The effects of exogenous E-cadherin inhibition in MCF-7 mammary epithelial cells.

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

2015

Natalie Miazga

School of Dentistry
Table of Contents

List of figures .......................................................................................................................... 7
List of tables .............................................................................................................................. 10
Abstract........................................................................................................................................ 11
Declaration.................................................................................................................................... 12
Copyright statement .................................................................................................................... 12
Acknowledgements .................................................................................................................... 13
Abbreviations ............................................................................................................................... 14

Chapter 1. Introduction .............................................................................................................. 16
  1.1 E-cadherin ....................................................................................................................... 17
  1.2 Structure of E-cadherin and the adherens junction .......................................................... 17
      1.2.1 Extracellular domain ............................................................................................. 17
      1.2.2 Intracellular domain .............................................................................................. 18
      1.2.3 The E-cadherin interactome .................................................................................... 21
  1.3 Loss of E-cadherin in cancer ............................................................................................. 22
  1.4 Mechanisms of E-cadherin loss ......................................................................................... 22
      1.4.1 Transcriptional repression ...................................................................................... 22
      1.4.2 Genetic mutation ..................................................................................................... 24
      1.4.3 Epigenetic silencing ............................................................................................... 26
      1.4.4 Post-translational modification .............................................................................. 26
      1.4.5 Endocytosis ............................................................................................................ 28
      1.4.6 Loss of E-cadherin binding partners ....................................................................... 28
      1.4.7 E-cadherin heterophilic interactions ...................................................................... 28
  1.5 Impact of E-cadherin loss on cell signal cascades ............................................................... 30
      1.5.1 Wnt signalling ......................................................................................................... 30
      1.5.2 Receptor Tyrosine Kinases ..................................................................................... 31
      1.5.3 sEcad ....................................................................................................................... 32
      1.5.4 Hippo signalling ...................................................................................................... 33
      1.5.5 Rho GTPases .......................................................................................................... 34
      1.5.6 Resistance to apoptosis .......................................................................................... 35
      1.5.7 Embryonic stem cell self-renewal ........................................................................... 35
  1.6 E-cadherin acts as a regulator of gene transcription ............................................................... 36
1.7 Expression of E-cadherin alters cellular protein localisation ..................37
1.8 Epithelial-Mesenchymal Transition ...........................................................38
  1.8.1 Overview of EMT .................................................................................39
  1.8.2 Developmental EMT ............................................................................41
  1.8.3 Cancer Metastasis ................................................................................42
  1.8.4 Inducers of EMT ..................................................................................45
1.9 Cancer stem cell hypothesis .................................................................47
  1.9.1 Origin of cancer stem cells .................................................................47
  1.9.2 Chemoresistance .................................................................................49
1.10 Retention of E-cadherin in cancer ............................................................50
1.11 The dysregulation of E-cadherin in neoplasia and tumorigenesis (DENT) hypothesis .................................................................51
1.12 Cadherins as therapeutic targets in cancer ............................................52
1.13 Summary .................................................................................................53
1.14 Project aims .............................................................................................54

Chapter 2. Materials and Methods .................................................................57
2.1 Cell culture .................................................................................................58
  2.1.1 Epithelial cell lines .................................................................................59
  2.1.2 Stem cells .............................................................................................59
  2.1.3 Exogenous inhibition of E-cadherin ....................................................59
  2.1.4 Small molecule inhibitors .....................................................................60
  2.1.5 Proliferation assay .................................................................................60
2.2 Flow cytometry ..........................................................................................60
  2.2.1 Cell surface staining ............................................................................60
  2.2.2 Intracellular staining ............................................................................62
  2.2.3 Acquisition and analysis ......................................................................62
  2.2.4 Annexin V/Propidium iodide assay ......................................................63
  2.2.5 Cell cycle analysis ................................................................................64
  2.2.6 Side population analysis ......................................................................65
2.3 Immunofluorescence staining ..................................................................67
  2.3.1 Cell lines .............................................................................................67
2.3.2 Tissue sections ...............................................................67
2.3.3 Image analysis .............................................................69
2.4 mRNA transcript analysis ..................................................70
  2.4.1 Isolation of RNA.........................................................70
  2.4.2 Purification of RNA......................................................70
  2.4.3 Reverse transcription of cDNA........................................70
  2.4.4 Reverse transcriptase PCR ..........................................71
  2.4.5 Real-time quantitative PCR .........................................71
2.5 Western blot ....................................................................77
  2.5.1 Sample preparation......................................................77
  2.5.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis .........77
  2.5.3 Membrane transfer.......................................................78
  2.5.4 Antibody incubation and visualisation............................78
  2.5.5 Densitometry...............................................................79
2.6 Apoptosis protein arrays ....................................................80
  2.6.1 Sample preparation......................................................80
  2.6.2 Array procedure.........................................................80
  2.6.3 Data analysis...............................................................81
2.7 Whole genome microarray analysis .......................................81
  2.7.1 Array procedure.........................................................81
  2.7.2 Bioinformatics analysis...............................................82
  2.7.3 Analysis of significantly altered transcripts .........................82
2.8 Imagen Biotech drug screen ...............................................83
2.9 Plasma membrane protein isolation.....................................84
  2.9.1 Biotinylation and lysis of proteins.....................................84
  2.9.2 Isolation of biotin labelled proteins...................................84
  2.9.3 Elution of biotinylated proteins.......................................85
  2.9.4 Mass spectrometry analysis.........................................85
  2.9.5 Data analysis...............................................................86
2.10 Statistical analysis ........................................................86
Chapter 3. Exogenous inhibition of E-cadherin in MCF-7 cells is insufficient to induce EMT or acquisition of a CD24\textsuperscript{low}/CD44\textsuperscript{high} CSC phenotype.

3.1 Introduction

3.2 Results

3.2.1 Treatment of MCF-7 cells with E-cadherin SHE7 8.7 neutralising antibody results in reversible inhibition of cell-cell contact.

3.2.2 Prolonged exposure of MCF-7 cells to an exogenous E-cadherin inhibitor is insufficient to induce EMT or a CSC phenotype.

3.2.3 E-cadherin SHE78.7-treated MCF-7 cells exhibit increased cell numbers in adherent monolayer and suspension culture.

3.2.4 Exogenous inhibition of E-cadherin induces a wide range of gene transcript alterations in MCF-7 cells.

3.3 Discussion

Chapter 4. E-cadherin acts as a positive regulator of p300-induced gene transactivation

4.1 Introduction

4.2 Results

4.2.1 Exogenous inhibition of E-cadherin alters gene transcripts related to multiple biological processes.

4.2.2 The E-cadherin nAb MCF-7 cell transcriptome exhibits similarities to naïve human embryonic stem cells.

4.2.3 Network analysis identifies core modulators of MCF-7+nAb transcriptome.

4.2.4 Expression of p300 in mammary epithelial cells.

4.2.5 Inhibition of p300 significantly alters proliferation and gene transcript expression.

4.3 Discussion

Chapter 5. Manipulation of cell surface E-cadherin alters cell signal transduction and enhances drug efficacy

5.1 Introduction
5.2 Results .................................................................................................................. 142

5.2.1 E-cadherin neutralising peptide reversibly inhibits E-cadherin mediated cell-cell contact in MCF-7 cells ................................................................. 142

5.2.2 Neutralisation of E-cadherin in MCF-7 cells using nPep increases cell numbers in adherent monolayer and suspension culture .......................... 142

5.2.3 Inhibition of E-cadherin mediated cell contact in MCF-7 cells enhances drug sensitivity .................................................................................. 151

5.2.4 Reduced efficacy of small molecule therapeutics in nPep-treated MCF-7 cells ................................................................................................. 156

5.2.5 Exogenous inhibition of E-cadherin increases sensitivity to VEGFR inhibitors and alters Akt phosphorylation .................................................. 158

5.2.6 Exogenous inhibition of E-cadherin using nPep induces differences in the plasma membrane proteome ......................................................... 160

5.3 Discussion ........................................................................................................... 162

Chapter 6. General discussion ............................................................................... 167

6.1 Introduction ....................................................................................................... 168

6.2 Exogenous inhibition of E-cadherin increases cell numbers due to increased proliferation and reduced apoptosis .............................................. 168

6.3 Prolonged exogenous inhibition of E-cadherin is insufficient to induce an EMT event or acquisition of a CSC phenotype ........................................ 170

6.4 E-cadherin regulates gene transcription and cell surface protein expression ........................................................................................................ 172

6.5 Manipulation of E-cadherin enhances the efficacy of cancer therapies . 173

6.6 Discrepancies between methods of E-cadherin inhibition ............................ 175

6.7 Final conclusions ............................................................................................. 177

6.8 Future directions ............................................................................................. 178

References .............................................................................................................. 198

Appendix ................................................................................................................. 198

A1: Supplementary data ........................................................................................ 199

A2: List of companies ........................................................................................... 200

A3: Publication ....................................................................................................... 203

Word count: 51,128
List of figures

Figure 1.1: Diagrammatic representation of E-cadherin mediated cell-cell adhesion. ..............................................................20
Figure 1.2: Functional classification of the E-cadherin interactome proteins ..........21
Figure 1.3: Transcriptional repression of E-cadherin ..............................................23
Figure 1.4: Proteolytic cleavage of E-cadherin .........................................................27
Figure 1.5: Schematic representation of the epithelial-mesenchymal transition and related phenotypes ........................................40
Figure 1.6: A diagrammatic representation of the role of epithelial-mesenchymal transition in metastasis ....................................43
Figure 1.7: Schematic diagram of methods of E-cadherin inhibition described within the literature ..................................................56
Figure 2.1: Flow cytometry analysis .......................................................................63
Figure 2.2: Annexin V/Propidium iodide assay quadrant statistics ..............................64
Figure 2.3: Gating strategy for side population analysis ...........................................66
Figure 2.4: Quantification of nuclear area ..................................................................69
Figure 2.5: Real-time quantitative PCR melt curve analysis .......................................73
Figure 2.6: Determination of primer efficiency ............................................................74
Figure 3.1: E-cadherin SHE78.7 neutralising antibody mediates reversible inhibition of E-cadherin mediated cell-cell contacts in MCF-7 cells ........................................92
Figure 3.2: Exogenous inhibition of E-cadherin is insufficient to induce an EMT event within 50 days .................................................94
Figure 3.3: Cellular localisation and total expression of β-catenin is unaffected by exogenous inhibition of E-cadherin.................................95
Figure 3.4: Exogenous inhibition of E-cadherin is insufficient to induce acquisition of a CD24\text{low}/CD44\text{high} CSC phenotype within 50 days .................................................96
Figure 3.5: Exogenous inhibition of E-cadherin increases growth and survival of MCF-7 cells in monolayer culture within 3 days ......................98
Figure 3.6: Exogenous inhibition of E-cadherin increases survival and alters expression of apoptosis related proteins ........................................100
Figure 3.7: Exogenous inhibition of E-cadherin increases proliferation but not survival in 3-dimensional culture. ................................................................. 102

Figure 3.8: Microarray analysis of cAb/nAb-treated MCF-7 cells after 3 days. .... 105

Figure 3.9: Loss of cell surface expression of E-cadherin correlates with cytoplasmic expression of EMP-1 in tumour tissue sections. ........................................ 109

Figure 3.10: Neutralisation of E-cadherin contributes to abnormal growth but is insufficient to induce EMT ................................................................. 113

Figure 4.1: Gene Ontology analysis of significantly altered gene transcripts. .... 119

Figure 4.2: Exogenous inhibition of E-cadherin alters gene transcripts related to multiple canonical pathways ......................................................... 120

Figure 4.3: Exogenous inhibition of E-cadherin is insufficient to induce a stem cell side population ................................................................. 123

Figure 4.4: Network analysis of the MCF-7 E-cadherin+nAb transcriptome. .... 125

Figure 4.5: Exogenous inhibition of E-cadherin reduces p300 expression. .... 127

Figure 4.6: Expression of p300 correlates with E-cadherin expression in breast tissue. ......................................................................................... 128

Figure 4.7: Garcinol reduces cell contact and decreases proliferation of MCF-7 cells. ................................................................................................. 132

Figure 4.8: Garcinol induces similar gene transcript changes to E-cadherin SHE78.7 and transcription of CSC-related genes ......................................................... 133

Figure 4.9: STAT3 pathway analysis in E-cadherin nAb-treated MCF-7 .......... 137

Figure 5.1: Application of E-cadherin neutralising peptide inhibited cell-cell contact in MCF-7 cells .............................................................................. 144

Figure 5.2: Application of nPep increases MCF-7 cell numbers. ...................... 145

Figure 5.3: Exogenous inhibition of E-cadherin increases cell survival in monolayer culture. ......................................................................................... 147

Figure 5.4: Exogenous inhibition of E-cadherin with nPep is insufficient to alter expression of apoptosis-related proteins in MCF-7 cells .................. 148

Figure 5.5: Culture of nPep-treated MCF-7 cells in a 3-dimensional environment increases cell numbers ................................................................. 150
Figure 5.6: E-cadherin neutralising peptide enhances efficacy of proteasome inhibitors in MCF-7 cells .......................................................... 125
Figure 5.7: Exogenous inhibition of E-cadherin increases efficacy of mitotic inhibitors ................................................................. 154
Figure 5.8: Increased efficacy of folate cycle inhibitors and anti-metabolite therapeutics in nPep-treated MCF-7 cells ................................. 155
Figure 5.9: Treatment of MCF-7 cells with nPep decreases drug efficacy. .............. 157
Figure 5.10: Differential response of nPep-treated MCF-7 cells to VEGFR inhibitors. ........................................................................ 159
Figure 5.11: Exogenous inhibition of E-cadherin alters the cell surface proteome. 161
Figure S3.1: MCF-10A and A549 cells express N-cadherin. .................................. 199
List of tables

Table 1.1 Percentages of observed non-silent somatic gene mutations by breast cancer subtype........................................................................................................................................25
Table 1.2: Examples of EMT events in embryonic development........................................42
Table 1.3:: Targets of EMT-inducing transcription factors ..............................................46
Table 2.1: Cell culture media. ..........................................................................................59
Table 2.2: Antibodies used for flow cytometry..................................................................61
Table 2.3: Antibodies used for immunofluorescence staining. .......................................68
Table 2.4: Reverse transcriptase PCR primer sequences..................................................72
Table 2.5: Real-time quantitative PCR primer sequences .................................................76
Table 2.6: Western blot 10% gel components.....................................................................77
Table 2.7: Antibodies used for Western blot ....................................................................79
Table 3.1: 20 most downregulated transcripts in nAb-treated MCF-7 cells compared to controls. .................................................................................................................106
Table 3.2: 20 most upregulated transcripts in nAb-treated MCF-7 cells compared to controls .........................................................................................................................107
Table 4.1: Comparison of the top 100 downregulated gene transcripts in MCF-7+nAb cells compared with primed vs. naïve human ES cells ............................................122
Table 4.2: Comparison of the top 100 upregulated gene transcripts in MCF-7+nAb cells compared with primed vs. naïve human ES cells.................................................122
Abstract

Understanding mechanisms that contribute to tumorigenesis and metastasis is important for developing more effective cancer therapies. Epithelial-mesenchymal transition (EMT) is associated with loss of the cell surface protein E-cadherin, increased tumour cell metastasis and acquisition of a cancer stem cell (CSC) phenotype. Whilst the process of EMT and aberrant E-cadherin expression during metastasis have been studied in detail, the role of exogenous inhibition of E-cadherin protein alone in epithelial cells remains to be elucidated. In this study the E-cadherin neutralising antibody SHE78.7 (nAb) and a peptide inhibitor of E-cadherin (nPep) have been used to assess how alterations in cell surface E-cadherin affect adenocarcinoma cell line MCF-7. MCF-7 cells exhibit an epithelial phenotype characterised by cell surface E-cadherin expression and lack of EMT marker expression, such as N-cadherin and Vimentin. Exogenous inhibition of cell surface E-cadherin using nAb in MCF-7 cells is a reversible process which induces increased cell numbers due to decreased apoptosis and cell proliferation. However, nAb treatment was insufficient to induce EMT or a CSC phenotype. Treatment of MCF-7 cells with nPep also induced increased cell numbers, but this was due to increased proliferation of the cells, with no changes in apoptosis observed. Microarray analysis of nAb-treated MCF-7 cells revealed >1000 gene transcript alterations compared to control Ab-treated cells, with changes associated with a wide range of cellular functions. Using an in silico network analysis approach, E-cadherin was identified as a positive regulator of the histone acetyltransferase p300. Exposure of MCF-7 cells to the p300 inhibitor garcinol resulted in increased CD44 and Slug, and decreased CD24 CSC associated transcripts. nPep treatment of MCF-7 cells and subsequent high content screening (HSC) analysis following exposure to a panel of cancer therapeutics demonstrated increased drug efficacy in combination with nPep for most of the therapeutics. Furthermore, nPep-treated MCF-7 cells exhibited a significantly altered plasma membrane-associated protein profile compared to control cells. Together, these results show that exogenous inhibition of E-cadherin in MCF-7 cells is a reversible event associated with increased proliferation, reduced apoptosis and significantly altered protein and transcript expression that may contribute to neoplasm formation in vivo. Furthermore, I show for the first time that inhibition of p300-dependent gene transactivation induces a CSC gene transcript expression profile in MCF-7 cells in vitro.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trade marks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DocuInfo.aspx?DocID=487), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.manchester.ac.uk/library/aboutus/regulations) and in The University’s policy on Presentation of Theses.
Acknowledgements

Firstly, I would like to thank my supervisors Chris and Cathy for their support and guidance over the last 4 years. To all past and present members of the Ward group, thank you for the insightful discussions, technical advice and keeping me sane over cups of tea. I would also like to thank Adam Stevens for supporting me with the world of network analysis.

I would like to acknowledge Karen Cosgrove for being my advisor for this project.

I am grateful to the BBSRC for funding this degree and my Policy Internship at the Centre for Science and Policy, University of Cambridge. Without their funding I would never have had these opportunities – thank you.

Finally, I would like to thank my family and friends for their support, encouragement and patience throughout.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>18S ribosomal RNA</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metallopeptidase</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens junction</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAb</td>
<td>Control antibody</td>
</tr>
<tr>
<td>CCC</td>
<td>Cytoplasmic cell adhesion complex</td>
</tr>
<tr>
<td>cPep</td>
<td>Control peptide</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DENT</td>
<td>Dysregulation of E-cadherin in neoplasia and tumorigenesis</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EC</td>
<td>Extracellular cadherin</td>
</tr>
<tr>
<td>E-cad/CTF</td>
<td>E-cadherin C-terminal fragment</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMP-1</td>
<td>Epithelial membrane protein 1</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>EP300</td>
<td>E1A binding protein p300</td>
</tr>
<tr>
<td>EPLIN</td>
<td>Epithelial lost in neoplasm</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FN1</td>
<td>Fibronectin-1</td>
</tr>
<tr>
<td>FOXC2</td>
<td>Forkhead box protein C2</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>gp130</td>
<td>Glycoprotein 130</td>
</tr>
<tr>
<td>HAV</td>
<td>Histidine-Alanine-Valine</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>Insulin-like growth factor binding protein-3</td>
</tr>
<tr>
<td>JMD</td>
<td>Juxtamembrane domain</td>
</tr>
<tr>
<td>KLF8</td>
<td>Kruppel-like factor 8</td>
</tr>
<tr>
<td>KLRG1</td>
<td>Killer cell lectin-like receptor G1</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>LIFR</td>
<td>Leukemia inhibitory factor receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-epithelial transition</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MrpL19</td>
<td>Mitochondrial ribosomal protein L19</td>
</tr>
<tr>
<td>nAb</td>
<td>Neutralising antibody</td>
</tr>
<tr>
<td>NCOA3</td>
<td>Nuclear receptor coactivator 3</td>
</tr>
<tr>
<td>nPep</td>
<td>Neutralising peptide</td>
</tr>
<tr>
<td>p120&lt;sup&gt;ctn&lt;/sup&gt;</td>
<td>p120-catenin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</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>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sEcad</td>
<td>Soluble E-cadherin</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIP1</td>
<td>Smad interacting protein 1</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interference RNA</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TAZ</td>
<td>Transcriptional coactivator with PDZ binding motif</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell factor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>YAP</td>
<td>Yes associated protein</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide</td>
</tr>
<tr>
<td>ZEB</td>
<td>Zinc finger E-box binding homeobox</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction
1.1 E-cadherin

Cell adhesion is crucial for maintenance of tissue integrity. Cell-cell contacts are mediated by a large superfamily of glycoproteins known as cadherins. Cadherins are typically present at the cell surface of multiple cell types and mediate homotypic cell-cell interactions. Members of the cadherin superfamily include Classical (type I), non-classical (type II), desmosomal and proto-cadherins (Nollet et al., 2000). The classical cadherin subfamily represent the best characterised of the cadherins, which includes Epithelial cadherin (E-cadherin) and Neural cadherin (N-cadherin).

Initially named Uvomorulin, E-cadherin was first identified as an important protein in embryonic development; antibodies raised against E-cadherin demonstrated the necessity of E-cadherin for compaction of mouse morulae and embryonal carcinoma cells (Hyafil et al., 1981, Hyafil et al., 1980, Peyriéras et al., 1983). In fact, E-cadherin is essential for embryonic development, which was demonstrated by the lethality of homozygous E-cadherin\(^{-/-}\) knock out experiments where embryos were unable to maintain cell polarity or develop trophectoderm epithelium (Larue et al., 1994, Riethmacher et al., 1995). E-cadherin is present not only in the developing embryo but also in almost all human epithelial tissues (Shimoyama et al., 1989). High levels of conservation are observed between mouse and human E-cadherin with 82% and 83% homology observed for nucleotide and amino acid sequence respectively (Mansouri et al., 1988).

1.2 Structure of E-cadherin and the adherens junction

1.2.1 Extracellular domain

Encoded by the CDH1 gene, E-cadherin is a single pass transmembrane glycoprotein belonging to the classical (Type I) cadherin subfamily. All classical cadherins are defined by an ectodomain comprising of 5 extracellular cadherin (EC) repeats, a transmembrane and an intracellular domain (Nollet et al., 2000). Intercalated calcium ion binding sites are located between the EC domains and are required to
form a rigid conformation (Pokutta et al., 1994). E-cadherin is classified as a Type I classical cadherin due to the presence of a conserved Histidine-Alanine-Valine (HAV) domain at residues 79-81 within EC1 (Nollet et al., 2000). The HAV domain is also known as the cadherin recognition sequence, peptide targeting of the HAV sequence in 8-stage mouse embryos prevented compaction and identified this sequence as crucial for cell adhesion (Blaschuk et al., 1990). As the principal component of adherens junctions (AJs), E-cadherin mediates lateral adhesion of epithelial cells (Figure 1.1). AJs are formed through the homodimerization of E-cadherin with a second E-cadherin protein on the same cell (cis-homodimerization), cell-cell contact occurs as a result of two cis-homodimers from adjacent cells interacting to generate a trans-homodimer (Harrison et al., 2011). The HAV domain forms a hydrophobic pocket which enables insertion of Tryptophan-2 (Trp2) from an adjacent cadherin, the significance of this interaction was demonstrated by targeted mutations which inhibited the HAV-Trp2 interaction and prevented trans-homodimerization (Pertz et al., 1999). This strand swap interaction mediates the formation of an adherens junction, formation of weaker, X-shaped cadherin dimers (X-dimers) that are mediated by the EC1-EC2 region have also been described (Harrison et al., 2010). However, these are believed to be required for disassembly of adherens junctions (Harrison et al., 2010, Hong et al., 2011). Given that the E-cadherin neutralising antibody DECMA-1, which targets EC5, is sufficient to cause disaggregation of epithelial cells it is possible that other domains of E-cadherin also contribute to cell adhesion (Vestweber and Kemler, 1985).

1.2.2 Intracellular domain

Within the intracellular domain, the Juxtamembrane domain (JMD) and β-catenin binding domain represent conserved regions that are present among all classical cadherins (Nollet et al., 2000). Binding of β-catenin to the cytoplasmic domain of E-cadherin is promoted and stabilised by phosphorylation of serine residues 840, 846 and 847 within the β-catenin binding domain of E-cadherin (McEwen et al., 2014). Interaction of β-catenin with monomeric α-catenin contributes to the formation of
the Cytoplasmic Cell adhesion Complex (CCC), and anchoring of E-cadherin to the cortical actin cytoskeleton (Ozawa and Kemler, 1992). Epithelial lost in neoplasm (EPLIN) also contributes to the CCC and facilitates anchorage of E-cadherin to F-actin to stabilise the adherens junction while p120-catenin (p120\(ctn\)) binds to the JMD to stabilise the CCC (Abe and Takeichi, 2008, Ishiyama et al., 2010, Hong et al., 2013). The presence of \(\alpha\)-catenin strengthens the binding of p120\(ctn\), which inhibits clathrin-mediated endocytosis of E-cadherin by denying endocytic machinery access to a dileucine motif within the JMD (Miyashita and Ozawa, 2007, Troyanovsky et al., 2011). Amino acid residues Lys401 and Asn478 have been identified as crucial for the binding of p120ctn to the JMD since mutation of either residue eliminated p120\(ctn\) binding (Ishiyama et al., 2010). The JMD also contains an overlapping region for binding of Presenilin 1 (PS1), which competes with p120\(ctn\) for the binding site (Baki et al., 2001). Upon binding, PS1 complexes with \(\beta\)-catenin to stabilise the adherens complex but PS1 also regulates \(\gamma\)-secretase-like cleavage of E-cadherin that can be triggered by apoptosis (Baki et al., 2001, Marambaud et al., 2002). The \(\beta\)-catenin binding domain also facilitates binding of the type I\(\gamma\) phosphatidylinositol phosphate kinase (PIPKI\(\gamma\)) and Protein Tyrosine Phosphatase-\(\mu\) (Brady-Kalnay et al., 1998, Ling et al., 2007). PIPK\(\gamma\) was originally identified as an important factor for successful trafficking and formation of the adherens junction (Ling et al., 2007), however, multiple isoforms of PIPK\(\gamma\) exist which exert different effects when bound to E-cadherin. For example, isoform 2 is associated with assembly and trafficking whereas isoform 5 has been associated with increased disassembly and lysosomal degradation of E-cadherin (Schill et al., 2014).
Chapter 1 | Introduction

Figure 1.1: Diagrammatic representation of E-cadherin mediated cell-cell adhesion

Lateral adhesion of epithelial cells is mediated by formation of adherens junctions. Intercalated binding of calcium (Ca2+) ions between the extracellular cadherin domains are required for formation of a rigid E-cadherin structure and cell adhesion. E-cadherin functions as a dimer by interacting with E-cadherin molecules on the adjacent cell. Binding of β-catenin to the cytoplasmic domain anchors E-cadherin to the actin cytoskeleton through α-catenin and Epithelial lost in neoplasm (EPLIN), which form the cytoplasmic cell adhesion complex (CCC). The binding of p120-catenin functions to stabilise the CCC and the expression of E-cadherin at the cell membrane by preventing clathrin-mediated endocytosis.
1.2.3 The E-cadherin interactome

E-cadherin associated protein interactions are complex and not limited to proteins that bind directly to E-cadherin. A recent analysis described 561 proteins identified within the cytoplasmic region of E-cadherin (the interactome), of these, α, β and p120-catenin were among the most abundant proteins associated with E-cadherin and the majority of interactions were not reliant upon cell adhesion (Guo et al., 2014). The authors identified proteins associated with several molecular functions, these included adapter proteins (15%), actin binding adaptors (8%) metabolic activity (5%), GTPase regulation (5%) and transmembrane proteins (3%). Figure 1.2 represents a complete classification of the molecular functions described.

Figure 1.2: Functional classification of the E-cadherin interactome proteins
A recent analysis identified 561 proteins within close proximity to E-cadherin (Guo et al., 2014). A bacterial biotin ligase (BirA) was conjugated to the C-terminus of E-cadherin (E-cad-BirA), which localised to adherens junctions following the generation of a stable MKN28-E-cad-BirA human gastric carcinoma cell line. E-cadherin associated proteins were isolated with Streptavidin and analysed by mass spectrometry. Functional classifications of identified proteins were determined using UniProt, Entrez Gene and the primary literature. Figure taken from Guo et al. (2014).
1.3 Loss of E-cadherin in cancer

The majority of cancers are of epithelial origin and are characterised by common hallmarks that include evasion of apoptosis, self sustained growth, increased proliferation, insensitivity to growth suppressive signals and invasive capability (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). Expression of E-cadherin is crucial for inducing epithelial polarity and maintaining tissue integrity, loss of E-cadherin is often correlated with tumour progression and more aggressive tumour phenotypes (Cavallaro and Christofori, 2004). This is supported by a recent study in breast cancer, where expression of E-cadherin in lymph node metastases was significantly reduced compared to expression in the primary breast tumour (Fulga et al., 2015). Acceleration of in vivo invasion and metastasis following loss of E-cadherin has been demonstrated by studies in RipTag2 mice, which develop cancer in pancreatic islets (Perl et al., 1998). Conversely, forced expression of E-cadherin was sufficient to suppress tumour invasiveness in vivo (Vleminckx et al., 1991). Expression of E-cadherin can be modulated by a number of mechanisms in cancer. Experimental evidence has also demonstrated that loss of E-cadherin affects not only cell adhesion but also cell signalling cascades that can contribute to tumorigenesis (Gottardi et al., 2001, Kim et al., 2011, Lu et al., 2014, Qian et al., 2004).

1.4 Mechanisms of E-cadherin loss

Multiple mechanisms are known to regulate the expression of E-cadherin, which include genetic, epigenetic and post-translational modifications.

1.4.1 Transcriptional repression

E-box binding transcription factors Snail (Snai1), Slug (Snai2) and E47 function as direct repressors of E-cadherin (Figure 1.3), by binding to the E2 box sequence 5’-CA(G/C)(G/C)TG-3’ in the promoter region of E-cadherin to prevent transcription (Bolós et al., 2003, Cano et al., 2000). Other direct repressors of E-cadherin include
Zinc finger E-box binding homeobox 1 (ZEB1/deltaEF1), Smad interacting protein 1 (SIP1/ZEB2) and Kruppel-like factor 8 (KLF8; Eger et al., 2005, Comijn et al., 2001). Twist mediated repression of E-cadherin is a controversial subject with reports in the literature describing Twist as both a direct and indirect repressor of E-cadherin (Vesuna et al., 2008, Yang and Weinberg, 2008). Other indirect repressors include Goosecoid, E2-2 and Forkhead box protein C2 (FOXC2; Hartwell et al., 2006, Mani et al., 2007, Sobrado et al., 2009). In addition to E-cadherin, these factors repress transcription of other epithelial genes such as cell junction components or polarity genes such as Crumbs (Vandewalle et al., 2005, Whiteman et al., 2008). Activation of these transcriptional repressors lie downstream of many signalling cascades including Epidermal growth factor (EGF), Transforming growth factor β-1 (TGFβ1), Fibroblast growth factor (FGF), Nuclear factor kappa B (NFκB) and hypoxia (Lu et al., 2003, Peinado et al., 2003, Lau et al., 2013, Imai et al., 2003, Chua et al., 2006).

