum PKC inhibitor), Go6976 (1 µM) (cPKC-specific inhibitor) or vehicle alone followed by PMA stimulation (0.65 ng/ml for 60 hours). The cells were subsequently stained with Hoechst 33528, APC-anti-CD44, PE-anti-CD24 cocktail and FACS analysis was performed to observe effect on CD44\textsuperscript{high}CD24\textsuperscript{low} sub-population. This experiment revealed that bisindolylmaleimide-I completely abrogated the formation of the CSC-like subpopulation in PMA-induced CSC model of MCF-7 cells (Figure 4.3 B). On the other hand, treatment with Go6976 resulted in around ~2 fold increase in the PMA-induced CSC-like subpopulation (Figure 4.3 C).

The mammosphere assay was performed, where cells were first treated with bisindolylmaleimide-I (1 µM) for 1 hour followed by PMA stimulation (0.65 ng/ml) for 60 hours. Mammosphere numbers were counted in a full well of 6-well plate and images were taken with a phase contrast microscope. The mammosphere assay (as described in section 2.2.8) displayed that bisindolylmaleimide-I was able to block the PMA-induced increase in mammosphere numbers (Figure 4.4).

In summary, the broad spectrum PKC inhibitor bisindolylmaleimide-I inhibited both the PMA-induced EMT process and the formation of the CSC-like subpopulation in MCF-7 cells. On the other hand, treatment with the cPKC-specific inhibitor, Go6976, increased the EMT changes and CSC-like subpopulation formation. Collectively, these results suggest that PKC activity is important in EMT and CSC formation processes.
Figure 4.3 Bisindolylmaleimide-I inhibits PMA-induced CSC-like subpopulation, whilst Go6976 increases CSC-like subpopulation formation

MCF-7 cells were either pre-incubated (A) with vehicle alone, (B) with bisindolylmaleimide-I (1 μM) or (C) Go6976 (1 μM), prior to PMA (0.65 ng/μl for 60 hours) stimulation (ST) or left untreated, non-stimulation (NS). Cells were subsequently stained with Hoechst 333528, APC-anti-CD44 and PE-anti-CD24 for 20 minutes on ice and subjected to FACS analysis. Circles on FACS plot indicate appropriate gating of CD44^{hi} / CD24^{low} CSC-like subpopulation and % CSC-like subpopulation is shown above the gates respectively.
Figure 4.4  Bisindolylmaleimide-1 reduce mammosphere formation induced by PMA

Mammosphere assay was performed with 4 x 10^4 MCF-7 cells/well in an ultra low attachment 6 well plates. MCF-7 cells were pre-incubated either with vehicle alone or bisindolylmaleimide-1 (1μM for 1 hr) prior to PMA stimulation (0.65 ng/ml for 6 days) (ST) or left non-stimulated (NS). Phase contrast microscopic images of mammospheres were taken after 6 days of assay and only mammospheres larger than 60 μm were counted.
4.3 Chromatin-associated PKC family members regulate the EMT process

Data presented in the previous section of this chapter demonstrated that PKC activity is essential for both the PMA-induced epithelial to mesenchymal transition and the formation of cancer stem cell-like sub-population, consequently it was hypothesized that PKC family members are recruited to the promoter regions of EMT-related inducible genes and regulate the PMA-induced EMT in MCF-7 cells. The occupancy of two PKC isozymes, PKC-θ (member of subfamily nPKC) and PKC-β (member of subfamily cPKC), at the promoter of the uPAR gene in non-stimulated and stimulated MCF-7 cells was investigated by the ChIP assay (as described in section 2.7.1), using anti-PKC-θ and anti-PKC-β antibodies. The levels of PKC-θ occupancy at the promoter regions of the uPAR gene were found to be higher in the non-stimulated state than in the stimulated state (Figure 4.5 A), while PKC-β occupancy increased in the stimulated state relative to the non-stimulated state (Figure 4.5 B). Here, it is important to mention that the antibodies used against PKC-θ and PKC-β in this preliminary experiment, were intended not to differentiate between non-active and active (phosphorylated) form of PKCs.

A subsequent investigation was performed to discriminate between the active or non-active forms of PKC that are involved in regulating the process of EMT. To achieve this, ChIP assays were performed using antibodies against the active forms of PKC-θ and PKC-β. To detect the active form of PKC-θ, anti-PKC-θ (phospho) antibody (phosphorylated at serine-695) (Sutcliffe et al., 2011) was used and to detect PKC-β activity indirectly, anti-phospho-H3T6 (histone H3 phosphorylated at threonine 6) antibody was selected which has been shown to be phosphorylated exclusively by PKC-β (Metzger et al., 2010). In stimulated cells, phosphorylated PKC-θ enrichment on uPAR gene promoter was massively elevated in comparison to non-stimulated cells (Figure 4.5 C). A similar enrichment of phosphorylated H3T6 was evident on the uPAR gene promoter (Figure 4.5 D). Antibody against an active transcription mark, RNA Pol II
Figure 4.5

PKC family members and RNA Pol II are associated with chromatin at the promoter region of uPAR gene in PMA-induced EMT.

MCF-7 cells were either non-stimulated (NS) or stimulated with PMA (ST) (0.65 ng/μl for 60 hours). ChIP assays were subsequently performed on immuno-precipitated DNA with antibodies (A) anti-PKC-θ, (B) anti-PKC-β, (C) anti-PKC-θ (phospho), (D) anti-H3T6 or (E) anti-Pol II. Real time PCR analysis was carried out on these immuno-precipitated DNA using uPAR promoter primers. The data is shown graphically as ChIP enrichment ratio of immuno-precipitated DNA relative to the nil antibody control and normalized against the total input DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. Fold change was calculated with respect to the non-stimulated sample, which was set as 1. IP represents the immuno-precipitated signal and No AB represents the no-antibody or negative control signals. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
was also used to perform ChIP assay using both non-stimulated and stimulated cells and results showed that recruitment of RNA Pol II was greater in stimulated cells than non-stimulated cell (Figure 4.5 E).

4.4 Effect of a broad spectrum PKC inhibitor on the recruitment of PKC and other regulators of EMT on the uPAR promoter

MCF-7 cells were pre-incubated with 1μM bisindolylmaleimide-I for one hour and either stimulated with PMA (0.65 ng/ml) for 60 hours or left untreated (non-stimulated) and ChIP assays (refer section 2.7.1 for details) were subsequently carried out using antibodies against PKC-θ, PKC-β, Zeb1, LSD1, H3K4Me2 and H3K4Me1 (refer to table 2.3); and primers specific for uPAR gene promoter (refer to table 2.2) were used to perform real-time PCR analysis. In the absence of bisindolylmaleimide-I treatment, recruitment of PKC-θ on the uPAR promoter was greater in non-stimulated cells in comparison to stimulated cells (consistent with previous results Figure 4.5), however, pre-incubation of MCF-7 cells with bisindolylmaleimide-I resulted in up-regulation of PKC-θ recruitment in both stimulated and non-stimulated MCF-7 cells to an extent above that of the level of uninhibited (bisindolylmaleimide-I untreated) non-stimulated cells (Figure 4.6 A). A similar pattern was observed in recruitment of PKC-β, where recruitment of PKC-β on the uPAR promoter increased upon treatment of bisindolylmaleimide-I in both stimulated and non-stimulated cells (Figure 4.6 B). The level of Zeb1 recruitment to the uPAR promoter decreased in stimulated cells when compared with non-stimulated cells. Upon treatment with bisindolylmaleimide-I, Zeb1 occupancy on the uPAR promoter in stimulated cells becomes highly enriched (Figure 4.6 C). The enrichment profiles of LSD1, H3K4Me2 and H3K4Me1 on the uPAR gene promoter paralleled that of Zeb1 recruitment, as shown in Figure 4.6 D, Figure 4.6 E and Figure 4.6 F respectively. Taken together, these results suggest that at the chromatin level, the broad spectrum PKC inhibitor, bisindolylmaleimide-I, reverses the effect of PMA stimulation on the recruitment of EMT related proteins and PKCs in MCF-7 cells.
Figure 4.6 Bisindolylmaleimide-I modulates the binding of PKCs and other epigenetic markers across the uPAR promoter following PMA stimulation of MCF-7 cells

MCF-7 cells that were first pre-incubated for 1 hr with vehicle alone or with bisindolylmaleimide-I (1 μM) and subsequently either left untreated, non-stimulated (NS) or stimulated (ST) with PMA (0.65 ng/μl for 60 hours). ChIP assays were subsequently performed on immuno-precipitated DNA with antibodies (A) anti-PKC-θ, (B) anti-PKC-β, (C) anti-Zeb1, (D) anti-LSD1 (E) anti-H3K4Me2, and (F) anti-H3K4Me1. Real time PCR analysis was carried out on these immuno-precipitated DNA using uPAR promoter primers. The data is plotted as ChIP enrichment ratio of immuno-precipitated DNA relative to the nil antibody control and normalized against the total input DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. No AB represents the no-antibody or negative control signals. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
4.5 Proteins that interact on the chromatin template of key EMT promoters

Sequential ChIP experiments (as described in section 2.7.2) were undertaken on non-stimulated and stimulated MCF-7 cells to determine whether PKCs co-exist with other proteins on the EMT-induced gene uPAR promoter. Sequential ChIP results demonstrated higher co-occupancy of PKC-θ and Zeb1 in non-stimulated state on the uPAR gene promoter and their co-existence decreased in stimulated cells (Figure 4.7 A). Sequential ChIP performed for co-occupancy of Pol II and Zeb1 showed that these proteins co-exist on the uPAR promoter in the non-stimulated state and this association also decreases upon PMA stimulation (Figure 4.7 B). In contrast, Pol II and PKC-θ are co-occupants of the uPAR gene promoter in the non-stimulated state but their association increases in the stimulated state (Figure 4.7 C). It should be noted here that anti-PKC-θ antibody used was insensitive to the phosphorylation states of PKC-θ.

4.6 Different roles for chromatin associated PKC-θ and PKC-β during the epithelial to mesenchymal transition and cancer stem cell-like subpopulation formation

The experiments involving PKC isozyme specific knockdown using siRNA strategy were designed to understand the role of specific PKC isozyme proteins after finding the differential effects of various inhibitor treatments (inhibition of kinase activity) on EMT and CSC formation as well as differential chromatin binding of PKC isozymes. MCF-7 cells were transfected with either mock siRNA or PKC-θ siRNA, followed by either PMA stimulation (0.65 ng/ml for 60 hours) or left untreated. Images of the transfected cells were taken from a phase contrast microscope and FACS analysis was performed to determine the CD44$^{high}$/CD24$^{low}$-CSC-like sub-population. As expected, mock siRNA transfected stimulated cells showed EMT like changes in comparison to mock siRNA transfected non-stimulated cells (Figure 4.8 A). At the same time stimulation of PKC-θ siRNA transfected cells showed similar but attenuated EMT-like morphological changes and PKC-θ siRNA transfected non-stimulated cells did not

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PKC-θ physically interacts with Pol II and Zeb1 on uPAR promoter during PMA induced EMT in MCF7 cells

Sequential ChIP was performed on either non-stimulated (NS) or PMA stimulated (ST) (0.65 ng/ml for 60 hours) MCF-7 cells. First primary chromatin immunoprecipitation was carried out, and then secondary chromatin immunoprecipitation was performed on chromatin recovered from the primary immunoprecipitation. The antibodies used were (A) Primary ChIP with anti-PKC-θ antibody and secondary ChIP with anti-Zeb1 antibody, (B) Primary ChIP with anti-Pol II antibody and secondary ChIP with anti-Zeb1 antibody and (C) Primary ChIP with anti Pol II antibody and secondary ChIP with anti-PKC-θ antibody. Real time PCR analysis was performed on the immuno-precipitated DNA using uPAR promoter directed primers. The data is shown graphically as ChIP enrichment ratio of immuno-precipitated DNA relative to the no antibody control and normalized against the total input DNA. Fold change was calculated with respect to the non-stimulated sample, which was set as 1. “No antibody” (No AB) refers to the sample where no antibody (neither of primary or secondary) have been added to the cross-linked DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
Figure 4.8

Knockdown of PKC-θ gene by siRNA strategy has no effect on PMA-induced EMT-like morphological changes in MCF-7 cells

MCF-7 cells were transfected with either (A) Mock siRNA (mock) or (B) PKC-θ siRNA for 48 hr and subsequently left untreated, non-stimulation (NS) or stimulated with PMA (ST) (0.65 ng/ml for 60 hours). Phase contrast microscopy images of cells were captured using 10 x magnification of Olympus 17X1 microscope.
show any EMT changes (Figure 4.8 B). This result indicates that the PKC-θ siRNA knockdown did not abrogate PMA-induced EMT. Next, siRNA transfected cells were stained with Hoechst 33528, APC-anti-CD44, and PE-anti-CD24 cocktail for 20 minutes on ice and FACS analysis was carried out to monitor the effect of PKC-θ knockdown on the formation of the CD44\(^{\text{high}}\)/CD24\(^{\text{low}}\) or CSC-like sub-population. This experiment showed that as anticipated, both mock siRNA and PKC-θ siRNA transfected non-stimulated cells did not display a CSC-like sub population (Figure 4.9 A). Mock siRNA transfected stimulated cells showed around 5% CSC-like subpopulation as shown in Figure 4.9 B (consistent with results shown previously in Figure 4.3 A), but conversely, cells stimulated but transfected with PKC-θ siRNA showed significant reduction in CSC-like subpopulation in PMA induced CSC model of MCF-7 cells (Figure 4.9 B). Experiments were performed subsequently to observe the effect of PKC-β siRNA on CSC-like subpopulation regulation. Surprisingly, PKC-β knockdown not only increased the CD44\(^{\text{high}}\)/CD24\(^{\text{low}}\) subpopulation in non-stimulated MCF-7 cells in comparison to non-stimulated mock siRNA treated cells (Figure 4.10 A) but there was a substantial amplification of CD44\(^{\text{high}}\)/CD24\(^{\text{low}}\) subset in PMA stimulated PKC-β siRNA transfected cells (Figure 4.10 B).