![Figure 1.3: Transcriptional repression of E-cadherin](image)

Direct repression of E-cadherin gene transcription is mediated by KLF8, E47, Snail (Snai1), Slug (Snai2), Zeb1 (deltaEF1) and Zeb2 (SIP1). E2-2, Goosecoid and FOXC2 are indirect repressors of E-cadherin gene transcription but Twist has been described as both a direct and indirect repressor of E-cadherin.
1.4.2 Genetic mutation

Truncated or non-functional expression of E-cadherin in endometrial, ovarian and lobular breast carcinoma has been attributed to non-sense and mis-sense gene mutations (Risinger et al., 1994). Evidence has also been presented to suggest carriers of germline mutations in the E-cadherin gene (CDH1) are predisposed to develop hereditary diffuse gastric carcinoma (Brooks-Wilson et al., 2004). In contrast, germline mutations are uncommon in patients with early onset or family history of lobular breast cancer (Schrader et al., 2011). The prevalence of mutation appears to be specific to particular cancer subtypes, for instance, non-sense and frame-shift E-cadherin mutations in lobular breast cancer have been reported to result in truncation of E-cadherin, yet mutations were absent in medullary and ductal carcinoma (Berx et al., 1995). Although E-cadherin gene mutations have been documented the frequency of these are somewhat uncommon compared to other gene mutations, a comprehensive analysis of breast cancer tumours described TP53 (p53) and PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase) among the most frequently mutated genes (The Cancer Genome Atlas Network, 2012). The prevalence of non-somatic gene mutations were categorised according to breast cancer molecular subtype (Table 1.1), these data further highlight the heterogeneity between different cancer subtypes.
### Table 1.1: Percentages of observed non-silent somatic gene mutations by breast cancer subtype

Frequencies of non-silent somatic (truncated and missense) gene mutations in Luminal A (ER\(^+\) and/or PR\(^+\), HER2\(^-\); n=225), Luminal B (ER\(^+\) and/or PR\(^+\), HER2\(^+/\-\); n=126), HER2 enriched (ER\(^-\), PR\(^-\), HER2\(^+\); n=57) and Basal-like (ER\(^-\), PR\(^-\), HER2\(^-\) [also known as triple negative]; n=93) breast cancer tumour samples. Table adapted from The Cancer Genome Atlas Network (2012).

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>All</th>
<th>Luminal A</th>
<th>Luminal B</th>
<th>HER2 Enriched</th>
<th>Basal-like</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIK3CA</td>
<td>36%</td>
<td>45%</td>
<td>29%</td>
<td>39%</td>
<td>9%</td>
</tr>
<tr>
<td>TP53</td>
<td>37%</td>
<td>12%</td>
<td>29%</td>
<td>72%</td>
<td>80%</td>
</tr>
<tr>
<td>MAP3K1</td>
<td>8%</td>
<td>13%</td>
<td>5%</td>
<td>4%</td>
<td>0%</td>
</tr>
<tr>
<td>MAP2K4</td>
<td>4%</td>
<td>7%</td>
<td>2%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>GATA3</td>
<td>11%</td>
<td>14%</td>
<td>15%</td>
<td>2%</td>
<td>2%</td>
</tr>
<tr>
<td>MLL3</td>
<td>7%</td>
<td>8%</td>
<td>6%</td>
<td>7%</td>
<td>5%</td>
</tr>
<tr>
<td>CDH1</td>
<td>7%</td>
<td>9%</td>
<td>5%</td>
<td>5%</td>
<td>0%</td>
</tr>
<tr>
<td>PTEN</td>
<td>3%</td>
<td>4%</td>
<td>4%</td>
<td>2%</td>
<td>1%</td>
</tr>
<tr>
<td>PIK3R1</td>
<td>3%</td>
<td>0.40%</td>
<td>2%</td>
<td>4%</td>
<td>0%</td>
</tr>
<tr>
<td>AKT1</td>
<td>2%</td>
<td>4%</td>
<td>2%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>RUNX1</td>
<td>4%</td>
<td>5%</td>
<td>2%</td>
<td>4%</td>
<td>0%</td>
</tr>
<tr>
<td>CBFB</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>TBX3</td>
<td>3%</td>
<td>3%</td>
<td>4%</td>
<td>0%</td>
<td>1%</td>
</tr>
<tr>
<td>NCOR1</td>
<td>3%</td>
<td>5%</td>
<td>2%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>CTCF</td>
<td>3%</td>
<td>4%</td>
<td>2%</td>
<td>2%</td>
<td>1%</td>
</tr>
<tr>
<td>FOXA1</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>SF3B1</td>
<td>2%</td>
<td>3%</td>
<td>0%</td>
<td>4%</td>
<td>1%</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>RB1</td>
<td>2%</td>
<td>0.40%</td>
<td>3%</td>
<td>0%</td>
<td>4%</td>
</tr>
<tr>
<td>AFF2</td>
<td>3%</td>
<td>1%</td>
<td>2%</td>
<td>5%</td>
<td>4%</td>
</tr>
<tr>
<td>NF1</td>
<td>3%</td>
<td>2%</td>
<td>4%</td>
<td>0%</td>
<td>2%</td>
</tr>
<tr>
<td>PTPN22</td>
<td>1%</td>
<td>0.40%</td>
<td>2%</td>
<td>5%</td>
<td>0%</td>
</tr>
<tr>
<td>PTPRD</td>
<td>2%</td>
<td>2%</td>
<td>4%</td>
<td>4%</td>
<td>1%</td>
</tr>
</tbody>
</table>
1.4.3 Epigenetic silencing

Although mutation of E-cadherin has been described, the prevalence is uncommon when compared to the prevalence of epigenetic silencing of the CDH1 gene (Strathdee, 2002). Dense methylation of cytosine residues in cytosine-guanosine (CpG) islands within the promoter region of E-cadherin results in epigenetic inactivation of the gene by decreased transcription or reduced fidelity of gene transcription. Hypermethylation of the E-cadherin promoter region has been described in breast, prostate, gastric and ovarian cancers (Jing et al., 2014, Graff et al., 1995, Wu et al., 2014). The prevalence of hypermethylation of the E-cadherin promoter was determined to be in 94% of 50 ductal breast and 51% of 53 gastric carcinoma samples tested (Shargh et al., 2014, Tamura et al., 2000). The significance of E-cadherin promoter hypermethylation had been demonstrated by application of the demethylating agent 5-aza-2-deoxycytidine, which restored expression of E-cadherin, cell adhesion and decreased growth and motility of breast and ovarian cell lines (Wu et al., 2014, Nam et al., 2004).

1.4.4 Post-translational modification

A soluble 80kDa extracellular fragment of E-cadherin (sE-cad) and up to 3 cytoplasmic fragments known as E-cadherin cytoplasmic C-terminal fragment 1 (Ecad/CTF1), E-cad/CTF2 and E-cad/CTF3 have been described as a result of proteolytic cleavage (Craig and Brady-Kalnay, 2011). Cleavage of the E-cadherin extracellular domain to form sE-cad and E-cad/CTF1 is mediated by an alpha-secretase mechanism (Figure 1.4), which includes matrix metalloproteinases (MMPs) MMP-3, MMP-7, and A Disintegrin and Metalloproteinase (ADAM) 10 and ADAM 15 (Maretzky et al., 2005, Najy et al., 2008, Noe et al., 2001, Symowicz et al., 2007). Cleavage of the E-cadherin intracellular domain is performed by a γ-secretase and caspase 3 (Marambaud et al., 2002, Steinhusen et al., 2001). Elevated levels of sE-cad are frequently observed in cancer patients and despite no longer being anchored to the cell membrane sE-cad is capable of binding full length E-
cadherin molecules to further disrupt cell adhesion (Wheelock et al., 1987, Noe et al., 2001, Iacopino et al., 2012, Katayama et al., 1994, Fang et al., 2014).

**Figure 1.4: Proteolytic cleavage of E-cadherin**

The extracellular domain of E-cadherin can be subjected to proteolytic cleavage by an α-secretase mechanism to generate an 80kDa soluble E-cadherin (sEcad) fragment and a 40kDa membrane anchored E-cadherin cytoplasmic C-terminal fragment 1 (Ecad/CTF1). Interaction of sE-cad with full length E-cadherin facilitates further abrogation of E-cadherin mediated cell-cell contact. Ecad/CTF1 can be further cleaved on the cytoplasmic side by a γ-secretase and Caspase 3 to generate Ecad/CTF2 and Ecad/CTF3 respectively.
1.4.5 Endocytosis

Internalisation of E-cadherin by endocytosis represents an additional method to regulate the function of E-cadherin and this mechanism can be high-jacked in cancer (Mosesson et al., 2008). For example, decreased expression of Protein phosphatase 2A (PP2A) has been reported in breast cancers (Suzuki and Takahashi, 2006), PP2A recruits IQGAP to Rac1-bound E-cadherin and functions to connect E-cadherin to the actin cytoskeleton and, therefore, stabilise membrane expression. This observed decrease in PP2A expression suggests the loss of E-cadherin in cancer is partly attributed to increased endocytosis. Aberrant regulation of Epidermal growth factor receptors (EGFRs) is commonly observed in cancer and EGF signalling in breast cancer cells is capable of inducing endocytosis of E-cadherin through activation of Arf6, a regulator of membrane trafficking (Xu et al., 2015, Fujita et al., 2002).

1.4.6 Loss of E-cadherin binding partners

Binding of p120^{ctn} to the cytoplasmic domain is believed to protect E-cadherin from endocytosis, therefore, loss of p120^{ctn} could contribute to loss of E-cadherin mediated cell adhesion (Miyashita and Ozawa, 2007). Loss of α-catenin also rendered lung carcinoma cells unable to mediate cell adhesion despite the presence of both E-cadherin and β-catenin (Watabe et al., 1994). Transfection of α-catenin was sufficient to restore cell-cell contacts and apical-basal polarity, therefore, demonstrating the importance of E-cadherin binding partners for functional cell adhesion.

1.4.7 E-cadherin heterophilic interactions

Formation of AJs requires homotypic binding of E-cadherin proteins on adjacent cells but heterotypic interactions between E-cadherin and alternative molecules have been documented in the literature (Mengaud et al., 1996, Nakamura et al., 2009, Cepek et al., 1994, Phan et al., 2007). Expression of killer cell lectin-like
Chapter 1

Introduction

receptor G1 (KLRG1) on natural killer and T-cells is known to complex with monomeric E-cadherin and this interaction is mediated by the EC1 domain (Nakamura et al., 2009). Consequently KLRG1-E-cadherin binding prevents cell adhesion (Banh et al., 2009), however, this is thought to protect E-cadherin positive cells from lysis by natural killer cells (Ito et al., 2006). Natural killer and T-cells have important roles in tumour immunosurveillance, therefore, when T-cells meet tumour cells lacking E-cadherin the protective effects of the KLRG1-E-cadherin will not come into effect. However, given that some tumour cells up-regulate N-cadherin, which is also recognised by KLRG1, tumour cells may use this to their advantage. Integrin αEβ7 is expressed on intraepithelial lymphocytes and is also reported to interact with E-cadherin (Cepek et al., 1994). In contrast to KLRG1, mutation analysis suggested this interaction with αEβ7 integrin is distinct from homophilic E-cadherin interactions and unlikely to disrupt cell adhesion (Karecla et al., 1996). Interestingly, the αEβ7-E-cadherin interaction is thought to increase susceptibility to the cytolytic function of T-cells (Floc'h et al., 2007). A subsequent study proposed the T-cells that express both αEβ7 integrin and KLRG1 may co-engage E-cadherin to result in KLRG1 suppression of αEβ7 integrin (Li et al., 2009).

The food borne bacterium *Listeria monocytogenes* mediates entry into epithelial cells through the bacterial surface protein Internalin (In1A), which targets E-cadherin to induce phagocytosis and disrupt cell adhesion (Mengaud et al., 1996). This facilitates passage across epithelial, placental and blood-brain barriers. Interestingly, mouse E-cadherin is not a receptor for Internalin due to a single amino acid substitution at residue 16 within the EC1 domain (Lecuit et al., 1999). Similarly, the fungus *Candida albicans* uses the adhesin Als3 to target E-cadherin and gain entry into oral epithelial cells by imitating the EC1-EC2 domains of E-cadherin (Phan et al., 2007). This results in the enhanced cleavage of E-cadherin and internalisation of both E-cadherin and the fungus (Frank and Hostetter, 2007). Together these observations illustrate the ability of E-cadherin to mediate heterotypic interactions, which may abrogate E-cadherin mediated cell adhesion and have consequences for downstream signalling cascades.
1.5 Impact of E-cadherin loss on cell signal cascades

1.5.1 Wnt signalling

Formation of adherens junctions sequesters β-catenin at the cell membrane as part of the E-cadherin interactome but β-catenin also plays a central role in canonical Wnt signalling (Heuberger and Birchmeier, 2010). Glycogen synthase kinase 3β (GSK3β), in coalition with adenomatous polyposis coli (APC) protein and axin form the destruction complex which target cytoplasmic β-catenin for degradation in the absence of Wnt signalling (Hinoi et al., 2000). Activation of Frizzled and LRP5/6 co-receptors in the presence of Wnt inhibits GSK3β, which enables cytosolic accumulation of β-catenin by impeding phosphorylation of serine/threonine residues in the N-terminal region. Subsequent nuclear translocation of β-catenin facilitates transactivation of target genes in association with T-cell factor (TCF)/Lef (Huber et al., 1996, Behrens et al., 1996). Wnt signalling targets a plethora of genes associated with cancer, these include MMP-7, CD44, Survivin (Brabletz et al., 1999, Herbst et al., 2014, Torres et al., 2007). E-cadherin is, therefore, termed a tumour suppressor since its expression suppressed transactivation of β-catenin-TCF/Lef target genes (Gottardi et al., 2001). These authors also reported E-cadherin constructs that mediated cell adhesion but lacked the β-catenin binding domain failed to suppress growth.

The regulation of β-catenin in cell adhesion and signalling is a widely debated subject. The competitive binding model suggests E-cadherin and Wnt signal transduction compete for the β-catenin protein. The model assumes the loss of E-cadherin results in decreased cell adhesion and increased transactivation of β-catenin-TCF/Lef target genes, conversely, activated Wnt signalling reduces cell adhesion and increases transactivation of β-catenin-TCF/Lef target genes. Support for this theory is evidenced by the observed decrease in β-catenin-TCF/Lef transactivation in cells over expressing E-cadherin and increased β-catenin-TCF/Lef transactivation following short-hairpin knock down of E-cadherin (Orsulic et al., 1999, Lau et al., 2011, Onder et al., 2008). It is important to note that inhibition of
the destruction complex would also be required to enable cytosolic accumulation of β-catenin in the absence of E-cadherin. In contrast to the competitive binding model, inactivation of E-cadherin was not associated with elevated β-catenin-TCF/Lef transactivation in gastric, pancreatic or non-small cell lung cancers (Caca et al., 1999, Asnaghi et al., 2010). The existence of separate functional pools of β-catenin has been proposed as an alternative theory (Gottardi and Gumbiner, 2004), and generation of a specific form of β-catenin that preferentially binds TCF, as opposed to the cytoplasmic domain of E-cadherin, has been demonstrated following activation of Wnt signalling. Analysis of β-catenin expression in wild type and E-cadherin+/− mouse embryonic stem cells demonstrated that loss of E-cadherin reduced membrane and total expression of β-catenin yet expression of nuclear β-catenin was comparable between both cell lines (Soncin et al., 2011). The loss of E-cadherin also failed to induce β-catenin-TCF/Lef transactivation, therefore providing further support for the existence of separate functional pools of β-catenin.

1.5.2 Receptor Tyrosine Kinases

Localisation of E-cadherin and EGFR (also known as Human epidermal growth factor receptor 1 [HER1] and ErbB) has been observed in breast epithelial cells and recent proteomics analysis identified EGFR among the most abundant proteins in the E-cadherin interactome (Guo et al., 2014). Phosphorylation of E-cadherin by receptor tyrosine kinases (RTKs) is sufficient to induce endocytosis of E-cadherin but E-cadherin mediated cell adhesion can also negatively regulate activation of EGFRs (Fujita et al., 2002). Application of an E-cadherin neutralising antibody was found to be sufficient to relieve E-cadherin mediated repression of EGFR as this induced phosphorylation but not dimerisation of EGFRs. Complex formation of E-cadherin with multiple RTKs including EGFR, Neu /HER2 and insulin-like growth factor-1 receptors (IGF-1R) has been reported to inhibit activation of these receptors, which required presence of the E-cadherin extracellular domain and calcium (Qian et al., 2004). Therefore, it seems a reciprocal relationship exists between E-cadherin expression and EGFR activation. A more recent study described the interaction of
full length E-cadherin with HER2, HER3 but not HER1 (Brouxhon et al., 2014c). Elevated expression and constitutive activation of RTKs are commonly observed in cancer and have important roles in proliferation, survival and migration, and are attractive targets for cancer therapeutics (Hanahan and Weinberg, 2000). Interestingly, transfection of E-cadherin in non small cell carcinoma cell lines enhanced efficacy of the EGFR inhibitor gefitinib (Witta et al., 2006). Downstream of RTKs, conflicting reports have described E-cadherin as an activator and repressor of phosphorylated Akt, which is associated with increased cell survival (Pece et al., 1999, Lau et al., 2011, De Santis et al., 2009).

1.5.3 sEcad

Discharge of the soluble ectodomain of E-cadherin (sEcad) into the extracellular environment has been associated with entry of sEcad into the bloodstream and poor prognosis of gastric carcinoma patients (Chan et al., 2003). Much like full length E-cadherin, association of sEcad with multiple members of Epidermal growth factor receptor (EGFR) family, including HER1, HER2, HER3 and insulin-like growth factor-1 receptors (IGF-1R) has been reported (Brouxhon et al., 2014b, Brouxhon et al., 2014c, Teng et al., 2015). This resulted in enhanced dimerisation and signal transduction associated with proliferation, migration and resistance to apoptosis through activation of mitogen-activated protein (MAP) kinase, phosphatidylinositol 3-kinase (PI3K)/AKT and ERK1/2 signalling (Najy et al., 2008, Brouxhon et al., 2014b, Inge et al., 2011, Brouxhon et al., 2014c, Brouxhon et al., 2013). In fact, sEcad displayed enhanced interaction with RTKs compared to the interactions observed with full length E-cadherin (Brouxhon et al., 2014c). Invasive activity of cancer cells is also reinforced by sEcad activation of MMP-2 and MMP-9 (Nawrocki-Raby et al., 2003, Brouxhon et al., 2014b). Reduced sEcad-mediated tumorigenic activities have been demonstrated following antibody targeted neutralisation of sEcad, the authors described suppression of inhibitor of apoptosis proteins, activation of tumour suppressor proteins (such as p53) and, therefore, suggest neutralisation of
sEcad as a therapeutic strategy for skin squamous cell carcinoma (Brouxhon et al., 2014a).

1.5.4 Hippo signalling

Regulation of tissue growth and apoptosis are controlled by the Hippo pathway, which is commonly disrupted in cancer (Harvey et al., 2013). Yes associated protein (YAP) and Transcriptional co-activator with PDZ binding motif (TAZ) represent core effectors of this pathway, activation of Hippo signalling causes phosphorylation of YAP and TAZ to promote cytoplasmic retention and repression of transcriptional activity. Inactivation of the Hippo pathway enables PP1A mediated dephosphorylation and nuclear translocation of YAP and TAZ (Wang et al., 2011a, Liu et al., 2011). Over expression or activation of YAP has been linked to increased proliferation of different tissue types (Dong et al., 2007b, Schlegelmilch et al., 2011, Camargo et al., 2007), furthermore, tumours that resembled squamous cell carcinoma formed following prolonged activation (42 days) of YAP in mice (Schlegelmilch et al., 2011). Resistance to apoptosis has also been described following YAP over expression, which resulted in elevated expression of inhibitor of apoptosis proteins (IAPs) including Survivin (Dong et al., 2007b). Impaired function of adherens junctions, via application of the calcium chelator EGTA or knock down of E-cadherin, resulted in nuclear translocation, and thus activation, of YAP (Schlegelmilch et al., 2011, Yang et al., 2015). Similarly, expression of E-cadherin in endogenously negative MDA-MB-231 cells promoted cytoplasmic localisation of YAP, which required binding of β-catenin to the cytoplasmic domain of E-cadherin (Kim et al., 2011). Expression of α-catenin is also associated with cytoplasmic retention of YAP since α-catenin knockdown induces nuclear translocation in multiple mammalian cell lines, including MCF-7 cells (Schlegelmilch et al., 2011, Kim et al., 2011, Yang et al., 2015). Similar results have been observed in breast epithelial cells where β-catenin knock down also induced nuclear translocation of YAP (Kim et al., 2011). Subsequently, active Hippo signalling was found to inhibit Wnt signalling through binding of phosphorylated YAP to β-catenin, which
sequestered β-catenin in the cytoplasm (Imajo et al., 2012). Microarray gene expression data were analysed to determine gene expression patterns of high or low activation of Hippo signalling, expression of β-catenin target genes were found to be elevated in colorectal cancer samples where activation of Hippo signalling was considered low (Imajo et al., 2012). In contrast, expression levels of β-catenin target genes were found to be lower in normal colon samples where Hippo signalling was predicted to be high. The loss of E-cadherin may, therefore, not only result in reduced Hippo signalling and nuclear accumulation of YAP and TAZ but also prevent cytoplasmic sequestration of β-catenin to enable nuclear transactivation of β-catenin/TCF target genes following Wnt activation.

1.5.5 Rho GTPases

Connections between E-cadherin and the actin cytoskeleton are mediated by Ras homologous (Rho) GTPases. Members of the Rho family of GTPases include Rac, Rho and cdc42, which act as binary molecular switches that flip between a GDP-bound inactive and a GTP-bound active state. In the active state, Rho GTPases bind effector molecules to modulate multiple pathways including proliferation, cell survival and migration. Up-regulation of Rac, Rho and cdc42 expression have been observed in multiple cancers and these are associated with formation of stress fibres, filipodia and lamellipodia, which contribute to a more migratory phenotype (Fritz et al., 1999, Nobes and Hall, 1995). Formation of adherens junctions is typically associated with activation of Rac1 and inhibition of Rho, more specifically activation of Rac1 stimulates translocation of p190RhoGAP to adherens junctions where interaction with p120^{ctn} inhibits Rho (Wildenberg et al., 2006). Mutation of E-cadherin to result in deletion of exon 8, as commonly observed in gastric carcinomas, reduced the ability of E-cadherin to activate Rac1 and inhibit Rho (Deplazes et al., 2009). The authors noted reduced membrane localisation of p120^{ctn}, which likely accounted for the reduced inhibition of Rho. Localisation of Rho associated protein kinase 1 (ROCK1), an effector of Rho, at adherens junctions is also thought to be mediated by E-cadherin-bound p120^{ctn} (Smith et al., 2012).
1.5.6  **Resistance to apoptosis**

Programmed cell death, known as apoptosis, is a crucial process for normal embryonic development and homeostasis of adult tissues. Resistance to apoptosis is considered a hallmark of cancer, which enables abnormal cells to survive and contribute to tumorigenesis (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). Apoptosis can be activated by extrinsic cell signalling through DR4 (TRAIL-R1) and DR5 (TRAIL-R2) cell surface death receptors (Chaudhary et al., 1997). Upon activation the death inducing signal complex (DISC) is formed, which also requires Fas-associated death domain (FADD) and Caspase-8 and functions to activate additional Caspases 3 and 7 to commit to apoptosis (Gonzalvez and Ashkenazi, 2010). The EC1 domain of E-cadherin has been identified as crucial for the interaction of E-cadherin with ligated DR4 and DR5 (Lu et al., 2014). Furthermore, these authors described the depletion or antibody mediated inhibition of E-cadherin was sufficient to impair DR4/DR5 mediated apoptosis and, therefore, increased resistance to apoptosis. Lack of E-cadherin is also thought to hinder apoptotic cell extrusion, which mediates the removal of cells targeted for apoptosis from the epithelium (Lubkov and Bar-Sagi, 2014).

1.5.7  **Embryonic stem cell self-renewal**

Embryonic stem cells are defined by their limitless potential to self-renew whilst maintaining a pluripotent state, which enables differentiation into any cell type from each of the three germ layers (endoderm, mesoderm, ectoderm). The transcription factors OCT-3/4, SOX2 and NANOG act as a core foundation of pluripotency (Chambers and Tomlinson, 2009). In mouse ES cells expression of this core circuitry of transcription factors can be maintained by Leukaemia inhibitory factor (LIF) and the requirement for E-cadherin this process has previously been demonstrated in our lab (Soncin et al., 2009). Removal of LIF induced differentiation of mouse ES cells but, interestingly, abrogation of E-cadherin by genetic knock out, RNAi or peptide neutralisation and removal of LIF did not result in a differentiated phenotype. Instead these cells maintained pluripotency via Activin/Nodal and self-
renewal by FGF2 (Soncin et al., 2009), which is similar to that employed by human ES cells (Vallier et al., 2005). Furthermore, E-cadherin negative proliferating stem (ENPS) cells, which exhibit methylation of the E-cadherin promoter, also maintain pluripotency and self-renew by Activin/Nodal/FGF2 signalling (Hawkins et al., 2014). Co-localisation of E-cadherin with the LIF-Gp130 co-receptor complex has been described and abrogation of E-cadherin in ES cells is associated with reduced downstream STAT3 phosphorylation (Hawkins et al., 2014, Arulanandam et al., 2009, del Valle et al., 2013). These observations demonstrate that loss of E-cadherin mediated cell-cell contact is sufficient to alter growth factor dependency and cell signal transduction. Since abrogation of E-cadherin is commonly observed in cancer cells it is possible growth factor dependency is also altered in this context. Loss of E-cadherin has also been associated with increased proliferation and motility of mouse ES cells and increased motility of human ES cells and the authors suggest that ES cells may provide a practical model to study EMT events in vitro (Eastham et al., 2007, Spencer et al., 2007).

1.6 E-cadherin acts as a regulator of gene transcription

The effect of E-cadherin loss on the gene transcriptome has been assessed in E-cadherin−/− mouse ES cells, where 2265 significant gene transcript changes were observed compared to wild-type D3 ES cells (Soncin et al., 2011). Notably gene expression changes were not limited to cell attachment, with changes in gene expression related, but not limited, to metabolism and apoptosis also reported. Similarly, microarray analysis of whole gene transcript changes in ENPS cells highlighted alterations in cell signalling pathways such as ERK/MAPK and molecular mechanisms associated with cancer (Hawkins et al., 2014). These were in addition to functions related to cell adhesion such as tight junction signalling and Rho GTPases.

The loss of E-cadherin mediated cell-cell contact via dominant negative expression of E-cadherin has also been described as sufficient to induce global changes in gene
expression, transcript changes included repression of multiple cytokeratin transcripts related to epithelial morphology (Onder et al., 2008). In parallel these authors also performed whole genome analysis following shRNA knockdown of E-cadherin in human immortalised mammary epithelial (HMLE) cells, this induced a greater number of gene transcript alterations when compared to dominant negative expression of E-cadherin and was sufficient to induce an epithelial-mesenchymal transition (EMT) event.

Conversely, transfection of E-cadherin into E-cadherin negative MDA-MB-231 resulted in decreased transcription of genes related to motility including Rho GDP dissociation inhibitor beta but increased transcription of genes related to signal transduction, cell cycle and apoptosis including JUNB proto oncogene, Vascular endothelial growth factor C (VEGFC) and Early growth response 1 (EGR1) (Sarrió et al., 2009).

E-cadherin is largely described as an up-stream regulator of gene transcription but a direct role of cleaved E-cadherin fragments in nuclear signalling has been reported. Ferber and colleagues (2008) observed the nuclear translocation of cleaved E-cad/CTF2 in association with p120ctn, this resulted in binding and inactivation of the transcriptional repressor kaiso, and increased transcription of MMP-7 and cleavage activity. More recently, 4% of the proteins identified as part of the E-cadherin interactome had molecular functions associated with ‘DNA/transcription’ and 8% with ‘RNA/ribosome/translation’ but were not associated with cell junctions (Guo et al., 2014). This suggests that protein interactions with E-cadherin take place in cellular locations other than at the cell membrane.

1.7 Expression of E-cadherin alters cellular protein localisation

Spontaneous differentiation of mouse and human ES cells has been associated with an EMT event and increased membrane expression of ST4 oncofetal antigen (Eastham et al., 2007, Spencer et al., 2007). Application of E-cadherin SHE78.7 or DECMA-1 neutralising antibodies to human or mouse ES cells respectively induced
translocation of 5T4 oncofetal antigen from the cytoplasm to the cell membrane and this could be reversed upon removal of the neutralising antibody (Eastham et al., 2007, Spencer et al., 2007). The 5T4 oncofetal antigen was also detected at the cell membrane of E-cadherin−/− mouse ES cells, although transfection of E-cadherin was insufficient to alter cellular localisation (Spencer et al., 2007). The 5T4 oncofetal antigen is a glycoprotein that is commonly up-regulated in several types of cancer including gastric, colorectal and non-small cell lung carcinoma and has been linked to poorer patient prognosis (Damelin et al., 2011, Starzynska et al., 1992, Naganuma et al., 2002). The results observed in ES cells are corroborated by observations in epithelial cells since over expression of the 5T4 antigen resulted in decreased expression of E-cadherin and dissolution of cell-cell contacts (Carsberg et al., 1996). Together these results indicate the membrane expression of the 5T4 antigen is dependent upon loss of E-cadherin mediated cell adhesion. Membrane expression of E-cadherin has also been shown to influence membrane localisation of the EphA2 receptor, the authors demonstrated the loss of E-cadherin expression was associated with perinuclear expression of EphA2 receptor (Orsulic and Kemler, 2000).

1.8 Epithelial-Mesenchymal Transition

The concept of the epithelial-mesenchymal transition (EMT) was pioneered by Elizabeth Hay in 3-dimensional culture of chick embryos when a process by which cells exchange their epithelial tissue architecture for a mesenchymal phenotype was described (Greenburg and Hay, 1982, Hay, 1995). The process of EMT is a normal physiological process, which has central roles in embryonic development and wound healing. More recently, EMT has been offered as a mechanism to describe the complexities of cancer metastasis (Thiery, 2002). In both development and disease, the defining features of EMT include the loss of epithelial cell-cell contacts and apico-basal polarity. Instead cells adopt a more spindle-like mesenchymal morphology and front-back polarity, gene expression signatures are
also reconfigured to enhance mesenchymal morphology, motility and invasion (Figure 1.5).