Transcript analysis of CD44, uPAR, Zeb1 and E-cadherin in response to PKC-θ siRNA is shown in Figure 4.11 A. mRNA expression of CD44 in both non-stimulated mock siRNA and PKC-θ siRNA treated cells remained at basal levels but, in contrast, CD44 expression was strikingly reduced in stimulated PKC-θ siRNA treated cells compared to stimulated mock siRNA transfected cells (Figure 4.11 A). uPAR (Figure 4.11 B) and Zeb1 (Figure 4.11 C) expression levels followed a similar pattern as CD44 expression levels. E-cadherin mRNA expression (Figure 4.11 D) did not show that intense decrease in PKC-θ siRNA treated stimulated cells but rather, E-cadherin expression levels in stimulated cells were not altered much. It remains unclear here that if this difference in transcript pattern between CD44 or related genes (uPAR, Zeb1) and E-cadherin, is related to specific CSC-like changes or EMT.
Figure 4.9  PKC-θ knockdown results in abolishment of PMA-induced CSC-like subpopulation in MCF-7 cells

MCF-7 cells were transfected with either mock siRNA (mock) or PKC-θ siRNA for 48 hr and followed by either (A) left untreated, non-stimulation (NS) or (B) PMA stimulation (ST) (0.65 ng/ml for 60 hr). FACS analysis was subsequently carried out by staining cells with Hoechst, APC-anti-CD44 and PE-anti-CD24 antibodies stain cocktail. Circles on FACS plots indicate appropriate gating of CSC-like subpopulation and % CSC-like subpopulation is shown in a representative FACS plot from one of the two independent experiments.
Figure 4.10  PKC-β knockdown enhances PMA-induced CSC-like subpopulation in MCF-7 cells

MCF-7 cells were transfected with either mock siRNA (mock) or PKC-β siRNA for 48 hr and followed by either (A) left untreated, non-stimulation (NS) or (B) PMA stimulation (ST) (0.65 ng/ml for 60 hr). FACS analysis was subsequently carried out by staining cells with Hoechst, APC-anti-CD44 and PE-anti-CD24 antibodies stain cocktail. Circles on FACS plots indicate appropriate gating of CSC-like subpopulation and % CSC-like subpopulation is shown in a representative FACS plot from one of the two independent experiments.
Figure 4.11

PKC-θ siRNA knockdown decreases transcription of PMA-induced genes in the CSC-like subset in MCF7 cells

MCF-7 cells were transfected with either mock siRNA (mock) or PKC-θ siRNA followed by either non-stimulation (NS) or PMA stimulation (ST) (0.65 ng/ml for 60 hr) 48 hr post-transfection. Taqman® real time PCR for EMT/CSC genes- (A) CD44, (B) uPAR, (C) Zeb1 and (D) E-cadherin was performed on cDNA synthesized from total RNA isolated from cells. Threshold cycle (Ct) values generated for each time points were converted to arbitrary copy number and normalized to Cyclophilin A reference levels. Data represent the mean ± standard error (SE) of three independent experiments.
The data prompted the question of whether knock-down of PKC-θ would have any influence on chromatin association of proteins, including PKC-β, Zeb1, LSD1 and the active histone modifications-H3K4Me2 and H3K4Me1. This experiment was imperative to determine the specific role of PKC-θ in the EMT/CSC process in comparison to the broad spectrum PKC inhibition by bisindolylmaleimide-I. In ChIP assays using anti-PKC-θ, anti-PKC-β, anti-Zeb1, anti-LSD1, anti-H3K4Me2 or anti-H3K4Me1 antibodies, chromatin binding of PKC-θ in stimulated mock siRNA treated cells was decreased in relation to non-stimulated mock treated cells and this trend was similar in PKC-θ siRNA treated cells as well (Figure 4.12 A). Conversely, PKC-β chromatin binding increased in mock siRNA treated stimulated cells (Figure 4.12 B) relative to mock treated non-stimulated cells (similar to results presented in Figure 4.5) but chromatin associated of PKC-β was reduced in stimulated cells when compared with non-stimulated cells upon PKC-θ siRNA treatment (Figure 4.12 B). In contrast to PKC-β ChIP enrichment ratio, chromatin association of Zeb1 in mock siRNA treated stimulated cells was less than non-stimulated mock siRNA treated cells (similar to results presented in Figure 4.6), however, PKC-θ siRNA treatment reverses this effect by showing more chromatin association of Zeb1 in stimulated cells than non-stimulated cells (this pattern of Zeb1 association is in parallel to it transcript trend upon PKC-θ knock-down as presented in Figure 4.11 C). ChIP assay using anti-LSD1 antibody showed that LSD1 binding to chromatin decreases upon stimulation in mock siRNA treated cells (similar to results presented in Figure 4.6 D). At the same time, PKC-θ knock-down also results in less chromatin association of LSD1 after stimulation (Figure 4.12 D). Further, stimulation of mock siRNA treated cells leads to less enrichment of the H3K4Me2 mark) in contrast to non-stimulated cells (similar to results presented in Figure 4.6). PKC-θ siRNA treatment however results in decreased enrichment of this histone modification upon stimulation, compared to non-stimulation but still it was relatively higher than enrichment in mock siRNA treated cells (Figure 4.2 E). The other histone modification investigated, H3K4Me1, was less enriched in the stimulated state in both PKC-θ siRNA knockdown and in mock siRNA treated cells (Figure 4.12 F).
Figure 4.12 PKC-θ knockdown modulates chromatin association of PKC-β and other key EMT related epigenetic markers across the uPAR promoter

MCF-7 cells were transfected with either Mock siRNA (mock) or PKC-θ siRNA followed by, either non-stimulation (NS) or PMA stimulation (ST) (0.65 ng/ml for 60 hr) 48 hr post-transfection. ChIP assays were subsequently performed on immuno-precipitated DNA with antibodies (A) anti-PKC-θ, (B) anti-PKC-β, (C) anti-Zeb1, (D) anti-LSD1 (E) anti-H3K4Me2, or (F) anti-H3K4Me1. Real time PCR analysis was performed on these immuno-precipitated DNA by using uPAR promoter primer. The data is shown graphically as ChIP enrichment ratio of immuno-precipitated DNA relative to the nil antibody control and normalized against the total input DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. IP represents the immuno-precipitated signal and No AB represents the no-antibody or negative control signals. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
Discussion

The PMA-induced experimental model for epithelial to mesenchymal plasticity in MCF-7 cells appears to be mediated by the PKC pathways because bisindolylmaleimide-I (broad spectrum PKC inhibitor) inhibition results in abrogation of EMT. However, Go6976 (cPKC inhibitor) treatment resulted in increased EMT as illustrated by both EMT related morphological changes (Figure 4.1) and up-regulation of inducible genes such as laminin-5, uPAR and CD44 (Figure 4.2). The expression of two cancer stem cell markers: CD44 and CD24 on MCF-7 cells were also affected after PKC pathway inhibition (Figure 4.3). Inhibition of PKC pathway using a broad spectrum PKC inhibitor showed striking disappearance of cells with CSC-like phenotype, which was characterized by CD44<sup>high</sup> and CD24<sup>low</sup> markers (Figure 4.3 B) and reduced mammosphere formation (Figure 4.4). In contrast, inhibition of only cPKCs showed increased CSC-like subpopulation formation (Figure 4.3 C).

Chromatin immuno-precipitation (ChIP) assays demonstrated that both PKC-θ and PKC-β dock onto the promoter of the inducible EMT characteristic gene, uPAR in MCF-7 cells (Figure 4.5 A & B). Furthermore, active (phosphorylated) forms of these PKCs were highly enriched on the promoter of the uPAR gene in the stimulated or mesenchymal state (Figure 4.5 C & D) and, moreover, the data also suggest a positive correlation of these active forms with active mark Pol II recruitment (Figure 4.5 E). These results were validated by using the broad spectrum PKC activity inhibitor, bisindolylmaleimide-I, which resulted in the resetting of epithelial epigenetic tags as observed by increased recruitment of PKC-θ, Zeb1 and LSD1 in stimulated state after pre-incubating MCF-7 cells with bisindolylmaleimide-I (Figure 4.6 A, C and D). Chromatin association of PKC-β, which showed increased recruitment upon stimulation in preceding section (Figure 4.5), after bisindolylmaleimide-I treatment was not changed between non-stimulated and stimulated state (Figure 4.6 B). Taken together, these results suggested that
inhibition of PKC activity resets PMA-induced mesenchymal epigenetic tags back to epithelial tags in MCF-7 cells. This data also correlates with reduced EMT (Figure 4.1 A and Figure 4.2 A) and reduced CSC-like phenotype in presence of broad spectrum PKC activity inhibitor (Figure 4.3 A and Figure 4.4).

Data presented in Figure 4.6 also illustrated a negative correlation of PKC activity with known EMT repressive proteins such as Zeb1 and LSD1, or in other words induction of epithelial to mesenchymal transition by increased PKC activity (induced by PMA), leads to decreased association of Zeb1 and LSD1 on the uPAR promoter. LSD1 activity is very specific for only mono- and dimethylated H3K4 through a flavin-dependent mechanism (Forneris et al., 2005; Shi et al., 2004). Therefore, ChIP enrichment ratio of LSD1 mediated methylation marks; H3K4Me2 and H3K4Me1 were observed to examine PKC directed effects on LSD1 activity during PMA-induced EMT. Results of this experiment were very exciting as inhibition of PKC activity prevented the demethylation of these two histone modifications (Figure 4.6 E & F). This data certainly raises a very interesting question as to how LSD1 and PKC family members interact in regulating histone modifications. Indeed, two of the major queries in today's epigenetic field is “how do the various epigenetic players interact?” and “what mechanisms convey the sequence specificity to the enzymes involved (Portela and Esteller, 2010)?” Thus, the next question was if PKC-θ interacts with EMT-related repressing transcription factor, Zeb1, or Pol II? Sequential ChIP data presented in Figure 4.7 not only revealed that these epigenetic tags interact with each other during EMT but also that interaction of PKC-θ-Zeb1 and Pol II-Zeb1 decreases in the mesenchymal state (stimulated state), while interaction between Pol II-PKC- θ increases in mesenchymal state across the uPAR gene promoter (Figure 4.7).
Specific roles for PKC-θ and PKC-β were identified in the in EMT and CSC formation process. This study provides evidence that in absence of PKC-θ the CSC-like subpopulation is reduced (Figure 4.9 and 4.11), while transient absence of PKC-β leads to the opposite affect by increasing CSC-like subpopulation (Figure 4.10). The data shown in the Figure 4.8, 4.9 and 4.11 are not contradictory. As shown in Figure 4.8, PKC-θ siRNA treatment although does not reduce EMT-like morphological changes, however, it results in decreased CSC-like sub-population specifically (Figure 4.9). This result was confirmed by transcript analysis (Figure 4.11) of CSC-specific genes CD44, uPAR and Zeb-1 which showed decrease upon PKC-θ siRNA treatment, however, E-cadherin, an EMT characteristic gene did not changed much upon PKC-θ knockdown. In contrast, PKC-β siRNA results in increased CSC-like sub-population formation (Figure 4.10). Therefore, both the PKC isoforms are important for EMT.

Bisindolylmaleimide-I (broad spectrum PKC inhibitor) inhibition results in abrogation of EMT and CSC but Go6976 (cPKC inhibitor) treatment resulted in increased EMT and CSC-like subset (Figure 4.1 and 4.2). If Go6976 does not work, there would have been no effect on CSC population formation and the EMT/CSC genes would be unchanged. Clearly this is not the case. In fact treatment of Go6976, cPKC inhibitor which inhibits PKC-β, increases CSC-like sub-population (Chapter 4, Figure 4.3 C). Therefore, these results correlate closely with the PKC-θ and PKC-β knockdown studies, in which knockdown of PKC-θ results in decreased CSC-like sub-population and PKC-β knockdown increases CSC-like sub-population respectively.

ChIP assays showed that non-phosphorylated PKC-θ predominated in the epithelial state (Figure 4.5 A); however the phosphorylated PKC-θ form predominated in the mesenchymal state of MCF-7 cells (Figure 4.5 C). In contrast, recruitment of PKC-β predominated in the mesenchymal state (Figure 4.5 B). It should be noted that although chromatin association of
PKC-β increases upon stimulation during EMT, however, in absence of PKC-θ (upon PKC-θ knockdown), PKC-β could not tether to the promoter of the inducible gene uPAR (Figure 4.11 B). In summary, as mentioned earlier in this section, two PKC isozymes namely, PKC-θ and PKC-β demonstrate distinctly opposing chromatin binding trends upon stimulation and also reveal opposing influence on regulation of CSC-like subpopulation. Hence, the current study provides an indication that specific PKC isozymes could play different roles in the same tissues. Interestingly, knockdown of PKC-θ decreases the binding of PKC-β and LSD1 on active gene promoters but fails to decrease binding of Zeb1, which could be a possible mechanism to regulate EMT and cancer stem cells process in human breast cancers.

In the next chapter, an investigation of the role of LSD1 in the PMA-induced EMT and CSC formation will be described along with the description of an investigation into the interaction of LSD1 with PKC-θ, Pol II and Zeb-1. Previous studies have demonstrated that Zeb1 is a major player in the regulation of EMT through microRNAs (Gregory et al., 2008) and, in next chapter, the role of PKC-θ in regulating microRNAs is also explored.
Chapter Five

Chromatin anchored PKCs associate with LSD1, DNMT1 and Zeb1 on promoters of EMT inducible genes and microRNAs during the epithelial to mesenchymal transition and cancer stem cell formation processes
Chapter 5: Chromatin anchored PKCs associate with LSD1, DNMT1 and Zeb1 on promoters of EMT inducible genes and microRNAs during the epithelial to mesenchymal transition and cancer stem cell formation processes

Introduction

As described earlier, various layers of epigenetic modifications regulate gene transcription (refer to section 1.3 for details) and chromatin modification is considered to be a principal epigenetic regulator of gene expression in eukaryotes (Jaenisch and Bird, 2003; Jenuwein and Allis, 2001). It has been demonstrated also that during cancer, DNA promoter hypermethylation and chromatin modifications (both active and repressive marks in combinatorial manner) could lead to either activation (Shi et al., 2004) or repression of genes (Baylin and Ohm, 2006). The combination of chromatin marks at a given promoter partially determine open active or closed repressive status of the gene (Baylin and Ohm, 2006; Jenuwein and Allis, 2001; Lachner et al., 2003). Among the many histone modifications identified so far, lysine methylation provides for a rich array of biological readouts (Kouzarides, 2007; Martin and Zhang, 2005) as it is linked with either active or repressive signals. Histone methylation is a dynamic process which is regulated by addition of methyl groups by histone methyltransferases (writers) and removal of methylation by lysine-specific demethylases and JmjC domain demethylases (erasers) (Klose et al., 2006b; Shi et al., 2004; Tsukada et al., 2006; Whetstine et al., 2006; Yamane et al., 2006). LSD1, which is also known as BHC110 (Lee et al., 2006; Shi et al., 2004), is specifically associated with demethylation of both H3K4Me2 and H3K4Me1 and its activity has been shown to be linked with transcriptional repression (Huang
et al., 2007c). Another study describes the role of transient LSD1-mediated demethylation of H3K4 in driving local DNA oxidation followed by the assembly of Myc-induced transcriptional initiation complexes (Amente et al., 2010). LSD1 has also been shown to be highly expressed in estrogen receptor-negative breast cancers (Hayami et al., 2010) and also been proposed as a biomarker for aggressive tumors (Lim et al., 2010). Recently, DNA methyltransferase 1 (DNMT1), an enzyme responsible principally for maintaining DNA methylation patterns during DNA replication, has been shown to be a novel substrate for LSD1 (Wang et al., 2009). Furthermore, this same study demonstrated that LSD1 plays a role in providing stability to DNMT1 (Wang et al., 2009). Aberrant DNA methylation of CpG-island-containing promoters leads to permanent silencing of genes in both physiologically normal and pathological contexts, most particularly in cancer cells (Baylin and Ohm, 2006).

A recent study provided evidence that phosphorylation of H3T6 by PKC-β1 inhibits LSD1 mediated demethylation of the adjacent H3K4Me2 mark during androgen-receptor-dependent gene activation (Metzger et al., 2010). Subsequently, in the same year another group demonstrated that LSD1 is essential for Snail1-mediated transcriptional repression during EMT (Lin et al., 2010a). The results presented in the preceding chapter clearly demonstrated that either inhibition of PKC family members activity (Figure 4.6) or transient knockdown of PKC-θ (Figure 4.12) increased the chromatin binding of LSD1 on the uPAR promoter during PMA induced EMT. Consequently, it was important to identify the role of LSD1 in regulating the cancer stem like cell formation. In this chapter, it was explored (i) whether LSD1 was essential for controlling PMA-induced EMT and CSC-like subpopulation formation subsequent to PMA stimulation of MCF-7 cells, and (ii) whether LSD1 is required for chromatin tethering of epigenetic marks including PKCs. LSD1 knockdown by the siRNA strategy (as described in section 2.5.4) was performed first, followed by transcript analysis to find out whether LSD1
plays a role in PMA-induced EMT and CSC formation process. Sequential ChIP was then performed to determine if LSD1 co-existed with PKC-θ, Pol II or Zeb-1 on chromatin during the PMA-induced EMT and, finally ChIP assays were carried out to understand the effect of LSD1 siRNA treatment on the chromatin association of PKC-θ, PKC-β, H3K4Me2, H3K4Me1 and DNMT1 across the EMT inducible gene uPAR.

One of the most studied microRNA groups in relation to breast cancers and the EMT are the miR 200 family members and two recent reports have identified a new role of miR 200 family members through direct regulation of the transcription factors Zeb1 and Zeb2 (Gregory et al., 2008; Korpal et al., 2008; Park et al., 2008). Elevated expression of the miR 200 family has been shown to correlate with epithelial phenotypes of several cancer cell lines (Gregory et al., 2008; Korpal et al., 2008; Park et al., 2008). Additionally, miR 200c has been shown to suppress the ability of both normal mammary stem cells to form mammary ducts and, also, tumor formation driven by breast cancer stem cells in vivo (Shimono et al., 2009). In a recent study, using ChIP-on-ChIP analysis, it has been shown that the variant PKC isozyme PKC-θ binds to promoter regions of microRNA encoding genes following stimulation in T cells (Sutcliffe et al., 2011). In that study, PKC-θ was also shown to participate in signaling complexes that negatively regulate miRNA expression and, consistent with previous findings, transfection of pre-miR-200c markedly repressed Zeb1 transcription in T cells (Sutcliffe et al., 2011).

In this chapter, the role of chromatin-tethered PKCs in regulating microRNA 200c expression is described. ChIP assays were performed to observe the pattern of chromatin association of PKC-θ and PKC-β on the miR 200c promoter. Sequential ChIP assays were carried out to observe the co-occupancy of PKCs with other epigenetic modifiers such as LSD1 on the miR 200c promoter. Subsequently, ChIP assays were used to identify the effect of PKC-θ knockdown and PKC activity inhibition on chromatin binding of these epigenetic marks on the miR 200c promoter.
during EMT. Additionally, miR 200c, miR 200b and Zeb1 transcripts were monitored to understand the role of Zeb1 in this process. Ultimately, the importance of miR 200b and miR 200c was explored for regulating EMT and CSC formation processes by over-expression of these microRNA 200 family members in MCF-7 cells.

5.1 LSD1 plays major role in regulating the process of cancer stem cell formation

Firstly, MCF-7 cells were transfected with mock siRNA or LSD1 siRNA followed either by non-stimulation or PMA stimulation (0.65 ng/ml) for 60 hours. Next, siRNA transfected cells were stained with the Hoechst 33528, APC-anti-CD44, PE-anti-CD24 cocktail and FACS analysis (as described in section 2.3.3) was carried out to monitor the effect of LSD1 knockdown on the formation of the CD44\textsuperscript{high}/CD24\textsuperscript{low} CSC-like sub-population. Stimulation of LSD1 siRNA transfected MCF-7 cells resulted in approximately ~3 fold increase in the CSC-like subpopulation compared to mock-siRNA-transfected stimulated cells (Figure 5.1 B). In contrast, mock siRNA and LSD1 siRNA transfected non-stimulated MCF-7 cells did not show formation of a CSC-like sub population as judged by low CD44 and low CD24 staining observed in FACS analysis (Figure 5.1 A). Consistent with the FACS data, transcript analysis also demonstrated that both mock and LSD1 transfected non-stimulated cells did not result in an increase of the CD44 transcript but LSD1 knockdown by siRNA resulted in substantial increase in CD44 transcript levels in stimulated cells (Figure 5.2 A) in comparison to mock-siRNA transfected stimulated cells. Another EMT marker, uPAR followed same pattern as CD44 (Figure 5.2 B) with its transcript levels increasing upon stimulation in LSD1 siRNA treated cells. Taken together, these results show that LSD1 knockdown results in changes in gene expression that leads to increased cancer stem-like cell subpopulation.
Figure 5.1

LSD1 siRNA treatment enhances the PMA-induced CSC-like subpopulation in MCF-7 cells

MCF-7 cells were transfected with either mock siRNA (mock) or LSD1 siRNA followed by either (A) non-stimulation (NS) or (B) PMA stimulation (ST) (0.65 ng/ml for 60 hr) 48 hr post-transfection. Cells were then stained with Hoechst, APC-anti-CD44 and PE-anti-CD24 antibodies stain cocktail and subsequently subjected to FACS analysis. Circles on FACS plots indicate appropriate gating of CSC-like subpopulation and % CSC-like subpopulation is shown in a representative FACS plot from one of two independent experiments.
Figure 5.2  

LSD1 knockdown enhances the transcription of PMA-induced EMT/CSC genes in MCF-7 cells  

MCF-7 cells were transfected with either mock siRNA (mock) or LSD1 siRNA followed by either non-stimulation (NS) or PMA stimulation (ST) (0.65 ng/ml for 60 hr) 48 hr post-transfection. Taqman® real time PCR for EMT/CSC genes- (A) CD44 and (B) uPAR was performed on cDNA synthesized from total RNA isolated from cells. Cycle threshold (Ct) values generated for each time points were converted to arbitrary copy number and normalized to Cyclophilin A reference levels. Data represent duplicates from three independent experiments with results expressed as the mean ± standard error (SE).
5.2 The interplay between LSD1, chromatin anchored PKC-θ and other EMT regulators including Pol II and Zeb1 on chromatin template

To examine the contribution of LSD1 in cancer stem cell formation, an investigation was made to determine if LSD1 interacts with chromatin-associated PKC-θ, the key active transcription mark-Pol II and key EMT repressor Zeb1 during the EMT process. Zeb1 was included as a potential co-regulator in these studies as it has been demonstrated previously in developmental biology that a LSD1-Zeb1 complex regulates growth hormone (GH) gene repression in lactotropes (Wang et al., 2007) and, in the preceding chapter of this thesis it has been shown that there is a close association of PKC with Zeb1 during EMT (Figure 4.7 A). To test the hypothesis that LSD1 collaborates with PKC-θ, Pol II and Zeb1 in regulating the EMT process, sequential ChIP was performed using antibodies against each of the above target proteins. Sequential ChIP demonstrates that PKC-θ and LSD1 co-exist on the EMT inducible uPAR gene promoter in non-stimulated cells and this association decreases upon stimulation (Figure 5.3 A). Similarly, sequential ChIP analysis showed reduced co-occupancy of active mark-Pol II and LSD1 in PMA-induced MCF-7 cells on the uPAR promoter (Figure 5.3 B). In contrast, LSD1 co-occupancy with EMT repressor-Zeb1 increased in stimulated state (Figure 5.3 C). Collectively, these findings demonstrate that LSD1 co-exist with PKC-θ and Pol II to greater extent in non-stimulated MCF-7 cells and this co-relation is reduced upon induction of the EMT.

Next, ChIP assays using anti-LSD1 and anti-PKC-θ antibodies were performed on FACS sorted CSC-like and non-CSC (NCSC) subpopulations (as described in section 3.7) after treating cells with either mock siRNA or LSD1 siRNA (detailed siRNA strategy described in section 2.5.4). Results of the ChIP assays showed that LSD1 siRNA treatment not only decreases the tethering of LSD1 but also reduces tethering of PKC-θ to the uPAR promoter in the epithelial state (non-stimulated) in comparison to mock transfected cells (Figure 5.4). Chromatin association of LSD1 and PKC-θ on the uPAR promoter was very low in cancer stem cells for both mock and LSD1 siRNA transfected cells.
(Figure 5.4). LSD1-siRNA transfected non-cancer stem cells demonstrated lower recruitment of LSD1 on the uPAR promoter when compared with mock siRNA transfected cells, however; chromatin association of PKC-θ in non-cancer stem cells did not change upon LSD1 siRNA treatment in comparison to mock transfection (Figure 5.4). The ChIP results also showed that the active histone modifications, H3K4Me2 and H3K4Me1 were loosely associated on uPAR promoter in cancer stem cells in comparison to non-cancer stem cells but they were highly associated with chromatin in non-stimulated cells (Figure 5.5). However when compared in between cells types, it was observed that both histone marks showed reduced chromatin association in non-stimulated-LSD1 siRNA transfected cells than non-stimulated-mock siRNA transfected cells. Cancer stem-like cell subpopulation (CSC) of mock siRNA transfected cells and LSD1 siRNA transfected cells displayed no difference in H3K4Me2 (Figure 5.5). A similar pattern of H3K4Me2 was observed in non-cancer stem cell like subpopulation (NCSC). In contrast, H3K4Me1 demonstrated lower recruitment on the uPAR promoter in both CSC-like and NCSC subpopulation in LSD1 siRNA transfected cells than mock-transfected cells (Figure 5.5). Chromatin associated DNMT1 on the uPAR promoter was observed to be less in all of the three LSD1 siRNA transfected cell types (NS, CSC, and NCSC) than their respective mock siRNA transfected MCF-7 cells (Figure 5.5). The chromatin association of these epigenetic marks on the CD44 gene promoter was also assessed as described for the uPAR promoter. The results on CD44 promoter mostly mirrored the pattern for uPAR in association of LSD1 and PKC-θ (Figure 5.6). Both the histone methylation modifications on the CD44 promoter demonstrated a similar chromatin binding trend to that on the uPAR promoter, however, chromatin association of DNMT1 on CD44 promoter in mock and LSD1 siRNA transfected cells did not differ in non-stimulated mock and LSD1 siRNA treated cells but both CSC and NCSC subpopulations displayed lower chromatin binding on CD44 promoter upon LSD1 siRNA treatment (Figure 5.7).
Figure 5.3

LSD1 interacts with PKC-θ, Pol II and Zeb1 on uPAR promoter during PMA induced EMT in MCF-7 cells

MCF-7 cells were either left untreated, non-stimulated (NS) or PMA stimulated (ST) (0.65 ng/ml for 60 hours). Sequential ChIP was performed by first carrying out primary chromatin immunoprecipitation and then secondary chromatin immunoprecipitation was performed on chromatin recovered from the primary immunoprecipitation. The antibodies used were (A) First ChIP with anti-PKC-θ antibody and secondary ChIP with anti-LSD1 antibody, (B) Primary ChIP with anti-Pol II antibody and secondary ChIP with anti-LSD-1 antibody and (C) Primary ChIP with anti LSD1 antibody and secondary ChIP with anti-Zeb1 antibody. Real time PCR analysis was performed on immuno-precipitated DNA using uPAR promoter specific primers. The data is shown graphically as ChIP enrichment ratio of immuno-precipitated DNA relative to the no antibody control and normalized against the total input DNA. Fold change was calculated with respect to the non-stimulated sample, which was set as 1. “No antibody” (No AB) refers to the sample where no antibody (neither of primary or secondary) have been added to the cross-linked DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
**Figure 5.4**

LSD1 knockdown effects chromatin association of PKC-θ across the uPAR promoter

MCF-7 cells were transfected with either mock siRNA (mock) or LSD1 siRNA followed by either non-stimulation (NS) or PMA stimulation (0.65 ng/ml for 60 hr) prior to FACS sorting of cancer stem-like cells (CSC) and non-cancer stem-like cells (NCSC) subpopulations. ChIP assays were subsequently performed on immuno-precipitated DNA with anti-LSD1 or anti-PKC-θ antibodies. Real time PCR analysis was performed on these for immuno-precipitated DNA using primers specific for the uPAR promoter primer. The data is plotted as ChIP enrichment ratio of immuno-precipitated DNA relative to the nil antibody control and normalized against the total input DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. No AB represents the no-antibody or negative control signals. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
Figure 5.5

**LSD1 knockdown modulates recruitment of epigenetic marks across the uPAR promoter**

MCF-7 cells were transfected with either mock siRNA (mock) or LSD1 siRNA followed by either non-stimulation (NS) or PMA stimulation (0.65 ng/ml for 60 hr) prior to FACS sorting of cancer stem-like cells (CSC) and non-cancer stem-like cells (NCSC) subpopulations. ChIP assays were subsequently performed on immuno-precipitated DNA with anti-H3K4Me2, anti-H3K4Me1 or anti-DNMT1 antibodies. Real time PCR analysis was performed for these cases for immuno-precipitated DNA using primers specific for the uPAR promoter primer. The data is plotted as ChIP enrichment ratio of immuno-precipitated DNA relative to the nil antibody control and normalized against the total input DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. No AB represents the no-antibody or negative control signals. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student's t test).
Figure 5.6

LSD1 knockdown affects chromatin association of PKC-θ across the CD44 promoter

MCF-7 cells were transfected with either mock siRNA (mock) or LSD1 siRNA followed by either non-stimulation (NS) or PMA stimulation (0.65 ng/ml for 60 hr) prior to FACS sorting of cancer stem-like cells (CSC) and non-cancer stem-like cells (NCSC) subpopulations. ChIP assays were subsequently performed on immuno-precipitated DNA with anti-LSD1 or anti-PKC-θ antibodies. Real time PCR analysis was performed for these cases for immuno-precipitated DNA using primers specific for the CD44 promoter primer. The data is plotted as ChIP enrichment ratio of immuno-precipitated DNA relative to the nil antibody control and normalized against the total input DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. No AB represents the no-antibody or negative control signals. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
Figure 5.7

LSD1 knockdown modulates the recruitment of epigenetic marks across the CD44 promoter

MCF-7 cells were transfected with either mock siRNA (mock) or LSD1 siRNA followed by either non-stimulation (NS) or PMA stimulation (0.65 ng/ml for 60 hr) prior to FACS sorting of cancer stem-like cells (CSC) and non-cancer stem-like cells (NCSC) subpopulations. ChIP assays were subsequently performed on immuno-precipitated DNA with anti-H3K4Me2, anti-H3K4Me1 or anti-DNMT1 antibodies. Real time PCR analysis was performed for these cases for immuno-precipitated DNA using primers specific for the CD44 promoter primer. The data is plotted as ChIP enrichment ratio of immuno-precipitated DNA relative to the nil antibody control and normalized against the total input DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. No AB represents the no-antibody or negative control signals. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
5.3 Interplay of PKC pathway with miR 200 family members in PMA-induced EMT model

Consistent with the previous observations, microRNA 200 family member expression also decreases in PMA-induced EMT (as shown in preceding chapter Figure 3.7). ChIP assays were carried out, to examine whether PKC-θ, PKC-β and EMT related epigenetic marks associated with chromatin template on miR 200c promoter in the PMA-induced model of EMT in MCF-7 cells. The ChIP results presented in Figure 5.8 A demonstrated that chromatin association of PKC-θ across the miR 200c promoter was greater in the non-stimulated state than the stimulated state; however PKC-β binding on miR 200c promoter was greater in stimulated cells in comparison to non-stimulated cells (Figure 5.8 B). Phosphorylated forms of both PKC-θ and H3T6 demonstrated more chromatin binding in stimulated cells (Figure 5.8 C & D) across the miR 200c promoter. However, chromatin association of Pol II on miR 200c promoter was diminished upon stimulation (Figure 5.8 E).

Increased chromatin association of Pol II at miR 200c promoter observed in non-stimulated or epithelial state suggested that Pol II might be associated with a repressor complex containing PKC-θ, LSD1 and Zeb1 during induction of mesenchymal state. Consequently, sequential ChIP was carried out on non-stimulated and PMA stimulated MCF-7 cells. Results from sequential ChIP with antibodies against Pol II and PKC-θ revealed the co-existence of these proteins on miR 200c promoter and this association was increased in stimulated cells relative to non-stimulated cells (Figure 5.9 A). Sequential ChIP results revealed that Pol II and Zeb1 also co-existed on the miR 200c promoter and that their co-occupancy increased upon stimulation (Figure 5.9 B). Additionally, Pol II and LSD1 co-existed together on miR 200c promoter and their co-occupancy also followed similar increasing trend upon stimulation (Figure 5.9 C), as observed by Pol II-PKC-θ and Pol II-Zeb1. Next, sequential ChIP was performed with antibodies against LSD1 and PKC-θ;
Figure 5.8

Phosphorylated (active) form of PKCs physically associate with promoter region of miR 200c in EMT.