1.8.1 Overview of EMT

The first step in the process of EMT is the dissolution of cell-cell contacts, which include adherens junctions, tight junctions and desmosomes, with loss of E-cadherin expression is considered a defining feature of EMT. In exchange, the less adhesive mesenchymal-associated N-cadherin is often upregulated, which represents the classical E-to-N-cadherin switch that is more conducive to migration and invasion of tumour cells. Epithelial cells typically express cytokeratin intermediate filaments, however, these are downregulated and replaced with Vimentin. This exchange alters the trafficking of membrane proteins and organelles to favour a more mesenchymal morphology (Toivola et al., 2005). Repression of apico-basal polarity occurs as mesenchymal cells favour anterior-posterior polarity, with the polarity proteins Crumbs and Scribble, which have been described as repressors of EMT, being downregulated during an EMT event (Karp et al., 2008, Elsum et al., 2013). Epithelial cells seek contact with the basement membrane through α6β4 integrins but as EMT progresses, these integrins are epigenetically silenced and this contact is lost (Yang et al., 2009). Expression and interaction of β1-containing integrins with Collagen Type I is also increased, which is sufficient to dissociate E-cadherin from the cytoskeleton and increase tumour cell proliferation (Koenig et al., 2006). Furthermore, Collagen Type I has been shown to decrease E-cadherin gene transcript levels through upregulation of Snail, Slug and Zeb1 transcriptional repressors (Cheng and Leung, 2011). Increased invasive potential is further provided by the upregulation of matrix metalloproteinases (MMPs), which act to degrade extracellular matrix proteins but also cleave the extracellular fragment of E-cadherin to release sE-cad and increase proliferation (Noe et al., 2001, Lynch et al., 2010, Maretzky et al., 2005) Up-regulation of additional MMPs, including MMP-2 and MMP-9, are subsequently induced by sE-cad (Nawrocki-Raby et al., 2003, Brouxhon et al., 2014b), further reinforcing the acquisition of an invasive phenotype.
Figure 1.5: Schematic representation of the epithelial-mesenchymal transition and related phenotypes

Epithelial cells typically express E-cadherin and multiple cytokeratins. Together epithelial cells make up a well connected epithelial layer through formation of cell-cell contacts, which include desmosomes, adherens and tight junctions. These characteristics induce apical-basal polarity and prevent motility and invasion. The epithelial-mesenchymal transition (EMT) describes a process whereby epithelial cells convert to a mesenchymal phenotype by repressing epithelial morphology in exchange for mesenchymal proteins such as N-cadherin, Vimentin and matrix metalloproteinases (MMPs). Cells exhibit front-back polarity, reduced cell-cell contacts and increased invasive activity.
1.8.2 Developmental EMT

Cadherins play important roles in cell sorting during tissue formation of the developing embryo with expression of E-cadherin observed as early as the 2-cell embryo stage (Larue et al., 1994). Differential patterns of cadherins, including E-cadherin, N-cadherin and Placental (P)-cadherin, have been described at different stages of development. This switching of cadherins is crucial for correct segregation of cell types and tissue formation (Wheelock et al., 2008). E-cadherin expression is required to segregate the inner cell mass from the trophectoderm in blastocyst stage embryos and genetic knockout of E-cadherin (E-cadherin−/−) is embryonic lethal with these embryos failing to form trophectoderm epithelium or implant (Larue et al., 1994). The importance of E-cadherin in development is further highlighted by the observation that 14% of the gene transcript changes that occur in E-cadherin−/− mouse ES cells are related to multicellular organism development (Soncin et al., 2009). Similarly, 13% of the up- and 16% of the downregulated gene transcript changes observed in mouse ENPS cells were also related to the multicellular organism development category (Hawkins et al., 2014).

Successive rounds of EMT and its reversal via mesenchymal-epithelial transition (MET) are required at different stages of development for tissue formation (Thiery et al., 2009). Examples of EMT events during development are listed in Table 1.2. Reduced expression of E-cadherin is observed during gastrulation when the 3 germ layers are formed (endoderm, mesoderm and ectoderm). An EMT event facilitates ingression of the primitive streak to form the mesoderm layer (Williams et al., 2012). However, the loss of E-cadherin is only transient as some cells emerging from ingression of the primitive streak proceed to form the gut endoderm epithelium following an MET event. An EMT event is also needed for the formation of the neural crest and subsequent differentiation events enable formation of the neurons. A succession of EMT-MET events are also required for the formation of the heart valves, and secondary palate (Yang and Weinberg, 2008). Therefore, EMT and MET are essential parts of development and demonstrate the importance of cadherin proteins in this process.
Chapter 1 | Introduction

<table>
<thead>
<tr>
<th>Developmental process</th>
<th>Transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesoderm formation</td>
<td>Primitive ectoderm to mesoderm</td>
</tr>
<tr>
<td>Neural crest formation</td>
<td>Neural epithelium to neural crest cells</td>
</tr>
<tr>
<td>Secondary palate formation</td>
<td>Palatal shelf epithelial cells to palatal mesenchymal cells</td>
</tr>
<tr>
<td>Pancreas development</td>
<td>Pancreatic bud to endocrine cells</td>
</tr>
<tr>
<td>Liver development</td>
<td>Liver diverticulum to hepatoblast</td>
</tr>
<tr>
<td>Cardiac valve formation</td>
<td>Endocardial cells to cardiac septa and valves</td>
</tr>
</tbody>
</table>

**Table 1.2: Examples of EMT events in embryonic development**

1.8.3 Cancer Metastasis

Primary tumours originating from epithelial tissues begin with excessive proliferation (Hanahan and Weinberg, 2000), these are initially restricted to the site of initiation due to the maintenance of cell-cell contacts and lack of invasive capabilities (Figure 1.6A). Detachment of epithelial cells typically induce anoikis, a form of programmed cell death, but EMT has been shown to grant anoikis resistance (Kumar et al., 2011). Acquisition of more motile and invasive mesenchymal traits promotes subsequent invasion of the basement membrane and surrounding tissues by cancer cells (Figure 1.6B). This is followed by their intravasation into the lymphatic vasculature and blood stream enables dissemination of metastatic tumour cells. At distant sites tumour cells exit the blood stream by extravasation where they must survive and adapt to the new microenvironment in order to colonize and establish secondary tumours (Figure 1.6C). This is associated with a period of dormancy as micrometastases adapt to their new environment and it is believed that these disseminated cells must revert to an epithelial morphology in order to integrate and establish a secondary tumour (Tsai et al., 2012). This spread of the tumour mass is known as metastasis, which is the primary cause of cancer-associated deaths. The concept of metastasis is complex but the theory of an EMT event offers an explanation as to how metastasis occurs. Although EMT is observed in embryonic development (Type 1), and wound healing, tissue regeneration and organ fibrosis (Type 2), the role of EMT in
metastasis is likely to be much more complex, involving genetically abnormal cells which allow induction of a type 3 EMT event (Kalluri and Weinberg, 2009).

Figure 1.6: A diagrammatic representation of the role of epithelial-mesenchymal transition in metastasis
Accumulation of genetic and epigenetic abnormalities in epithelial cells induces abnormal proliferation and initiation of a localised primary tumour (A). The process of EMT bestows cells with an increased invasive and migratory capacity, which enables tumour cells to disseminate by invading the basement membrane and entering the circulation by intravasation (B). Cells exit the blood stream by extravasation and integrate within a secondary site (C), possibly by reverting to an epithelial phenotype by a mesenchymal-epithelial transition, to recapitulate the heterogeneity of the primary tumour.
The EMT theory of metastasis was long criticised by sceptics due to the lack of human in vivo evidence (Tarin, 2005). The identification of tumour cells that have undergone EMT has been challenged by the presence of stromal cells in tumours making it difficult to distinguish EMT-induced mesenchymal cells and stromal fibroblasts. In addition, it is believed that cells that have undergone EMT and successfully metastasized subsequently revert to an epithelial phenotype in a mesenchymal-epithelial transition (MET) in order to integrate at secondary sites and establish a new tumour mass. This theory is supported by the transient hypermethylation of the CDH1 gene promoter observed in primary tumours, which is subsequently demethylated in metastases resulting in re-expression of E-cadherin (Graff et al., 2000). Similarly, introduction of tumour cells to a new microenvironment through co-culture of MDA-MB-231 cells with hepatocytes was sufficient to induce demethylation of the E-cadherin promoter and protein re-expression (Chao et al., 2010). A potential cause for the reversion to an epithelial phenotype is the lack of EMT-inducing signals at the disseminated site. Much of the early EMT research was performed using in vitro cell lines, however, the first human in vivo evidence in support of the EMT theory of metastasis was provided by Yu et al. (2013). Circulating tumour cells (CTCs) were isolated and characterised from breast cancer patients undergoing chemotherapy. The authors observed a reduction of CTCs expressing mesenchymal markers in patients responsive to therapy, in contrast, patients unresponsive to therapy exhibited increased levels of mesenchymal CTCs.

Successive EMT and MET represents just one theory to explain the role of EMT in metastasis. The heterogenic nature of tumours highlights the potential for EMT to be a partial, reversible and multifaceted event, whereby, not all tumour cells complete and maintain the EMT process. Support for this theory is provided by the observed retention of E-cadherin expression in multiple in vitro breast cancer cell lines that exhibited features of EMT (Hollestelle et al., 2013). In addition, induction of Twist resulted in dissemination of normal mammary epithelial cells, retention of cytokeratin and membrane expression of E-cadherin were observed but
modifications in gene expression associated with cell-matrix adhesion were also reported (Shamir et al., 2014). In vivo, cells of mixed epithelial and mesenchymal morphology were observed from the primary tumour and draining lymph nodes in breast cancer patients (Yu et al., 2013). As an alternative theory to partial EMT events, Tsuji et al. (2009) proposed tumour cells that have undergone EMT represent the invasive front of tumours, which is supported by the observations of mesenchymal tumour cells at the leading edge (Brabletz et al., 2001). Invasion of surrounding tissues and intravasation was enabled by EMT induced cells, yet these cells failed to integrate at secondary sites but non-EMT induced cells succeeded (Tsuji et al., 2009). This led to the suggestion that metastasis is achieved by cooperation of EMT and non-EMT induced cells whereby EMT-induced cells lead the way for non EMT-induced cells to disseminate and metastasize at distant sites. Although much evidence supports the role of EMT in metastasis, clear evidence to suggest EMT is dispensable in tumour invasion and metastasis has been presented. Expression of podoplanin induced reconfiguration of the cytoskeleton and tumour cell invasion without loss of epithelial marker expression or acquisition of mesenchymal traits (Wicki et al., 2006). Furthermore, loss of E-cadherin in mouse or human ES cells was sufficient to induce a mesenchymal phenotype in the absence of a characteristic EMT event (Eastham et al., 2007, Spencer et al., 2007).

1.8.4 Inducers of EMT

Activation of multiple cell signalling pathways have been implicated with induction of EMT including transforming growth factor β (TGFβ), Wnt-β-catenin, Akt, Epidermal growth factor (EGF), Hepatocyte growth factor (HGF) and Nuclear factor kappa B (NFκB), which have been reviewed by Lamouille et al. (2014). The tumour microenvironment is thought to play an important role in generation of EMT inducing signals through recruitment of multiple cells types, including fibroblasts, lymphocytes and mesenchymal stem cells, to form a ‘reactive’ stroma (Chaffer and Weinberg, 2011). Downstream of cell signalling cascades, EMT-associated changes in gene expression are orchestrated by a plethora of transcription factors with
overlapping activity. These may also be influenced by the genetic and epigenetic alterations acquired as part of the primary tumour formation. It is important to note that many of these transcription factors act as direct repressors of E-cadherin or activate additional transcription factors with direct E-cadherin suppressive action (Table 1.3). More recently, the covalent attachment of small ubiquitin-like modifier (SUMO) proteins to lysine residues of transcription factors has been implicated in driving EMT-MET transitions, as reviewed by Bogachek et al. (2015). For example, Zeb2 is well documented as a transcriptional repressor of E-cadherin but this suppressive action was relieved following SUMOylation of Zeb2 (Long et al., 2005).

Expression of certain microRNA sequences have also been correlated with EMT, more specifically, over expression of miR-10b was sufficient to induce an EMT event in laryngeal carcinoma cells (Zhang et al., 2015). Direct targeting of E-cadherin by miR-10b was described but the expression of protein and gene transcripts for EMT inducing transcription factors Slug, Snail and Twist remained unchanged.

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Repression</th>
<th>Activation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail &amp; Slug</td>
<td>E-cadherin, Claudins, Occludins, Crumbs3, Cytokeratins.</td>
<td>Fibronectin, N-cadherin, MMPs, Twist, Zeb1, Zeb2.</td>
<td>(Bolós et al., 2003, Whiteman et al., 2008, Martínez-Estrada et al., 2006, Cano et al., 2000)</td>
</tr>
<tr>
<td>Twist</td>
<td>E-cadherin, Claudins, Occludins.</td>
<td>α5 integrin, Fibronectin, N-cadherin.</td>
<td>(Yang et al., 2004)</td>
</tr>
<tr>
<td>Zeb1 &amp; Zeb2</td>
<td>E-cadherin, Crumbs3</td>
<td>N-cadherin, MMPs.</td>
<td>(Aigner et al., 2007, Vandewalle et al., 2005)</td>
</tr>
<tr>
<td>KLF8</td>
<td>E-cadherin</td>
<td>MMP-9</td>
<td>(Wang et al., 2007, Wang et al., 2011b)</td>
</tr>
<tr>
<td>FOXC2</td>
<td>E-cadherin</td>
<td>Fibronectin, N-cadherin, Vimentin</td>
<td>(Mani et al., 2007)</td>
</tr>
<tr>
<td>ZNF703</td>
<td>E-cadherin</td>
<td>Cytokeratins, N-cadherin, Snail, Vimentin.</td>
<td>(Slorach et al., 2011)</td>
</tr>
<tr>
<td>TBX2</td>
<td>E-cadherin</td>
<td></td>
<td>(Wang et al., 2012)</td>
</tr>
<tr>
<td>Fra-1</td>
<td>E-cadherin</td>
<td>Slug, Zeb1, Zeb2.</td>
<td>(Bakiri et al., 2015)</td>
</tr>
</tbody>
</table>

Table 1.3: Targets of EMT-inducing transcription factors
1.9 Cancer stem cell hypothesis

Heterogeneity within tumours and the differential capacity of tumour cells to initiate new tumours in vivo is well documented. It is hypothesised that tumour growth and metastatic capability are driven by a small population of cancer stem cells (CSCs) or tumour initiating cells, which are defined by the ability to self-renew and initiate a heterogeneous cancer cell population reminiscent of the parental tumour (Clarke et al., 2006). Cancer stem cells were first described in acute myloid leukaemia (AML), a small subpopulation of cells that exhibited a CD34^+ /CD38^- cell surface expression profile were able to initiate AML in severe combined immune-deficient (SCID) mice (Lapidot et al., 1994). Cancer stem cells were later identified in multiple tumours from the brain, breast and colon with varying cell surface expression profiles, which can be exploited for isolation (Al-Hajj et al., 2003, Singh et al., 2004, O'Brien et al., 2007). Support for the CSC hypothesis was evidenced by the observation that fewer than 100 breast CSCs with a CD44^+ /CD24^-/low expression profile were capable of forming heterogeneous tumours in immunocompromised mice, yet thousands of tumour cells of a CD44^-/CD24^+ phenotype failed to generate tumours (Al-Hajj et al., 2003).

1.9.1 Origin of cancer stem cells

The origin and existence of such CSCs has been a hotly debated topic (Gupta et al., 2009a). Indeed, CSCs are akin to normal endogenous stem cells in their ability to divide asymmetrically and give rise to progenitor cells. Many CSCs also share common markers with their normal endogenous counterparts, which has led to the suggestion that CSCs arise from transformation of these normal cells. Alternatively, it is hypothesised that CSC characteristics are acquired in differentiated cells following a transdifferentiation event such as EMT. It has also been suggested that the prevalence of CSCs may be a combination of intrinsic and induced CSCs (Chaffer and Weinberg, 2011), with the intrinsic CSCs arising from normal endogenous stem or progenitor cells and induced CSCs arising from an EMT event.
Generation of CSCs has been demonstrated in human mammary epithelial (HMLE) cells following induction of EMT by exposure to TGFβ1, or over expression of Twist or Snail (Mani et al., 2008). Cells acquired a fibroblast-like morphology and a CD44\textsuperscript{high}/CD24\textsuperscript{low} cell surface expression profile, typical of breast CSCs (Al-Hajj et al., 2003). The ability to form mammospheres consisting of heterogeneous cell populations with expression markers from basal, luminal or both lineages were also documented. The authors went on to determine whether the EMT process could confer these properties onto transformed epithelial cells, HMLEs were transfected to constitutively express HER2/neu or V12H-RAS oncogene to form HMLEN and HMLER cells respectively. Induction of EMT via over expression of Snail or Twist resulted in >10 fold increase in tumour spheres in HMLEN cells compared to controls. Injection of HMLER cells into immune deficient mice resulted in elevated CD44\textsuperscript{high}/CD24\textsuperscript{low} expressing cells and mammosphere formation. Tumour formation occurred after injection of 10\textsuperscript{3} HMLER cells expressing Snail or Twist, yet ineffective tumour formation was described upon injection of 10\textsuperscript{5} HMLER cells deficient in Snail or Twist (Mani et al., 2008). These findings were consolidated by Morel et al. (2008) who also confirmed acquisition of a CD44\textsuperscript{high}/CD24\textsuperscript{low}, mesenchymal phenotype in HMLER cells and extended these findings to V12K-RAS transformed MCF-10A cells.

To further support the hypothesis of EMT-mediated generation of CSCs, the EMT induced transcription factor FOXC2 has been identified as sufficient to drive the expression of a CSC phenotype in HMLER cells (Hollier et al., 2013). Cells exhibited a CD44\textsuperscript{+/−}/CD24\textsuperscript{−/low} cell surface expression profile, which was accompanied by increased mammosphere forming capacity \textit{in vitro}. Furthermore, metastasis of HMLER-FOXC2 cells was achieved within 28 days of their injection \textit{in vivo}, whereas vector controlled cells failed to metastasize (Hollier et al., 2013). Twist induced generation of a breast CSC phenotype in mammary epithelial cells has also been described, however, only transient expression of Twist is required to stabilise CSC traits (Vesuna et al., 2009, Schmidt et al., 2015, Li and Zhou, 2011). Emergence of a CSC phenotype in MCF-7 cells was reported prior to an EMT event, which
demonstrates EMT may not be a prerequisite for generation of CSCs (Vesuna et al., 2009).

Much like ES cells are governed by a ‘transcriptional foundation of pluripotency’ comprised of Oct-4, SOX-2 and Nanog (Chambers and Tomlinson, 2009), endogenous mammary stem and breast cancer stem cells are regulated by Slug and SOX9 (Guo et al., 2012). These authors demonstrated the simultaneous expression of Slug and SOX-9 in differentiated luminal epithelial cells was sufficient to confer mammary stem cell (MaSC) capabilities, which enabled complete recapitulation of cleared mammary glands in mouse fat pad transplantation assays when as few as 100 cells were injected. In contrast, injection of 1x10^4 control vector cells failed to reconstitute mammary ductal trees (Guo et al., 2012). Interestingly, Slug and SOX-9 were found to act co-operatively with neither factor able to drive a MaSC phenotype alone. These findings were extended to breast cancer stem cells, knock down of either Slug or SOX9 in MDA-MB-231 was found to inhibit tumour formation and growth, and metastatic capability following tail vein injection in mice. In non-metastatic MCF-7ras cells, expression of Slug and SOX9 increased the occurrence of metastases by ~26 fold (Guo et al., 2012).

1.9.2 Chemoresistance

Expression of EMT and CSC associated proteins, and loss of epithelial proteins are associated with poor clinical outcome in breast cancer (Oon et al., 2015). A growing body of evidence suggests that EMT and CSCs are associated with resistance to cancer therapeutics (Gupta et al., 2009b), which are thought to be responsible for tumour resurgence. For example, MCF-7 cells that have undergone EMT demonstrate increased resistance to paclitaxel and tamoxifen (Cheng et al., 2007, Hiscox et al., 2006). Both normal and cancer stem cells can be identified by their increased ability to efflux Hoechst 33342 dye through ABC transporters using the side population method developed by Goodell et al. (1996) in haematopoietic stem cells. ABC transporters are typically localised to the cell membrane and act to shield cells from potentially damaging substances such as toxins and xenobiotics.
Expression of P-glycoprotein (also known as multidrug resistant protein 1 [MDR1]) and breast cancer resistant protein (also known as ABCG2) have been associated with the efflux of Hoechst 33342 but also correlate with resistance to cancer therapeutics (Scharenberg et al., 2002, Kim et al., 2002b, Mechetner et al., 1998). Increased aldehyde dehydrogenase (ALDH) activity has also been observed in CSCs, with the role in chemoresistance demonstrated by the enhanced sensitivity of breast cancer cells to paclitaxel and doxorubicin therapeutics following inhibition of ALDH (Croker and Allan, 2012).

1.10 Retention of E-cadherin in cancer

The loss of E-cadherin expression in cancer metastasis has been well documented, however, retention of E-cadherin is becoming increasingly recognised as advantageous for tumour progression. The importance of E-cadherin for integration and colonization of tumour cells in a new microenvironment has already been discussed and the conferred survival advantage, in the form of chemoresistance, has been reported in breast and prostate cancer cell lines in vitro following re-expression of E-cadherin (Chao et al., 2012).

High expression of E-cadherin protein has been documented in inflammatory breast carcinoma and E-cadherin has been directly shown to facilitate aggregation of inflammatory breast carcinoma cells, which enables the cumulative metastasis of multiple tumour cells (Dong et al., 2007a, Tomlinson et al., 2001). The authors demonstrated the advantages of retained E-cadherin expression via use of dominant negative mutants, neutralising antibodies and Ca\(^{2+}\) deficient media, which inhibited invasiveness and induced dissolution of cellular aggregates. E-cadherin is not thought to be required for motility of cells collectively migrating but to provide overall polarity and direction to the migrating cells, for which positive feedback between E-cadherin and Rac is required (Cai et al., 2014).

The loss of E-cadherin expression in tumour cells is thought to be largely overestimated due to the use of antibodies that detect extracellular E-cadherin
epitopes only (David and Rajasekaran, 2012). As a result, the prevalence of E-cadherin ectodomain shedding may also be underestimated. The authors advise the use of multiple antibodies, specific to both the intracellular and extracellular domains of E-cadherin, should be employed to more accurately characterise expression.

1.11 The dysregulation of E-cadherin in neoplasia and tumorigenesis (DENT) hypothesis

Twist and Snail function as inducers of EMT but also as repressors of E-cadherin (Yang et al., 2004, Mani et al., 2008), which lends the possibility that the generation of CSCs observed by Mani et al. (2008) may be attributed to the loss of E-cadherin as opposed to an EMT event. Mohamet and colleagues (2011) proposed the ‘dysregulation of E-cadherin in neoplasia and tumorigenesis (DENT) hypothesis’. The authors suggest loss of E-cadherin function contributes to neoplasm formation by altering the response of cells to growth factors to promote proliferation and evasion of apoptosis, without an EMT event. Early re-expression of E-cadherin is hypothesised to restore epithelial integrity and reverse the process, alternatively, persistent proliferation and transformation, and acquisition of stem cell traits precedes establishment of a tumour cell mass. Cell signals in favour of proliferation and apoptotic resistance are required in order to establish the tumour cell mass, whereas antiproliferative and proapoptotic signals prevent establishment of the tumour cell mass.

An EMT event has been demonstrated following prolonged expression of dominant negative E-cadherin in A431 human vulvar epidermoid adenocarcinoma cells although this was a slow process (Andersen et al., 2005). The DENT hypothesis, therefore, suggests the aberrant expression of E-cadherin may not be sufficient to induce metastasis but the altered growth factor response may amplify metastatic potential by facilitating expression of EMT-related factors.
1.12 Cadherins as therapeutic targets in cancer

Devemy and Blaschuk (2009) described a novel peptide that is an effective inhibitor of both E- and N-cadherin. These authors also hypothesised that such a peptide could aid drug delivery across permeability barriers. Indeed the N-Ac-CHAVC-NH2 peptide (also known as ADH-1 and Exherin™) targeting N-cadherin demonstrated reduced tumour growth and metastasis of pancreatic cancer in vitro and in mouse models (Shintani et al., 2008). In phase I clinical trials ADH-1 showed promise in the treatment of gynaecological cancers in patients with N-cadherin positive solid tumours, doses were well tolerated with disease control observed (Perotti et al., 2009). Suppression of tumour growth was increased by >30 fold following intravenous administration of ADH-1 with melphalan for the treatment of melanoma in rat preclinical studies (Augustine et al., 2008). In phase I trials the combined administration of ADH-1 in combination with melphalan exceeded expectations in patients with solid tumours, 50% demonstrated a complete response, 12.5% showed a partial response, 6.5% had disease control and disease progression was observed in 31% (Beasley et al., 2009). The authors concluded that combined administration of ADH-1 represents a promising method to overcome melphalan chemoresistance in melanoma patients. Unfortunately, subsequent phase II trials in 45 patients with advanced extremity melanoma did not demonstrate a complete response rate that was significantly different from patients who were treated with melphalan alone (Beasley et al., 2011). The authors also conducted gene expression analysis which revealed >100 altered genes in 10 patients where matched biopsy samples were taken before and after ADH-1 treatment. Gene ontology analysis identified regulators of cell adhesion to be among the altered genes, these included increased protocadherin γ C4 and tight junction component Claudin 3, and decreased Plakophilin-2 (Beasley et al., 2011).

Much like E-cadherin, N-cadherin is a classical cadherin comprising of 5 EC domains, which also has a HAV motif within the EC1 domain (Nollet et al., 2000). Expression of N-cadherin has been associated with more aggressive cancers and maintenance
of the tumour vasculature, which provides an ideal target for therapy (Blaschuk and Devemy, 2009). Given that tumours are composed of a mixed population of cells that may express E-cadherin from their epithelial origin or N-cadherin following an EMT event, a dual E- and N-cadherin antagonist may provide a valuable tool to disaggregate the tumour mass and enhance drug delivery. In support of this theory, the E-cadherin SHE78.7 neutralising antibody has been shown to increase uptake and improve efficacy of paclitaxel and vinblastine in HT29 colon carcinoma cell grown in 3-dimensional culture (Green et al., 2004).

1.13 Summary

E-cadherin is a single pass transmembrane glycoprotein and a core component of adherens junctions that mediates lateral adhesion of epithelial cells. Loss of E-cadherin protein expression or function is well documented in cancer and has been associated with an EMT event, which has been proposed as a mechanism to explain cancer metastasis and is thought to bestow tumour cells with CSC capabilities. Induction of EMT represses expression of E-cadherin but also mediates a plethora of additional changes. The role of E-cadherin protein loss and expression of dominant negative mutants to abrogate cell-cell adhesion in EMT have been investigated. However, given the potential for full length E-cadherin to interact with alternative extracellular binding partners, such as sEcad, the exact role of exogenous inhibition of E-cadherin merits investigation but has been largely overlooked. Induction of EMT has been linked to the emergence of a CSC population but the DENT hypothesis suggests aberrant expression of E-cadherin may contribute to altered proliferation and generation of CSCs in the absence of an EMT event (Mohamet et al., 2011). Since E-cadherin is implicated in several cell signalling cascades, exogenous inhibition of E-cadherin may also modify these cascades and, ultimately, alter gene transcription. Gene transcript profiles have been well characterised in ES cells lacking E-cadherin revealing large changes in gene expression with functions not limited to cell adhesion (Soncin et al., 2011, Hawkins et al., 2014), and if these changes were to translate to adult epithelial cells
these changes could impact upon cancer progression. Limited studies have revealed changes in cell surface localisation of EphA2 and ST4 oncofetal protein following loss of E-cadherin (Eastham et al., 2007, Spencer et al., 2007, Orsulic and Kemler, 2000). Given the complex composition of the cell membrane it is likely that localisation of additional proteins are affected and this may alter response to growth factors or xenobiotics.

### 1.14 Project aims

In accordance with the DENT hypothesis (Mohamet et al. 2011), I hypothesised that exogenous inhibition of E-cadherin would be sufficient to increase proliferation, resistance to apoptosis and acquisition of a CSC phenotype. Similar to previous observations in our lab using embryonic stem cells (Eastham et al., 2007, Spencer et al., 2007), exogenous inhibition of E-cadherin alone would be insufficient to induce an EMT event. In addition, exogenous inhibition of E-cadherin would result in altered expression of whole genome transcripts, cell surface protein expression and response to cytotoxic therapies.

The overall aim of this research was to investigate the effects of exogenous E-cadherin inhibition in epithelial cells and assess any changes. E-cadherin mediated cell-cell contact was inhibited using E-cadherin SHE 78.7 neutralising antibody or peptide. Attempts to transfect E-cadherin into E-cadherin negative MDA-MB-231 cells were unsuccessful. A schematic representation of how exogenous inhibition differs from previous methods of E-cadherin loss of function studies is illustrated in Figure 1.7.

The first aim was to investigate the dysregulation of E-cadherin in neoplasia and tumorigenesis (DENT) hypothesis proposed by Mohamet et al. (2011). MCF-7 mammary epithelial cells were selected for these experiments due to the retained characteristics of differentiated epithelium and absence of mesenchymal markers. The E-cadherin SHE78.7 neutralising antibody (nAb) was added to MCF-7 mammary epithelial cells and induction of EMT, survival and acquisition of a cancer stem cell
phenotype were assessed. The effects of exogenous E-cadherin inhibition were further explored by performing microarray analysis to determine changes in whole genome transcript expression.

The second aim was to identify key regulators of the gene transcript profile induced by exogenous inhibition of E-cadherin. An in silico network modelling approach was employed to interrogate microarray data from MCF-7 cells treated with an E-cadherin neutralising antibody to identify key regulators of this phenotype.

Finally, exogenous inhibition of E-cadherin was achieved using a neutralising peptide (nPep) in MCF-7 cells to investigate the effects on growth and survival and compared to results observed with the E-cadherin SHE78.7 neutralising antibody. As a combination therapy, the use of nPep was hypothesised to improve drug delivery (Devemy and Blaschuk, 2009). This was addressed by screening a small panel of small molecule cancer therapeutics in combination with nPep to assess effects on drug efficacy in MCF-7 cells. MCF-7 cells were also treated with a VEGFR inhibitor to assess the effects on proliferation and determine the mechanisms associated with this process. In addition, the cell surface proteome of nPep-treated cells was characterised to address whether exogenous inhibition of E-cadherin was sufficient to alter expression of cell membrane protein localisation.
Figure 1.7: Schematic diagram of methods of E-cadherin inhibition described within the literature
Formation of adherens junctions are mediated by interaction of E-cadherin dimers on adjacent epithelial cells (A). Expression of a truncated dominant negative form of E-cadherin was employed by Onder et al. (2008) to induce disaggregation of mammary epithelial cells (B). Genetic knock-out or siRNA knock down of E-cadherin results in loss of E-cadherin expression, inhibition of cell-cell contact and liberation of E-cadherin binding proteins (C). Application of E-cadherin neutralising antibodies (nAb) or peptides (nPep) enables disaggregation of epithelial cells while expression of full length E-cadherin remains (D).
Chapter 2. Materials and Methods
2.1 Cell culture

2.1.1 Epithelial cell lines

MCF-7, MCF-10A, A549 and MDA-MB-231 were cultured in 6-well tissue culture grade plates (Greiner Bio-One, Gloucestershire UK) with 3mls of complete cell culture media. See Table 2.1 for cell culture media specific to each cell line. Cells were maintained at sub-confluence and passaged every 2-3 days, cell culture media was removed and cells were washed 1X with 1ml PBS (Sigma-Aldrich, Dorset UK) prior to addition of 1ml Trypsin-EDTA solution (Sigma-Aldrich). Trypsin-EDTA was removed and after 5 minutes cells were re-suspended in pre-warmed cell culture media before being transferred to a new cell culture vessel. For MCF-10A cell culture, the Trypsin-EDTA incubation time was extended to 15 minutes.