ChIP assays were performed on MCF-7 cells either non-stimulated (NS) or stimulated with PMA (ST) (0.65 ng/μl for 60 hr). Real time PCR analysis was performed on immuno-precipitated DNA recovered with (A) anti-PKC-θ, (B) anti-PKC-β, (C) anti-PKC-θ (phospho), (D) anti-H3T6 or (E) anti-Pol II antibody using miR 200c promoter primer. The data is shown graphically as ChIP enrichment ratio of immuno-precipitated DNA relative to the nil antibody control and normalized against the total input DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. Fold change was calculated with respect to the non-stimulated sample, which was set as 1. IP represents the immuno-precipitated signal and No AB represents the no-antibody or negative control signals. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
Figure 5.9

Pol II associates with PKC-θ and other EMT marks such as Zeb1 and LSD1 on the miR 200c promoter during PMA-induced EMT in MCF-7 cells

Sequential ChIP was performed on either non-stimulated (NS) or PMA stimulated (ST) (0.65 ng/ml for 60 hours) MCF-7 cells, by first carrying out chromatin immunoprecipitation partly and then in the second stage carrying out the chromatin immunoprecipitation performed on DNA recovered from the first stage immuno-precipitation. The antibodies used were (A) Primary ChIP with anti-Pol II antibody and secondary ChIP with anti-PKC-θ antibody, (B) Primary ChIP with anti-Pol II antibody and secondary ChIP with anti-Zeb1 antibody and (C) Primary ChIP with anti Pol II antibody and secondary ChIP with anti-LSD-1 antibody, (D) Primary ChIP with anti-LSD1 antibody and secondary ChIP with anti-PKC-θ antibody, (E) Primary ChIP with anti-LSD1 antibody and secondary ChIP with anti-Zeb1 antibody. Real time PCR analysis was performed on immuno-precipitated DNA using miR 200c specific promoter primers. The data is shown graphically as ChIP enrichment ratio of immuno-precipitated DNA relative to the no antibody control and normalized against the total input DNA. Fold change was calculated with respect to the non-stimulated sample, which was set as 1. "No antibody" (No AB) refers to the sample where no antibody (neither of primary or secondary) have been added to the cross-linked DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
LSD1 and Zeb1 to understand the co-occupancy of these proteins with LSD1 on miR 200c promoter. The results illustrated that LSD1 and PKC-θ co-existed on the miR 200c promoter and their co-existence was stronger in stimulated cells (Figure 5.9 D). LSD1 and Zeb1 also showed stronger co-existence on miR 200c promoter in stimulated cells (Figure 5.9 E). Collectively, the sequential ChIP results supported the hypothesis that in mesenchymal state, Pol II is recruited on the miR 200c promoter but its activity is blocked as it may be "captured" in a repressive complex that includes PKC-θ.

5.4 Chromatin tethered PKC-θ plays a crucial role in regulating the interaction of EMT related epigenetic marks on the miR 200c promoter

The results presented in previous chapter collectively revealed the importance of chromatin associated PKC-θ in the transcriptional regulation of EMT and CSC associated genes such as uPAR and CD44. In the previous section of this chapter (section 5.3) it was shown that PKC-θ containing epigenetic complexes assemble across the miR 200c promoter during PMA-induced EMT. The role of PKC-θ in the interaction of these epigenetic complexes with miR 200c was examined; ChIP assays were performed with antibodies against PKC-β, Zeb1, LSD1, DNMT1, Set7/9, H3K4Me2, H3K4Me1 and PKC-θ on cells following either mock siRNA or PKC-θ directed siRNA transfection. The results of ChIP assay showed that in mock siRNA transfected cells, PKC-β accumulation on the miR 200c promoter was observed to a greater extent in stimulated cells than non-stimulated cells (consistent with previous results, Figure 5.8 B). However, PKC-β recruitment on miR 200c promoter diminished in both non-stimulated and stimulated states after knockdown of PKC-θ by PKC-θ siRNA (Figure 5.10 A). It should be noted here that PKC-θ knockdown was also linked to inhibition of cancer stem cell like subpopulation (Figure 4.10). The ChIP data also showed a very striking result that the chromatin association of Zeb1 on miR 200c promoter goes down upon stimulation in mock siRNA transfected cells but markedly
Figure 5.10 continued-

(E) Set7/9

(F) H3K4Me2

(G) H3K4Me1

(H) PKC-θ

Figure 5.10  PKC-θ knockdown affects chromatin association of PKC-β and other EMT related epigenetic markers across the miR 200c promoter

MCF-7 cells were transfected with either mock siRNA (mock) or PKC-θ siRNA and subsequently subjected to either Non-stimulation (NS) or PMA stimulation (ST) (0.65 ng/ml for 60 hr). Then ChIP assays were performed on immuno-precipitate DNA with antibodies (A) anti-PKC-β, (B) anti-Zeb1, (C) anti-LSD1 (D) anti-DNMT1, (E) anti-set7/9 (F) anti-H3K4Me2, (G) anti-H3K4Me1, or (H) anti-PKC-θ. Real time PCR analysis was performed for these cases for immuno-precipitated DNA by using miR 200c promoter primer. The data is plotted as ChIP enrichment ratio of immuno-precipitated DNA relative to the nil antibody control and normalized against the total input DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. No AB represents the no-antibody or negative control signals. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
increases in PKC-θ siRNA transfected cells in stimulated cells (Figure 5.10 B). Chromatin association of LSD1 further decreased upon stimulation in PKC-θ treated cells in comparison to mock siRNA treated cells (Figure 5.10 C). Chromatin association patterns of H3K4Me2 and H3K4Me1 on the miR 200c promoter followed the same trend as LSD1 (Figure 5.10 F & G). Upon PKC-θ knockdown, DNMT1 chromatin binding on the miR 200c promoter was also decreased in both non-stimulated and stimulated cells in comparison to mock siRNA transfected cells but PKC-θ knockdown resulted in greater accumulation of DNMT1 in stimulated cells than non-stimulated cells (Figure 5.10 D).

Chromatin association of Set7/9 was reduced upon PKC-θ siRNA treatment in both non-stimulated and stimulated cells, however there was no difference in its chromatin association upon stimulation (Figure 5.10 E). As expected, the chromatin association of PKC-θ on the miR 200c promoter decreased in both the non-stimulated and stimulated cells upon PKC-θ knockdown in comparison to mock siRNA transfected cells (Figure 5.10 H).

The importance of PKC activity for the assembly of miR 200c associated chromatin associated enzyme complex was examined by using PKC specific inhibitor-bisindolylmaleimide-I for ChIP assays. MCF-7 cells were either pre-treated with bisindolylmaleimide-I or vehicle alone followed by PMA stimulation (as detailed in section 4.1). Pre-treatment of cells with bisindolylmaleimide-I not only resulted in reduced PKC-β binding on miR 200c promoter in non-stimulated cells but it also prevented PMA-induced increased recruitment of PKC-β across miR 200c promoter (Figure 5.11 A). Pre-incubation with bisindolylmaleimide-I resulted in reduced recruitment of epigenetic marks such as Zeb1, LSD1, H3K4Me2, H3K4Me1 and PKC-θ (Figure 5.11 B, C, F, G and H respectively) in both PMA stimulated and non-stimulated cells across the miR 200c promoter than cells not treated with inhibitor, with a further decrease in stimulated cells when compared with non-stimulated cells upon bisindolylmaleimide-I treatment. Conversely, Set7/9 showed
Figure 5.11 Bisindolylmaleimide-I effects chromatin association of PKC-β, PKC-θ and other EMT related epigenetic markers across the miR 200c promoter in a similar fashion as PKC-θ knockdown

MCF-7 cells were pre-treated with either vehicle alone or bisindolylmaleimide-I (1 μM for 1 hr) and subsequently either left untreated, non-stimulation (NS) or PMA stimulated (ST) (0.65 ng/ml for 60 hr). Then ChIP assays were performed on immuno-precipitate DNA with antibodies (A) anti-PKC-β, (B) anti-Zeb1, (C) anti-LSD1 (D) anti-DNMT1, (E) anti-set7/9 (F) anti-H3K4Me2, (G) anti-H3K4Me1, or (H) anti-PKC-θ. Real time PCR analysis was performed for these cases for immuno-precipitated DNA by using miR 200c promoter primer. The data is plotted as ChIP enrichment ratio of immuno-precipitated DNA relative to the nil antibody control and normalized against the total input DNA. All chip assays represent mean ± standard error (SE) of three independent experiments and analysed in duplicate by real-time PCR. No AB represents the no-antibody or negative control signals. (* p < 0.05; ** p < 0.01; *** p < 0.001; p-values determined by two tailed student’s t test).
increased recruitment on miR 200c promoter upon bisindolylmaleimide-I treatment in comparison to inhibitor untreated (vehicle alone) cells (Figure 5.11 E). In the absence of bisindolylmaleimide-I treatment, recruitment of DNMT1 on miR 200c promoter was same in the non-stimulated and stimulated cells. However, pre-incubation of cells with bisindolylmaleimide-I resulted in diminished DNMT1 recruitment in stimulated cells in comparison to non-stimulated cells (Figure 5.11 D), although overall DNMT1 association with miR 200c promoter was decreased in both stimulated and non-stimulated cells. Cumulatively, these results suggest PKC activity is required for recruitment of EMT associated epigenetic marks across miR 200c during PMA-induced EMT.

Recent studies show that the major target of miR 200c is EMT related transcription factor, Zeb1. At the same time, Zeb1 has been reported to suppress transcription of miR 200c family members directly (Bracken et al., 2008; Burk et al., 2008). The results in preceding section show that PKC-θ knockdown resulted in the accumulation of Zeb1 on miR 200c promoter in stimulated cells (Figure 5.10 C) and also reduction of CSC-like cells (Figure 4.10). Following these observations, the effect of PKC-θ knockdown on mRNA expression of miR 200 family members as well as its correlation with Zeb1 was explored. After mock siRNA treatment, miR 200b and miR 200c expression were noticed more in non-stimulated cells and their expression went down upon stimulation (consistent with previous results, Figure 3.6 A & B). Surprisingly, PKC-θ siRNA treated stimulated cells showed higher miR 200b & miR 200c expression than PKC-θ siRNA treated non-stimulated cells, although knockdown of PKC-θ by the siRNA technique resulted in decreased miR 200b and miR 200c expression in both stimulated and non-stimulated cells in comparison with mock siRNA transfected cell (Figure 5.12 A and B). At the same time Zeb1 expression level was observed to be higher upon stimulation in mock siRNA
transfected cells (consistent with previous results, Figure 3.5 C) but PKC-δ SiRNA transfection did not result in much increase of Zeb1 expression in comparison to mock siRNA (Figure 5.12 C).

Experiments were carried out by pre-incubating cells with the PKC specific inhibitor, bisindolylmaleimide-I to investigate the effect of broad spectrum PKC phosphorylation inhibition on miR 200b, miR 200c and Zeb1 expression. Consistent with the previous results (Figure 3.6 A & B and 5.12 A), miR 200b and miR 200c expression levels were reduced upon stimulation without bisindolylmaleimide-I treatment (Figure 5.13 A and B). Pre-incubation of cells with bisindolylmaleimide-I resulted in less expression of miR 200b and miR 200c in both non-stimulated and stimulated cells (Figure 5.13 A and B); and was not able to improve expression of both miR 200b and miR 200c after stimulation (Figure 5.13 A and B). At the same time bisindolylmaleimide-I treatment inhibited induction of Zeb1 upon stimulation (Figure 5.13 B), unlike the cells which were not treated with bisindolylmaleimide-I (consistent with previous results, Figure 3.5 C and 5.12 B). It should be noted here that treatment of bisindolylmaleimide-I also resulted into complete inhibition of EMT (Figure 4.1 and 4.2) and CSC processes (Figure 4.3 and 4.4).

5.5 Over-expression of microRNA 200 family members reduce PKC pathway induced epithelial to mesenchymal transition but not cancer stem cell-like sub-population formation

First MCF-7 cells were transfected with either mock pre-miR, pre-miR 200b, pre-miR 200c or pre-miR 200c*(anti-sense miR 200c), followed by either non-stimulation or PMA stimulation (0.65 ng/ml) for 60 hours. Next, phase contrast microscopy was performed followed by staining with Hoechst 33528, APC-anti-CD44, PE-anti-CD24 cocktail and FACS analysis (as described in section 2.3.3) was carried out to monitor the effect of pre-miR
Figure 5.12  PKC-θ RNAi knockdown alters transcription of miR 200b, miR 200c and Zeb1

MCF-7 cells were transfected with either Mock siRNA (mock) or PKC-θ siRNA followed by either Non-stimulation (NS) or PMA stimulation (ST) (0.65 ng/ml for 60 hr) 48 hr post-transfection. Taqman® microRNA assay were performed for (A) miR 200b, (B) miR 200c and (C) Taqman® real time PCR for Zeb1 was performed on cDNA synthesized from total RNA isolated from cells. Cycle threshold (Ct) values generated for each time points were converted to arbitrary copy number. mRNA levels are expressed as arbitrary copies normalized to either RNUB in case of miR 200c or Cyclophilin A reference levels in case of Zeb1. Data represent the mean ± standard error (SE) of three independent experiments.
Figure 5.13  Bisindolylmaleimide-I alters transcription of miR 200b, miR 200c and Zeb1

MCF-7 cells were either pre-incubated with vehicle alone or bisindolylmaleimide-I (1μM for 1 hr) and subsequently either left untreated, non-stimulation (NS) or stimulated with PMA (0.65 ng/ml for 60 hr). Taqman® microRNA assay were performed for (A) miR 200b, (B) miR 200c and (C) Taqman® real time PCR for Zeb1 was performed on cDNA synthesized from total RNA isolated from cells. Cycle threshold (Ct) values generated for each time points were converted to arbitrary copy number. mRNA levels are expressed as arbitrary copies normalized to either RNUB in case of miR 200c or Cyclophilin A reference levels in case of Zeb1. Data represent the mean ± standard error (SE) of two independent experiments.
transfection on the formation of CD44\textsuperscript{high}/CD24\textsuperscript{low} CSC-like sub-population. Results of phase contrast microscopy showed that both pre-miR 200b and pre-miR 200c reduced the EMT-like phenotypic changes induced by PMA (Figure 5.14). In contrast, pre-miR 200c\textsuperscript{*} increased PMA-induced EMT (Figure 5.14). FACS analysis demonstrated that pre-miR 200b and 200c did not affect formation of CD44\textsuperscript{high}/CD24\textsuperscript{low} CSC-like sub-population as but pre-miR 200c\textsuperscript{*} transfection resulted in a significant increment of PMA-induced CSC-like subset (Figure 5.15). Transcript analysis demonstrated that upon stimulation mRNA expression of Zeb1 is reduced by pre-miR 200b transfection in comparison to mock miR probe (Figure 5.16 A), however, pre-miR 200c did not affect Zeb1 transcript (Figure 5.17 A). A similar effect on transcript expression was followed by E-cadherin (Figure 5.16 D and Figure 5.17 D) upon pre-miR 200b and miR 200c transfection. CD44 transcript level was higher upon stimulation after pre-miR 200b and 200c transfection in comparison to mock miR transfected cells (Figure 5.16 B and Figure 5.17 B). Other CSC-related genes such as uPAR (Figure 5.16 C and Figure 5.17 C), Fibronectin (Figure 5.16 E and Figure 5.17 E) and Integrin-β (Figure 5.16 F and Figure 5.17 F) also showed same transcript pattern as CD44 upon pre-miR 200b and 200c transfection. In contrast, pre-miR 200c\textsuperscript{*} or anti-sense miR 200c transfection significantly increased mRNA expression of Zeb1 upon stimulation (Figure 5.18 A). Other EMT genes such as CD44, uPAR, E-cadherin, fibronectin and integrin-β also followed same trend as Zeb1 (Figure 5.18 B, C, D, E and F respectively). Taken together, these results indicate that ectopic expression of miR 200 family members reduce PKC pathway induced EMT but they are unable to effect the formation of cancer stem-like cells induced by PKC pathway. In a strikingly contrasting observation, inhibition of miR 200c activity by anti-sense miR 200c leads to increased CSC-like subset which is suggestive of miR 200 family in formation of PMA-induced CD44\textsuperscript{high}/CD24\textsuperscript{low} CSC-like sub-population.
Figure 5.14  Over-expression of miR 200 family members prevent PMA-induced EMT changes whilst over-expression of anti-sense miR 200c (miR 200c*) enhances PMA-induced EMT-like morphological changes in MCF-7 cells

Transfection of MCF-7 cells using Lipofectamine 2000 reagent was carried out with either mock miR (mock), pre-miR 200b, pre-miR 200c or anti-sense miR 200c (miR 200c*) prior to non-stimulation (NS) or PMA stimulation (0.65 ng/ml PMA for 60 hours). Phase contrast images were subsequently captured using 10 x magnification of Olympus 17X1 microscope.
Figure 5.15 PMA-induced CSC-like subpopulation is unaffected by over-expression of miR 200 family members in MCF-7 cells but anti-sense miR 200c (miR 200c*) significantly enhances CSC-like subset.