2.1.2 Stem cells

Mouse embryonic stem (ES) cells-(D3, strain 129S2/SvPas, ATCC number CRL-11632 [wtD3]) and E-cadherin knockout D3 (129Sv [Ecad−/−] Mouse ES cells, donated by Dr R. Kemler) were cultured in gelatin-coated 6-well cell culture plates. Cells were maintained at 70-80% confluence in 3mls of complete media. Cells were passaged every 2 days, cell culture media was removed and cells were washed 1X with PBS before addition of Trypsin-EDTA. Upon removal of Trypsin-EDTA cells were incubated for 5 minutes prior to resuspension in pre-warmed cell culture media before being transferred to a new cell culture vessel.

Bone marrow mesenchymal stem cells (MSCs; Lonza, Cambridge UK) were cultured on gelatin-coated cell culture plastic in complete media, cells were passaged with Trypsin-EDTA at ~90% confluence at a ratio of 1:5. For details of all cell culture media see Table 2.1
### Materials and Methods

#### Cell line Media

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-10A</td>
<td>MEGM bullet kit (Lonza) + 100ng/ml Cholera toxin (Sigma-Aldrich)</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Eagle’s Minimum Essential Medium (Sigma-Aldrich) + 10% FBS (Gibco by Life Technologies)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>RPMI-1640 (Sigma-Aldrich) + 10% FBS</td>
</tr>
<tr>
<td>A549</td>
<td>DMEM (Sigma-Aldrich) + 10% FBS</td>
</tr>
<tr>
<td>Mouse ES cells</td>
<td>Knockout DMEM (Gibco by Life Technologies) + 10% FBS + 2mM L-glutamine + 1% non-essential amino acids (PAA Laboratories) + 50µl β-mercaptoethanol (Gibco by Life Technologies) + 1000U/ml LIF (Chemicon International).</td>
</tr>
<tr>
<td>Bone marrow MSCs</td>
<td>MesenPRO RS basal media (Gibco by Life Technologies).</td>
</tr>
</tbody>
</table>

**Table 2.1: Cell culture media**

#### 2.1.3 Exogenous inhibition of E-cadherin

To abolish E-cadherin mediated cell-cell contact in adherent culture MCF-7 cells were cultured with an E-cadherin SHE78.7 neutralising antibody (nAb; Invitrogen by Life Technologies, Paisley UK) at a final concentration of 2µg/ml or an E-cadherin neutralising peptide (nPep; Bachem, Bubendorf Switzerland) at a final concentration of 0.5mM. The E-cadherin nPep consisted of 12 amino acids (H-Ser-Trp-Glu-Leu-Tyr-Tyr-Pro-Leu-Arg-Ala-Asn-Leu-NH₂) as described by Devemy and Blaschuk (2009). Control populations were treated with a normal mouse IgG control antibody (cAb; 2µg/ml; Life Technologies), a control peptide (cPep; H-Ser-Arg-Glu-Leu-Tyr-Try-Pro-Leu-Arg-Ala-Asn-Leu-NH₂) at an equivalent concentration or an equivalent volume of H₂O (Ambion by Life Technologies) used to solubilise the E-cadherin neutralising peptide as a vehicle control. The concentration of E-cadherin neutralising agents were increased for MCF-7 cells grown in ultra-low attachment plates (Corning, Amsterdam, The Netherlands). Cells were cultured with E-cadherin SHE78.7 nAb (3µg/ml) or E-cadherin nPep (1mM) and control treatments were adjusted accordingly.
2.1.4 Small molecule inhibitors

Garcinol (10µM; Santa Cruz Biotechnology) and SU5402 (2µM; Tocris Bioscience) were added to inhibit p300 and vascular endothelial growth factor receptor (VEGFR) respectively. Stock concentrations were serially diluted into cell culture media and control populations were treated with an equivalent volume of DMSO (≤0.1%).

2.1.5 Proliferation assay

MCF-7 cells were seeded at $1.25 \times 10^4$ cells/cm$^2$ in cell culture plates (Greiner Bio-One, Gloucestershire UK) and allowed to adhere prior to addition of exogenous inhibitors. Cells grown in suspension were grown in monolayer culture with exogenous inhibitors for 3 days prior to seeding at $4 \times 10^4$ cells/ml in ultra-low attachment plates with exogenous inhibitors. Cells were passaged at specified time-points and cell counts were acquired using a Countess® automated cell counter (Invitrogen by Life Technologies) and Trypan blue (Sigma-Aldrich) to determine cell viability. Cumulative cell counts were obtained in triplicate over 9 days in monolayer or 6 days in 3-dimensional culture to account for each cell passage. Doubling Time Software was used to calculate cell population doubling times (Roth V. 2006, http://www.doubling-time.com/compute.php). Three independent experiments were conducted using this method, data are presented as mean ± standard deviation from one representative experiment.

2.2 Flow cytometry

2.2.1 Cell surface staining

Following removal of cell culture media, cells were washed 1X with of PBS (1ml/well). Cell dissociation buffer (1ml/well; Gibco by Life technologies) was applied for 10 minutes at 37°C. Cell dissociation buffer was removed, cells were collected in PBS (1ml/sample) and transferred to 1.5ml microcentrifuge tubes
(STARLAB, Milton Keynes UK). Cell pellets formed after centrifugation at 280 x g for 5 minutes, the supernatant was removed and cell pellets were re-suspended in flow cytometry buffer (0.2% BSA [w/v] + 0.1% Sodium azide [w/v] + PBS) containing the primary antibody (total volume 100µl/sample), for antibody dilutions see Table 2.2. Samples were incubated for 20 minutes on ice before centrifugation at 280 x g for 5 minutes and removal of the supernatant. Samples were washed 1X with PBS (100µl/sample) and centrifuged at 280 x g for 5 minutes. The supernatant was removed and appropriate fluorescent conjugated secondary antibodies (Santa Cruz Biotechnology, Heidelberg Germany) were diluted (1:100) in buffer and incubated with the cells in the dark for 30 minutes on ice. Samples were centrifuged at 220 x g for 5 minutes and washed 1X with PBS after removal of the supernatant. Cell pellets were re-suspended in 1% (w/v) paraformaldehyde (500µl/sample; Sigma-Aldrich) prior to analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cellular localisation</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-E-cadherin SHE78.7 IgG&lt;sub&gt;2a&lt;/sub&gt;</td>
<td>Cell surface</td>
<td>Invitrogen</td>
<td>Antibody prepared to 500µg/ml 1:100</td>
</tr>
<tr>
<td>Rabbit anti-N-cadherin IgG</td>
<td>Cell surface</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit anti-CD24 IgG</td>
<td>Cell surface</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit anti-CD44 IgG</td>
<td>Cell surface</td>
<td>Abcam</td>
<td>1:30</td>
</tr>
<tr>
<td>Rabbit anti-EMP-1 IgG</td>
<td>Cell surface</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit anti-ki67 IgG</td>
<td>Intracellular</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit anti-p27&lt;sup&gt;Kip1&lt;/sup&gt; IgG</td>
<td>Intracellular</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100</td>
</tr>
<tr>
<td>Chicken anti-mouse IgG PE</td>
<td>-</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG PE</td>
<td>-</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100</td>
</tr>
</tbody>
</table>

Table 2.2: Antibodies used for flow cytometry
2.2.2 Intracellular staining

Cells were washed 1X with PBS (1ml/well; Sigma-Aldrich) and incubated with cell dissociation buffer (1ml/well; Gibco by Life technologies) for 10 minutes at 37°C. Cell dissociation buffer was removed and samples were collected in 1ml PBS and centrifuged at 220 x g for 5 minutes. Cell pellets were re-suspended and fixed in 1% paraformaldehyde (w/v; Sigma-Aldrich) for 10 minutes prior to centrifugation at 220 x g for 5 minutes, samples were washed 1X with PBS and centrifuged at 220 x g for 5 minutes. The cell membrane was permeated with 70% methanol (v/v) at -20°C overnight, following centrifugation cell pellets were washed 2X with PBS. Samples were incubated in flow cytometry buffer (0.2% BSA [w/v; Sigma-Aldrich] + 0.1% Sodium azide [w/v; Sigma-Aldrich] + PBS) for 30 minutes, prior to centrifugation at 220 x g for 5 minutes and re-suspension in blocking buffer containing optimal concentrations of primary antibody (Table 2.2). Samples were incubated for 60 minutes at room temperature, following centrifugation at 220 x g for 5 minutes cells samples were washed 1X with PBS and centrifugation was repeated. Appropriate fluorescent conjugated secondary antibodies were diluted in flow cytometry buffer and incubated with samples in the dark for 30 minutes at room temperature. Samples were centrifuged at 220 x g for 5 minutes and washed 1X with PBS before centrifugation was repeated. Samples were re-suspended in PBS (500µl/sample) prior to analysis.

2.2.3 Acquisition and analysis

Samples were analysed using a Becton Dickinson FACS Calibur™ (BD Biosciences, Oxford UK) through excitation with a 488nm laser, data was collected using the appropriate emission filters and a live cell gating scheme. Single viable cells were identified using forward and side scatter (Figure 2.1A), live cells were gated (Figure 2.1B) and data were collected from 10,000 events within the gated area. Data was collected from control (Figure 2.1C) and test samples, an increase in fluorescence on the X axis was indicative of increased expression of the target protein (Figure
2.1D), in order to distinguish differences between control and test samples the plots were overlaid (Figure E).

![Figure 2.1: Flow cytometry analysis](image)

Cells were separated based on forward and side scatter (A), live cells were gated (B) and data were collected from isotype control (C) and test (D) samples. To determine an increase in fluorescence above isotype control levels the control and test plots were overlaid (E).

### 2.2.4 Annexin V/Propidium iodide assay

Cell viability was determined using an Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Molecular Probes by Life Technologies) according to the manufactures instructions, reagents were provided with the kit unless otherwise specified. Cell culture media was removed and cells were washed 1X with PBS (1ml/well; Sigma-Aldrich). Cell dissociation buffer (Gibco by Life Technologies) was applied for 10 minutes at 37°C, cells were collected in PBS and a sample was removed for counting. Cells were centrifuged at 130 x g for 5 minutes and the
supernatant was removed. The cell pellet was re-suspended in 100µl 1X Annexin binding buffer containing 5µl Alexa Fluor® 488 Annexin V and 1µl propidium iodide (PI) (100µg/ml) at a cell density of 1x10⁶ cells/ml. Samples were incubated in the dark for 15 minutes at room temperature, 400µl 1X Annexin-binding buffer was added and samples were placed on ice and analysed immediately using a Becton Dickinson FACS Calibur™ (BD Biosciences) and excited with a 488nm laser. Data are represented from 1x10⁶ cells. Quadrant statistics were performed using Cell Quest Software v6.0 to quantify the percentage of live (Annexin V⁻/PI⁻), dead/necrotic (Annexin V⁺/PI⁺) and apoptotic (Annexin V⁺/PI⁺) populations (Figure 2.2).

![Figure 2.2: Annexin V/Propidium iodide assay quadrant statistics](image)

Quadrant statistics were performed to quantify the percentage of live (Annexin V⁻/PI⁻), dead/necrotic (Annexin V⁺/PI⁺) and apoptotic (Annexin V⁺/PI⁺) populations.

2.2.5 Cell cycle analysis

DNA quantification using a DNA binding dye, Propidium iodide (PI), was performed to classify cells into different phases of the cell cycle. Cells were harvested at specified time-points with Trypsin-EDTA (Sigma Aldrich) and washed twice with wash buffer (0.1% BSA [w/v; Sigma-Aldrich] + PBS) between centrifugations at 220 x g for 5 minutes. In a 15ml V-bottomed tube, 1x10⁶ cells were re-suspended in 1ml
Chapter 2 | Materials and Methods

wash buffer, 3ml cold 100% ethanol was added to cells dropwise while vortexing and stored overnight at -20°C. Cells were centrifuged at 220 x g for 5 minutes and washed with wash buffer, this was repeated for a total of two washes. After the final centrifugation, cells were re-suspended in 1ml staining solution (3.8mM Sodium citrate [Sigma-Aldrich] + 40μg/ml PI [Sigma-Aldrich] in PBS) plus RNase A (Roche, Welwyn UK) to prevent intercalation of PI with RNA. Samples were incubated at 4°C for 3 hours in the dark and not washed prior to analysis. Samples were analysed using a Becton Dickinson FACS Calibur™ (BD Biosciences). Using FlowJo software v.7.6.5, DNA histogram data was fitted to the Dean-Jett Fox mathematical model, which fitted G1, G2-phase data to Gaussian curves and the S-phase to a second-degree polynomial.

2.2.6 Side population analysis

Hoechst side population analysis can be used to identify populations of stem cells due to the passive uptake of Hoechst 33342 DNA dye in live cells and the ability of stem cells to pump out the dye using ATP-Binding Cassette (ABC) transporters. Side population analysis was performed based on the experiments by Goodell et al. (1996). Cells were washed 1X with PBS and incubated with Trypsin-EDTA prior to being resuspended in DMEM + 2% FBS + 1mM HEPES + 5µg/ml Hoechst 33342. Samples were incubated with ± 50µM verapamil, an ABC transporter inhibitor, for 90 minutes at 37°C in a shaking incubator. Following centrifugation at 130 x g for 5 minutes cells were resuspended in PBS + 2μg/ml PI as a marker of viability. Samples were analysed on a BD Fortessa cytometer (BD Biosciences) using a UV laser, dead and doublet cells were excluded from analysis and data from a total of 100,000 cells were recorded. A step by step gating strategy is described in Figure 2.3.
Figure 2.3: Gating strategy for side population analysis
All cells were gated based on forward (FSC) and side (SSC) scatter (A), dead cells positive for Propidium iodide (PI) and doublet cells were excluded from analysis (B-C). Side population analysis was performed on single and viable cells based on Hoeschst red and Hoeschst blue emission, side population cells were gated (D). Percentages of cells in each population were calculated (E).
2.3 Immunofluorescence staining

2.3.1 Cell lines

Cells were fixed with 4% paraformaldehyde (w/v; Sigma-Aldrich) for 20 minutes at room temperature prior to 3X washes with PBS. Cells were incubated with blocking buffer (0.1% BSA [w/v; Sigma-Aldrich]) + 1% goat serum [v/v; Sigma-Aldrich] + PBS) for 1 hour at room temperature. For intracellular staining 0.1% Triton X-100 (Sigma-Aldrich) was added to blocking buffer to permeate the cell membrane. Blocking buffer was removed before primary antibodies were added at the optimal dilution (Table 2.3) in blocking buffer and incubated for 2 hours on a platform rocker at room temperature. Cells were washed 3X with PBS and appropriate Alexa Flour® conjugated secondary antibodies were added in blocking buffer (all 1:500 dilution; Life Technologies) for 1 hour in the dark at room temperature. Cells were washed 4X with PBS and coverslips were applied following addition of Vectorshield with 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Peterborough UK). Images were acquired using a Leica DM5000 B fluorescence microscope (Leica Microsystems, Milton Keynes UK).

2.3.2 Tissue sections

Breast fibroadenoma, hyperplasia (male) and invasive ductal carcinoma paraffin embedded tissue sections were ethically obtained by Abcam (Cambridge, UK) under the Human Tissue Act (2004), license number 12506, awarded by the Human Tissue Authority. To de-wax and rehydrate the tissue slides were immersed in Xylene for 2X 5 minutes prior to immersion in 100% ethanol for 2X 5 minutes. To block endogenous peroxidase, slides were immersed in methanol containing 3% hydrogen peroxide for 20 minutes and washed 1X in PBS for 5 minutes on a platform rocker. Heat induced antigen retrieval was performed prior to E1A binding protein p300 (p300) staining, citrate antigen retrieval buffer (10mM Tri-sodium citrate + 0.05% Tween 20 + diH2O pH6.0) was heated to 100°C prior to the addition of the tissue sections. Tissue samples were removed from the heat source after 15
minutes and incubated in the hot citrate buffer for a further 20 minutes. Samples were washed 1X in water for 5 minutes before blocking buffer was applied to cells for 30 minutes at room temperature. Primary antibodies E-cadherin (1:100, BD Biosciences), EMP-1 (1:100; Abcam) and p300 (1:2000; Abcam) were diluted in blocking buffer and incubated with the tissue sections overnight at 4°C. Slides were washed 2X with PBS in a vertical rack on a platform rocker for 5 minutes. Alexa Flour fluorescent conjugated secondary antibodies (1:500; Life Technologies) were diluted in blocking buffer and applied to tissue sections for 1 hour in the dark at room temperature. For a complete list of antibodies see Table 2.3. Slides were washed 2X with PBS followed by 1X with diH₂O in a vertical rack on a platform rocker for 5 minutes and mounted using Vectorshield with DAPI. Images were acquired using a Leica DM5000 B fluorescence microscope.

<table>
<thead>
<tr>
<th>Type</th>
<th>Application</th>
<th>Antibody description</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>Cell lines</td>
<td>Rabbit anti-EP300 IgG</td>
<td>Sigma Aldrich</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Cell lines</td>
<td>Rabbit anti-Vimentin IgG</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Cell lines</td>
<td>Mouse anti-E-cadherin SHE78.7 IgG₂a</td>
<td>Invitrogen</td>
<td>Antibody prepared to 500μg/ml 1:100</td>
</tr>
<tr>
<td></td>
<td>Cell lines</td>
<td>Rabbit anti-beta-catenin IgG</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Cell lines</td>
<td>Rabbit anti-Fibronectin-1 IgG</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Cell lines</td>
<td>Rabbit anti-p27Kip1 IgG</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Cell lines</td>
<td>Rabbit anti-N-cadherin IgG</td>
<td>Santa Cruz Biotechnology</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Tissue</td>
<td>Mouse anti-E-cadherin IgG₂a</td>
<td>BD Biosciences</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Tissue</td>
<td>Rabbit anti-EMP1 IgG</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Tissue</td>
<td>Rabbit anti-p300/KAT3B IgG</td>
<td>Abcam</td>
<td>1:2000</td>
</tr>
<tr>
<td>Secondary</td>
<td>Cell lines &amp; tissue</td>
<td>Alexa Fluor -488 goat anti-mouse IgG₂a</td>
<td>Invitrogen</td>
<td>1:500</td>
</tr>
<tr>
<td></td>
<td>Cell lines &amp; tissue</td>
<td>Alexa Fluor -488 goat anti-rabbit IgG</td>
<td>Invitrogen</td>
<td>1:500</td>
</tr>
<tr>
<td></td>
<td>Cell lines &amp; tissue</td>
<td>Alexa Fluor-546 goat anti-rabbit IgG</td>
<td>Invitrogen</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Table 2.3: Antibodies used for immunofluorescence staining
2.3.3 Image analysis

All images were analysed using ImageJ v1.48 software (Schneider et al., 2012). Images were imported to the ImageJ software and the scale was set using the image scale bar applied by the Leica DM5000 B fluorescence microscope software. To account for smaller or rounded up cells appearing to have higher levels of staining corrected total cell fluorescence (CTCF) was calculated using the following formula:

$$\text{CTCF} = \text{Integrated Density} - (\text{Area of selected cell} \times \text{Mean fluorescence of 5 background readings}).$$

Fluorescence was determined from 3 biological replicates and 4 fields of view. For nuclear quantification images were converted to a binary format and individual nuclei were added to the region of interest manager (Figure 2.3), partial nuclei at the edge of images were excluded from analysis.

![Figure 2.4: Quantification of nuclear area](image-url)

Images of DAPI stained nuclei were imported into ImageJ software (A). Images were converted to a binary format and mean nuclear area ($\mu m^2$) was quantified from whole nuclei only, partial nuclei at the edge of images were excluded (B).
2.4 mRNA transcript analysis

2.4.1 Isolation of RNA

Cell culture media was removed and cells were washed 1X with 1ml PBS which was aspirated prior to the addition of 500µl TRIZOL (Sigma-Aldrich). Samples were collected in 1.5ml microcentrifuge tubes (STARLAB) and RNA was either isolated immediately or stored at -80°C prior to extraction. Samples were thawed on ice, 50µl chloroform (Sigma-Aldrich) was added to the homogenate and shaken for 15 seconds prior to incubation on ice (5 minutes). Samples were centrifuged at 10,000 x g for 15 minutes, the supernatant (200µl) was removed and placed in a new microcentrifuge tube. Samples were incubated on ice for 15 minutes following the addition of 200µl isopropanol, samples were then centrifuged at 12,000 x g for 8 minutes. The supernatant was removed and the pellet was washed with 70% ethanol (v/v; 800µl) and centrifuged at 7,500 x g for 15 minutes. This was repeated and pellets were allowed to air dry before being resuspended in 40µl nuclease free water (Ambion by Life Technologies).

2.4.2 Purification of RNA

A DNA digest was performed to remove any DNA contaminants. A typical reaction consisted of 40µl RNA + 5µl 10X DNA1 buffer (Promega, Southampton UK) + 1.5µl nuclease free H2O (Ambion by Life Technologies) + 2.5µl DNase1 (Promega) + 1µl RNAsin (Promega), which was incubated at 37°C for 90 minutes. The reaction was terminated by addition of 10µl 10X termination mix (Promega) + 50µl phenol (Sigma-Aldrich) + 30µl chloroform (Sigma-Aldrich). After centrifugation at 14,000 x g for 10 minutes the top layer (50µl) was extracted and transferred to a new microcentrifuge tube (STARLAB). Centrifugation was repeated after addition of 55µl chloroform (Sigma-Aldrich). The top layer (50µl) was transferred to a new microcentrifuge tube, RNA was precipitated by addition of 5µl 3M sodium acetate pH 4.8 + 150µl 95% ethanol [v/v] + 1µl glycogen (Roche) and incubation on ice for 10 minutes. Samples were centrifuged at 14,000 x g for 15 minutes, the
supernatant was removed and the pellet washed with 50µl 80% ethanol (v/v). Centrifugation was repeated, the supernatant was removed and the pellet was air dried prior to re-suspension in 20µl nuclease free H₂O (Life Technologies). RNA was quantified and the quality determined by assessing the 260/280 ratio using an ND-1000 nanodrop (Thermo Fischer Scientific, Loughborough UK).

### 2.4.3 Reverse transcription of cDNA

RNA was reverse transcribed to cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems by Life Technologies) according to the manufacturer’s instructions. Briefly, 1µg RNA was made up to a total volume of 9µl in nuclease free H₂O (Ambion by Life Technologies) and added to 10µl RT buffer (2X) + 1µl RT enzyme mix (20X). To identify DNA contaminants control samples were prepared without the reverse transcriptase enzyme (-RT). The reaction was performed at 37°C for 1 hour and terminated by heating the samples to 95°C for 5 minutes in a 2720 Thermocycler (Applied Biosystems by Life Technologies), samples were stored at -20°C.

### 2.4.4 Reverse transcriptase PCR

A typical reverse transcriptase PCR reaction contained 1µl cDNA + 4µl nuclease free H₂O + 4µl Reddymix 2X PCR Mastermix (Thermo Scientific) + 50 pmol/µl primer pairs. Primers were purchased from Life Technologies and sequences are supplied in Table 2.4. Control samples were prepared which replaced cDNA with nuclease free H₂O (Ambion by Life Technologies). The following PCR reaction was completed in a 2720 Thermocycler (Applied Biosystems by Life Technologies):

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>05:00</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>00:30</td>
<td></td>
</tr>
<tr>
<td>55/60</td>
<td>00:40</td>
<td>25</td>
</tr>
<tr>
<td>72</td>
<td>00:45</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>07:00</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Continuous</td>
<td>1</td>
</tr>
</tbody>
</table>
Chapter 2  Materials and Methods

Products were separated by agarose gel (2%; Lonza) electrophoresis at 90V for 30 minutes. Fragment size was determined with Hyperladder IV (Bioline, London UK) and visualised using Safeview (NBS Biologicals, Huntingdon UK) and a transilluminator with UV Proplatinum software (UVItec, Cambridge UK).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-tubulin</td>
<td>F: ACATCCAGGCTGGTCAGTGT</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>R: CCCAGGTTCTAGATCCACCA</td>
<td></td>
</tr>
<tr>
<td>CD24</td>
<td>F: TGAAGAACATGTGAGAGTTTGAC</td>
<td>208</td>
</tr>
<tr>
<td></td>
<td>R: GAAAACTGAATCTCCATTCCACAA</td>
<td></td>
</tr>
<tr>
<td>CD44</td>
<td>F: GGCTTTCAATAGCACCTTGC</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>R: ACACCCCTGTTTGTCTTTC</td>
<td></td>
</tr>
<tr>
<td>E-cadherin</td>
<td>F: TGCCCAGAAAATGAAAAAGG</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>R: GTGTATGTGGCAATGCGTTC</td>
<td></td>
</tr>
<tr>
<td>Fibronectin-1</td>
<td>F: CAGTGGGAGACCTGAGAAG</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>R: TCCCTCGGAACATCGAACA</td>
<td></td>
</tr>
<tr>
<td>FOX-C2</td>
<td>F: GCTAAGGACCTGGAGAGC</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>R: TTGACGAAGCCTCGTGTGAG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: ACCCAGAAGACTGGATGGG</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>R: TCTAGAGCGGAGTCAGGTC</td>
<td></td>
</tr>
<tr>
<td>N-cadherin</td>
<td>F: CCAGCGATGGATGAAGAAGCC</td>
<td>438</td>
</tr>
<tr>
<td></td>
<td>R: TTGCAGCCATGCGAAGAC</td>
<td></td>
</tr>
<tr>
<td>SIP1</td>
<td>F: TTCTGGGCTACGACATAC</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>R: TGTGCCCATCAAGCAATTC</td>
<td></td>
</tr>
<tr>
<td>Slug</td>
<td>F: TCGGACCCACACATTACC</td>
<td>528</td>
</tr>
<tr>
<td></td>
<td>R: TCTCTCAATCTAGCCATAGC</td>
<td></td>
</tr>
<tr>
<td>Snail</td>
<td>F: CCTCCGTGCTAGTGGAGGAC</td>
<td>234</td>
</tr>
<tr>
<td></td>
<td>R: CCAGGCTGAGTTATCTTG</td>
<td></td>
</tr>
<tr>
<td>Twist</td>
<td>F: GAGATCCGCAGTCTTACGAG</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>R: TCTGGAGGACCTGGTAGAGG</td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>F: GAGAACCTTGGCGTTGAAGGC</td>
<td>163</td>
</tr>
<tr>
<td></td>
<td>R: GCTTCTGTAGGTTGGCAATTC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4: Reverse transcriptase PCR primer sequences
2.4.5 Real-time quantitative PCR

SYBR Green is a fluorescent dye that intercalates with double-stranded DNA and can, therefore, be used to quantify amplicons. PCR primers were designed using the Universal Probe Library System (Roche; www.roche-applied-science.com) and purchased from Life Technologies or custom designed by Primer Design (Primer Design, Southampton UK). Specificity of primer sequences were confirmed using basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Melt curve analysis was performed and primers used displayed a single peak to indicate amplification of a single PCR product (Figure 2.5).

![Real-time quantitative PCR melt curve analysis](image)

**Figure 2.5: Real-time quantitative PCR melt curve analysis**
An example of an accepted primer that exhibited a melt curve with a single peak to represent amplification of a single product (A), multiple peaks in the melt curve indicated a lack of primer specificity and these primers were rejected (B).

### 2.4.5.1 Primer efficiency

In an ideal, 100% efficient, PCR reaction the number of target amplicons should double after each cycle. To determine primer efficiency a standard curve of template DNA was generated and Ct values were plotted against the log concentration of target DNA (Figure 2.6A). A linear trend line was fitted to the data and the slope of the line was used to calculate primer efficiency using the following calculation:

\[
\text{Efficiency} = 10^{(-1/\text{slope})}
\]
A trend line with a slope of -3.32 would provide an efficiency of 2 (100%), primers with a reaction efficiency of 1.9-2.1 (90-110%) were accepted. Primer efficiencies are listed in Figure 2.6B.

![Graph showing trend line and data points](image)

**Figure 2.6: Determination of primer efficiency**
Threshold cycle values were plotted against a standard curve of template DNA and trendlines fitted to the data, human IGFBP-3 is shown as an example (A). The slope of the trendline was used to calculate efficiency of primer pairs using Efficiency=10^{-1/Slope}, primers with a reaction efficiency of 1.9-2.1 (90-110%) were accepted (B).
### 2.4.5.2 Real-time quantitative PCR reaction

Each PCR reaction contained 12.5µl SYBR Green PCR mastermix (Sigma-Aldrich) + 300nM primer pairs + 2ng/µl cDNA + RNAse free H₂O to a total volume of 25µl in 96-well MicroAmp Optical Reaction plates (Applied Biosystems by Life Technologies). Primer sequences are listed in Table 2.5. Samples lacking the reverse transcriptase enzyme were run alongside to eliminate the possibility of detecting contaminating genomic DNA. Samples were analysed from three independent biological replicates and technical replicates were performed in triplicate for each condition. Plates were analysed using StepOnePlus™ Real-time PCR system (Applied Biosystems by Life Technologies) using the following amplification protocol:

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (minutes)</th>
<th>Function</th>
<th>No. cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>10:00</td>
<td>Initial denaturation</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>00:30</td>
<td>Denaturation</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>00:30</td>
<td>Annealing</td>
<td>40</td>
</tr>
<tr>
<td>72</td>
<td>00:35</td>
<td>Extension and data collection</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10:00</td>
<td>Final extension</td>
<td>1</td>
</tr>
<tr>
<td>95</td>
<td>00:15</td>
<td>Dissociation/Melt curve analysis</td>
<td>1</td>
</tr>
<tr>
<td>60</td>
<td>01:00</td>
<td>Dissociation/Melt curve analysis</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>00:15</td>
<td>Dissociation/Melt curve analysis</td>
<td></td>
</tr>
</tbody>
</table>

### 2.4.5.3 Analysis

Gene expression was assessed by analysing threshold cycle (Ct) values generated by the PCR system. The Ct value represents the cycle at which SYBR Green fluorescence reaches a pre-determined threshold, hence, lower Ct values represent higher target expression. Expression of target genes were determined alongside multiple reference genes (endogenous controls), which displayed a mean standard deviation of <0.5 Ct between all experimental conditions. The relative fold change for each gene was calculated using the ΔΔCt method, where ΔΔCt = (Ct gene − Ct reference gene)_{test sample} − (Ct gene − Ct reference gene)_{control sample}. Expression of
target genes were normalised to multiple reference genes by calculating the mean of the reference genes (Vandesompele et al., 2002). The relative amount of unknown in a test sample relative to the amount of unknown in a control sample was calculated by $2^{-\Delta\Delta C_{T}}$. Data are represented as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD24</strong></td>
<td>F: TAAAAGTGGGCTTGATTCTGC</td>
<td>85</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>R: ACTTGGGGAGTGTCTATTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CD44</strong></td>
<td>F: TCCCACCTCAGACCCACTCA</td>
<td>112</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>R: GGATGGAAAAACCTTGGACAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E-cadherin</strong></td>
<td>F: CATGAGTGTCCTCCCGGTATC</td>
<td>89</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>R: CAGTATCAGCCGCTTTCAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EMP-1</strong></td>
<td>F: GAGGGCAAGGCCACAAATTA</td>
<td>76</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>R: ACAAAGTTTACCTCCCACAGTTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EP300</strong></td>
<td>F: TCTGTGAAGTGCGTCTCCAA</td>
<td>77</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>R: GCGGCCTTAAACTCTCATCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>F: ACCCAGAAGACTGTGGATGG</td>
<td>200</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>R: TCTAGACGCAGGTGTCGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IGFBP3</strong></td>
<td>F: GTCAACGCTAGTGCCGTCAG</td>
<td>175</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>R: GGTGGAACCTTGGGATCAGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S100A9</strong></td>
<td>F: GTGCAGGAAGATCTGCAAAA</td>
<td>103</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>R: TCAGCTGCTTGTCTGCATTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Slug</strong></td>
<td>F: ACTCCGAAAGCCAAATGACAAA</td>
<td>119</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>R: CTCTCTCTGGGTGTTGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vimentin</strong></td>
<td>F: TTCTCTGCTCCTCCAAAACCTTT</td>
<td>137</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>R: CGTTGATAACCTGGCAGCCTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>YWHAZ</strong></td>
<td>F: ACTTTTGTTACATTGGGCTTCAA</td>
<td>94</td>
<td>Human</td>
</tr>
<tr>
<td></td>
<td>R: CGGAGGACAAACACAGTAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>18S rRNA</strong></td>
<td>F: GTAACCGGTGAAACCCATT</td>
<td>68</td>
<td>Human &amp; Mouse</td>
</tr>
<tr>
<td></td>
<td>R: CCATCCAATGGGATAGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MrpL19</strong></td>
<td>F: ACCCCTATGCACGGATGGAAA</td>
<td>60</td>
<td>Mouse</td>
</tr>
<tr>
<td></td>
<td>R: TCCCTGATCCTTGATGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IGFBP3</strong></td>
<td>F: CGCGAGAGAATGGAGGACA</td>
<td>139</td>
<td>Mouse</td>
</tr>
<tr>
<td></td>
<td>R: CGCTTTCTGCCTTGGAGAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EMP-1</strong></td>
<td>F: GGAAGAATCTGACTGGTGTT</td>
<td>167</td>
<td>Mouse</td>
</tr>
<tr>
<td></td>
<td>R: GAACCGGTTTCCTTCCTCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5: Real-time quantitative PCR primer sequences
Chapter 2 Materials and Methods

2.5 Western blot

2.5.1 Sample preparation

Western blot analysis was performed to resolve expressed proteins by molecular weight. Whole cell lysates were harvested with Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Sigma-Aldrich [50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS]) containing a protease cocktail inhibitor tablet (Roche). Samples were incubated on ice for 30 minutes prior to centrifugation at 14,000 x g for 5 minutes, cell debris was discarded and cell lysate was stored at -80°C if not used immediately. For analysis of phospho-proteins, small molecules were added in cell culture medium and incubated for 30 minutes at 37°C with 5% CO₂. Small molecules and media were replenished and incubated for a further 30 minutes, cells were washed with ice-cold PBS prior to harvesting cell lysates.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Separating gel</td>
<td>3.3ml 30% acrylamide/bis-acrylamide solution (Bio-Rad) + 2.5ml 1.5M TrisHCl pH 8.8 / 0.4% (w/v) SDS + 4.2ml dH₂O + 50µl 10% (v/v) APS + 10µl TEMED</td>
</tr>
<tr>
<td>Stacking gel</td>
<td>670µl 30% acrylamide/bis-acrylamide solution + 1.0ml 0.5M TrisHCl pH 6.8 /0.4% (w/v) SDS + 2.3ml dH₂O +30µl 10% (v/v) APS + 5µl TEMED</td>
</tr>
</tbody>
</table>

Table 2.6: Western blot 10% gel components

2.5.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Acrylamide (10%) gels with a top stacking region were cast using reagents described in Table 2.6. For detection of p300 experiments were performed using 8% acrylamide gels and the concentration of reagents described in Table 2.6 were adjusted accordingly. Cell lysates were mixed 1:1 with 2X Laemmli buffer (Sigma Aldrich [4% Sodium dodecyl sulphate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125M TrisHCl, pH 6.8]) and incubated for 5 minutes at 95°C to denature proteins and provide an overall negative charge. Lysates from 50,000 cells were loaded into gels inserted into the Mini-PROTEAN tank (Bio-Rad
Laboratories, Hertfordshire UK) filled with 500ml running buffer (25mM Tris base + 190mM glycine + 0.1% [w/v] SDS; all Sigma-Aldrich). ColorPlus pre-stained protein standards (New England BioLabs, Hertfordshire UK) were loaded beside protein lysates to determine protein molecular weight and confirm antibody specificity. Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 100V through the stacking gel, the voltage was then increased to 125V until the bromophenol dye front reached the bottom of the gel.

2.5.3 Membrane transfer

Proteins were electrotransferred from the SDS gel to Amersham Hybond ECL nitrocellulose membrane (GE Healthcare, Buckinghamshire UK) using a Trans blot SD20 blotter (Jencons, West Sussex UK). Membrane and 12 pieces of filter paper (Sigma-Aldrich) were pre-soaked in cold transfer buffer (25mM Tris base [w/v] + 190mM [w/v] glycine + 20% [v/v] methanol), the membrane was then applied to the SDS gel and sandwiched between the filter paper. The transfer was performed at 15V for 60 minutes. For detection of p300 proteins were transferred using a Mini Trans-Blot (Bio-Rad Laboratories), the gel and membrane were sandwiched between 6 pieces of filter paper and loaded into the Mini-PROTEAN filled with transfer buffer (25mM Tris base [w/v] + 190mM [w/v] glycine + 0.1% [w/v] SDS + 10% [v/v] methanol). The transfer was performed at 60V for 2 hours.

2.5.4 Antibody incubation and visualisation

The membrane was incubated in blocking buffer for 1 hour prior to antibody addition in blocking buffer (Table 2.7), antibodies were incubated overnight at 4°C on a platform rocker. Membranes were washed 3X with TBS-T (20mM Tris base + 500mM NaCl + 10% [v/v] Tween20; all Sigma-Aldrich) for 5 minutes prior to addition of relevant HRP-conjugated secondary antibodies (Table 2.7) in blocking buffer for 60 minutes. Membranes were washed 3X with TBS-T for 5 minutes and chemiluminescence ECL detection reagents (GE Healthcare) were used to visualise
protein bands. The membrane was exposed to an autoradiographic film in an X-ray cassette and manually developed using Kodak GBX developer and fixer solutions (Sigma Aldrich).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-alpha tubulin IgG₁</td>
<td>Sigma-Aldrich</td>
<td>1:2000</td>
</tr>
<tr>
<td>Rabbit anti-Vimentin IgG</td>
<td>Abcam</td>
<td>1:4000</td>
</tr>
<tr>
<td>Rabbit anti N-cadherin IgG</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-Fibronectin-1 IgG</td>
<td>Abcam</td>
<td>1:2000</td>
</tr>
<tr>
<td>Rabbit anti-beta-catenin IgG</td>
<td>Abcam</td>
<td>1:2000</td>
</tr>
<tr>
<td>Chicken anti-Cytokeratin-8 Igγ</td>
<td>Abcam</td>
<td>1:10000</td>
</tr>
<tr>
<td>Mouse anti-E-cadherin SHE78.7 IgG₂a</td>
<td>Invitrogen</td>
<td>Antibody prepared to 500µg/ml 1:500</td>
</tr>
<tr>
<td>Rabbit anti-CD44 IgG</td>
<td>Abcam</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-CD24 IgG</td>
<td>Santa Cruz Biotechnology</td>
<td>1:200</td>
</tr>
<tr>
<td>Rabbit anti-alpha-tubulin IgG</td>
<td>Abcam</td>
<td>1:5000</td>
</tr>
<tr>
<td>Rabbit anti-phospho-Akt (Ser473) IgG</td>
<td>Cell Signaling Technology</td>
<td>1:500</td>
</tr>
<tr>
<td>Rabbit anti-Akt IgG</td>
<td>Cell Signaling Technology</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-EMP-1 IgG</td>
<td>Abcam</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse anti-p53 IgG₁</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat anti-rabbit IgG HRP</td>
<td>DAKO</td>
<td>1:2000</td>
</tr>
<tr>
<td>Goat anti-mouse IgG HRP</td>
<td>DAKO</td>
<td>1:2000</td>
</tr>
<tr>
<td>Anti-Mouse IgG1 VHH Single Domain Antibody HRP</td>
<td>Abcam</td>
<td>1:10000</td>
</tr>
<tr>
<td>Rabbit anti-chicken Igγ</td>
<td>Abcam</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Table 2.7: Antibodies used for Western blot

2.5.5 Densitometry

Developed X-ray films were imaged using an Intelligent dark box II (Fujifilm, Bedford UK) with an LAS-1000 camera (Fujifilm) using Image reader LAS-1000 Pro v.2.6 software. Pixel density was determined using Aida image analyser v3.44 and background pixel density was subtracted from sample density values.
2.6 Apoptosis protein arrays

2.6.1 Sample preparation

MCF-7 cells were cultured with E-cadherin nAb, nPep or relevant controls for 3 days. Expression of apoptosis-related proteins was determined using a Proteome Profiler Human Apoptosis Array Kit (R&D Systems, Abingdon UK) according to the manufacturer’s instructions. Whole cell lysates were harvested at $1 \times 10^7$ cells/ml with Lysis buffer 15 (R&D Systems) containing a protease cocktail inhibitor tablet (Roche). Lysates were rocked at 4°C for 30 minutes prior to centrifugation at 14,000 x g for 5 minutes. The supernatant was transferred into a new micro-centrifuge tube and stored at -80°C.

2.6.2 Array procedure

All reagents were provided with Proteome Profiler Human Apoptosis Array Kit (R&D Systems) unless otherwise specified and prepared according to the manufacturer’s instructions. Arrays were blocked with Array Buffer 1 for 1 hour on a platform rocker prior to addition of protein lysates. Protein samples were quantified using the Quick Start™ Bradford protein assay (Bio-Rad Laboratories, Hertfordshire UK) and each array was incubated with 275µg protein mixed with Array Buffer 1 to a final volume of 1.5ml. Lysates were incubated overnight on a rocking platform at 4°C. Arrays were washed 3X with 1X wash buffer (20ml) for 10 minutes. Detection Antibody Cocktail was reconstituted in Array Buffer 2/3, of which 1.5ml was added to each array and incubated on a rocking platform for 1 hour at room temperature. Arrays were washed 3X with 1X wash buffer (20ml) for 10 minutes. Streptavidin-HRP was diluted in Array Buffer 2/3, of which 2ml was incubated with each array for 30 minutes on a rocking platform at room temperature. Arrays were washed 3X with 1X wash buffer (20ml) for 10 minutes and chemiluminescence reagents were applied to membranes to detect protein binding. Membranes were exposed to autoradiographic film in an X-ray cassette and manually developed using Kodak GBX developer and fixer solutions (Sigma-Aldrich).
2.6.3 Data analysis

Developed X-ray films were imaged and pixel density determined as described in section 2.5.5. Mean pixel density, standard deviation and standard error of the mean were calculated for replicate spots and relative pixel density was calculated to compare control and E-cadherin neutralised samples. Mean relative fold change was calculated by neutralised pixel density/control pixel density and plotted graphically, results are displayed as Mean±SEM from 3 biological replicates.

2.7 Whole genome microarray analysis

MCF-7 cells were cultured in 6-well plates with a normal mouse IgG cAb or E-cadherin SHE78-7 nAb at a final concentration of 2µg/ml in cell culture media for a total of 3 days. RNA was harvested and purified as described in section 2.4. Microarray and bioinformatics analysis were carried out by the Genomic Technologies Core Facility (The University of Manchester, UK).

2.7.4 Array procedure

RNA quality was checked using the RNA 6000 Nano Assay, and analysed on an Agilent 2100 Bioanalyser (Agilent Technologies, Stockport UK). Quantification of RNA was carried out using a Nanodrop ultra-low-volume spectrophotometer (Nanodrop Technologies by Thermo Fisher Scientific). Affymetrix Human Genome U133 Plus 2.0 Array GeneChips were run according to the manufacturer’s instructions. cDNA synthesis was performed using a two cycle synthesis kit from 10ng RNA, biotin-labelled cRNA was synthesised from cDNA by performing an in vitro transcription (IVT) reaction. cRNA was cleaned up and quantified prior to fragmentation of 15ng cRNA at 95°C for 35 minutes in fragmentation buffer. Hybridization cocktails containing the fragmented and labelled cRNA were prepared according to the manufacturer’s instructions and allowed to hybridise with Affymetrix Human Genome U133 Plus 2.0 Array GeneChips on a rotational platform at 45°C. After 16 hours, the GeneChips were washed and stained with Streptavidin.
Phycoerythrin (SAPE) in an automated process using a fluidics station. The GeneChip Scanner 3000 (Agilent Technologies) and GeneChip Operating Software (GCOS) were used to analyse the GeneChips.

2.7.5 Bioinformatics analysis

Quality control and outlier analysis was performed with dChip (v2005) ([www.dchip.org](http://www.dchip.org)) using the default settings (Li and Wong, 2001). Background correction, quantile normalization, and gene expression analysis were performed using RMA in Bioconductor (Bolstad et al., 2003). Principle components analysis (PCA) was performed with Partek Genomics Solution v6.5 Copyright 2010 (Partek Inc., St. Charles, MO, USA) to establish relationships and compare variability (Quackenbush, 2001). Differential expression between cAb and nAb-treated samples was determined with Limma using the functions lmFit and eBayes (Smyth, 2004). False discovery rate of differentially expressed genes were controlled for using the method of QVALUE (Storey and Tibshirani, 2003).

2.7.6 Analysis of significantly altered transcripts

A threshold of \( p<0.05 \) and a fold-change of \( \geq \pm 1.2 \) were selected as statistically significant. Significantly altered genes were compared using Venny (Oliveros, 2007-2015). The PANTHER (Protein Analysis THrough Evolutionary Relationships) Classification System was used to classify significant gene transcript alterations according to biological processes using Gene Ontology Classification terms (Mi et al., 2013, The Gene Ontology Consortium, 2013). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Huang et al., 2008, Huang et al., 2009). Canonical pathway analysis was performed using QIAGEN’s Ingenuity® Pathway Analysis (IPA®; QIAGEN Redwood City). Network analysis was performed using Cytoscape v2.8.3 with the Moduland plugin and BioGRID v3.2.117 as a reference to identify overlapping network modules and
assign modular hierarchy (Szalay-Bekő et al., 2012). Further interrogation of the network was performed using IPA®.

2.8 Imagen Biotech drug screen

Cell count and cell viability assays were performed by Imagen Biotech (Cheshire, UK) following treatment of MCF-7 cells with small molecule cancer therapeutics. Cells were seeded at a density 500 cells/well in 384-well plates and incubated with E-cadherin neutralising peptide or H₂O as a vehicle control for 2 days prior to addition of small molecules (10⁻¹⁻¹⁻³nM) or an equivalent volume of DMSO. DRAQ7 is a fluorescent marker of viability that binds nuclear double-stranded DNA in cells with a permeated membrane, which can represent cytotoxicity induced by small molecules. After 3 days cells were stained with 0.6µM DRAQ7 and nuclei were counter stained with 20µg/ml Hoechst 33342 (Sigma-Aldrich) in PBS for 30 minutes. Cells were washed 1X with PBS and fixed with 0.1% formaldehyde (v/v) immediately before reading. Staining was visualised using a Cellomics® ArrayScan® HCS Reader (Thermo Scientific), a single image was acquired from the well centre at 5X magnification from 2 channels (channel 1 [386nmEM], channel 2 [650nmEM]). Image analysis was performed using the Compartmental Analysis Bio-Application protocols (V2 Version; Thermo Scientific), cells were identified by positive Hoechst 33342 staining and DRAQ7 fluorescence intensity was measured in the cellular region. Samples were analysed in quadruplicate, cell number and percentage of DRAQ7 positive cells were plotted graphically.
2.9 Plasma membrane protein isolation

Cell membrane lysates were harvested using a Pierce cell surface protein isolation kit (Thermo Fischer Scientific) according to the manufacturer’s instructions, materials and reagents were provided with the kit unless otherwise specified. MCF-7 cells were grown in 4X T75 cm$^2$ cell culture flasks (Corning) with E-cadherin neutralising peptide or an equivalent volume of H$_2$O (vehicle control) for 3 days.

2.9.7 Biotinylation and lysis of proteins

Cell culture media was removed and cells were washed X2 with ice-cold PBS. Following removal of PBS, sulfo-succinimidyl-2-(biotinamido)ethyl-1,1,3-dithiopropionate (Sulfo-NHS-SS) Biotin solution (10 ml/flask) was added to cells and incubated for 20 minutes at 4°C on a platform rocker. The reaction was quenched with Quenching solution (500µl/flask) and cells were scraped into solution. The cell suspension was transferred into a single 50ml centrifuge tube (Corning) and all four flasks were washed with 10ml TBS, which was then added to the cell suspension and centrifuged at 500 x g for 3 minutes. The supernatant was discarded and 500µl Lysis buffer containing a protease inhibitor cocktail tablet (Roche) was added to the cell pellet. Low power sonication was performed and samples were incubated on ice for 30 minutes, vortexing at 5 minute intervals and an additional sonication after 15 minutes were performed during incubation. Lysates were centrifuged at 10,000 x g for 2 minutes at 4°C and the supernatant transferred to a new tube.

2.9.8 Isolation of biotin labelled proteins

NeutraAvidin Agarose resin (500µl) was added to a column and centrifuged at 1000 x g for 1 minute, the flow through was discarded from the collection tube. The column was washed 2X with wash buffer and centrifuged at 1000 x g for 1 minute. Cell lysate was added to the column, which was capped and incubated on a rocking platform for 60 minutes at room temperature to isolate Sulfo-NHS-SS-Biotin bound proteins. Column was centrifuged at 1000 x g for 1 minute, the flow through
containing non-biotinylated (intracellular) proteins was collected in a microcentrifuge tube (STARLAB) and stored at -20°C.

### 2.9.9 Elution of biotinylated proteins

The column was washed 3X with 500µl wash buffer containing protease inhibitor cocktail tablet and centrifuged at 1000 x g for 1 minute. To release the Sulfo-NHS-SS-Biotin bound proteins, 400µl SDS-PAGE sample buffer containing 50mM Dithiothreitol (DTT) was added to the capped column and incubated on a rocking platform for 60 minutes at room temperature. The un-capped column was placed in a new collection tube and cell membrane proteins were eluted by centrifugation at 1000 x g for 2 minutes. Protein lysates were heated to 95°C for 5 minutes and stored at -20°C.

### 2.9.10 Mass spectrometry analysis

Polyacrylamide gel electrophoresis was performed as previously described for western blot analysis, triplicate samples for each condition were loaded into 10% acrylamide gels without a stacking region and the current was applied for 5 minutes. Gels were stained with Coomassie blue to visualise protein bands. Subsequent analysis was performed by the Protein Mass Spectrometry Core Facility (The University of Manchester) using the following procedure. Protein bands were excised and dehydrated with acetonitrile and subjected to vacuum centrifugation. Reduction and alkylation of dried gel pieces was achieved using 10mM DTT and 55mM iodoacetamide respectively. Alternate washes were performed with 25mM ammonium bicarbonate and acetonitrile, which was repeated prior to vacuum centrifugation to dry gel pieces. Trypsin digestion was performed overnight at 37°C.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on digested samples using an UltiMate® 3000 Rapid Separation LC (Dionex Corporation, Sunnyvale, CA) coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Peptide mixtures were separated using a gradient from
92% A (0.1% formic acid [FA] in water) and 8% B (0.1% FA in acetonitrile) to 33% B, in 120 min at a flow rate of 300 nL min⁻¹, using a 250 mm x 75 μm i.d. 1.7μM Ethylene Bridged Hybrid C18, analytical column (Waters, Hertfordshire UK). Peptides were selected for fragmentation automatically by data dependant analysis.

2.9.11 Data analysis

Mass spectrometry data were searched using Mascot v.2.2.06 (Matrix Science, London UK), against The UniProt Knowledgebase (UniProtKB) database v.2013-05 with taxonomy of human selected. Samples were scored according to the matched number of peptides:

- 1 Possible identification
- 2-3 Probable identification
- ≥4 Almost certain identification

Proteins that were present in control samples and absent in test samples or vice versa with a score of ≥2 were selected for further investigation. Mass spectrometry results were confirmed by performing western blot analysis on cell membrane lysates or cell-surface flow cytometry.

2.10 Statistical analysis

Data from control and E-cadherin neutralised samples were statistically analysed using an un-paired Student’s t-test. Where 3 or more conditions exist data were statistically analysed using a One-way ANOVA and the means were compared using Tukey’s comparison test. Statistical significance was set at p<0.05 for all experiments. Data are expressed as p<0.05*, p<0.01**, p<0.001*** and p<0.0001****.
Chapter 3. Exogenous inhibition of E-cadherin in MCF-7 cells is insufficient to induce EMT or acquisition of a CD24<sub>low</sub>/CD44<sub>high</sub> CSC phenotype.
3.1 Introduction

Epithelial architecture is highly organised and lateral adhesion is mediated by the single pass transmembrane glycoprotein E-cadherin, encoded by the CDH1 gene. As a major component of adherens junctions, E-cadherin forms homophilic interactions with E-cadherin molecules on adjacent cells and at the intracellular level binds multiple proteins, including catenins (α, β and p120) and epithelial lost in neoplasms, which form the cytoplasmic cell adhesion complex and link E-cadherin to the actin cytoskeleton (Cavallaro and Christofori, 2004). Epithelial cells typically display apical-basal polarity and are anchored to the basal lamina, which restricts mobility. In contrast, mesenchymal cells exhibit front-back polarity and express less adhesive N-cadherin, Vimentin and Fibronectin. Epithelial-mesenchymal transition (EMT) is a normal physiological process that occurs during embryonic development and has also been proposed as a theory to explain the metastatic spread of epithelial tumours, which correlates with increased aggressiveness and poor patient prognosis (Thiery, 2002). During EMT, epithelial cells lose their epithelial characteristics by down-regulation of E-cadherin protein and acquire more motile mesenchymal characteristics, which may enable carcinoma cells to break away from the primary tumour, invade the basement membrane and enter the circulation to disseminate at secondary sites (Yu et al., 2013). Interestingly, despite the requirement for loss of E-cadherin protein expression in tumour metastasis the gene is mutated in few cancers (Berx et al., 1998). Acquisition of a cancer stem cell (CSC) phenotype has been linked to EMT following activation of the Ras-MAPK pathway (Morel et al., 2008). Similarly, Mani et al. (2008) described the induction of EMT and acquisition of a CSC phenotype following ectopic expression of Twist or Snail in human immortalised mammary epithelial (HMLE) cells.

Additional roles for E-cadherin have emerged since its discovery as a cell adhesion protein, for example, data from our lab has shown that inhibition of E-cadherin protein in mouse embryonic stem cells results in increased proliferation of the cells and alterations in >2000 gene transcripts associated with a range of biological functions (Soncin et al., 2011). Similarly, recent derivation of E-cadherin negative
proliferating stem (ENPS) cells demonstrated >2000 gene transcript alterations, many of which were associated with tumorigenic events, such as neoplasia and metastasis (Hawkins et al., 2014). In addition, inhibition of E-cadherin mediated cell-cell contact in mES cells through genetic depletion, RNA interference or treatment with a neutralising peptide has been linked to altered self-renewal signalling pathways in these cells (Soncin et al., 2009). However, inhibition of E-cadherin in both mouse and human ES cells does not induce an EMT event (Eastham et al., 2007, Spencer et al., 2007). The lack of EMT upon loss of E-cadherin in ES cells is in contrast to several studies performed in adult carcinoma cell lines. Andersen et al. (2005) concluded that EMT could be induced in the human vulvar epidermoid adenocarcinoma cell line A431 by expression of a dominant negative mutant form of E-cadherin, although this was described as a slow and gradual process. Onder et al (2008) found that inhibition of E-cadherin using RNAi in immortalised HMLE cells induced EMT, invasiveness and anoikis resistance and led to upregulation of the E-cadherin repressor Twist.

A further process in which cell surface E-cadherin protein is regulated is via metalloproteinases. For example, E-cadherin is proteolytically cleaved by ADAM10, resulting in shedding of a soluble 80kDa ectodomain fragment (sE-cad) and loss of E-cadherin mediated cell-cell contact (Maretzky et al., 2005). The sE-cad fragment retains the ability to interact with full length E-cadherin molecules on neighbouring cells, thus preventing formation of a functional adherens junction, leading to activation of the epidermal growth factor receptor (Inge et al., 2011). However, sE-cad has also been observed to bind cells lacking cell surface E-cadherin protein and promote tumour growth, motility and invasion, the latter via upregulation of matrix metalloproteinase-2 (MMP-2) and MMP-9 (Brouxhon et al., 2014b). Therefore, the result of exogenous abrogation of cell surface E-cadherin protein alone in tumour cells has not been elucidated.

A role for E-cadherin in early tumorigenesis has recently been proposed, the dysregulation of E-cadherin in neoplasia and tumorigenesis (DENT) hypothesis, in which exogenous inhibition of E-cadherin via inhibiting binding proteins, altered
growth factor expression and/or sE-cad protein fragments results in increased proliferation of the cells and acquisition of a CSC phenotype in the absence of EMT (Mohamet et al., 2011). In this study the exogenous inhibition of E-cadherin using the E-cadherin neutralising antibody SHE78.7 has been investigated. The data show this antibody binds only to E-cadherin, in the MCF-7 breast cancer cell line, which was found to be one of few cell lines to exhibit a characteristic epithelial phenotype and lack of EMT marker expression, to assess induction of EMT, cell survival and CSC formation.
3.2 Results

3.2.1 Treatment of MCF-7 cells with E-cadherin SHE78.7 neutralising antibody results in reversible inhibition of cell-cell contact

The breast cancer cell line MCF-7, the non-tumorigenic mammary epithelial cell line MCF-10A and the lung epithelial cell line A549 were assessed for epithelial morphology. Only MCF-7 cells exhibited characteristic epithelial morphology, expression of E-cadherin and the epithelial cell-specific intermediate filament protein Cytokeratin-8, and absence of N-cadherin, CD44, Vimentin and Fibronectin proteins (Figure 3A[i-v]) and were therefore utilised for further study. MCF-10A and A549 cells were excluded from further study due to expression of N-cadherin (Figure S3.1). The E-cadherin SHE78.7 neutralising antibody (nAb) was assessed by western blot in MCF-7 cells to confirm immunoreactivity and specificity with E-cadherin protein (Figure 3B). Treatment of MCF-7 cells with the E-cadherin nAb induced loss of cell-cell contact (Figure 3C[i]) but did not repress cell surface E-cadherin protein (Figure 3C[ii]-[iii]) or transcript (Figure 1C[iv-v]) expression. Exposure of MCF-7 cells to the E-cadherin nAb for up to 50 days maintained inhibition of E-cadherin mediated cell-cell contacts (Figure 3D; +cAb/+nAb). At days 20, 30, 40 and 50 following exposure of MCF-7 cells to cAb/nAb, the treatment was removed (Figure 3D; -cAb/-nAb) and the post-nAb treated cells reverted to an adherent ‘cobblestone-like’ morphology. These results demonstrate that exposure of MCF-7 cells to nAb inhibits cell-cell contact but does not repress E-cadherin transcript or protein expression.
**Figure 3.1: E-cadherin SHE78.7 neutralising antibody mediates reversible inhibition of E-cadherin mediated cell-cell contacts in MCF-7 cells**

MCF-7 cells were characterised for presence of epithelial E-cadherin and cytokeratin-8 proteins, absence of mesenchymal proteins N-cadherin, Fibronectin-1 (FN-1), Vimentin and cancer stem cell phenotype CD24\textsuperscript{low}/CD44\textsuperscript{high} by flow cytometry (A[i-iv]) and western blot (A[v]). Specificity of E-cadherin SHE78.7 neutralising antibody (nAb) was confirmed by western blot, which produced a single band representing the molecular weight of full length E-cadherin (B). Application of SHE78.7 nAb inhibited E-cadherin mediated cell-cell contact, phase contrast images displayed disaggregation of nAb but not control antibody (cAb)-treated MCF-7 cells (C[i]), Images acquired using a 20X objective, scale bars represent 50μm. Immunofluorescence staining revealed expression of E-cadherin at the cell membrane (C[iii]) on day 3. Images acquired using a 40X objective, scale bars represent 50μm. Cell surface expression of E-cadherin was confirmed by flow cytometry (C[iii]) and gene transcript expression was determined by reverse transcriptase (C[iv]) and quantitative (C[v]) PCR. Addition of nAb inhibited E-cadherin cell-cell contact up to 50 days, upon removal of cAb/nAb (-cAb/-nAb) at specified time-points cell-cell contacts were re-established in -nAb populations (D).
3.2.2 Prolonged exposure of MCF-7 cells to an exogenous E-cadherin inhibitor is insufficient to induce EMT or a CSC phenotype.

To determine whether exogenous inhibition of E-cadherin in MCF-7 cells could induce an EMT phenotype, expression of epithelial and mesenchymal markers were assessed between 3 and 50 days of induction of cAb/nAb treatment. At each time point assessed cAb and nAb treated MCF-7 cells populations expressed epithelial E-cadherin and Cytokeratin-8 and lacked expression of mesenchymal-associated proteins N-cadherin, Vimentin and Fibronectin-1, as determined by flow cytometry (Figure 3.2A[i]) and western blot (Figure 3.2A[ii]). RT-PCR analysis of epithelial and EMT-associated gene transcripts revealed no significant alterations between cAb- and nAb-treated MCF-7 cells throughout the 50d time period (Figure 3.2B). Expression of Fibronectin-1 (FN1) and Vimentin were also undetected in cAb/nAb-treated populations by immunofluorescence (Figure 3.2C[i-iv]). Since β-catenin is required for E-cadherin mediated EMT (Onder et al., 2008), the cellular localisation of β-catenin protein was assessed using immunofluorescence analysis (Figure 3.3A) and total protein expression levels were determined by western blot (Figure 3.3B) in MCF-7 treated cells. β-catenin was concentrated at the cell membrane in both cAb- and nAb-treated cells and no differences in total protein expression were detected.
Figure 3.2: Exogenous inhibition of E-cadherin is insufficient to induce an EMT event within 50 days

Presence of epithelial E-cadherin (E-cad) and absence of mesenchymal N-cadherin (N-cad) cell surface proteins were observed by flow cytometry in cAb and nAb-treated MCF-7 cells after 3-50 days (A). Expression of E-cadherin and Cytokeratin 8 and absence of N-cadherin, Vimentin and Fibronectin-1 were detected by western blot in cAb and nAb-treated populations after 3-50 days (B). Gene transcript analysis failed to detect loss of epithelial and gain of mesenchymal transcripts within 50 days of nAb treatment, GAPDH and β-tubulin gene expression were determined as endogenous controls. (C). Vimentin and Fibronectin-1 (FN1) were absent by immunofluorescence in cAb and nAb-treated MCF-7 cells (D[i-iii]), bone marrow mesenchymal stem cells (D[iii]) and MDA-MB-231 (D[iv]) were used as a positive control for immunofluorescence detection of FN1 and Vimentin respectively. Images were acquired with a 20X objective, scale bars represent 100µm.
Mani et al. (2008) have demonstrated induction of EMT and acquisition of a CSC phenotype following ectopic expression of Twist or Snail in immortalised human mammary epithelial (HMLE) cells. Untreated HMLE cells were characterised as CD44<sup>low</sup>/CD24<sup>high</sup> and, upon EMT induction with Twist or Snail, exhibited a CD44<sup>high</sup>/CD24<sup>low</sup> CSC phenotype. The DENT hypothesis suggests that this transformation to a CSC phenotype may be a direct result of E-cadherin repression in these cells. To test this hypothesis, expression of CD24 and CD44 was assessed in MCF-7 cells treated with cAb or nAb over 50 days using flow cytometry (Figure 3.4A) and western blot (Figure 3.4B) analysis. Both cAb- and nAb-treated MCF-7 cells exhibited a cell surface CD44<sup>low</sup>/CD24<sup>high</sup> expression profile throughout the time period (Figure 3.4A), and this was confirmed in the western blot analysis (Figure 3.4B), with no evidence of transition to a CSC phenotype. In addition, gene
transcript analysis revealed no changes in CD24 or CD44 gene transcription following treatment with cAb or nAb (Figure 3.4C).