MCF-7 cells were transfected with either mock miR (mock), pre-miR 200b, pre-miR 200c or pre-anti-sense miR 200c (miR 200c*) followed by non-stimulation (NS) or PMA stimulation (ST) (0.65 ng/ml for 60 hr) 48 post-transfection. Cells were then stained with Hoechst, APC-anti-CD44 and PE-anti-CD24 antibodies and subjected to FACS analysis. Circles on FACS plots indicate appropriate gating of CSC-like subpopulation and % CSC-like subpopulation is shown in a representative FACS plot from one of the two independent experiments.
Figure 5.16  Effect of miR 200b over-expression on EMT/CSC gene transcription

MCF-7 cells were transfected with either Mock miR (mock) or pre-miR 200b followed by either non-stimulation or PMA stimulation (ST) (0.65 ng/ml for 60 hr) 48 hr post-transfection. Taqman® real time PCR for EMT/CSC genes- (A) Zeb1, (B) CD44, (C) uPAR, (D) E-cadherin, (E) Fibronectin and (F) Integrin-β was performed on cDNA synthesized from total RNA isolated from cells. Cycle threshold (Ct) values generated for each time points were converted to arbitrary copy number and normalized to Cyclophilin A reference levels. Data represent the mean ± standard error (SE) of two independent experiments.
Figure 5.17  Effect of miR 200c over-expression on EMT/CSC gene transcription

MCF-7 cells were transfected with either Mock miR (mock) or pre-miR 200c followed by either non-stimulation or PMA stimulation (ST) (0.65 ng/ml for 60 hr) 48 hr post-transfection. Taqman® real time PCR for EMT/CSC genes - (A) Zeb1, (B) CD44, (C) uPAR, (D) E-cadherin, (E) Fibronectin and (F) Integrin-β was performed on cDNA synthesized from total RNA isolated from cells. Cycle threshold (Ct) values generated for each time points were converted to arbitrary copy number and normalized to Cyclophilin A reference levels. Data represent the mean ± standard error (SE) of two independent experiments.
Figure 5.18

Effect of miR 200c* (anti-sense miR 200c) over-expression on EMT/CSC gene transcription

MCF-7 cells were transfected with either Mock miR (mock) or antisense-miR 200c (miR 200c*) followed by either non-stimulation or PMA stimulation (ST) (0.65 ng/ml for 60 hr) 48 hr post-transfection. Taqman® real time PCR for EMT/CSC genes- (A) Zeb1, (B) CD44, (C) uPAR, (D) E-cadherin, (E) Fibronectin and (F) Integrin-β was performed on cDNA synthesized from total RNA isolated from cells. Cycle threshold (Ct) values generated for each time points were converted to arbitrary copy number and normalized to Cyclophilin A reference levels. Data represent the mean ± standard error (SE) of two independent experiments.
Discussion

LSD1 plays an important role in negatively regulating formation of cancer stem cells, since knockdown of LSD1 leads to an increase in CD44<sup>high</sup>/CD24<sup>low</sup> cell population in the PMA-induced CSC-like model (Figure 5.1). Furthermore, it led to an increase in PMA induced transcription of EMT/CSC related genes (uPAR and CD44) upon LSD1 siRNA treatment (Figure 5.2), which provided further evidence for the role of LSD1 in regulation of CSC-like sub-population formation. It appears that the association between LSD1, PKC-θ and Pol II on the uPAR promoter is stronger in the non-stimulated (epithelial) state than the stimulated (mesenchymal) state (Figure 5.3 A and B). However, Zeb1 and LSD1 co-existed more strongly across the uPAR promoter in stimulated cells in comparison to non-stimulated state (Figure 5.3 C). Knockdown of LSD1 resulted in less binding of PKC-θ on uPAR or CD44 promoter (Figure 5.4 and 5.6) but did not have much effect on chromatin binding of histone methylation marks (Figure 5.5 and 5.7). Collectively, these findings support the fact that LSD1 and Pol II co-exist with the PKC-θ containing repressor complex on uPAR promoter in the epithelial state and Pol II is relieved from PKC-θ containing repressor-complex in mesenchymal state leading to more transcription of uPAR gene.

Chromatin tethering of EMT associated epigenetic marks on the miR 200c promoter was examined and results displayed that although both PKC-θ and PKC-β associate with the chromatin across the miR 200c promoter in non-stimulated state, but after PMA stimulation, the active or phosphorylated form of PKC-θ was present in the mesenchymal state (Figure 5.8 C). Total chromatin binding of PKC-θ decreased in stimulated state (Figure 5.8 A), whilst the opposite binding pattern of PKC-β was observed as PKC-β recruitment on miR 200c promoter was higher in stimulated state (Figure 5.8 B). Pol II chromatin association on the miR 200c promoter decreased in the PMA-stimulated cells, which gave a clue to correlate its presence
with greater expression of miR 200c in non-stimulated (epithelial) state and repression in stimulated cells (mesenchymal) (Figure 3.7). Although, Pol II was strongly associated with chromatin in non-stimulated cells (epithelial state) (Figure 5.8 E), it also complexed with PKC-θ associated epigenetic complex that included PKC-θ, Zeb1, LSD1 and the association of Pol II with PKC-θ, Zeb1 and LSD1 on miR 200c promoter also increases in mesenchymal state (Figure 5.9 A, B and C). It appears that the switching of the repressor complex on the miR 200c promoter in epithelial and mesenchymal state is opposite to that of on the promoters of an EMT related inducible genes such as uPAR. Additionally, it was demonstrated that PKC-θ knockdown helped in dampening the mesenchymal epigenetic marks, especially PKC-β on the miR 200c promoter (Figure 5.10) and, therefore, could be linked with the increased miR 200c expression upon PKC-θ knockdown (Figure 5.12 B) leading to repression of cancer stem-like cell subpopulation (Figure 4.9). Inhibition of PKC activity, however seems to prevent loss of epithelial marks on the miR 200c promoter (Figure 5.11) but does not appear to have a role in regulation of miR 200c expression (Figure 5.13 A), while general PKC activity is important in both regulating EMT and CSC formation processes (Figure 4.1 and 4.3).

Ectopic expression of miR 200b and miR 200c inhibited PMA-induced EMT (Figure 5.14) but failed to have any effect on PMA-induced CSC formation (Figure 5.15, Figure 5.16 and Figure 5.17). Conversely, inhibition of miR 200c activity by anti-sense miR 200c* increased PMA-induced CSC formation (Figure 5.16 and Figure 5.18), which is suggestive of positive role of microRNA 200 family in preventing PKC pathway induced CSC process.

In conclusion, the results presented in this chapter provide evidence that LSD1, Zeb-1 and DNMT1 play a collectively important role in regulating cancer stem-like cell formation. The chromatin tethering of epigenetic tags on miR 200c promoter was explored and the effect of
PKC-θ siRNA and pharmacological inhibition of PKC activity on this association was discussed. In the next chapter all of the experimental observations form results chapters are summarized and their relevance to the literature is discussed.
Chapter Six

Final discussion
Chapter 6: Final discussion

Introduction

The epithelial to mesenchymal transition (EMT) is a key step in cancer progression and metastasis. The principal focus of this research was to unravel the epigenetic signatures underlying the processes of epithelial to mesenchymal transition and cancer stem cell generation. A major effort has been to develop an inducible human in vitro model suitable for studying the epigenetic signatures occurring in the EMT and CSC formation process. Using this model, it was discovered that the PKC pathway is involved in the initial triggering of EMT/CSC processes and that chromatin-associated PKCs play key role in these processes. Different isoforms of PKCs, namely: PKC-θ and PKC-β were found to play crucial but opposing roles in controlling the process of EMT. Additionally, it was also identified that the interplay between chromatin tethered PKCs, histone demethylases and transcription factors have an important role in regulating the inducible gene transcription program and microRNAs involved in the EMT and CSC formation processes.

6.1 PMA induced EMT model in MCF-7 cells is an ideal system to study epigenetic signature underpinning EMT and CSC-like changes

There is very little published work on the influence of epigenetic mechanisms on the expression of genes involved in EMT and CSC processes. The major contributing factor for this is the lack of appropriate in vitro human inducible models of EMT/CSCs. Hence, a major focal point of this thesis was the development of a human in vitro inducible system amenable to epigenetic analysis. The human breast cancer epithelial cell line, MCF-7 was used to develop such a model by stimulating cells with PMA, referred as the “MCF-7-PMA-EMT model”. This MCF-7-PMA-EMT model showed that EMT characteristic genes such as uPAR, laminin-5, Zeb1, and E-cadherin were robustly induced. MicroRNA profiling supported the nature of this EMT model as miR 200 family members were highly repressed in
this system following stimulation, which is a characteristic of epithelial to mesenchymal transition. It is noteworthy that rapid induction (within 4-48 hour) of EMT related genes is a feature of the MCF-7-PMA-EMT model system (Figure 3.6); hence the initial events triggering EMT might easily be studied with this model as manipulation of this system is easily achievable using pharmacological blockade, transient gene knockdown with siRNAs or over-expression. It should be noted here that in a prior in vitro human inducible model of EMT using TGF-β (Mani et al., 2008), EMT induction takes place in 12-22 days and therefore TGF-β model cannot be manipulated easily. Although a TGF-β induced rapid EMT model using a dog cell line (MDCK cells) is available but such a model is non-human (Gregory et al., 2008). The MCF-7-PMA-EMT model also provides an opportunity to unravel the epigenetic changes that distinctly occur prior to and after the cell division.

In addition, the MCF-7-PMA-EMT model results in the generation of a significant cancer stem-like cell (CSCs) subpopulation and EMT-like (unconverted CSC subset or Non-cancer stem cell subset/NCSC) cells within the same system (schematic picture shown in Figure 6.1). For monitoring CSC-like subpopulations, the surface markers CD44\textsuperscript{high}/CD24\textsuperscript{low} (Figure 3.10), transcript analysis (Figure 3.14) and microRNA profiling (Figure 3.15), along with mammosphere assays (Figure 3.12) were used in the present work. Thus this model has an edge over other pre-existing CSC model systems, as with the latter it is not possible to study non-cancer stem cells in same system, as all the cells get converted into CSC-like cells upon treatment with TGF-β or either over-expression of snail-1 or Twist (Mani et al., 2008).

It was discovered that basal epithelial cells, CSC-like and non-CSC (NCSC) subpopulations in MCF-7-PMA-EMT/CSC model are distinct in their transcriptional programs (Figure 3.14) and microRNA profiles (Figure 3.15). EMT/CSC-associated inducible genes such as uPAR (Figure 3.14 B) and CD44 (Figure 3.14 A) were highly enriched in the CSC-like subset specifically, while genes such as Integrin-β (Figure 3.14 F ) and fibronectin (Figure 3.14 E) were highly inducible but did not show a difference between CSC-like
and non-CSC-like subset. MicroRNAs were highly repressed in both CSC-like and NCSC sub-populations (Figure 3.15). Thus, MCF-7-PMA-EMT model provided a powerful approach to unravel the epigenetic and gene expression programs that are specifically triggered in CSC-like subset compared to EMT-like (unconverted CSC subset or NCSC) cells.

Several explanations could account for the conversion of a small but reproducible CSC subset in the PMA-MCF7-model. The criterion for optimal concentration of stimulus was based on achieving maximal conversion of EMT with minimal cell death. Extensive optimization experiments showed 0.65 ng/ml of PMA (Figure 3.4), to be optimal for EMT conversion (as measured by wound healing assays, transcript profile of classical EMT induced genes). Interestingly, experiments carried out using higher range of stimulus (For example, 20ng/ml PMA) produced greater formation of CSC-like sub-population, increasing up to 26-30%. However, this concentration of stimulus was not pursued further as this concentration of PMA also results in a larger percentage of cell death. In addition, miR 200c knockdown within this model results in greater recovery of CSCs, which is consistent with recently published data from the Struhl laboratory (Iliopoulos et al., 2011)

Gene expression profile of CSC-like subpopulation from MCF-PMA-EMT model matches that of human primary cancer stem cells. Recently, global transcriptome analysis using the Affymetrix platform on CSCs and NCSCs generated using MCF-PMA-EMT model has been carried out successfully. Importantly, we have compared this data set with the transcriptome analysis of primary human derived breast cancer stem cells (Shipitsin et al., 2007). We have recently carried out Affymetrix microarray profiling of the CSCs and NCSCs generated via our MCF-7 CSC/NCSC model. Importantly, bioinformatic analysis revealed high degree of similarity with key breast cancer gene clusters: 85%: Gene Signature for CD44+PROCR+ cluster; 70% cell markers or TGFB pathway components; 64% stem markers 70% differentiation markers. In particular, recent publications show Aldehyde Dehydrogenase (ALDH) is a marker of cancer stem cells (Croker et al., 2009; Charafe-Jauffretet al., 2010; Neumeister et al., 2010;
Marcato et al., 2011) and our microarray data also shows that ALDH transcript levels altered in the PMA-induced CD44<sup>high</sup>CD24<sup>low</sup> (CSC-like) sub-population. Currently, we are in the process of refining the current FACS strategy of CSC isolation that involves the use of ALDH as an additional CSC marker.

It is accepted in this emerging field of CSC biology that several criteria ultimately identify the presence of this CSC subset, the majority of which have been utilized in this thesis. As the field emerges, further criteria will be included as more cellular and molecular markers of these cancer stem cells are identified.

Several lines of evidence suggest that MCF-PMA-EMT model results in generation of the distinct CSC-like subpopulation: (i) only after PMA stimulation, does the CD44<sup>high</sup> CD24<sup>low</sup>-CSC-like population emerge and in non-stimulated cells, this sub-population is absent (ii) transcript analysis which showed distinct gene patterns in CSC versus non-CSCs, (iii) mammosphere assay which showed that PMA treatment increased mammosphere generation and (iv) bioinformatics analysis which showed that MCF-PMA-EMT model correlates closely with the published transcript profiling of human primary breast cancer stem cells.

A key question remained as to whether the PMA-induced mesenchymal/ CSC-like cells in this model have the capacity to form primary tumors and metastasis in mice. Therefore, mice tumorigenesis assays were performed but the preliminary results did not show a difference between non-stimulated cells and PMA-stimulated cells in their abilities to form tumors (Data not presented in this thesis). This issue remains to be addressed in subsequent studies.

Overall the data presented in this thesis clearly indicates that the PMA induced model of EMT/CSC remains a superior model to study epigenetic changes occurring during EMT; especially to study the initial EMT triggering events and role of PKCs in regulation of EMT/CSC process. In the following sections the major role played by the PKC pathway in EMT and cancer stem cell formation is reviewed.
Figure 6.1 MCF-7-PMA-EMT model results in rapid conversion of epithelial to mesenchymal phenotype and simultaneously generates CSC-like and NCSC cells in the same system.

Upon PMA induction, EMT changes occur in majority of cells (all the epithelial cells indicated by green circle convert in mesenchymal phenotype indicated by yellow circle). However, 3-10% of this EMT population acquires properties of CSC-like cells (depicted as purple circle), which are characterized by CD44^{high}/CD24^{low} surface markers. The rest of EMT-like cells (around 80-95%) do not acquire CSC-like phenotype, remain mesenchymal and are referred as NCSC or non-cancer stem-like cells (depicted as blue circle).
6.2 PKC activation is sufficient to drive PMA-induced EMT-like changes and CSC formation

This thesis presents data showing for the first time that exposure of MCF-7 to the PKC pathway inducer Phorbol 12-myristate 13-acetate (PMA), results in the rapid conversion of majority of epithelial cells into mesenchymal phenotype (Figure 3.2f). Additionally, blocking PKC pathway with broad spectrum PKC specific inhibitor completely abolished PMA-induced EMT/CSC-like changes (Figure 4.1 B). These two key findings, presented in this thesis, strongly support the involvement of the PKC pathway in the PMA-driven EMT and CSC formation processes.

In the Chapter 3 of this thesis, it was clearly outlined that PMA treatment of MCF-7 cells induces EMT in a significant proportion of the epithelial cells as judged by EMT-like morphological changes (Figure 3.2 F), increased wound healing (Figure 3.5 A), transcript changes in EMT-associated genes (Figure 3.6) and microRNA profiles (Figure 3.7). Following PMA stimulation, a significant proportion of these MCF-7 cells undergoing EMT, also acquire characteristics of cancer stem-like cells such as CD44^{high}/CD24^{low} profile (Figure 3.10), enhanced ability to form mammospheres in \textit{in vitro} cultures (Figure 3.12), characteristic transcriptional patterns (Figure 3.14) and microRNA repression (Figure 3.15). Experiments with Bisindolylmaleimide-I, which is a broad spectrum PKC inhibitor, were carried out to determine whether PKC pathway is involved in the PMA-induced EMT and CSC formation processes. The results showed that this PKC specific inhibitor blocked the EMT process entirely as inferred from the abolishment of PMA-induced morphological EMT changes (Figure 4.2 A), inhibition of increase in mRNA expression of PMA-induced genes such as uPAR, laminin-5 and CD44 (Figure 4.2 A). This inhibitor also blocked development of PMA-induced CD44^{high}/CD24^{low}-CSC-like subpopulation formation (Figure 4.3 B) and mammosphere formation (Figure 4.4). Collectively, this data correlates the PKC pathway with EMT and cancer stem cell formation.
6.3 Chromatin associated Protein Kinase C family members regulate the EMT and CSC formation processes

An emerging pool of evidence suggests a dual role of signaling kinases such as PKCs in both (i) cytoplasmic-signaling role and (ii) nuclear-chromatin associated role. The focus of this thesis has mainly been on the chromatin associated role of two of the PKC isozymes, PKC-θ and PKC-β in regulating EMT and CSC formation process. This role of PKC isoforms as chromatin associated enzymes in EMT and CSC processes has not been reported anywhere and this is a novel finding. Chromatin immuno-precipitation (ChIP) assays were performed to identify if specific PKC isoforms namely: PKC-θ and PKC-β tethered on to the promoter of an archetypal EMT-associated inducible gene, uPAR. As discussed earlier, PKC activity pathway inhibition (by PKC specific inhibitor) resulted in decreased EMT and CSC formation and PKC activity might be important in regulating EMT and CSC processes. In contrast with these observations, the phosphorylated form of PKC-θ (which represents active form of PKC-θ) and phosphorylated histone H3 at threonine 6 or H3T6 (which is phosphorylated exclusively by PKC-β) were also investigated for their recruitment on uPAR gene promoter to understand the role of PKC activity in this process. ChIP assays revealed that non-phosphorylated PKC-θ predominated in the epithelial state across the promoter of uPAR (Figure 4.5 A), whilst the phosphorylated PKC-θ form predominated in the mesenchymal state of MCF-7 cells (Figure 4.5 C). Schematic representation of non-phosphorylated and phosphorylated state of PKC-θ recruitment is shown in Figure 6.2.

In contrast to PKC-θ, recruitment of PKC-β on the uPAR promoter predominated in the mesenchymal state with little enrichment detected in the epithelial state (Figure 4.5 B). Interestingly, the PKC-β binding profile correlated closely with phosphorylated H3T6, which was also found to be highly recruited in the stimulated (mesenchymal) state in comparison to
Figure 6.2  Non-phosphorylated and phosphorylated state of PKC-θ recruitment in different transcriptional states.

(A) Epithelial transcriptional state (non-stimulated state) is characterized by increased chromatin association of non-phosphorylated PKC-θ (dark blue circle) on uPAR promoter. (B) Mesenchymal transcription state is characterized by association of phosphorylated PKC-θ (dark blue circle with pink star as phosphorylation). It remains unclear if non-phosphorylated PKC-θ cannot recruit in mesenchymal state or if non-phosphorylated PKC-θ converts into phosphorylated PKC-θ in mesenchymal state.
non-stimulated state (epithelial) (Figure 4.5 D). H3T6 has been shown previously to be specifically phosphorylated by phosphorylated-PKC-β and is a surrogate indication of active PKC-β (Metzger et al., 2010), therefore it is proposed that phosphorylated PKC-β is also responsible for H3T6 phosphorylation in the MCF-7-PMA-EMT model. A schematic representation of PKC-β recruitment on uPAR promoter is shown in Figure 6.3. Future studies need to be undertaken to include isoform specific PKC-β kinase assays and ChIP assays with active PKC-β (phosphorylated PKC-β) forms to confirm that PKC-β is present in its active form in the mesenchymal state.

The important finding that chromatin association of PKC-β isoform is dependent upon PKC-θ has been established for the first time in this thesis. Chromatin association of PKC-β increases upon stimulation during EMT but in absence of PKC-θ (upon PKC-θ knockdown), PKC-β could not tether to the promoter of the inducible gene uPAR (Figure 4.11 B). This result suggests that PKC-θ might be important for bringing PKC-β on to the promoter of uPAR during the transition of epithelial cells to mesenchymal cells; however it is unclear whether these two isoforms exist together in same complex across uPAR promoter.

Conversely, in the absence of PKC-β (knockdown PKC-β by siRNA), the CSC-like subset increased (Figure 4.10), which suggests that PKC-β is a negative regulator of cancer stem cell-like subpopulation formation and its presence is important to keep CSC-like subset under control i.e. the function of PKC-β is dampening CSC formation. However, this raises a question as to why does more PKC-β associate with the chromatin template during the formation of mesenchymal state?
Figure 6.3 Non-phosphorylated and phosphorylated state of PKC-β recruitment in different transcriptional states.

(A) Epithelial transcriptional state (non-stimulated cells) displays lower chromatin association of non-phosphorylated PKC-β (green circle) on uPAR promoter. (B) Mesenchymal transcriptional state is characterized by greater association of phosphorylated PKC-β (green circle with pink star as phosphorylation) and phosphorylation of histone H3T6 (orange circle) across uPAR promoter.
Collectively these data indicate that phosphorylated forms of both PKC-θ and PKC-β dock onto the promoter of inducible EMT signature gene uPAR in the mesenchymal state, which is suggestive of their chromatin associated role in controlling transcription during EMT/CSC processes. Therefore, PKC isozymes might have a “dual role” consisting of a signal transduction role and a chromatin-associated signature role. Furthermore, these data also imply that PKC activity is important in their chromatin-associated role of regulating transcription of EMT-associated inducible genes and that the two isoforms of PKCs are interdependent on each other.

6.4 Interplay of chromatin associated PKC family members with EMT-associated epigenetic tags

The chromatin association of two key repressive proteins involved in EMT was investigated, namely the transcription factor-Zeb1 and an EMT-associated histone demethylase LSD1, during epithelial to mesenchymal transition along with the effect of a broad spectrum PKC activity inhibitor. The results were rather interesting as both LSD1 and Zeb1 recruitment on uPAR promoter decreased in the mesenchymal state upon PMA stimulation (Figure 4.6 C and D). Inhibition of PKC activity by use of a general PKC inhibitor not only prevented PMA-induced decrease in recruitment of these key EMT proteins but also drastically increased the recruitment of both LSD1 and Zeb1 on uPAR promoter (Figure 4.6 C and D). Specific histone methylation marks H3K4Me2 and H3K4Me1 which are mediated by LSD1, also followed the same pattern as LSD1 (Figure 4.6 E and F), which was indicative of the fact that PKC activity might be important for “setting” of mesenchymal epigenetic tags, specifically removal of LSD1 and Zeb1. The most dramatic result of pharmacological inhibition of PKC activity by broad spectrum PKC inhibitor is its effect on PKC-θ and PKC-β chromatin association on uPAR
promoter, where both PKC-θ (Figure 4.6 A) and PKC-β (Figure 4.6 B) were highly recruited on the uPAR promoter in the non-stimulated state (epithelial state).

The active transcription mark, RNA polymerase II (Pol II) showed high chromatin association on the uPAR promoter in the mesenchymal state in comparison with the epithelial state (Figure 4.5 E). Sequential ChIP results revealed that co-occupancy of Pol II with PKC-θ increased upon stimulation (Figure 4.7 C) but co-existence of PKC-θ with the EMT-associated repressive transcription factor, Zeb1, decreased whilst Pol II decreased its co-existence with Zeb1 on uPAR promoter in the mesenchymal state (Figure 4.7 A and B). The PKC-θ antibody used in sequential ChIP was insensitive to the phosphorylation state of PKC-θ but data presented earlier in this thesis suggest that in the mesenchymal state, the phosphorylated form of PKC-θ and PKC-β are recruited (Figure 4.5, Figure 6.2 and 6.3). Therefore, this result implies that active transcription mark Pol II complexes with both phosphorylated PKC-θ and phosphorylated PKC-β on uPAR promoter in the mesenchymal state.

The results presented in this thesis demonstrate that in the breast cancer cell line-MCF-7, PKC-θ associates with chromatin template in both the non-activated epithelial (basal transcriptional state) and the mesenchymal states (active transcriptional state) in different phosphorylated forms. However, PKC-θ switches its partners on inducible EMT gene promoters when cells transform from the epithelial (non-stimulated) to the mesenchymal (stimulated) state. The inactive form of PKC-θ complexes with LSD1 and Zeb1 (Figure 5.3 A and Figure 4.7 A) on the chromatin template in epithelial cells, whilst in mesenchymal cells, the active mark Pol II is the major component of phosphorylated or active PKC-θ (Figure 4.3 C) containing transcription complex. At the same time co-occupancy of Pol II with LSD1 decreases in the mesenchymal
state on the uPAR promoter. A schematic depiction of the proposed model for transcriptional control of EMT inducible gene-uPAR by PKC isoforms is shown in the Figure 6.4.

It is important to reiterate that, upon stimulation, H3T6 becomes phosphorylated in the PMA-induced EMT model (Figure 4.5 D) and, as mentioned earlier, a previous study has shown that phosphorylation of H3T6 is linked with active PKC-β during tumorigenesis (Metzger et al., 2010). Furthermore, PKC-β-mediated-H3T6 phosphorylation has been demonstrated to prevent association of LSD1 on the chromatin template (Metzger et al., 2010). Therefore, it appears that in the PMA-induced EMT model of MCF-7 cells also; phosphorylation of H3T6 mediated by PKC-β in the mesenchymal state prevents LSD1 from binding on the uPAR promoter and consequently provides a “code” for PKC-β to switch its partners during epithelial to mesenchymal transition. Knockdown of PKC-θ inhibits the recruitment of PKC-β on uPAR promoter; therefore there is another possibility for this scenario that phosphorylated PKC-θ in the mesenchymal state might be writing a histone modification “X” which might be working as a code for phosphorylated PKC-β to get recruited on the uPAR promoter. However, in the absence of the data about which code is written first, further investigation is required to understand which of the phosphorylated PKC isoform functions as the major driver of the histone code and ultimately EMT process. A schematic illustration of this interplay is shown in Figure 6.4.
Figure 6.4  PKC-θ partner’s switch during the process of epithelial to mesenchymal transition.

(A) In the epithelial state, non-phosphorylated PKC-θ (dark blue circle) complexes with LSD1 and Zeb1 across the uPAR promoter. However, it is unclear if PKC-β (green circle) is present in this same complex. (B) During the mesenchymal state, phosphorylated PKC-θ (dark blue circle with pink star as phosphorylation) writes a code “X” (brown circle) on uPAR promoter, which allows recruitment of phosphorylated PKC-β and also phosphorylates H3T6 (orange circle) that ultimately prevents association of LSD1 on uPAR promoter. PKC-θ also complexes with the active transcription mark, Pol II on the uPAR promoter in mesenchymal state, hence switches its partners during EMT.
Previously, it has been shown that the signal transduction kinase, PKC-θ forms part of an active transcription complex with MSK-1, Pol II, 14-3-3ζ, and LSD1 on the promoters of inducible genes in T cells upon stimulation of cells but PKC-θ does not associate with the chromatin template in non-stimulated T cells (Sutcliffe et al., 2011). Another group illustrated that the stress-induced MAP kinase, Hog-1; associates with the transcription factors bound at target promoters upon exposure to osmotic stress (Proft et al., 2006) in S. cerevisiae. In both these studies the respective kinases associate with chromatin upon activation signals only and form part of active transcription complexes. The results presented in this thesis reveals for the first time that unlike T cells and S. cerevisiae, in the breast cancer cell line-MCF-7, PKC-θ forms part of both inactive (epithelial state/non-stimulated) and active (mesenchymal) complexes. However, PKC-θ switches its partners on inducible EMT gene promoters when cells transform from epithelial (non-stimulated/ inactive) to mesenchymal (stimulated/active) state. A very recent study, also points out the ability of chromatin modifiers to switch between different roles under different environmental conditions through interaction with different complexes (Wu et al., 2011).

Histone methylation and DNA methylation have been linked and shown to act co-operatively in the regulation of chromatin structure and gene activity (Esteve et al., 2009; Jackson et al., 2002; Lehnertz et al., 2003; Tamaru and Selker, 2001; Vire et al., 2006) but how these two systems synchronize with each other is not very clear. Figure 5.5 and 5.7 of this thesis gave a clue that LSD1 might have a role in regulating DNA methylation in EMT/CSC process as knockdown of LSD1 results in decreased chromatin binding of DNMT1 on uPAR (Figure 5.5) and CD44 (Figure 5.7) promoters. A recent study of embryogenesis provides a mechanistic link between these two processes where it is highlighted that LSD1 demethylates DNA methyltransferase 1 (DNMT) and thereby stabilizes DNMT1 in embryonic stem cells (Wang et
al., 2009). In summary, preliminary ChIP experiments on uPAR and CD44 promoter suggest that absence of LSD1 might lead to instability of DNMT1 in a similar way as it behaves in embryonic stem cells.

Collectively, the kinase activity of PKC-θ appears to be important for the association of PKC-complexes with the chromatin template and epigenetic regulatory proteins in the mesenchymal state. Importantly, this study suggests that phosphorylated PKC-θ is required for PKC-β recruitment in the mesenchymal state.

6.5 Different PKC isoforms play novel but opposing roles in regulating EMT and CSC-like changes

In spite of many studies describing the signal transduction role of PKCs during cancer progression (refer to section 1.4.2 for details), to date only very few studies have demonstrated the chromatin associated roles of PKCs, although it has been known for a long time that PKC isozymes may reside in the nucleus (Martelli et al., 1999) and have the ability to phosphorylate histones in vitro (Inoue et al., 1977; Metzger et al., 2010; Yu et al., 1998). Additionally, a comparatively old study described, that PMA-induced and activated PKC isoforms translocate from cytoplasm into the nucleus (Disatnik et al., 1994). Sutcliffe et al., demonstrated recently that in T cells, one of the PKC family members, PKC-θ, is an integral component of transcription complexes assembled at control regions of immune genes and microRNAs (Sutcliffe et al., 2011).