Figure 3.4: Exogenous inhibition of E-cadherin is insufficient to induce acquisition of a CD24\textsuperscript{low}/CD44\textsuperscript{high} CSC phenotype within 50 days
Application of E-cadherin SHE78.7 nAb for 3-50 days failed to induce a CD24\textsuperscript{low}/CD44\textsuperscript{high} cancer stem cell (CSC) phenotype as determined by flow cytometry (A), western blot (B) and gene transcript expression (C). Data are representative of 3 biological replicates (n=3).
3.2.3 E-cadherin SHE78.7-treated MCF-7 cells exhibit increased cell numbers in adherent monolayer and suspension culture

Proliferation of cAb or nAb-treated MCF-7 cells was monitored over 9 days in adherent culture and revealed a statistically significant increase in cell numbers in nAb-treated populations from 3 days (Figure 3.5A[i]), which was maintained throughout the duration of the experiment. Population doubling times of nAb-treated cells was 30.4±0.46h compared to 33.7±0.63h for cAb-treated cells. Removal of nAb (-nAb) or cAb (-cAb) after 9 days of treatment led to a reduction in cell numbers in –nAb cells proliferation to that of control cells (-cAb), with a decreased population doubling time of 35.1±0.7h (Figure 3.5A[iii]).

To determine whether the increased cell numbers observed in nAb-treated MCF-7 populations in adherent culture was due to increased cell proliferation, expression of ki67 (Figure 3.5B), a proliferative marker, and analysis of the cell cycle was performed (Figure 3.5C[i-ii]). No increased ki67 expression was observed in nAb-treated MCF-7 cells compared to controls (Figure 3.5B) and no significant differences were observed in the percentage of cells in each phase of the cell cycle (Figure 3.5C[i-ii]). Increased expression of p27^Kip1 has been reported in thyroid carcinoma cells responsive to contact inhibition and reduced expression of p27^Kip1 was described following application of E-cadherin SHE78.7 nAb (Motti et al., 2005). Analysis of p27^Kip1 expression in cAb and nAb treated MCF-7 cells using fluorescent flow cytometry (Figure 3.5D[i]), western blot (Figure 3.5D[ii]) and immunofluorescence analysis (Figure 3.5D[iii]) revealed no evidence of decreased p27^Kip1 protein expression or altered cellular localisation in nAb-treated cells.
Figure 3.5: Exogenous inhibition of E-cadherin increases growth and survival of MCF-7 cells in monolayer culture within 3 days

MCF-7 cells were seeded at equal densities with either cAb or nAb and cell counts were obtained every 3 days for a total of 9 days (A), cAb and nAb treatments were removed after 9 days and cell counts were obtained every 3 days for a total of 9 days (B). Detection of ki67 (B) and cell cycle analysis (C[i-iii]) were performed by flow cytometry after 3 days to detect and increase in cellular proliferation. Expression of p27kip1 was also determined by flow cytometry (D[i]), western blot (D[iii]) and immunofluorescence (D[iii]), scale bars represent 50µm. Data from cAb and nAb-treated samples were statistically analysed using an un-paired Student’s t-test (n=3), p<0.05*, p<0.01**.
Annexin V/Propidium iodide (PI) apoptosis analysis was employed to determine whether the increased cell counts observed in nAb-treated MCF-7 cells was due to decreased apoptosis and/or cell death/necrosis (Figure 3.6A[i]). Treatment of MCF-7 cells with nAb for 3 days resulted in a significant increase (p=0.0006) in the percentage of live cells (Annexin V\^−/PI\^−) (Figure 3.6A[iii]), with the mean percentage of live cells in nAb-treated MCF-7 population 84.34±0.57% compared to 52.99±0.60% in cAb-treated cells. Conversely, the mean percentage of dead/necrotic cells (Annexin V\^+/PI\^+) was significantly decreased in nAb-treated populations (10.55±0.17% compared to 34.36±0.57% for cAb [p=0.0002]). A small but significant difference (p=0.0291) was also observed in apoptotic cells (Annexin V\^+/PI\^−) between the cAb- and nAb-treated populations (2.56±0.24% and 1.58±0.10%, respectively). These data suggests the increased cell counts observed in nAb-treated MCF-7 cells is attributable to increased numbers of viable cells, which suggests increased survival in culture. Application of E-cadherin nAb DECMA-1 in MCF-7 cells has been associated with increased expression of p53 and decreased expression of inhibitor of apoptosis proteins (IAPs), such as XIAP and Survivin (Brouxhon et al., 2013). To further investigate the effects of E-cadherin SHE78.7 apoptosis protein arrays were performed (Figure 3.6B[i-ii]). Mean relative fold changes in pixel density between cAb and nAb-treated populations are shown in Figure 3.6[i] and fold changes >1.5 are highlighted in Figure 3.6[ii]. Contrary to findings by Brouxhon et al., 2013, increased expressions of multiple IAPs were observed including Clusterin, Livin and Survivin. E-cadherin, in conjunction with Caveolin-1 represses the transcription of Survivin by β-catenin-TCF/Lef transactivation (Torres et al., 2007, Torres et al., 2006). Since no nuclear translocation of β-catenin was observed in this study, this suggests up-regulation of Survivin occurs through an alternative mechanism. Activation of phospho-p53 (S46) and phospho-p53 (S392) were increased 2.53±0.37 and 2.10±0.29 fold respectively, further investigation of p53 expression by western blot found p53 to be expressed in nAb-treated MCF-7 cells but absent in controls. These data suggest that E-cadherin mediated cell-cell contact is capable of regulating the expression of apoptosis-related proteins in MCF-7 cells.
Figure 3.6: Exogenous inhibition of E-cadherin increases survival and alters expression of apoptosis related proteins

The Annexin V/propidium iodide assay was used to determine cell viability as live, dead or apoptotic (apop.) in cAb and nAb-treated cells after 3 days (A[i-i]). Data from cAb and nAb-treated samples were statistically analysed using an un-paired Student’s t-test (n=3), p<0.05*, p<0.001***. Proteome arrays were performed to determine relative changes in expression of apoptosis related proteins between cAb and nAb-treated MCF-7 cells after 3 days (B[i]), fold changes >1.5 are highlighted (B[ii]). Expression of the tumour suppressor protein p53 was detected by western blot (C).
Once cells disaggregate within a tumour environment they must survive anoikis in order to proliferate (Guadamillas et al., 2011). To determine whether loss of E-cadherin mediated cell-cell contact increases resistance to anoikis, MCF-7 cells +/- nAb were cultured in ultra-low attachment plates and proliferation and cell viability assays performed (Figure 3.7), as described for adherent monolayer cultures with cell counts obtained every 2 days. MCF-7 cells treated with nAb exhibited a dispersed phenotype whereas cAb-treated cells formed discreet cellular aggregates (Figure 3.7A). Cumulative cell counts demonstrated a significant increase in nAb-treated MCF-7 cell numbers compared to controls (Figure 3.7B), with mean cell counts after 2 days of $2.62 \times 10^5 \pm 3.51 \times 10^4$ in nAb-treated cells compared to $1.35 \times 10^5 \pm 1.32 \times 10^4$ in cAb-treated cells ($p=0.0043$). After 6 days in culture, nAb-treated cell counts were $1.31 \times 10^6 \pm 2.72 \times 10^5$ compared to $2.89 \times 10^5 \pm 8.55 \times 10^4$ in cAb-treated cells ($p=0.0036$), representing population doubling times of 25.4±1.40h and 43.1±8.1h, respectively, and total cell number increase of 4.5-fold. Cell cycle analysis revealed a significantly increased percentage of proliferating cells in nAb-treated populations ($p=0.0001$), with 38.54±1.01% of nAb-treated cells in S-phase compared to 21.36±0.10% of control cells (Figure 3.7C[i-ii]). Conversely, the percentage of cells in the G1 phase of the cell cycle were significantly decreased ($p=0.0260$). Cell viability was determined using Annexin V/PI analysis after 2 days in culture (Figure 3.7D[i]). No significant difference was apparent between cAb- and nAb-treated cells in the viable ($p=0.4552$), dead/necrotic ($p=0.1974$) or apoptotic ($p=0.7053$) populations (Figure 3.7D[ii]), suggesting that differences in cell numbers between the cAb and nAb-treated populations was due to increased cell proliferation in the nAb population and decreased proliferation in the cAb-treated cells.
Figure 3.7: Exogenous inhibition of E-cadherin increases proliferation but not survival in 3-dimensional culture

MCF-7 cells grown in 3-dimensional culture with cAb formed aggregates whereas application of E-cadherin SHE78.7 inhibited cell-cell contact (A). A significant increase in cell numbers were observed in nAb-treated populations from 2 days in culture (B) and cell cycle analysis revealed a significant increase of nAb-treated cells in the S-phase of the cell cycle compared to controls (C[i-ii]). Annexin V/Propidium iodide analysis determined no significant differences between the percentages of live, dead/necrotic or apoptotic cells (C-D). Data from cAb and nAb-treated samples were statistically analysed using an un-paired Student’s t-test (n=3; p<0.05 *, p<0.01 ** and p<0.001 ***).
3.2.4 Exogenous inhibition of E-cadherin induces a wide range of gene transcript alterations in MCF-7 cells

Whole genome transcript analysis was conducted to determine early molecular changes as a result of exogenous inhibition of E-cadherin mediated cell-cell adhesion in MCF-7 cells. Principal component analysis (PCA) analysis demonstrated variations between cAb- and nAb-treated MCF-7 cell populations (Figure 3.8A). Tables 3.1-3.2 lists the 20 most up- and down-regulated transcripts in nAb-treated cells compared to cAb treatment. Functional annotation of gene transcript alterations revealed a wide range of cellular processes affected by exogenous E-cadherin inhibition. For example, upregulated transcripts were associated with pathways for fertilisation and progesterone-mediated oocyte meiosis, calcium signalling, cancer, ubiquitin-mediated proteolysis, xenobiotic metabolism and gp130, PDGF, TNF and TGFβ signalling. Downregulated genes were associated with pathways for cancer, fatty acid biosynthesis and metabolism, oxidative phosphorylation and various signalling pathways (adipocytokine, TGFβ, MAPK, neurotrophin). Confirmation of the microarray results was performed by assessing *Epithelial Membrane Protein-1* (**EMP-1**; -1.80 fold change), Insulin-like Binding Growth Factor 3 (**IGFBP-3**; 1.61 fold change), *Slug* (**SNAI2**; +1.32 fold change), *S100 calcium binding protein A9* (**S100A9**; -2.83 fold change) *Vimentin* (**VIM**; -1.37 fold change) expression using quantitative RT-PCR (Figure 3.8B). Although *Vimentin* gene transcripts were found to be repressed in all 3 biological replicates, this was not significant when statistically analysed.

Comparison of the MCF-7+nAb microarray analysis with that described for dominant negative expression of E-cadherin (DN-Ecad) and short-hairpin E-cadherin (shEcad) in immortalised human mammary epithelial (HMLE) cells (Onder et al., 2008) revealed little similarity (Figure 3.8C). Of the upregulated transcripts in MCF-7+nAb cells, only *CYP1B1* was shared with DN-Ecad and shEcad HMLE cells (Figure 3.8C[i]). Of the downregulated gene transcripts, *C1orf116, CEACAM6, DSC2, EDN1, KRT13, KRT15* and *S100A7* were shared between MCF-7+nAb and DN-Ecad/shEcad HMLE cells (Figure 3.8C[ii]). Previous microarray analysis in our lab has revealed
decreased expression of EMP-1 and increased expression of IGFBP-3 gene transcripts in E-cadherin\(^/-\) mouse ES cells and ENPS cells compared to controls (Soncin et al., 2011, Hawkins et al., 2014). Confirmation of alterations in EMP1 and IGFBP-3 in E-cadherin\(^/-\) ES cells compared to wild type D3 ES cells was performed using quantitative RT-PCR (Figure 3.8D), demonstrating a cross species correlation between E-cadherin, IGFBP-3 and EMP-1 expression. EMP-1 is a membrane spanning glycoprotein and has been recently described as an anti-proliferative and pro-apoptotic gene, with down-regulation in breast carcinoma correlating with poorer patient prognosis (Sun et al., 2014b). To determine whether exogenous inhibition of cell surface E-cadherin protein affected EMP-1 protein expression in MCF-7 cells, EMP-1 protein expression was assessed by western blot (Figure 3.8E) and fluorescent flow cytometry (Figure 3.8F) analysis in cAb- or nAb-treated MCF-7 cells. Whilst total EMP-1 protein expression levels did not alter following treatment of MCF-7 cells with nAb (Figure 3.8E[i-ii]), cell surface localisation of EMP-1 was significantly reduced in these cells (Figure 3.8F). This suggests that whilst downregulation of EMP-1 transcripts by nAb in MCF-7 cells does not affect total EMP-1 protein levels it is associated with altered cell surface localisation of this protein.
Figure 3.8: Microarray analysis of cAb/nAb-treated MCF-7 cells after 3 days

Whole genome transcript analysis was performed on MCF-7 cells following cAb/nAb-treatment for 3 days and principle component analysis (PCA) was conducted (A). Quantitative PCR confirmed upregulation of *IGFBP-3* and Slug (*SNAI2*), and repression *EMP-1, VIM* and *S100A9* transcripts (B). Upregulated (C[i]) and repressed (C[ii]) gene transcript changes were compared to results microarray obtained by Onder et al. (2008) in HMLE cells expressing a dominant negative form of E-cadherin (DN-Ecad) or short hairpin RNA against E-cadherin (shEcad). Quantitative PCR confirmed cross species correlation of *EMP-1* and *IGFBP-3* gene transcript alterations in wild type and E-cadherin-/- (E-cad-/-) mouse embryonic stem cells (D). No significant differences were observed in protein expression of EMP-1 in cAb/nAb-treated MCF-7 cells by western blot (E[i-ii]) but flow cytometry analysis revealed decreased expression of cell surface EMP-1 in nAb-treated MCF-7 cells compared to controls (F). Data were statistically analysed using an un-paired Student’s t-test (n=3; p<0.05*, p<0.01**, p<0.001***, p<0.0001****).
### Table 3.1: 20 most downregulated transcripts in nAb-treated MCF-7 cells compared to controls

<table>
<thead>
<tr>
<th>Affymetrix ID</th>
<th>Gene Symbol</th>
<th>Common name</th>
<th>p-value</th>
<th>Fold-Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>223484_at</td>
<td>C15orf48</td>
<td>Chromosome 15 open reading frame 48</td>
<td>2.00E-06</td>
<td>-3.21</td>
</tr>
<tr>
<td>213680_at</td>
<td>KRT6B</td>
<td>Keratin 6B</td>
<td>4.95E-06</td>
<td>-2.90</td>
</tr>
<tr>
<td>203535_at</td>
<td>S100A9</td>
<td>S100 calcium binding protein A9</td>
<td>4.13E-08</td>
<td>-2.83</td>
</tr>
<tr>
<td>205064_at</td>
<td>SPRR1A</td>
<td>Small proline-rich protein 1B</td>
<td>2.11E-06</td>
<td>-2.61</td>
</tr>
<tr>
<td>213796_at</td>
<td>SPRR1A</td>
<td>Small proline-rich protein 1A</td>
<td>2.97E-05</td>
<td>-2.29</td>
</tr>
<tr>
<td>229566_at</td>
<td>LOC645638</td>
<td>WDNM1-like pseudogene</td>
<td>1.78E-04</td>
<td>-2.23</td>
</tr>
<tr>
<td>209909_s_at</td>
<td>TGFB2</td>
<td>Transforming growth factor, beta 2</td>
<td>5.57E-07</td>
<td>-2.19</td>
</tr>
<tr>
<td>201141_at</td>
<td>GPNMB</td>
<td>Glycoprotein (transmembrane) nmb</td>
<td>1.35E-06</td>
<td>-2.07</td>
</tr>
<tr>
<td>219995_s_at</td>
<td>ZNF750</td>
<td>Zinc finger protein 750</td>
<td>7.19E-06</td>
<td>-2.06</td>
</tr>
<tr>
<td>201860_s_at</td>
<td>PLAT</td>
<td>Plasminogen activator, tissue</td>
<td>1.32E-05</td>
<td>-2.04</td>
</tr>
<tr>
<td>205916_at</td>
<td>S100A7</td>
<td>S100 calcium binding protein A7</td>
<td>3.75E-04</td>
<td>-2.03</td>
</tr>
<tr>
<td>218990_s_at</td>
<td>SPRR3</td>
<td>Small proline-rich protein 3</td>
<td>2.45E-04</td>
<td>-1.99</td>
</tr>
<tr>
<td>202644_s_at</td>
<td>TNFAIP3</td>
<td>Tumour necrosis factor, alpha-induced protein 3</td>
<td>1.79E-05</td>
<td>-1.97</td>
</tr>
<tr>
<td>221577_x_at</td>
<td>GDF15</td>
<td>Growth differentiation factor 15</td>
<td>3.08E-04</td>
<td>-1.89</td>
</tr>
<tr>
<td>202917_s_at</td>
<td>S100A8</td>
<td>S100 calcium binding protein A8</td>
<td>1.62E-05</td>
<td>-1.89</td>
</tr>
<tr>
<td>205786_s_at</td>
<td>ITGAM</td>
<td>Integrin, alpha M (complement component 3 receptor 3 subunit)</td>
<td>2.71E-05</td>
<td>-1.88</td>
</tr>
<tr>
<td>201324_at</td>
<td>EMP1</td>
<td>Epithelial membrane protein 1</td>
<td>9.43E-06</td>
<td>-1.80</td>
</tr>
<tr>
<td>221667_s_at</td>
<td>HSPB8</td>
<td>Heat shock 22kDa protein 8</td>
<td>5.65E-04</td>
<td>-1.75</td>
</tr>
<tr>
<td>228707_at</td>
<td>CLDN23</td>
<td>Claudin 23</td>
<td>3.60E-03</td>
<td>-1.70</td>
</tr>
<tr>
<td>209459_s_at</td>
<td>ABAT</td>
<td>4-aminobutyrate aminotransferase</td>
<td>1.92E-05</td>
<td>-1.65</td>
</tr>
<tr>
<td>Affymetrix ID</td>
<td>Gene Symbol</td>
<td>Common name</td>
<td>p-value</td>
<td>Fold change</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td>------------------------------------------------------------------------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>223254_s_at</td>
<td>G2E3</td>
<td>G2/M-phase specific E3 ubiquitin protein ligase</td>
<td>1.79E-02</td>
<td>1.74</td>
</tr>
<tr>
<td>228545_at</td>
<td>ZNF148</td>
<td>Zinc finger protein 148</td>
<td>4.29E-02</td>
<td>1.74</td>
</tr>
<tr>
<td>228260_at</td>
<td>ELAVL2</td>
<td>ELAV (embryonic lethal, abnormal vision, Drosophila)-like 2 (Hu antigen B)</td>
<td>5.02E-03</td>
<td>1.71</td>
</tr>
<tr>
<td>203176_s_at</td>
<td>TFAM</td>
<td>Transcription factor A, mitochondrial</td>
<td>1.08E-02</td>
<td>1.69</td>
</tr>
<tr>
<td>218729_at</td>
<td>LIXN</td>
<td>Laxxin</td>
<td>1.88E-02</td>
<td>1.67</td>
</tr>
<tr>
<td>236696_at</td>
<td>U2SURP</td>
<td>U2 snRNP-associated SURP domain containing</td>
<td>1.24E-02</td>
<td>1.66</td>
</tr>
<tr>
<td>205990_s_at</td>
<td>WNT5A</td>
<td>Wingless-type MMTV integration site family, member 5A</td>
<td>5.36E-04</td>
<td>1.66</td>
</tr>
<tr>
<td>202436_s_at</td>
<td>CYP1B1</td>
<td>Cytochrome P450, family 1, subfamily B, polypeptide 1</td>
<td>7.12E-03</td>
<td>1.65</td>
</tr>
<tr>
<td>243648_at</td>
<td>ZBED6</td>
<td>Zinc finger, BED-type containing 6</td>
<td>2.98E-02</td>
<td>1.64</td>
</tr>
<tr>
<td>210095_s_at</td>
<td>IGFBP3</td>
<td>Insulin-like growth factor binding protein 3</td>
<td>5.36E-05</td>
<td>1.61</td>
</tr>
<tr>
<td>201918_at</td>
<td>SLC25A36</td>
<td>Solute carrier family 25 (pyrimidine nucleotide carrier ), member 36</td>
<td>1.45E-02</td>
<td>1.59</td>
</tr>
<tr>
<td>231860_at</td>
<td>BRWD1</td>
<td>Bromodomain and WD repeat domain containing 1</td>
<td>1.43E-02</td>
<td>1.58</td>
</tr>
<tr>
<td>1557065_at</td>
<td>YLPM1</td>
<td>YLP motif containing 1</td>
<td>2.69E-02</td>
<td>1.58</td>
</tr>
<tr>
<td>232481_s_at</td>
<td>SLITRK6</td>
<td>SLIT and NTRK-like family, member 6</td>
<td>2.33E-02</td>
<td>1.57</td>
</tr>
<tr>
<td>203424_s_at</td>
<td>IGFBP5</td>
<td>Insulin-like growth factor binding protein 5</td>
<td>5.47E-03</td>
<td>1.56</td>
</tr>
<tr>
<td>214305_s_at</td>
<td>SF3B1</td>
<td>Splicing factor 3b, subunit 1, 155kDa</td>
<td>3.72E-02</td>
<td>1.56</td>
</tr>
<tr>
<td>233819_s_at</td>
<td>LTN1</td>
<td>Listerin E3 ubiquitin protein ligase</td>
<td>7.86E-03</td>
<td>1.55</td>
</tr>
<tr>
<td>235476_at</td>
<td>TRIM59</td>
<td>Tripartite motif containing 59</td>
<td>1.91E-02</td>
<td>1.54</td>
</tr>
<tr>
<td>208200_at</td>
<td>IL1A</td>
<td>Interleukin 1, alpha</td>
<td>4.07E-03</td>
<td>1.54</td>
</tr>
<tr>
<td>227639_at</td>
<td>PIGK</td>
<td>Phosphatidylinositol glycan anchor biosynthesis, class K</td>
<td>1.51E-03</td>
<td>1.53</td>
</tr>
</tbody>
</table>

Table 3.2 20 most upregulated transcripts in nAb-treated MCF-7 cells compared to controls
To determine whether this was also true in vivo the expression and localisation of E-cadherin and EMP-1 was assessed in biopsies from breast fibroadenoma (3.9A), hyperplasia (Figure 3.9B) and invasive ductal carcinoma patients (Figure 3.9C). E-cadherin was expressed in breast fibroadenoma but at very low levels in breast hyperplasia, suggesting that loss of E-cadherin is an early event during abnormal cell growth and can precede tumorigenesis and metastasis. EMP-1 expression was evident within the epithelium of hyperplastic tissue (Figure 3.9B), however, there was lack of discrete cell membrane localisation, with significant levels of the protein observed within the cytoplasm. Similarly, invasive ductal carcinoma biopsies lacked expression of E-cadherin protein and exhibited diffuse cytoplasmic expression of EMP-1 with little evidence of cell surface localisation (Figure 3.9C). These results suggest that localisation of EMP-1 protein, in combination with E-cadherin protein expression, may be a useful determinant of normal epithelial phenotype and alterations in EMP-1 localisation in cells may aid the identification of dysplastic and tumorigenic tissues within biopsies, although further analysis of additional patients will be required. Attempts to assess IGFBP-3 expression were thwarted by a lack of specific antibodies for detecting this protein.
Figure 3.9: Loss of cell surface expression of E-cadherin correlates with cytoplasmic expression of EMP-1 in tumour tissue sections

Expression of E-cadherin and EMP-1 were predominantly localised to the cell membrane in breast fibroadenoma (A) tissue sections although some cytoplasmic staining was observed in both cases. Absent or low expression of E-cadherin was associated with diffuse expression of EMP-1 in breast hyperplasia (B) and invasive ductal carcinoma (C) tissue sections. Images were acquired using a 63X objective, scale bars represent 50µm.
3.3 Discussion

Epithelial-mesenchymal transition (EMT) is thought to play an important role in the metastatic spread of cancer cells, with loss of E-cadherin considered a defining feature of this process, and has been studied in significant detail (Mani et al., 2008, Mani et al., 2007, Hartwell et al., 2006). However, the impact of inhibition of E-cadherin in epithelial cells has been largely overlooked. This is, in part, due to the requirement to induce EMT using exogenous factors (e.g. growth factors) making the study of E-cadherin repression in the complex EMT event extremely difficult. In agreement with observations in embryonic stem cells (Eastham et al., 2007, Spencer et al., 2007), application of an E-cadherin neutralising antibody alone was unable to induce an EMT event in MCF-7 cells. An EMT event has previously been demonstrated in epidermoid carcinoma cells expressing a dominant-negative form of E-cadherin, leading to increased expression of Vimentin, decreased expression of cytokeratins and altered gene expression (Andersen et al., 2005). Contrary to these findings, Onder et al. (2008) found dominant negative expression of E-cadherin in HMLE cells insufficient to induce an EMT event, whereas shRNA mediated knockdown of E-cadherin did induce an EMT event, with β-catenin signalling necessary, but not sufficient, to induce this process. Similar to observations in mouse EMT/6 cells treated with DECMA-1 (St. Croix et al., 1998), retention of β-catenin at the cell membrane following application of nAb is described here. This further supports notion that E-cadherin-β-catenin binding occurs independent of cell adhesion (Guo et al., 2014). Translocation of β-catenin to the nucleus has been linked with initiation of EMT (Kim et al., 2002a, Mukherjee et al., 2014), therefore, the retention of β-catenin at the cell membrane in MCF-7 cells treated with nAb may explain the lack of EMT in this study.

An EMT event is thought to bestow a small number of cells with CSC capabilities (Mani et al., 2008, Morel et al., 2008), therefore, the lack of an EMT event observed in this study may account for the failure of exogenous inhibition of E-cadherin to generate a CSC phenotype. Recently, it has been demonstrated that basal mammary epithelial cells more readily able to convert to a CSC phenotype than
luminal epithelial cells (Chaffer et al., 2013). The authors describe fundamental differences in the chromatin configuration of basal and luminal mammary epithelial cells treated with TGF-β, with 4 out of 5 basal cell lines able to convert chromatin at the Zeb1 promoter to an active conformation to acquire CSC characteristics. In contrast, chromatin conformation repressed acquisition of CSC properties in all luminal cell types tested (Chaffer et al., 2013). This indicates core discrepancies between basal and luminal cell biology and suggests increased plasticity of basal cells, which may explain the more aggressive nature of basal tumours and the lack of a CSC phenotype acquired by luminal MCF-7 cells in this study. It also illustrates the potential for neutralisation of E-cadherin mediated cell-cell contact to produce different results in a basal mammary epithelial cell line, necessitating further analysis of exogenous E-cadherin inhibition in additional epithelial cell lines.

Increased growth of E-cadherin nAb-treated MCF-7 cells were observed in both monolayer and 3-dimensional culture. In support of this finding, E-cadherin SHE78.7 stimulated cell growth has previously been reported in MCF-7 cells grown in 3-dimensional culture through increased \[^{3}H\]thymidine uptake (St. Croix et al., 1998). Increased growth has also been reported in mouse ES cells following addition of an E-cadherin neutralising antibody or genetic knockout in monolayer culture (Soncin et al., 2011, Spencer et al., 2007). Similar to results observed in this study, the authors failed to detect any cell cycle differences between wild type and E-cadherin\(^{-}\) ES cells (Soncin et al., 2011). E-cadherin has been linked to contact inhibition through up-regulation of p27\(^{\text{Kip1}}\), St. Croix et al. (1998) observed reduction of p27\(^{\text{Kip1}}\) expression in EMT/6 and MCF-7 cells grown in 3-dimensional culture following application of E-cadherin neutralising antibodies but the authors were unable to replicate the results in monolayer culture (St. Croix et al., 1998). However, reduced p27\(^{\text{Kip1}}\) expression has been described following application of E-cadherin SHE78.7 nAb to confluent populations of thyroid carcinoma cells grown on glass coverslips (Motti et al., 2005). Cells grown in monolayer culture experience decreased cell contact surface area when compared to 3-dimensional culture, which may result in decreased growth inhibitory signals and explain the unchanged p27\(^{\text{Kip1}}\) expression observed between cAb and nAb-treated populations described
here. Differential contact inhibitory signals may also explain the increased fold change in growth observed in 3-dimensional culture compared to monolayer culture. Cells within 3-dimensional aggregates may also suffer from decreased nutrient availability, which may account for the increase in doubling time observed in cAb-treated cells in 3-dimensional culture compared to monolayer. Alternatively, unlike experiments performed by Motti et al. (2005) cell populations in this study were maintained at sub-confluence and, therefore, still actively growing and this may have reduced growth inhibitory signals. In addition, this might account for the lack of differences observed in the cell cycle analysis and expression of Ki67.

Up-regulation of E-cadherin transcriptional repressors, Twist and Zeb1, have been described following loss of E-cadherin, suggesting initiation of a feedback loop whereby loss of E-cadherin stimulates up-regulation of transcriptional repressors to sustain E-cadherin loss (Onder et al., 2008). This study failed to detect up-regulation of E-cadherin transcriptional repressors, even after 50 days of nAb-treatment, and cells expressed E-cadherin at the cell surface and re-established cell-cell contacts following removal of the E-cadherin nAb. Few comparable gene transcript alterations are reported between HMLE and epidermoid carcinoma cells expressing a dominant negative form of E-cadherin (Onder et al., 2008, Andersen et al., 2005), in fact, more similarities were observed between shEcad HMLE cells and nAb-treated MCF-7 cells. Indeed MCF-7 cells are more advanced in their oncogenic state compared to HMLE cells, but the lack of similarities between DN-Ecad HMLE and epidermoid carcinoma cells highlights discrepancies between inhibition of E-cadherin mediated cell-cell contact by addition of an exogenous inhibitor and intracellular inhibition via expression of a dominant-negative mutant.