For the first time, in this thesis, it was established how two of the PKC isozymes, PKC-θ and PKC-β, play opposing roles in regulating transcription of inducible EMT genes unraveled by both pharmacological inhibition and siRNA experiments. Results presented in Chapter 4 clearly demonstrated that broad spectrum PKC pathway inhibition results in the abrogation of PMA-
induced EMT (Figure 4.1 B and Figure 4.2 A) and CSC formation processes (Figure 4.3 B and Figure 4.4). In contrast, a conventional PKC isozyme (cPKCs)-specific inhibitor caused an increase in both EMT (Figure 4.1 C and Figure 4.2 B) and CSC changes (Figure 4.3 C) in MCF-7-PMA-EMT model. These findings indicate that PKC isozymes exhibit specific differential regulation of EMT and CSC processes; thus isozyme specific siRNA strategy was carried out using PKC-θ siRNA and PKC-β siRNA. PKC-θ siRNA treatment abrogated formation of the PMA-induced CSC subpopulation (Figure 4.9 and 4.11) without effecting EMT, while knockdown of PKC-β using PKC-β siRNA resulted in significant increase in CSCs (Figure 4.10). Data from the siRNA experiments indicated that PKC-θ has a positive function in driving/initiating the process of cancer stem cell formation, whereas PKC-β somehow works to dampen the CSC formation (a possible model of how these two PKC isozymes regulate transcription is discussed in detail in section 6.4). The results of the siRNA knockdown experiments concur with the pharmacological inhibitor data suggesting that the PKC isoforms play opposing roles in regulation of EMT/CSC processes. Although, it is unclear from this data set if the effects of siRNA treatment are due to the absence of PKC protein or due to the absence of kinase activity of these isoforms.

Interestingly, there is already a published hypothesis suggesting that different PKC isozymes might have distinctive functions in developing malignant and aggressive tumor phenotypes (Koivunen et al., 2006). The results presented in this thesis strongly support the hypothesis proposed by Koivunen et al.

In summary, the current research clearly indicates that variation of PKC activation balance is an important factor in driving PMA-mediated EMT/CSC processes in MCF-7 cells.
6.6 Chromatin associated PKCs regulate epigenetic tagging of EMT on microRNA promoters during EMT regulation

Unlike inducible genes, certain microRNAs are repressed in the cells undergoing EMT (Figure 3.7) and CSC formation processes (Figure 3.15) during PMA-induced EMT, however there are not many studies directed at control of microRNA expression during the EMT and CSC-generation processes. Therefore it seemed obvious to address the question whether the PKC pathway also regulates microRNA. In the mesenchymal state (stimulated), PKC-θ exists in its non-phosphorylated form across the microRNA 200c promoter (Figure 5.8 A). Phosphorylated H3T6, (specifically phosphorylated by PKC-β), is also recruited to the miR 200c promoter in the mesenchymal state (Figure 5.8 D) and by implication phosphorylated PKC-β is also recruited in the mesenchymal state. Recruitment of Pol II to gene promoters and coding regions is often interpreted as a mark of active gene transcription. Data presented in this thesis shows for the first time that the association of Pol II across miR 200c promoter is greater in epithelial state (non-stimulated) in comparison to mesenchymal state (stimulated) (Figure 5.8E). Strikingly, sequential ChIP results revealed that not only does Pol II co-exist with PKC-θ in the mesenchymal state on the miR 200c promoter (Figure 5.9 A) but also with epigenetic tags such as LSD1 and Zeb-1 (Figure 5.9 B and Figure 5.9 C).

There are several reasons to believe that these results show lower PKC-θ recruitment in stimulated cells to the miR-200c promoter and its co-relation with RNA polymerase II:

(i) ChIP results showed diminished chromatin association of PKC-θ (Figure 5.8 A on page 161) and Pol II (Figure 5.8 E, page 161) across the miR 200c promoter in the stimulated state. However, phosphorylated forms of PKC-θ demonstrated more chromatin binding in stimulated cells across the miR 200c promoter (Figure 5.8 C, page 161).
(ii) Sequential ChIP analysis showed that the co-existence of Pol II and PKC-θ on miR 200c promoter was increased in stimulated cells (Figure 5.9 A, page 162). Here, it should be noted that the PKC-θ antibody used in the sequential ChIP experiments were intended not to differentiate between non-active and active (phosphorylated) form of PKCs.

(iii) Pol II also showed increased co-existence with the PKC-θ associated epigenetic complex that included Zeb-1 and LSD-1 in stimulated cells (Figure 5.9 B & C).

Collectively, these results show more binding of phosphorylated PKC-θ and its increased co-existence with the Pol II on the miR promoter. Although the PKC-θ recruitment to the miR-200c promoter in general is lower in stimulated cells, it does not mean that its co-enrichment with RNA polymerase II at the same promoter has to be lower. Along with the above phenomenon, co-occupancies of LSD1 with Zeb1 and LSD1 with PKC-θ were also increased on miR 200c promoter in the mesenchymal state (Figure 5.9 E and Figure 5.9 D). Taken together, these results suggest that Pol II co-exists with PKC-θ containing complex across the miR promoter in the mesenchymal state. It appears that “Pol II capturing” is occurring in the PKC-θ containing epigenetic complexes in the mesenchymal state on miR 200c promoter leading to blockade of Pol II activity. Repression of microRNA occurs in the mesenchymal state; therefore it seems that PKC-θ containing epigenetic complexes function as a repressive complex. This complex is referred to as “PKC-θ-containing repressive complex” from now onwards. Schematic depiction of the proposed model showing Pol II capturing in PKC-θ containing repressive complex on miR 200c promoter during epithelial to mesenchymal transition is shown in Figure 6.5.

Another novel finding of this thesis is that Pol II exists in different complexes across miR promoter (transcriptionally repressed genes during EMT) and inducible gene promoters (e.g. uPAR- transcriptionally active genes during EMT). On the EMT inducible signature uPAR
promoter, Pol II co-existed with PKC-θ in the mesenchymal state but not with the EMT-repressive epigenetic marks LSD1 and Zeb1 (refer to section 6.4 for PKC-θ complex on the uPAR gene), therefore Pol II remains free to move on uPAR promoter, leading to more transcription of uPAR gene. In contrast, on the transcriptionally repressed EMT signature gene-miR 200c promoter, co-existence of Pol II with PKC-θ, LSD1 and Zeb1 is higher during the mesenchymal state. Therefore, it appears that on miR 200c promoter, Pol II is captured in a tightly associated-PKC repressive complex, which blocks activity of Pol II in mesenchymal state. There could be two possible explanations for how “Pol II capturing” occurs on miR promoters. Either active site of Pol II is not free to transcribe because it is trapped in the PKC-associated repressive complex (Schematic depiction shown in Figure 6.5) or Pol II somehow becomes inactive by some conformational change or modification (“X” modification) in its structure and this modified form tightly associates with PKC-θ containing repressive complex (Schematic depiction shown in Figure 6.6) to become trapped in this complex on miR 200c promoter.

Although Pol II is known to be an active transcription mark, in embryonic stem cells it has been shown that Ser5P Pol II (Phosphorylation of serine 5 Pol II) is also located at silent genes (Guenther et al., 2007; Kim et al., 2005; Zeitlinger et al., 2007) that are repressed by Polycomb in a “promoter-proximal paused” configuration, which is a state where Pol II is transcriptionally engaged but becomes stalled by negative elongation factors (Brookes and Pombo, 2009; Core and Lis, 2008). Those studies indicated that regulation of the Pol II release from promoter-proximal pausing might be a universally important control point that primes genes for future activation, for example by promoting open chromatin states (Gilchrist et al., 2008; Lee et al., 2008). Therefore, it appears that “Pol II capturing” on the miR 200 c promoter (which is a repressed gene during EMT/CSC process) might equate to Pol II in a promoter-proximal paused state. However, further studies are needed to identify if Ser5P Pol II is present on miR 200c
promoter in the mesenchymal state. Interaction between the miR 200 family and Suz 12, a subunit of the Polycomb repressor complex has been shown to have a role in cancer stem cell formation promotes (Iliopoulos et al., 2010). A recent study by Brooks and Pombo discusses the need to investigate which are the enzymatic activities (kinases, phosphatases, other) that establish the different Pol II states (Brookes and Pombo, 2009). Importantly, Figure 5.11 of this thesis provides preliminary evidence that PKC activity might be one of the factors regulating the epigenetic tags on the miR 200c promoter as blockade of PKC activity resets the epigenetic tags on miR promoter. Therefore it would be of immense interest to know whether kinase activity of PKCs play a role in establishing the different states of Pol II on microRNA promoters during epithelial to mesenchymal transition and, hence is included in the future research section 6.9.
Figure 6.5 Proposed model demonstrating association of chromatin tethered PKCs on miR 200 family members promoter is different than inducible genes during EMT

Lower association (shown by dotted out-line) of PKC-\(\theta\) and PKC-\(\beta\) with Pol II, LSD1 and Zeb1 occurs across microRNA 200c promoter during epithelial state leading to increased transcription (red plus signs) of miR 200c. PKC-\(\theta\) and PKC-\(\beta\) along with other repressor epigenetic marks, LSD1 and Zeb1 associate highly with Pol II across microRNA promoter in mesenchymal state which results in repressed miR 200c expression (cross sign).
Figure 6.6 Proposed model demonstrating modification "X" of chromatin associated Pol II on miR 200c promoter during mesenchymal state.

Lower association of PKC-θ and PKC-β with Pol II, LSD1 and Zeb1 occurs across microRNA 200c promoter (shown by dotted out-line) during epithelial state leading to high expression of miR 200c (many red plus signs). During the mesenchymal state, "X" modification of Pol II occurs that causes "Pol II capturing" in between the PKC-θ containing epigenetic complexes on miR 200c promoter leading to lower transcription of microRNA 200c (cross sign).
6.7 The PKC pathway is crucial for controlling microRNAs repression in EMT/CSC processes

In order to investigate whether the cancer stem-like cell sub-population is inhibited by over-expression of microRNA 200 family, a preliminary experiment was performed where transfection of pre-miR 200b and pre-miR 200c were carried out in MCF-PMA-EMT/CSC model followed by monitoring of EMT changes and CSC-like sub-population. EMT was inhibited by both miR 200b and miR 200c (Figure 5.14) over-expression, however miR 200b and miR 200c failed to reduce the CSC-like subpopulations in MCF-PMA EMT/CSC model in comparison to mock (Figure 5.15). In contrast, the results of anti-sense miR 200c (miR 200c*) transfections demonstrated a massive increase in PMA-induced CSC formation (Figure 5.15 for miR 200c*), which indicates the positive role of the miR 200 family in CSC formation. There are two possible interpretations of this microRNA mediated regulation of EMT/CSC processes- (i) microRNA do not directly regulate CSC formation and PKCs form a transcriptional layer above microRNA, and (ii) presence of microRNA or microRNA activity is important for PKC pathway induced CSC formation since absence of microRNA or attenuation of microRNA activity (by anti-sense miR treatment) result in increased CSC formation. Previous studies have suggested that enforced expression of the microRNA 200 family alone is sufficient to prevent TGF-β mediated EMT (Gregory et al., 2008); however there is no evidence for inhibition of CSC formation by microRNAs in that study. An indirect link has been shown where over-expression of miR 200b resulted in decreased mammospheres, while transfection of anti-sense miR 200b leads to increased CD44-positive CSCs formation (Iliopoulos et al., 2010). The work presented in this thesis shows for the first time that PKC pathway is regulating miR repression in the EMT/CSC process. This leads to the question of whether PKC kinase activity is involved in regulating microRNA transcription. To address this, experiments were designed to block PKC
activity followed by observing its effect on binding of epigenetic tags on miR 200 promoter and on miR 200 transcription. Blocking PKC activity by using a broad spectrum PKC inhibitor resulted in decreased chromatin association of PKC-θ and PKC-β on the miR 200c promoter (Figure 5.11 H and A). PKC inhibition also resulted in decreased association of other epigenetic tags such as Zeb1, LSD1, DNMT1, H3K4Me2 and H3K4Me1 (Figure 5.11 B, C, D, F, G and H) in both stimulated and non-stimulated cells but in most cases epigenetic marks showed the same pattern between epithelial and mesenchymal states as inhibitor treated cells. Collectively, this data shows that PKC kinase activity is important for regulating recruitment of epigenetic tags on miR 200c promoter in mesenchymal state.

PKC-inhibition resulted in repression of both miR 200 b and miR 200c in stimulated and non-stimulated cells (Figure 5.13 A and B) but surprisingly, repression of microRNA 200 family members did not result in EMT (Figure 5.14) or CSC formation (Figure 5.15) in the MCF-7-PMA-EMT model. These results support the hypothesis that in MCF-PMA EMT/CSC model, chromatin-associated PKCs are positioned upstream of microRNAs and this is the reason why blockade of PKC pathway abolishes EMT/CSC even when microRNAs are repressed, while over-expression of microRNAs was not able to prevent PKC-induced CSC formation. Inhibition of PKC activity prevented PMA-induced increase in Zeb1 transcription (Figure 5.13 C), which suggests that Zeb1 might be an important EMT transcription factor in mediating effect of PKC pathway in miR 200 family regulation. Schematic representation of this scenario is shown in Figure 6.7.

In summary, PKC kinase activity appears to be important in the recruitment of PKC-θ containing repressor complex on microRNA promoters but whether PKC activity has a role in regulating microRNA transcription is not very clear.
Figure 6.7  Inhibition of PKC kinase activity decreases miR transcription but this decreased miR transcription does not result in EMT.

(A) Lower association of PKC-θ and PKC-β with Pol II, LSD1 and Zeb1 occurs across microRNA 200c promoter during epithelial state. DNMT1 and Set7/9 also associate on microRNA 200c promoter in both epithelial and mesenchymal states. PKC-θ and PKC-β along with other repressor epigenetic marks, LSD1 and Zeb1 associate highly with Pol II across microRNA 200c promoter in mesenchymal state which results into repression of miR 200c (cross sign). (B) Inhibition of PKC activity does not allow phosphorylation of PKC-θ and PKC-β (as shown by red crossed lines of both PKC isoforms on top of the Figure 6.8 B) and DNMT1 cannot associate on miR promoter. This inhibition of PKC activity does not allow cells to convert from epithelial phenotype to mesenchymal phenotype; however repression of miR 200c is not prevented by PKC activity inhibition.
6.8 PKC-θ has positive role in regulating CSC formation

The role of PKC-θ in regulating CSC formation was examined in this thesis using PKC-θ specific siRNA strategy. Knockdown of PKC-θ results in impaired recruitment of epigenetic tags such as PKC-β, LSD1, Set7/9, H3K4Me2, and H3K4Me1 (Figure 5.12 A, C, E, F, G) across the miR promoter in both stimulated and non-stimulated cells. Although PKC-θ knockdown does not prevent PMA-induced EMT (Figure 4.8) but significantly inhibits PMA-induced CSC formation (Figure 4.9). Interestingly, PKC-θ knockdown results in higher expression of miR 200 family members in the mesenchymal state in comparison to epithelial state, although knockdown of PKC-θ results in overall repression of miR 200b and miR 200c when compared to mock siRNA treatment (Figure 4.9). These results indicate a positive initiating role for PKC-θ in CSC formation as knockdown of PKC-θ results in decreased CSC formation.

Interestingly, Zeb1 transcription showed a marginal increase in mRNA expression in the mesenchymal state relative to the epithelial state upon PKC-θ knockdown (Figure 5.12 C). However, PKC-θ knockdown resulted in increased recruitment of Zeb1 on miR 200c promoter in the mesenchymal state in comparison to epithelial cells. This result suggests that Zeb1 might play an important role in PKC-θ mediated CSC formation. A further possible interpretation of this result could be that PKC-θ might be regulating another repressive transcription factor—“TF”, which in the presence of PKC-θ remains repressed and does not allow transcription of miR 200 family members and therefore cancer stem cell formation occurs. However, upon PKC-θ knockdown the repression is relieved from this “TF” and now “TF” becomes free to repress miR 200 transcription leading to decreased CSC formation. Schematic depiction of how PKCs and chromatin modifying enzymes form dynamic layers in regulation of EMT inducible genes and microRNAs during the process of EMT and CSC formation is shown in Figure 6.8. Taken together, the data presented in this thesis for the first time shows the PKC pathway is a central mechanism during regulation of microRNAs in epithelial to mesenchymal transition and cancer stem cell formation processes.
Figure 6.8  PKC-θ knockdown increases miR 200c transcription but decreased CSC formation.

(A) Lower association of PKC-θ and PKC-β with Pol II, LSD1 and Zeb1 occurs across microRNA 200c promoter during epithelial state. DNMT1 and Set7/9 also associate on microRNA 200c promoter in both epithelial and mesenchymal states. PKC-θ and PKC-β along with other repressor epigenetic marks, LSD1 and Zeb1 associate highly with Pol II across microRNA 200c promoter in mesenchymal state which results into repressed miR 200c expression (cross sign).  

(B) Knockdown of PKC-θ increases recruitment of Zeb1 and other transcription factors (TF-blue square) but prevents phosphorylation of H3T16. It also increases miR 200c transcription and now epithelial cells can convert into mesenchymal phenotype but cannot acquire cancer stem cell-like properties.
6.9 Future directions

As stated in the previous sections, the following specific investigations are desirable:

A recent study by He et al., demonstrated that PMA also induces EMT in the prostate cancer cell lines, ARCaPc (He et al., 2010). However, this study did not investigate the presence of cancer stem-like cells upon PMA stimulation in prostate cancer cells. Therefore, the findings from MCF-PMA-EMT/CSC breast cancer model could be translated into prostate cancer research to identify if PKC pathway is also involved in the cancer stem cell regulation in prostate cancer. At the same time, comparison of breast cancer and prostate cancer would be carried out to discover common and distinct transcriptional programs between the two cancer types, which might be helpful in the development of cancer therapeutics.

Findings presented in this thesis clearly suggested that PKC activity play crucial role for regulating the EMT and CSC processes. Therefore, it remains a subject of interest to confirm these results by over-expressing active and kinase defective forms of PKC-θ (PKC-θ-A/E and PKC-θ-K/R respectively); and PKC-β to study the effects of these mutants on EMT and CSC-processes to understand how important the PKC activity is in regulating EMT and CSC processes.

Importantly, the distinct nuclear and cytoplasmic roles of the PKC isoforms need to be examined in the future to separate the signaling from the chromatin associated functions of PKCs. Recently, a novel putative Nuclear Localization signal (NLS) sequence and a novel phosphorylated (Ser-Pro-Thr) SPT site in human PKC-θ sequence (unpublished work Sutcliffe et al., 2012) has been identified using bioinformatics approach. PKC-θ mutations of NLS and SPT have also been made which will help in delineating the relative importance of
nuclear translocated PKC-θ (unpublished work Sutcliffe et al., 2012) and the essential epigenetic role of PKC-θ in regulation of inducible gene regulation in cancer biology. In addition, PKC-θ and PKC-β isozyme specific kinase assays have also been developed, which along with confocal microscopy based studies which could be utilized to identify the contribution of PKCs in different cellular compartments (unpublished work Sutcliffe et al., 2012). Finally, novel in-situ nuclear transcription assay could be utilized to demonstrate if nuclear PKC-θ itself (without cytoplasmic function) can regulate the gene expression of cancer stem cell genes and microRNAs (unpublished work Sutcliffe et al., 2012). This study will develop a new understanding of the regulation of chromatin structure, inducible gene transcription programs and microRNAs during CSC processes. Furthermore, kinase function Vs chromatin tagging role of PKCs in nucleus need to be separated. It would be interesting to test whether PKC pathway and chromatin associated PKC isoforms also contribute to the modulating effects of other EMT associated signaling pathways such as Wnt, Notch and TGF-β (Peinado et al., 2004; Thiery et al., 2009; Yang and Weinberg, 2008).

Preliminary findings discussed under section 6.4 of this thesis, suggested that absence of LSD1 might lead to instability of DNMT1 on the promoter of EMT associated inducible genes in a similar way as in embryonic stem cells (Wang et al., 2009). Therefore, experiments are in progress to identify if PKC family members have a structural role, kinase activity role, or both in regulating the interplay of DNMT1 and LSD1.

It appears that the proposed “Pol II-captured state” on the miR 200 c promoter (discussed under section 6.6) might have Pol II in “promoter-proximal paused state”. Previous studies have demonstrated involvement of Serine-5 phosphorylation of Pol II in a promoter-proximal paused state (Guenther et al., 2007; Kim et al., 2005; Zeitlinger et al., 2007). Thus, further
studies need to be carried out to determine the presence of Ser5P Pol II isoforms on miR 200c promoter during mesenchymal state and whether PKC kinase activity has a role in establishing the different states of Pol II during the EMT process.

The data presented in this thesis are based on ChIP experiments carried out using primers designed for a small transcribed region. Recently experiments were carried out in collaboration with Beijing Genomics Institute, China using a ChIP sequencing technique (data not shown in this thesis) to explore the extent to which PKC-θ and PKC-β recruitment occurs across the human genome and to identify new gene targets on to which PKC-θ and PKC-β bind during the process of EMT. Analysis of the ChIP sequencing results and overlaying the ChIP sequencing data with microarray expression data is in progress. It would also be useful to perform studies for understanding the kinetics of PKC chromatin association to identify whether chromatin tethering of PKCs differ at different time points.

Finally, in vivo experiments are required to study the proposed mechanism of the interconnected functions of chromatin-associated enzymes, transcription factors and microRNAs using existing EMT and CSC mouse models (Mani et al., 2008; Stingl et al., 2006). Further studies need to be carried out on human mammary epithelial cells for translating the results of the PMA-induced EMT/CSC model to identify if similar mechanisms also exist in primary breast epithelial cells. CD44^{high}/CD24^{low} (CSC) and CD44^{low}/CD24^{high} (NCSC) cells could be isolated directly either from reduction mammaplasties or breast carcinomas, respectively, as described by Mani et al. (Mani et al., 2008).

6.10 Concluding summary

An inducible human in vitro model for studying epigenetic signatures underlying epithelial to mesenchymal transition and cancer stem cell formation processes has been developed. A novel
mechanism for the regulation of the epithelial to mesenchymal transition and cancer stem cell processes has been revealed involving new chromatin associated functions for Protein Kinase C family members. It is proposed that different PKC isozymes play opposing roles during regulation of EMT. The data illustrate the interconnected function of PKC family members with epigenetic enzymes including histone demethylases, DNA methyltransferases and transcription factors in regulating both inducible genes and microRNA expression during the epithelial to mesenchymal transition and cancer stem cells formation processes. Consequently, chromatin bound enzymes have been proposed as a new layer in the epigenetic regulation of epithelial to mesenchymal transition. This study provides the basis for developing a deeper understanding of the regulation of chromatin structure, inducible gene transcription programs and microRNAs during EMT and CSC processes, which could be utilized for developing novel targeted therapies for cancer metastasis.
References


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Luco, R.F., Pan, Q., Tominaga, K., Blencowe, B.J., Pereira-Smith, O.M., and Misteli, T. Regulation of alternative splicing by histone modifications. Science 327, 996-1000.


delta and -epsilon in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence, and tumorigenicity. J Biol Chem 268, 6090-6096.


APPENDIX 1: Reagent suppliers and Catalogue numbers

24-well flat-bottom polystyrene plates (Costar®, Corning Inc, Cat No. 3337)

6 well-ultra low adherent, flat bottom plates (Costar®, Corning Inc, Cat No. 3471)

96-well PVC Microtitre® U-bottom plates (Pathtec, Cat No.L 2101)

96-well V-bottom polystyrene plates (Corning Incorporated Costar®, Cat No.3898)

Acetic acid, anhydrous (Analytical UNIVAR, Ajax Finechem, Cat No. A796-2.5L GL)

Acetone (AR Pronalys, Cat No. 1090 2.5L)

Agarose Certified™Molecular Biology Grade (Bio-Rad Laboratories Inc., Cat No. 161-3102)

Bovine Serum Albumin powder (Sigma-Aldrich, Cat No.A-7906)

CaCl₂ (Sigma-Aldrich, Cat No. C-3306).

Calcium ionomycin- A23187 (Sigma-Aldrich, Cat No. C7522)

ChiP Dilution Buffer (Upstate Biotechnology, Cat No. 20-153)

Chloroform (Sigma-Aldrich, Cat No. C2432-500)

Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Cat No. 11697498001)

Cryotubes (NUNC Cat No. 377267)

DAPI (Sigma-Aldrich, Cat No. D-8417)

DEPC treated water, nuclease-free (Ambion, Cat No. 9920)

Distilled water DNAse, RNAnne free (Invitrogen Life Technologies, Cat No. 10977-015)

DMSO (Cambridge Isotope Laboratories, Inc., Cat No. CAS 2206-27-1)

DNase I, RNase-free (Roche Diagnostics, Cat No. 776 785)

dNTP Set 100mM (4 x 25 μmol) Kit (Astral Scientific Pty Ltd, Cat No. BIO-39025)

DTT (Sigma-Aldrich, Cat No.D9779-5G)
Dulbecco’s Phosphate-Buffered Saline (D-PBS) without CaCl$_2$ or MgCl$_2$ (Gibco, Invitrogen Corporation, Cat No. 14190-144)

Dulbecco’s Modified Eagle Medium (D-MEM) (1X, liquid, low glucose, Gibco, Cat No. 12320-32)

EDTA (Sigma-Aldrich, Cat No. E-5134)

EGTA (Sigma-Aldrich, Cat No. E-3889)

Ethanol, absolute (Analytical UNIVAR, Ajax Finechem, Cat No. A214-2.5L PL)

FACS 1.2 ml tubes (Corning Incorporated Costar®, Cat No. 4401)

Flat bottomed 6-well sterile plates (NUNC, Cat No. 140675)

Foetal calf serum (Sigma-Aldrich, Cat No. 12003-500ML)

FuGENE 6 (Roche Diagnostics, Mannheim, Cat No. 11 815 091 001)

GenElute™ Linear PolyAcrylamide (Sigma-Aldrich, Cat No. 5-6575)

Glycerol (Analytical UNIVAR, Ajax Finechem, Cat No. 242-500ML)

Glycine (AnalaR Merck, BDH, Cat No. 10119.0500)

HCl (Analytical UNIVAR, Ajax Finechem, Cat No. 1399-500ml)

HEPES (Sigma-Aldrich, Cat No. H-3375)

High Salt Immune Complex Wash Buffer (Upstate Biotechnology, Cat No. 20-155)

IGEPAL CA-630 Nonidet P-40 (Sigma-Aldrich, Cat No. I-8896)

Isopropanol/propan-2-ol (Analytical UNIVAR, Ajax Finechem, Cat No. 425-2.5L PL)

KCI (Sigma-Aldrich Cat No. P-9541)

L-glutamine (Sigma-Aldrich, Cat No. G-3126)

LiCl Immune Complex Wash Buffer (Upstate Biotechnology, Cat No. 20-156)

Lipofectamine LTX with Plus reagent (Invitrogen, Cat No. 15338-100)

Lipofectamine® 2000 (Invitrogen, Cat No. 11668-019)

Low Salt Immune Complex Wash Buffer (Upstate Biotechnology, Cat No. 20-154)
MG132 (Sigma-Aldrich, Cat No. C2211)

MgCl₂ (Sigma-Aldrich, Cat No. M8266)

Microtubes (Axygen Scientific, Inc., Cat No. MCT-150-C)

MycoAlert Q Mycoplasma detection kit (Lonza, Cat No. LT07-318)

Mycoplasma Plus™ PCR Primer Set (Stratagene, Cat No. 302008)

Na₂H₂PO₄·2H₂O (Merck, BDH, Cat No. 10383.4G)

NaCl (Merck, BDH, Cat No. 10241.3000) or NaCl (Sigma-Aldrich, Cat No. S-3014)

NaH₂PO₄·H₂O (Merck, BDH, Cat No. 10245.4R)

NaHCO₃ (Merck, BDH, Cat No. 10247.4V)

OptiMEM® I Reduced-Serum Medium (1X), liquid (Invitrogen, Cat No. 31985-062)

Penicillin G (MP Biomedical, LLC, Cat No. 156065)

PFA, 37% formaldehyde solution (Analytical UNIVAR, Ajax Finechem, Cat No. 809-2.5L PL)

Phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma-Aldrich, Cat No. P3803-400ML)

Phorbol 12-myristate 13-acetate (Sigma-Aldrich, Cat No. P8139)

Plastic disposable cuvettes 10 × 4 × 45 mm (Sarstedt, Cat No. 67.742)

Platinum® Taq DNA Polymerase Kit (Invitrogen Life Technologies, Cat No. 10966-018)

Poly-L-lysine (Sigma-Aldrich, Cat No. P1274)

Propidium iodide (Sigma-Aldrich, St. Louis, Cat No. 70335)

Proteinase K (solution), RNA grade (Invitrogen Life Technologies, Cat No. 25530-049)

QIAamp® DNA Blood Mini Kit (QIAGEN, Cat No. 51106)

RNase Zap (Ambion, Cat No. 9780.9782)

RPMI-1640 powder (Gibco, Invitrogen Corporation, Cat No. 31800-105)

Salmon Sperm DNA/Protein A Agarose (Upstate Biotechnology, Cat No. 16-157)
SDS (Sigma-Aldrich, Cat No. L-4390)
SDS Lysis Buffer (Upstate Biotechnology, Cat No. 20-163)
Sodium acetate buffer solution 3M, pH 5.2 (Sigma-Aldrich, Cat No. S-7899)
Sodium azide (Sigma-Aldrich, Cat No. S2002)
Sodium salicylate (Sigma-Aldrich, S3007)
Spermidine (Sigma-Aldrich, Cat No. S-2501)
Spermine (Sigma-Aldrich, Cat No. S-2876)
Sterile tissue culture flask 175 cm² (NUNC, Cat No. 159910)
Streptomycin sulphate (Sigma, Cat No. S6501-100G)
SuperScript™ III RNase H- Reverse Transcriptase Kit (Invitrogen Life Technologies, Molecular Research Center Inc., Cat No. 18080-044)
SYBR® Green 2 x PCR Master Mix (Applied Biosystems, Cat No. 4309155)
Taqman® MicroRNA Reverse Transcription kit (Applied Biosystems, Cat No. 4366596 and 4366597)
Taqman® Universal 2 x PCR Master Mix (Applied Biosystems, Cat No. 4304437)
TE Buffer (Upstate Biotechnology, Cat No. 20-157)
Tris (AnalaR Merck, BDH, Cat No. 1.08387.0500)
Tris AE Elution Buffer (Qiagen, Cat No. 19077)
Tris Assay Dilution Buffer (10 x TADB) (Upstate Biotechnology, Cat No. 20-181)
Tris-HEPES LongLife SDS Running Buffer (Gradiopore, BG-161)
Tris-HEPES-SDS 4 – 20% LongLife MiniGels (Gradiopore, Cat No. NH21-420)
Triton X-100 (Sigma-Aldrich, Cat No. T-8787)
TRIZMA® base pH 7.4 (Sigma-Aldrich, Cat No. T-6066)
TRizol® Reagent (Invitrogen Life Technologies, Molecular Research Center Inc., Cat No. 15596-018)

Trypan Blue 0.4% solution (Sigma-Aldrich, Cat No. T-8154)

0.05%Trypsin-EDTA, (1X), Phenol Red (Sigma-Aldrich, Cat No. 25300-054)

Tween 20 (Sigma-Aldrich, Cat No. P 1379)

Vectorshield anti-fade mounting medium (Vector Laboratories Inc., Cat No. H-1000)