The data presented in this study have allowed the DENT hypothesis as proposed by Mohamet et al. (2011) to be tested, as a result, an amended DENT hypothesis is proposed in Figure 3.10. It is clear from the data that loss of E-cadherin cell-cell contact in MCF-7 cells does not induce a CD24$^{\text{low}}$/CD44$^{\text{high}}$ CSC phenotype and additional EMT-inducing factors are likely to be required to achieve this. The induction of an EMT-like state has been demonstrated in A431 and DLD-1 cells.
following treatment with E-cadherin SHE78.7 nAb in combination with EGFR activation (Garnier et al., 2012). However, observations here support the role of E-cadherin repression in inducing proliferation/decreased apoptosis, allowing increased cell numbers that may contribute to formation of neoplasms. This is supported by the observations in hyperplastic tissue biopsies, where E-cadherin expression is significantly reduced and lacks cell surface localisation but tumour formation has yet to occur. Data presented here, suggest that this is a reversible process in which neoplastic cells only form an established tumour mass following exogenous factor stimulation (Figure 3.10; Mohamet et al. 2011). The data also raise the possibility of abnormal cell growth, via E-cadherin inhibition, occurring prior to oncogenic gene mutations, where the increased proliferation increases the likelihood of mutations occurring and subsequent formation of the tumour mass.

**Figure 3.10: Neutralisation of E-cadherin contributes to abnormal growth but is insufficient to induce EMT**

Exogenous inhibition of E-cadherin is sufficient to increase growth of epithelial cells, which may contribute to neoplasm formation in vivo. However, disaggregation of epithelial cells was insufficient to induce and EMT event or generate a CSC population. We suggest the necessity of additional signals such as Snail, Slug and/or Twist.

Expression of EMP-1 has been described as an anti-proliferative gene that inhibits tumour growth through induction of apoptosis (Sun et al., 2014b, Wang et al., 2003), decreased expression of EMP-1 has also been observed in nasopharyngeal and gastric carcinomas compared to controls, which was associated with poor patient prognosis (Sun et al., 2014c, Sun et al., 2014a). EMP-1 has also been described as essential for tight junction formation in bronchial epithelial cells (Durgan et al., 2014), with assembly of adherens junctions thought to be required...
for tight junction formation (Tunggal et al., 2005). Here, decreased gene transcription and cell surface expression of EMP-1 were observed upon nAb-treatment of MCF-7 cells. Cytoplasmic localisation of EMP-1 was also correlated with loss of E-cadherin membrane expression in the analysis of breast tissue biopsies. Furthermore, disruption of E-cadherin mediated cell-cell contact was associated with increased growth and survival of MCF-7 cells. This is the first correlation of EMP-1 expression with maintenance of E-cadherin mediated cell-cell contacts. Interestingly, EMP-1 has been described as a biomarker indicative of resistance to the lung cancer drug gefitinib (Jain et al., 2005), while another study reported that transfection of E-cadherin into non-small cell carcinoma cell lines enhanced the efficacy of gefitinib (Witta et al., 2006). This study raises the possibility that the improved sensitivity may be attributed to the repression of EMP-1 by E-cadherin in the non-small cell carcinoma cell lines and merits further investigation using additional cell lines and tissue samples.
Chapter 4. E-cadherin acts as a positive regulator of p300-induced gene transactivation
4.1 Introduction

The results in Chapter 3 described alterations in the MCF-7 gene transcriptome following exogenous E-cadherin inhibition using a neutralising antibody. Recent studies in our lab have also demonstrated significantly altered gene signatures in mouse E-cadherin$^{-/-}$ embryonic stem (ES) cells and E-cadherin Negative Proliferating Stem (ENPS) cells when compared to wild type controls (Soncin et al., 2011, Hawkins et al., 2014). These studies have highlighted that the function of E-cadherin stretches beyond cell adhesion and its loss can impact on multiple cellular processes including apoptosis and catabolism. Interestingly, the gene transcript alterations in E-cadherin$^{-/-}$ and ENPS cells showed striking similarities to cancer, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed loss of E-cadherin in E-cadherin$^{-/-}$ mouse ES cells was most associated with ‘pathways in cancer’ (Soncin et al., 2011) and in ENPS cells, ‘molecular mechanisms in cancer’ was identified as an enriched pathway and 124 gene transcripts were associated with ‘epithelial neoplasia’ (Hawkins et al., 2014).

In adult epithelial derived tumour cells, increased metastatic capability is commonly associated with loss of E-cadherin function and acquisition of a cancer stem cell (CSC) phenotype (Mani et al., 2008, Onder et al., 2008). Chapter 3 demonstrated the exogenous inhibition of E-cadherin was insufficient to induce a CD44$^{\text{high}}$/CD24$^{\text{low}}$ expression profile, which is commonly associated with breast CSCs (Al-Hajj et al., 2003). However, the acquisition of CSC phenotype is also associated with additional characteristics, such as the increased ability to efflux cancer therapeutics (Mechetner et al., 1998). The previous chapter identified >1000 gene transcript changes in nAb-treated MCF-7 cells, although E-cadherin SHE78.7 nAb was insufficient to induce a CD44$^{\text{high}}$/CD24$^{\text{low}}$ phenotype other stem cell characteristics may be induced.

Changes in the gene transcriptome are orchestrated by a multitude of transcription factors that function in concert to transcribe DNA. Following proteolytic cleavage of E-cadherin, nuclear translocation and a direct role for the E-cad/CTF2 fragment in
gene transcription has been identified (Ferber et al., 2008). These authors described the binding of E-cad/CTF2 to the transcriptional repressor kaiso, which resulted in the inactivation of kaiso and increased transcription of MMP-7. More recently, a comprehensive study of the E-cadherin interactome revealed 4% of the 561 proteins identified to have molecular functions related to ‘DNA/transcription’ (Guo et al., 2014). Upon further investigation, the authors determined that the proteins identified in this category were not localised to the cell membrane, which suggests these proteins may interact with E-cadherin in other cellular compartments. E-cadherin is largely localised to the cell membrane and anchored to the actin cytoskeleton through the cytoplasmic cell adhesion complex (Cavallaro and Christofori, 2004). This implies that expression of E-cadherin regulates downstream signalling events that control gene transcription. This could include altered transcription factor expression or epigenetic mechanisms that control gene transcript expression. These include expression of miRNA, which prevent translation of mRNA to protein, or patterns of acetylated and methylated DNA that determine whether transcription factors can gain access to DNA, which ultimately affects which genes are transcribed.

Meaningful interpretation of large microarray data sets can be challenging, gene ontology and pathway analysis can group gene transcript changes according to associated biological processes and canonical pathways but it is more difficult to identify core regulators of the phenotype. This Chapter further interrogates the microarray data described in Chapter 3 using an \textit{in silico} network analysis approach to identify core regulators associated with the loss of E-cadherin in MCF-7 cells.
4.2 Results

4.2.1 Exogenous inhibition of E-cadherin alters gene transcripts related to multiple biological processes.

Microarray analysis was performed on MCF-7 cells following treatment with E-cadherin SHE78.7 neutralising antibody (nAb) for 3 days. Data were normalised to MCF-7 cells treated with a control antibody (cAb) at an equivalent concentration. Chapter 3 confirmed the microarray results by demonstrating significant downregulation of EMP-1 and S100A9 and upregulation of IGFBP-3 and SNAI2 gene transcripts. Using the Protein Analysis THrough Evolutionary Relationships (PANTHER) analysis tool (Mi et al., 2013), Gene ontology analysis was performed on >1000 significantly up- and downregulated genes to categorise gene changes according to biological processes (Figure 4.1). Only 2% of the upregulated transcripts were related to biological adhesion, 2% were involved with apoptosis and within this category 66.7% were involved with the induction of apoptosis (GO:0006917) and 33.3% with repression (GO:0043066). Cellular processes accounted for 19% of the gene ontology analysis, this category included cell communication, cell cycle and proliferation (Figure 4.1A). Of the downregulated gene transcripts, 3% were involved in biological processes related to cell adhesion, 3% were related to apoptosis, with 56% of these genes involved with the induction of apoptosis and 44% with repression (Figure 4.1B). Additional gene ontologies affected included cellular and developmental processes.

Further KEGG pathway analysis revealed the most enriched term was ‘pathways in cancer’ with 35 terms identified in this pathway, including c-Jun, MMP-9 and STAT3 (Figure 4.2A). Other enriched pathways included tight junction and Wnt signalling pathways. Additional canonical pathway analysis was performed using Ingenuity Pathway Analysis (IPA; Figure 4.2B), which also identified tight junction signalling as an enriched pathway. The importance of E-cadherin in death receptor mediated apoptosis has recently been described (Lu et al., 2014). Interestingly, death receptor signalling was also identified in this analysis. Together, these data indicate
that exogenous inhibition of E-cadherin is sufficient to affect multiple biological processes that are not limited to cell adhesion.

Figure 4.1: Gene Ontology analysis of significantly altered gene transcripts
MCF-7 cells were cultured with E-cadherin SHE78.7 nAb for 3 days prior to microarray analysis. Significantly up- (A) and downregulated (B) transcripts using the criteria of fold change >1.2 and p<0.05 were analysed using the Protein AnalysisTHrough Evolutionary Relationships (PANTHER) analysis tool to classify significantly altered transcripts into Gene Ontologies (GO).
Chapter 4

Results

Figure 4.2: Exogenous inhibition of E-cadherin alters gene transcripts related to multiple canonical pathways

KEGG pathway analysis was performed on significantly altered gene transcripts (p<0.05, fold-change >1.2) using the Database for Annotation, Visualization and Integrated Discovery (DAVID) web based software (A), canonical pathways were also assessed using IPA (B).
4.2.2 The E-cadherin nAb MCF-7 cell transcriptome exhibits similarities to naïve human embryonic stem cells

Loss of E-cadherin in mouse ES cells results in significantly altered gene transcriptome with striking associations with cancer (Hawkins et al., 2014, Soncin et al., 2011). Given that cancer cells are also thought to reactivate stem cell pathways in order to survive and metastasize, this study also sought to determine whether this relationship was reciprocated following exogenous inhibition of E-cadherin in MCF-7 cells. Although experiments described in Chapter 3 found E-cadherin nAb insufficient to induce a cancer stem cell phenotype, as determined by expression of cell surface CD24 and CD44, a similar gene expression profile was observed when the MCF-7+nAb gene signature was compared to the naïve human ES cell signature described by Gafni et al. (2013). Comparison of the top 100 up- and downregulated gene transcripts with the E-cadherin+nAb transcriptome with naïve human ES cells revealed similarities of 42% and 31% respectively (Table 4.1-4.2).

Given that similarities were observed between the E-cadherin nAb MCF-7 and naïve human ES cell transcript signature, the potential for the E-cadherin nAb to induce a stem cell phenotype in MCF-7 cells was investigated using the side population analysis method (Figure 4.3A). Using this technique stem cell populations are characterised by the increased ability to efflux Hoechst dye. A stem cell population of 0.3% was identified in cAb-treated MCF-7 cells after 3 days (Figure 4.3B), this increased to 0.4% in populations treated with the ABCB1 inhibitor, verapamil, which suggests the results observed in cAb-treated cells may not be a genuine stem cell population. Treatment of MCF-7 cells with E-cadherin SHE78.7 nAb for 3 days reduced the ‘stem cell’ population to 0.03%, although this was not statistically significant, and verapamil had no effect on nAb-treated populations. Assuming the side population is genuine, these data suggest that exogenous inhibition of E-cadherin is insufficient to induce a CSC population in MCF-7 cells.
Table 4.1: Comparison of the top 100 downregulated gene transcripts in MCF-7+nAb cells compared with primed vs. naïve human ES cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>MCF-7+nAb</th>
<th>hES</th>
<th>MCF-7+nAb</th>
<th>hES</th>
<th>MCF-7+nAb</th>
<th>hES</th>
<th>MCF-7+nAb</th>
<th>hES</th>
<th>MCF-7+nAb</th>
<th>hES</th>
<th>MCF-7+nAb</th>
<th>hES</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL5orf48</td>
<td>↓</td>
<td>AQP3</td>
<td>-</td>
<td>PSMB9</td>
<td>-</td>
<td>MAFF</td>
<td>↓</td>
<td>SERPIN1A3</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRT6B</td>
<td>↓</td>
<td>KRT6A</td>
<td>-</td>
<td>TLR2</td>
<td>-</td>
<td>CLIC3</td>
<td>-</td>
<td>KRT23</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S100A9</td>
<td>-</td>
<td>APOBEC3B</td>
<td>↑</td>
<td>CALML5</td>
<td>-</td>
<td>MAFB</td>
<td>↑</td>
<td>KRT7</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPRR1B</td>
<td>-</td>
<td>PLA2G4C</td>
<td>-</td>
<td>RHEB</td>
<td>-</td>
<td>SGMS2</td>
<td>↓</td>
<td>KISS1R</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPRR1A</td>
<td>-</td>
<td>CYP4B1</td>
<td>-</td>
<td>TAP1</td>
<td>↑</td>
<td>RCAN1</td>
<td>↓</td>
<td>MAP3K8</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ2</td>
<td>-</td>
<td>DNAJ4A4</td>
<td>-</td>
<td>MATN3</td>
<td>-</td>
<td>IFT122</td>
<td>-</td>
<td>KIAA0513</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPNMB</td>
<td>-</td>
<td>COL12A1</td>
<td>↓</td>
<td>C1orf116</td>
<td>-</td>
<td>LINGO1</td>
<td>-</td>
<td>SPTBN1</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZNF750</td>
<td>-</td>
<td>CDH26</td>
<td>-</td>
<td>EDN1</td>
<td>↓</td>
<td>CLDN4</td>
<td>↓</td>
<td>TACSTD2</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLAT</td>
<td>-</td>
<td>MGLL</td>
<td>-</td>
<td>SOCS3</td>
<td>-</td>
<td>GABRP</td>
<td>↑</td>
<td>RELB</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S100A7</td>
<td>-</td>
<td>ITGB8</td>
<td>-</td>
<td>RAB3B</td>
<td>-</td>
<td>CEACAM5</td>
<td>-</td>
<td>COL4A6</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPRR3</td>
<td>-</td>
<td>C14orf182</td>
<td>-</td>
<td>MACC1</td>
<td>↑</td>
<td>KIAA0226L</td>
<td>↓</td>
<td>CYR61</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>-</td>
<td>EGR3</td>
<td>-</td>
<td>KRT16</td>
<td>-</td>
<td>SEC24D</td>
<td>-</td>
<td>LYN</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GDF15</td>
<td>↑</td>
<td>HEATR7B1</td>
<td>-</td>
<td>INHBA</td>
<td>↓</td>
<td>PCDHB6</td>
<td>-</td>
<td>TNFRSF11A</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S100A8</td>
<td>-</td>
<td>KRT15</td>
<td>-</td>
<td>RDH13</td>
<td>-</td>
<td>ATP11A</td>
<td>↑</td>
<td>PODXL</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITGAM</td>
<td>-</td>
<td>PAPLN</td>
<td>-</td>
<td>PRICKLE1</td>
<td>-</td>
<td>GDPD3</td>
<td>-</td>
<td>ASB9</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMP1</td>
<td>-</td>
<td>OASL</td>
<td>-</td>
<td>PRLR</td>
<td>↑</td>
<td>ZNF777</td>
<td>-</td>
<td>OCLN</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSPB8</td>
<td>-</td>
<td>MAP2</td>
<td>-</td>
<td>KIAA1211</td>
<td>-</td>
<td>KRT80</td>
<td>-</td>
<td>PKP1</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLDN23</td>
<td>-</td>
<td>TRIM16</td>
<td>-</td>
<td>TFI12</td>
<td>-</td>
<td>TFI1-A51</td>
<td>↑</td>
<td>ESYT2</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABAT</td>
<td>↑</td>
<td>CLDN7</td>
<td>↑</td>
<td>RAB11F1P1</td>
<td>-</td>
<td>DTNA</td>
<td>↓</td>
<td>STB5IA4</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC203A1</td>
<td>-</td>
<td>S100A4</td>
<td>-</td>
<td>B4GALT1</td>
<td>-</td>
<td>GPR39</td>
<td>-</td>
<td>PLAUR</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2: Comparison of the top 100 upregulated gene transcripts in MCF-7+nAb cells compared with primed vs. naïve human ES cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>MCF-7+nAb</th>
<th>hES</th>
<th>MCF-7+nAb</th>
<th>hES</th>
<th>MCF-7+nAb</th>
<th>hES</th>
<th>MCF-7+nAb</th>
<th>hES</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2E3</td>
<td>↑</td>
<td>VAV3</td>
<td>↑</td>
<td>PHF3</td>
<td>↑</td>
<td>MED31</td>
<td>↓</td>
<td>ASPM</td>
</tr>
<tr>
<td>ZNF418</td>
<td>↑</td>
<td>USP1</td>
<td>-</td>
<td>USP10</td>
<td>-</td>
<td>PTPLB</td>
<td>-</td>
<td>SMARCA2</td>
</tr>
<tr>
<td>ELAVL2</td>
<td>↑</td>
<td>ITPR1</td>
<td>-</td>
<td>PPFIBP1</td>
<td>-</td>
<td>SMARCE1</td>
<td>-</td>
<td>SLCA4A7</td>
</tr>
<tr>
<td>TFAM</td>
<td>-</td>
<td>ST3GAL5</td>
<td>-</td>
<td>UTP23</td>
<td>-</td>
<td>TRPS1</td>
<td>-</td>
<td>LHFP</td>
</tr>
<tr>
<td>LNX</td>
<td>-</td>
<td>TBL1XR1</td>
<td>↑</td>
<td>PUS7L</td>
<td>-</td>
<td>SCAMP1</td>
<td>↑</td>
<td>ID4</td>
</tr>
<tr>
<td>U2SURF</td>
<td>↑</td>
<td>RAD21</td>
<td>↑</td>
<td>FAM175A</td>
<td>↑</td>
<td>PPFIA1</td>
<td>-</td>
<td>FBXO9</td>
</tr>
<tr>
<td>WNT5A</td>
<td>↓</td>
<td>UBA6</td>
<td>-</td>
<td>THRAP3</td>
<td>-</td>
<td>LIFR</td>
<td>-</td>
<td>EML4</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>↓</td>
<td>TBC1D15</td>
<td>↑</td>
<td>FNB4</td>
<td>-</td>
<td>NBN</td>
<td>-</td>
<td>SOARL</td>
</tr>
<tr>
<td>ZBED6</td>
<td>-</td>
<td>ATP13A3</td>
<td>-</td>
<td>CEP57</td>
<td>-</td>
<td>ZC3H11A</td>
<td>↑</td>
<td>ABHD13</td>
</tr>
<tr>
<td>IGFBP3</td>
<td>-</td>
<td>NRP1</td>
<td>-</td>
<td>ARID4B</td>
<td>-</td>
<td>PLDN</td>
<td>-</td>
<td>UHMKI</td>
</tr>
<tr>
<td>SLC25A36</td>
<td>↑</td>
<td>SLC39A6</td>
<td>↑</td>
<td>TANK</td>
<td>-</td>
<td>GSTM4</td>
<td>-</td>
<td>LYPLA1</td>
</tr>
<tr>
<td>BRWD1</td>
<td>↑</td>
<td>HNMT</td>
<td>-</td>
<td>TIPPR</td>
<td>-</td>
<td>ENDO1</td>
<td>↓</td>
<td>MAP3K5</td>
</tr>
<tr>
<td>YLPM1</td>
<td>-</td>
<td>SEC231P</td>
<td>-</td>
<td>RNF13</td>
<td>-</td>
<td>ASNSD1</td>
<td>-</td>
<td>CASP14</td>
</tr>
<tr>
<td>SLITRK6</td>
<td>↑</td>
<td>MLF1</td>
<td>↑</td>
<td>ESCO2</td>
<td>-</td>
<td>NR3C1</td>
<td>-</td>
<td>SLC7A14</td>
</tr>
<tr>
<td>IGFBP5</td>
<td>-</td>
<td>CDC27</td>
<td>-</td>
<td>EIF1AX</td>
<td>-</td>
<td>DCPBD2</td>
<td>-</td>
<td>TNPO1</td>
</tr>
<tr>
<td>SF3B1</td>
<td>↑</td>
<td>FAM48A</td>
<td>↑</td>
<td>ZNF252P</td>
<td>-</td>
<td>ZNF415</td>
<td>-</td>
<td>ZFP91</td>
</tr>
<tr>
<td>LTN1</td>
<td>-</td>
<td>FAR1</td>
<td>-</td>
<td>INTS6</td>
<td>-</td>
<td>SIKE1</td>
<td>-</td>
<td>CTDSPL2</td>
</tr>
<tr>
<td>TRIM59</td>
<td>-</td>
<td>USP16</td>
<td>-</td>
<td>LUC7L3</td>
<td>-</td>
<td>RYBP</td>
<td>-</td>
<td>UGCG</td>
</tr>
<tr>
<td>IL1A</td>
<td>↓</td>
<td>CAV1</td>
<td>-</td>
<td>TXN1</td>
<td>-</td>
<td>VPS35</td>
<td>-</td>
<td>ZNF652</td>
</tr>
<tr>
<td>PIGK</td>
<td>-</td>
<td>PNRC2</td>
<td>-</td>
<td>ANAPC5</td>
<td>-</td>
<td>SKIL</td>
<td>-</td>
<td>C1orf60</td>
</tr>
</tbody>
</table>

Chapter 4

Results

122
Figure 4.3: Exogenous inhibition of E-cadherin is insufficient to induce a stem cell side population

MCF-7 cells were cultured with E-cadherin SHE78.7 nAb for 3 days prior to performing stem cell side population analysis (A). Cells were incubated with Hoechst 33342 in the presence or absence of the ABCB1 inhibitor verapamil, the percentage of side population cells were quantified using a BD Fortessa flow cytometer (B). Results were statistically analysed using a One-way ANOVA with a Tukey’s post test to compare the means (n=3), statistical significance was set at p<0.05.*
4.2.3 Network analysis identifies core modulators of MCF-7+nAb transcriptome

In order to identify core modulators of the MCF-7 E-cadherin nAb transcriptome, network analysis was performed. Significant gene transcript alterations were analysed using Cytoscape with the Moduland plugin to identify potential modulators of the gene signature. A total of 91 core central modulators (MetaNodes) were identified by community clustering (Figure 4.4A), EP300 was identified as a highly ranked MetaNode but also appeared in the top 10 nodes associated with 3 other MetaNodes JUN, SP1 and NCOA3, which were ranked alongside EP300 (Figure 4.4B). Interrogation of the model using IPA revealed connections between EP300 and additional MetaNodes within the network model including SOCS3 and GSK3β (Figure 4.4C). Furthermore, no previous connections between E-cadherin (CDH1) and EP300 have been observed. A similar network analysis approach to identify modulators of the mouse E-cadherin^-/-, ENPS and epiblast stem cell transcriptome also identified EP300 and NCOA3 as regulators of these phenotypes (Segal; unpublished data), suggesting species conservation between the mouse and human E-cadherin transcriptome and further supports the results of the human network analysis model presented here. In addition, decreased expression of EP300 gene transcripts were observed in nAb-treated MCF-7 cells in the microarray analysis. For these reasons, EP300 was selected for further study in MCF-7 cells.
Figure 4.4: Network analysis of the MCF-7 E-cadherin+nAb transcriptome
Community clustering analysis using Cytoscape with the Moduland plugin identified 91 central modulators (MetaNodes; A). JUN, EP300, SP1 and NCOA3 were among the highly ranked MetaNodes (B), within each of these modules EP300 was also identified as a closely related node. Interrogation of the model using Ingenuity Pathway Analysis revealed connections between EP300 and multiple nodes within the network but no connection between E-cadherin (CDH1) and EP300 was observed (C).
4.2.4  Expression of p300 in mammary epithelial cells

The *EP300* gene encodes the histone acetyltransferase E1A binding protein p300 (p300), which functions as an epigenetic modifier (Ogryzko et al., 1996). Decreased expression of p300 was observed in nAb-treated MCF-7 cells by microarray analysis (-1.23 fold-change) and this decrease in transcript expression was confirmed by quantitative real-time PCR (Figure 4.5A). To determine whether exogenous inhibition of E-cadherin affects protein expression of p300, western blot analysis was performed on E-cadherin nAb and cAb-treated MCF-7 cells after 3 days of treatment (Figure 4.5B[i]). Expression of p300 was observed in cAb and nAb-treated populations and no significant differences (p=0.2343) were observed following quantification (Figure 4.5B[ii]). Since p300 functions as a transcriptional co-activator in the nucleus, immunofluorescence microscopy was performed to assess cellular localisation of p300, which may affect protein function (Figure 4.5C[i]). Nuclear expression was observed in both cAb and nAb-treated MCF-7 cells after 3 days, which indicates exogenous inhibition of E-cadherin is insufficient to alter cellular localisation of p300. Although no significant decrease in p300 total protein was detected by western blot, p300 fluorescent staining appeared reduced in nAb-treated MCF-7 cells and this was confirmed by fluorescence quantification (p=0.0243; Figure 4.5C[ii]). Increased nuclear size has previously been associated with high grade breast tumours (Tan et al., 2001, Abdalla et al., 2012). Nuclear morphology of nAb-treated MCF-7 cells appeared smaller than control populations, to test this hypothesis mean nuclear area were quantified (Figure 4.5C[iii]). Mean nuclear area of nAb-treated cells was significantly smaller compared to cAb-treated cells (p=0.0005), which may be indicative of decreased cancerous activity. However, these results may also account for decreased tension across the cells following inhibition of cell-cell contact.

Expression of p300 was further investigated in breast fibroadenoma, hyperplasia and invasive ductal carcinoma tissue samples (Figure 4.6). Fluorescent staining revealed expression of p300 and E-cadherin in breast fibroadenoma samples (Figure 4.6A), however, expression of p300 was absent in hyperplasia and invasive ductal...
carcinoma samples where expression of E-cadherin was absent or low (Figure 4.6B-C). Although further analysis of additional patients is required, this suggests a positive correlation between E-cadherin and p300 expression in breast tissue in vivo.

Figure 4.5: Exogenous inhibition of E-cadherin reduces p300 expression
MCF-7 cells were cultured with E-cadherin SHE78.7 nAb for 3 days, $EP300$ gene transcript expression was quantified by PCR (A). Expression of p300 protein was detected in both cAb and nAb-treated MCF-7 cells (B[i]), densitometry was performed and statistically analysed using an un-paired Student’s t-test (n=3; B[ii]). Immunofluorescence staining was performed to determine cellular localisation of p300 (C[i]), scale bars represent 75µm. Mean nuclear area (C[iii]) and p300 cell fluorescence (C[iii]) were quantified using ImageJ software. Data were statistically analysed using an un-paired student t-test (n=3), statistical significance was set at p<0.05*. 

**Figure 4.5: Exogenous inhibition of E-cadherin reduces p300 expression**
MCF-7 cells were cultured with E-cadherin SHE78.7 nAb for 3 days, $EP300$ gene transcript expression was quantified by PCR (A). Expression of p300 protein was detected in both cAb and nAb-treated MCF-7 cells (B[i]), densitometry was performed and statistically analysed using an un-paired Student’s t-test (n=3; B[ii]). Immunofluorescence staining was performed to determine cellular localisation of p300 (C[i]), scale bars represent 75µm. Mean nuclear area (C[iii]) and p300 cell fluorescence (C[iii]) were quantified using ImageJ software. Data were statistically analysed using an un-paired student t-test (n=3), statistical significance was set at p<0.05*. 

127
Figure 4.6: Expression of p300 correlates with E-cadherin expression in breast tissue

Expression of p300 was observed in fibroadenoma tissue sections (A) by immunofluorescence staining but was absent in hyperplasia (B) and invasive ductal carcinoma (C) tissue samples. Expression of p300 correlated with expression of E-cadherin, which was found to be present in fibroadenoma samples and reduced or absent in hyperplasia and invasive ductal carcinoma samples respectively. Scale bars represent 50µM.
4.2.5 Inhibition of p300 significantly alters proliferation and gene transcript expression

In order to elucidate whether p300 modulates the E-cadherin nAb phenotype a small molecule inhibitor of p300, garcinol, was applied to MCF-7 cells. Following treatment of MCF-7 cells with cAb+garcinol for 3 days, MCF-7 cells adopted a more spindle-like morphology and displayed reduced cell-cell contacts compared to DMSO-treated controls (Figure 4.7A). Treatment with E-cadherin nAb+garcinol induced a disaggregated and spindle-like morphology, nAb+DMSO inhibited cell-cell contact but these cells lacked the spindle-like morphology. Although decreased cell-cell contacts were observed, flow cytometry analysis revealed E-cadherin was still present at the cell surface of garcinol and DMSO-treated control cells (Figure 4.7B). Furthermore, expressions of E-cadherin gene transcripts were not significantly altered between treatments (Figure 4.7C). Garcinol has previously been reported to decrease proliferation of MCF-7 cells (Ahmad et al., 2010), in this study similar results were also obtained (Figure 4.7D). As previously described, cell populations treated with nAb+DMSO showed significantly increased cell numbers compared to cAb+DMSO-treated populations (Figure 4.7D). Treatment with cAb+garcinol significantly decreased cell numbers to $9.83 \times 10^4 \pm 5.77 \times 10^3$, nAb+garcinol decreased cell numbers to $7.83 \times 10^4 \pm 1.26 \times 10^4$. However, no significant differences were determined between cAb+garcinol and nAb+garcinol populations. Garcinol has been reported to bind both the histone and the acetyl-coA binding sites within the p300 HAT domain to inhibit p300 activity (Arif et al., 2009). Immunofluorescence detection of p300 in nAb and cAb-treated MCF-7 cells cultured with garcinol or DMSO showed p300 remained localised to the nucleus (Figure 4.7E), this demonstrates that inhibition of p300 with garcinol does not alter cellular localisation of p300.

Inhibition of p300 in HeLa cells has been shown to predominantly repress gene transcript expression (Balasubramanyam et al., 2004, Mantelingu et al., 2007). To establish whether p300 is a regulator of the altered gene transcripts in nAb-treated MCF-7 cells, garcinol was added to MCF-7 cells and effects on transcription were
assessed. Following treatment of MCF-7 cells with E-cadherin SHE78.7 nAb, microarray analysis identified the increased expression of IGFBP-3 and SNAI2, and repression of EMP-1 and S100A9 gene transcripts, which were confirmed by quantitative PCR in Chapter 3 (Figure 3.8). Here, expression of IGFBP-3, SNAI2, EMP-1 and S100A9 were assessed in MCF-7 cells following application of E-cadherin nAb±garcinol or relevant controls (Figure 4.8A-C). Treatment of MCF-7 cells with E-cadherin nAb + DMSO induced a 2.39 fold decrease in EMP-1 transcript expression compared to cAb+DMSO-treated controls (Figure 4.8A), a similar level of repression (2.86 fold) was observed in populations treated with cAb+garcinol. Treatments with E-cadherin nAb+garcinol further repressed EMP-1 transcript expression by 3.33 fold. Similar trends were observed in S100A9 expression (Figure 4.8B), nAb + DMSO induced a -5.31 fold change and cAb+garcinol resulted in 5.89 fold decrease in transcript expression. Treatment with nAb+garcinol induced a 9.17 fold decrease compared to controls. A 2.01 fold increase in IGFBP-3 gene transcript expression was observed following treatment with nAb+DMSO (Figure 4.8C), cAb+garcinol induced a 1.64 fold increase and nAb+garcinol resulted in a 2.41 fold increase compared to cAb+DMSO-treated controls. Expression of SNAI2 gene transcripts were increased 1.79 fold in nAb+DMSO-treated MCF-7 cells compared to cAb+DMSO-treated controls (Figure 4.8D). Garcinol induced a 1.90 fold increase in SNAI2 gene transcripts in cAb-treated MCF-7 cells and treatment with nAb+garcinol induced a further increase of 2.58 fold. Together, these results suggest that E-cadherin positively regulates p300 function in MCF-7 cells and the E-cadherin nAb is able to inhibit this function. Interestingly, Slug (SNAI2) has been described as a key driver of the mammary stem cell state (Guo et al., 2012). To determine whether inhibition of p300 also acts as a driver of a stem cell phenotype, side population analysis was also performed on MCF-7 cells cultured with garcinol for 3 days (Figure 4.8E[i]). Interestingly, this increased the percentage of MCF-7 cells able to efflux Hoechst 33342 dye from 0.10% in DMSO-treated controls to 0.4% in garcinol-treated cells (Figure 4.8E[ii]). Incubation with verapamil inhibited the efflux of Hoechst 33342 dye in garcinol-treated cells, which validates the side population results although the increase observed between DMSO and garcinol-treated MCF-7
cells were not statistically significant. In order to further assess the ability of
garcinol to induce a CSC population the expression of *CD24* and *CD44* gene
transcripts were quantified by real-time PCR (Figure 4.8F[i-ii]). Application of E-
cadherin nAb+DMSO repressed CD24 gene transcripts by 1.30 fold (Figure 4.8F[i]),
this was further repressed by treatment with cAb+garcinol to 1.53. However,
treatment with nAb+garcinol exhibited similar repression to nAb+DMSO with a 1.25
fold decrease observed from cAb+DMSO controls. Treatment of MCF-7 cells with
nAb+garcinol had no significant effect when compared to cAb+DMSO controls but
treatment with cAb+garcinol or nAb+garcinol demonstrated significant increases of
1.50 and 1.53 respectively (Figure 4.8F[ii]). Together, these results demonstrate
that inhibition of p300 is sufficient to alter expression of CSC associated gene
transcripts.
Figure 4.7: Garcinol reduces cell contact and decreases proliferation of MCF-7 cells
MCF-7 cells were cultured with garcinol or an equivalent volume of DMSO (0.05%) as a control, images were acquired after 3 days (A) Scale bars represent 50µM. Flow cytometry analysis identified expression of E-cadherin at the cell surface following treatment with garcinol (B) and no significant differences in E-cadherin gene transcript expression were observed between treatments (C). Treatment with garcinol significantly reduced cell numbers compared to cAb+DMSO controls (D), One-way ANOVA statistical analysis was performed using data from 3 biological replicates (n=3), a Tukey’s post-test to compare the means to the control (cAb+DMSO; p<0.05 *, p<0.0001 ****). Immunofluorescence detection of p300 identified only nuclear staining in cAb and nAb cells treated with garcinol or DMSO (E). Scale bars represent 75µM.
Chapter 4 | Results

Figure 4.8: Garcinol induces similar gene transcript changes to E-cadherin SHE78.7 and transcription of CSC-related genes

MCF-7 cells were cultured in the presence of E-cadherin nAb ± garcinol or relevant controls for 3 days. Expression of EMP-1 (A), S100A9 (B), IGFBP-3 (C) and SNAI2 (D) gene transcripts were quantified by PCR and normalised to cAb+DMSO. Side population analysis was performed (E[i]) and quantified (E[ii]) to determine whether garcinol could drive a stem cell phenotype. Results were statistically analysed using a One-way ANOVA with a Tukey’s post test to compare the means (n=3), statistical significance was set at p<0.05*.

Expression of CD24 (F[i]) and CD44 (F[ii]) gene transcripts were quantified by PCR to further establish the acquisition of a stem cell phenotype. Data are representative of 3 experiments (n=3).
4.3 Discussion

This study has demonstrated that exogenous inhibition of E-cadherin induces a range of gene transcript alterations, which are not limited to functions related to adhesion. This is in agreement with previous observations in our lab in E-cadherin\(^{-/-}\) ES and ENPS cells (Soncin et al., 2011, Hawkins et al., 2014). Similar to results in mouse ES cells this study demonstrates the MCF-7 E-cadherin+nAb gene transcript changes are associated with canonical pathways such as ‘RAR activation’, ‘tight junction signalling’ and ‘molecular mechanisms of cancer’. Death receptor signalling was also among the most enriched canonical signalling pathways in the MCF-7+nAb array but array analysis of apoptosis related proteins in the previous chapter failed to detect differences in the expression of these receptors between cAb and nAb-treated populations (Figure 3.6B). In accordance with this finding antibody mediated neutralisation of E-cadherin has recently been shown to impair apoptosis mediated by the death receptors DR4 and DR5 (Lu et al., 2014). This chapter also describes similarities between the MCF-7+nAb and naïve human ES cells gene transcript signature described by Gafni et al. (2013). However, it seems that these stem cell similarities do not manifest at the protein level as a characteristic stem cell side population. This observation further supports the conclusion from Chapter 3, which suggests that exogenous inhibition of E-cadherin alone is unable to induce a CSC phenotype at the protein level. However, nAb-treated MCF-7 cells do exhibit some characteristics of a stem cell phenotype such as increased expression of Slug gene transcripts, and increased proliferation and resistance to apoptosis. Interestingly, treatment with garcinol did increase the percentage of side population cells observed and the quantitative PCR results demonstrated that garcinol mediated repression of p300 is sufficient to induce repression of CD24, and upregulation of CD44 and Slug CSC associated gene transcripts. These data suggest repression of p300 contributes to the acquisition of CSC traits.

A network modelling approach was applied to identify drivers of the E-cadherin nAb transcriptome in MCF-7 cells, which identified p300 as a core modulator. Exogenous inhibition of E-cadherin in MCF-7 cells was insufficient to alter cellular localisation
of p300 and conflicting results were obtained for total expression by western blot and immunofluorescence. This is in contrast to observations in E-cadherin−/− mouse ES cells in our lab, which exhibited decreased expression of p300 compared to wild-type controls (Segal; unpublished data). Similarly, only nuclear expression of p300 was observed in wild-type mouse ES cells whereas E-cadherin−/− mouse ES cells displayed expression of p300 in the cytoplasm and in the nucleus, which suggests loss of E-cadherin mediated cell-cell contact leads to repression of p300 nuclear activity. Preliminary results in human ES cells also indicate that p300 is localised exclusively to the nucleus and antibody mediated manipulation of E-cadherin is insufficient to alter this (Segal; unpublished data), which may indicate species differences in the regulation of p300. However, p300 functions as a co-activator with over 400 binding partners identified for p300 and its paralog CREB binding protein (CBP, CREBBP) in the literature, which can modify p300 activity (Bedford et al., 2010). Therefore, decreased p300 protein expression or altered cellular localisation may not be necessary to alter the gene transcriptome. Small molecule inhibition of p300 with garcinol induced gene transcript alterations similar to those observed following application of E-cadherin nAb. This suggests that the exogenous inhibition of E-cadherin results in the inhibition of p300. In the literature p300 is associated with transcription initiation, therefore, nAb or garcinol-induced inhibition of p300 should prevent transcription of p300 target genes and result in down-regulated gene expression compared to controls. In support of this theory, previous studies have demonstrated that inhibition of p300 is generally associated with repressed gene transcription (Balasubramanyam et al., 2004, Mantelingu et al., 2007). Repression of EMP-1, S100A9 and CD24 gene transcripts following inhibition of p300 with garcinol has been demonstrated here but direct interaction of p300 with the promoter region of these genes has yet to be confirmed. Chromatin immunoprecipitation (ChIP) experiments should be performed to confirm these interactions but due to time constraints this was not conducted in this study. Increased expressions of IGFBP-3 and SNAI2 gene transcripts were also observed following E-cadherin nAb-treatment or inhibition of p300 with garcinol, which suggests inhibition of p300 relieves transcriptional repression of these
transcripts. As an alternative theory, treatments of MCF-7 control populations with garcinol reduced cell-cell contact, this loss of cell-cell contact may have mimicked nAb treatment and induced similar gene transcript alterations. Ideally, more gene transcript alterations (particularly upregulated genes) need to be investigated and this should be accompanied by ChIP experiments to determine whether p300 acts as a regulator of the E-cadherin nAb-induced transcriptome or if the changes are due to loss of cell adhesion. It also remains a possibility that inhibition of p300 may alter the conformation and activity of transcriptional complexes, which may explain the upregulation of gene transcripts observed here. This is supported by IPA analysis, which identified no direct interactions between p300 and any of the transcripts significantly altered by nAb or garcinol treatment with the exception of CD44 (Figure 4.9A), which binds p300 in association with and has been linked to transcription of CSC-associated genes (Su et al., 2011). Interestingly, previous studies have linked p300 with STAT3 activation (Wang et al., 2005), STAT3 was identified as a closely related node in all modules that also identified p300 as either a MetaNode or closely related node (Figure 4.4B). Experiments in mouse ES cells have demonstrated decreased phosphorylation of STAT3 following loss of E-cadherin expression (Hawkins et al., 2014). Similarly, treatment of D3 mouse ES cells with garcinol also reduced STAT3 activation (Segal, unpublished data). Due to time constraints, it was not possible to investigate the extent of STAT3 activation in E-cadherin nAb and garcinol-treated cells. However, garcinol has been associated with decreased total expression and phosphorylation of STAT3 (Ahmad et al., 2012) and IPA analysis of the microarray data from this study indicate that STAT3 activation would be reduced in nAb-treated MCF-7 cells (Figure 4.9B). It would certainly be interesting to elucidate whether E-cadherin mediated cell-cell contact regulates STAT3 activation in humans and whether this is affected by inhibition of p300.

This study selected p300 for further investigation due to the identification of p300 as a MetaNode and a node within 3 other of the highly ranked modules in the network model, and because experiments in our lab in E-cadherin−/− mouse ES cells
have also identified E-cadherin as a regulator of p300 (Segal, unpublished data). However, it would also be interesting to investigate the role of other prominent nodes in the network such as ELAV like RNA binding protein 1 (ELAVL1), which binds to the 3’UTR of mRNA to increase their stability and was identified as a node in 3 core modules.

In conclusion, these data demonstrate that exogenous inhibition of E-cadherin is sufficient to induce gene transcript changes with functions not limited to cell adhesion. Although similarities were observed between the gene transcript signatures of MCF-7+nAb cells and naive human ES cells, this was not sufficient to induce a characteristic CSC phenotype by side population analysis. Network analysis identified E-cadherin mediated cell-cell contact as a positive regulator of p300 and MCF-7+nAb associated gene transcripts. This is supported by the correlated expression of p300 with E-cadherin in breast tissue sections and decreased p300 expression following exogenous inhibition of E-cadherin in MCF-7 cells by immunofluorescence. Although, additional experiments are required to investigate p300 interaction with target DNA or the potential conformational differences between transcriptional complexes upon p300 repression.

Figure 4.9: STAT3 pathway analysis in E-cadherin nAb-treated MCF-7
Ingenuity Pathway Analysis (IPA) revealed a direct interaction between EP300 and CD44 but not IGFBP3, EMP-1, SNAI2, S100A9 or CD24 (A). Further IPA analysis of the microarray data identified repressions of multiple components in the STAT3 pathway (green), no upregulation was observed (B).
Chapter 5. Manipulation of cell surface E-cadherin alters cell signal transduction and enhances drug efficacy.
5.1 Introduction

E-cadherin is a single pass transmembrane glycoprotein and is the core component of the adherens junction. The ectodomain of E-cadherin forms homophilic interactions with E-cadherin dimers on adjacent epithelial cells to mediate cell adhesion (van Roy and Berx, 2008). Stabilisation and tethering of E-cadherin to the actin cytoskeleton is achieved by the cytoplasmic cell adhesion complex (CCC), which is comprised of multiple proteins that bind via the E-cadherin cytoplasmic domain, including α, β and p120-catenin (Ozawa and Kemler, 1992, Ishiyama et al., 2010, Hong et al., 2013). A recent comprehensive analysis of the E-cadherin interactome described >500 proteins associated with E-cadherin with various functions, including metabolic enzymes, DNA transcription and RNA translation (Guo et al., 2014).

E-cadherin is commonly described as a tumour suppressor due to its role in contact dependant growth inhibition (Kim et al., 2011, St. Croix et al., 1998). In accordance with this theory, loss of E-cadherin mediated cell-cell contact has been associated with increased proliferation of human cancer and mouse embryonic stem (ES) cells (St. Croix et al., 1998, Soncin et al., 2011). E-cadherin has been implicated in several other signalling cascades, for example, co-localisation of E-cadherin with multiple receptor tyrosine kinases, including EGFR, Neu (Erb-B2) and IGF-1R has been described (Qian et al., 2004, Guo et al., 2014). Furthermore, antibody mediated neutralisation of E-cadherin was shown to be sufficient to induce phosphorylation of EGFRs but not active dimerisation of the receptors (Qian et al., 2004, Guo et al., 2014). Similarly, localisation of E-cadherin with the death receptors DR4 and DR5 has been shown to positively regulate apoptotic signalling with loss of E-cadherin mediated cell-cell contact contributing to resistance to apoptosis (Lu et al., 2014). In mouse ES cells, the interaction of the E-cadherin extracellular domain with Leukaemia inhibitory factor (LIF) receptor has been described (del Valle et al., 2013). Self-renewal of mouse ES cells is typically mediated through LIF (Niwa et al., 2009), yet LIF is dispensable in E-cadherin−/− mouse ES cells or wild type D3 mouse
ES cells treated with E-cadherin RNA interference (RNAi) or neutralising peptides (Soncin et al., 2009). These cells maintain pluripotent self-renewal using Activin/Nodal and FGF2 signalling cascades in the absence of LIF whereas wild-type ES cells expressing full length E-cadherin differentiate. Limited studies have also described the ability of E-cadherin to influence cellular localisation of proteins, with translocation of the ST4 oncofetal protein from the cytoplasm to the cell surface observed in human and mouse ES cells in response to exogenous inhibition of E-cadherin using neutralising antibodies (Eastham et al., 2007, Spencer et al., 2007). Similarly, co-localisation of E-cadherin with Eph receptor A2 was observed at cell contact regions of embryonic stem cells, however, Eph receptor A2 translocated to the perinuclear region in the absence E-cadherin or following dominant negative expression of E-cadherin lacking the extracellular domain (Orsulic and Kemler, 2000).

Therapeutic targeting of cadherins for treatment of cancer is being increasingly described in the literature (Blaschuk and Devemy, 2009). The N-cadherin neutralising peptide ADH-1 (also known as CHAVC and Exherin™) is being tested in clinical trials after showing promise for treatment of pancreatic cancers in in vitro cell lines and in vivo mouse models (Perotti et al., 2009, Shintani et al., 2008). Furthermore, combined dosing of ADH-1 with melphalan for treatment of melanoma was shown to enhance cytotoxicity and suppress tumour growth in rat preclinical models and phase I clinical trials in patients with N-cadherin positive tumours (Augustine et al., 2008, Beasley et al., 2009). Given that tumours likely consist of heterogeneous populations of cells that express both E- and N-cadherin, targeting E-cadherin may prove valuable to disaggregate the tumour mass and improve drug delivery when used as a combination therapy. A proof of concept study has been performed in human HT29 colon carcinoma cells using an E-cadherin neutralising antibody where increased uptake and efficacy of treatments, including paclitaxel and vinblastine, were observed (Green et al., 2004).

Experiments in Chapter 3 demonstrated exogenous inhibition of E-cadherin using a neutralising antibody in MCF-7 mammary epithelial cells increased cell growth and
survival within 3 days of application when compared to populations treated with a control antibody. It is, therefore, hypothesised that the disruption of E-cadherin mediated cell-cell contacts may alter endogenous cell signalling and response to small molecule therapeutics. In this study, exogenous inhibition of E-cadherin was achieved using a 12-mer E-cadherin neutralising peptide (nPep) to assess whether this effects proliferation and apoptosis, efficacy of cancer therapeutics and plasma membrane protein expression.
5.2 Results

5.2.1 E-cadherin neutralising peptide reversibly inhibits E-cadherin mediated cell-cell contact in MCF-7 cells

MCF-7 cells were treated with E-cadherin neutralising peptide (nPep; Devemy and Blaschuk, 2009), a control peptide (cPep) or H$_2$O as a vehicle control. Cells treated with H$_2$O or cPep maintained an adherent ‘cobble stone’ like morphology whereas nPep-treated cells exhibited loss of cell-cell contact (Figure 5.1A[i]). Analysis of E-cadherin protein expression was assessed by immunofluorescence microscopy (Figure 5.1A[ii]), western blot (Figure 1B) and flow cytometry (Figure 5.1C) revealed no significant changes between the treatments. Removal of nPep from MCF-7 cells led to reversion to the adherent ‘cobble stone’ morphology demonstrating loss of cell-cell contact was a reversible process (Figure 5.1D).

5.2.2 Neutralisation of E-cadherin in MCF-7 cells using nPep increases cell numbers in adherent monolayer and suspension culture

MCF-7 cells were treated with nPep, cPep or H$_2$O and cell numbers were monitored every 3 days for a total of 9 days (Figure 5.2A). Cell counts were significantly higher with nPep compared to H$_2$O and cPep from 3 days (Figure 5.2A). Cell counts remained significantly higher at all time-points and after 9 days nPep total cell numbers exhibited a 1.55 fold increase compared to H$_2$O and a 1.47 fold increase compared to cPep. Cell doubling time of the nPep-treated MCF-7 population decreased to 30.96±0.17h compared to 33.64±0.60h and 34.07±0.39h in cPep and H$_2$O-treated populations respectively. Following removal nPep from MCF-7 cells, the cell numbers returned to control population levels and no significant differences were observed between control and nPep populations (Figure 5.2B).

To establish whether the increased cell counts observed in nPep-treated populations were attributed to increased proliferation, expression of the proliferative marker, ki-67, was assessed following application of nPep to MCF-7 cells for 3 days (Figure 5.2C). However, no differences were observed between
nPep and control-treated cells. Analysis of the percentage of cells in each phase of the cell cycle was also performed but no significant differences were observed in the percentage of the cells in G1, S-phase or G2 phase of the cell cycle (Figure 5.2D). Upregulation of p27^Kip1 has been described in cell populations responsive to contact inhibition (Motti et al., 2005), but western blot and flow cytometry analysis revealed no significant differences in the expression of p27^Kip1 in nPep or control populations (p=0.5512; Figure 5.2E-F). Immunofluorescence staining showed p27^Kip1 was localised exclusively to the nucleus in all populations (Figure 5.2G[i]). Similar to observations in nAb-treated MCF-7 cells described in Chapter 4, nuclear area of DAPI staining appeared smaller in nPep-treated MCF-7 cells compared to controls, to confirm this nuclear area was quantified (Figure 5.2G[ii]). No significant differences were observed between the mean nuclear area of H2O and cPep-treated populations but nPep-treated MCF-7 cells exhibited significantly smaller nuclear area compared to H2O and cPep nuclei.
Chapter 5 | Results

Figure 5.1: Application of E-cadherin neutralising peptide inhibited cell-cell contact in MCF-7 cells

MCF-7 cells were treated E-cadherin neutralising peptide (nPep), a control peptide (cPep) or H₂O as a vehicle control, phase contrast images were acquired (A[i]; scale bars represent 50µm). Expression of E-cadherin was detected by immunofluorescence (A[ii]; scale bars represent 25µm), western blot (B) and flow cytometry (C). Phase contrast images were acquired 48h after removal of H₂O, cPep and nPep treatments (D; scale bars represent 50µm).
**Figure 5.2: Application of nPep increases MCF-7 cell numbers**

MCF-7 cell were treated with H₂O, cPep or nPep and cell numbers were assessed every 3 days for a total of 9 days (A), after 9 days H₂O, cPep and nPep were removed and cell numbers were assessed for a further 9 days (B). To detect increases in proliferation expression of ki67 (C) and cell cycle analysis (D) were assessed by flow cytometry. Expression of cell cycle inhibitor p27\(^{Kip1}\) was determined by flow cytometry (E), western blot (F[i-ii]) and immunofluorescence (G[i]). Scale bars represent 50µm. Mean nuclear area of H₂O, cPep or nPep-treated MCF-7 cells were quantified. Data were statistically analysed using a One-way ANOVA with a Tukey’s post-test (G[ii]), \(p<0.05^*\), \(p<0.0001^{****}\). All data are representative of 3 biological replicates (n=3).
To determine whether the increased cell counts were due to decreased apoptosis, cell viability was determined by staining cell populations with Annexin V and Propidium iodide (Figure 5.3A[i]). Similar to observations in MCF-7 cells treated with E-cadherin SHE78-7 neutralising antibody (nAb), nPep-treated cells displayed an increased viable (Annexin V/PI⁻) population, 81.83±0.55% compared to 62.71±0.58% and 62.34±0.72% in cPep and H₂O-treated populations respectively (Figure 5.3A[ii]). A decreased number of dead/necrotic (Annexin V⁺/PI⁺) cells were also observed. Decreased apoptosis (Annexin V⁺/PI⁻) was observed in nPep-treated populations compared to controls. In nPep-treated populations 1.03±0.07% were apoptotic compared to 1.83±0.16% and 2.35±0.19 in cPep and H₂O-treated populations respectively. However, this was not statistically significant. No significant differences were observed in the percentage of viable, dead/necrotic or apoptotic cells between cPep and H₂O-treated populations. This suggests the increased cell numbers observed in nPep-treated MCF-7 cells were due to increased survival in culture. Although no significant differences were observed between cPep and H₂O-treated cells with respect to cell numbers, viability or morphology, Biacore analysis has revealed a weak interaction between cPep and E-cadherin (Segal, personal communication). This suggests the use of cPep, which differs from nPep by a single amino acid (Tryptophan → Arginine) may not serve as an adequate control. Since application of a scrambled version of nPep resulted in cell death (data not shown), H₂O (vehicle control) was employed as the main control for the following experiments.

Extracellular inhibition of E-cadherin using the E-cadherin neutralising antibody DECMA-1 has been shown to increase expression of p53 and decrease expression of inhibitor of apoptosis proteins (IAPs), including Survivin, in MCF-7 cells (Brouxhon et al., 2013). To further investigate the effect on apoptosis, apoptosis proteome arrays were performed on nPep and control-treated populations of MCF-7 cell after 3 days of treatment (Figure 5.4A[i-ii]). Differences >1.5 fold between control and nPep populations were lacking for all apoptosis-related proteins. Expression of p53 was also absent by western blot (Figure 5.4B). This is in contrast to findings described in
Chapter 3 where MCF-7 cells treated with E-cadherin SHE78.7 nAb for 3 days (Figure 3.6B-C), which resulted in an >2 fold increase in expression of phospho-p53 proteins and the IAP Survivin (Figure 3.6B). In addition, increased expression of p53 was observed by western blot in nAb-treated but not control cells (Figure 3.6C).

**Figure 5.3: Exogenous inhibition of E-cadherin increases cell survival in monolayer culture**

MCF-7 cell were treated with H2O, cPep or nPep and the Annexin V/Propidium iodide was performed after 3 days to determine cell viability (A[i]). The percentage of live, dead and apoptotic cells were quantified and statistically analysed (A[ii]). Statistical significance was determined using a One-way ANOVA with a Tukey’s post test to compare the means to the controls (n=3; p<0.0001***).
Figure 5.4: Exogenous inhibition of E-cadherin with nPep is insufficient to alter expression of apoptosis-related proteins in MCF-7 cells

Expression of apoptosis-related proteins were determined using proteome arrays following treatment of MCF-7 cells with nPep or vehicle control for 3 days (A[i]), fold changes > ±1.5 previously identified with nAb-treatment in Chapter 3 are highlighted (A[ii]). Expression of p53 was determined in MCF-7 cells cultured with E-cadherin nPep for 3 days (B). MDA-MB-231 cells were used as a positive control for expression of p53.
Since 3-dimensional culture of cells is considered to be more representative of an *in vivo* environment, the effects of the E-cadherin nPep treatment on cell number and apoptosis were determined in ultra-low attachment plates. Control-treated MCF-7 cells formed aggregates that could not be dispersed with a pipette, whereas nPep-treated cells grew as near single cells that were easily dispersed (Figure 5.5A). Cell numbers were assessed every second day for a total of 6 days, with significantly higher cell numbers in MCF-7 cells treated with nPep compared to control-treated cells (Figure 5.5B). For example, on day 6 nPep-treated MCF-7 populations exhibited a mean cell count of $1.57 \times 10^6 \pm 1.21 \times 10^5$ compared to $3.00 \times 10^5 \pm 2.00 \times 10^4$ in control populations ($p<0.001$). These cell numbers represented population doubling times of $33.57 \pm 0.89$ h in nPep-treated MCF-7 cells compared to $75.73 \pm 3.84$ h in controls, which suggests loss of E-cadherin mediated cell-cell contact is a growth advantage in a 3-dimensional environment. Cell cycle analysis was performed to determine whether the increased cell numbers were due to increased proliferation of nPep-treated cells (Figure 5.5C[i]). A significant decrease in the percentage of cells in the G1 phase of the cell cycle was observed in nPep-treated MCF-7 cells compared to controls ($p=0.046$), the percentage of nPep-treated MCF-7 cells in the S-phase of the cell cycle was double the percentage observed in control populations ($p=0.015$) but no significant differences were observed between the percentage of cells in the G2 phase of the cell cycle ($p=0.482$). Together these data suggest exogenous inhibition of E-cadherin enables increased proliferation of MCF-7 cells in a 3-dimensional environment.

Cell viability of nPep and control-treated populations in 3-dimensional culture were determined using the Annexin V/Propidium iodide assay (Figure 5.5D[i-ii]). Similar to our observations in cAb/nAb-treated populations no significant differences were observed between E-cadherin neutralised or control populations in the percentage of Annexin V−/PI− live or Annexin V+/PI− apoptotic cells ($p=0.105$ and $p=0.192$ respectively). A small but significant ($p=0.017$) difference was, however, observed between the two treatments, with the percentage of Annexin V+/PI+ dead/necrotic cells, the mean percentage of dead cells $1.48 \pm 0.12\%$ (nPep) compared to
3.56±0.52% (control). These data suggest increased survival of E-cadherin neutralised MCF-7 population in a 3-dimensional environment. However, the differences observed in the percentage of dead cells was very small so it is likely that the main reason for the increased cell numbers observed was due to an increase in proliferation as evidenced by the increased number of cells in the S-phase of the cell cycle and decreased doubling times of nPep-treated cells.

Figure 5.5: Culture of nPep-treated MCF-7 cells in a 3-dimensional environment increases cell numbers
MCF-7 cells were cultured in ultra-low attachment plates with nPep or H$_2$O as a control (A), cell numbers were assessed every second day for a total of 6 days (B). To detect changes in proliferation cell cycle analysis was performed (C[i]) and the percentage of cells in each phase of the cell cycle was quantified (C[ii]). Annexin V/Propidium iodide (PI) assay was performed after 2 days to assess cell viability (D[i]) and the percentage of live, dead and apoptotic cells were quantified (D[ii]). Control and nPep data were statistically analysed using an un-paired Student’s t-test (n=3, $p<0.05^*$, $p<0.01^{**}$, $p<0.001^{***}$).
Chapter 5 | Results

5.2.3 Inhibition of E-cadherin mediated cell contact in MCF-7 cells enhances drug sensitivity

Due to the altered growth and survival characteristics of nPep-treated MCF-7 cells, loss of E-cadherin mediated cell-cell contact was hypothesised to induce alterations in cell signalling and cells may, therefore, respond differently to exogenous stimuli. In addition, use of the E-cadherin neutralising peptide has been hypothesised to improve drug delivery as a combination therapy (Devemy and Blaschuk, 2009). To test these hypotheses, we screened a panel of anti-cancer drugs against MCF-7 cells cultured in the presence and absence of E-cadherin neutralising peptide. Cell count and cell death analysis were performed and results were analysed according to drug mode of action (MOA).

5.2.3.1 Protease inhibitors

Proteasome inhibitors bortezomib and carfilzomib displayed increased cell death and decreased cell numbers in nPep-treated populations at almost all concentrations tested (Figure 5.6A-B). MG132 showed no observable difference between 0.1nM-1µM (Figure 5.6C[i]), however, at a concentration of 10µM an increase in cell death was observed although this was not statistically significant. Cell numbers of E-cadherin nPep-treated populations cultured with MG132 at doses of 1-100nM were significantly higher than controls (Figure 5.6C[ii]), however these numbers decreased at doses of 100nM and above. Given that all 3 drugs have the same MOA, these data suggest that disruption of E-cadherin mediated cell-cell contacts increases sensitivity of MCF-7 cells to proteasome inhibitors.
Figure 5.6: E-cadherin neutralising peptide enhances efficacy of proteasome inhibitors in MCF-7 cells

MCF-7 cells were cultured with nPep or vehicle control for 2 days prior to addition of bortezomib (A), carfilzomib (B) and MG132 (C) proteasome inhibitors at concentrations of 1-10³ nM for 3 days. Cell death was assessed by positive DRAQ7 staining and cell numbers were determined by nuclear counts after 3 days. Control and nPep data were statistically analysed using an un-paired Student’s t-test (n=4, \( p<0.05 \), \( p<0.01 \), \( p<0.001 \)).
5.2.3.2 Mitotic inhibitors

The efficacies of multiple anti-mitotic therapeutics were also increased when added in combination with nPep. Docetaxel significantly increased the percentage of cell death and decreased cell numbers at all concentrations tested (Figure 5.7A[i-ii]). Epothilone B also enhanced cell death in E-cadherin nPep-treated MCF-7 cells at all concentrations tested but the effects on cell number were less profound (Figure 5.7B[i-ii]). Paclitaxel, vinblastine and vincristine significantly decreased E-cadherin nPep-treated cell numbers at all concentrations tested (Figure 5.7C[ii], D[ii] and E[ii]). However, only vinblastine enhanced cell death of nPep-treated populations at some concentrations and paclitaxel and vincristine failed to demonstrate a significant increase in drug efficacy when compared to control treatments at any concentration (Figure 5.7C[i], D[i] and E[i]).

5.2.3.3 Folate cycle and Metabolite inhibitors

Pralatrextate, an anti-folate therapeutic, had little impact on cell death at all concentrations tested in control treated populations (Figure 5.8A[i]). When applied in combination with nPep, significant increases in cell death at 10-10³nM were observed compared to controls. Significant decreases in nPep-treated cell numbers were also observed at pralatrexate concentrations of 1-1000nM (Figure 5.8A[ii]). Doses of an alternative anti-folate therapeutic, methotrexate, at 1-10nM induced no significant differences in the percentage of cell death between nPep and control-treated MCF-7 cells (Figure 5.8B[i]). However, at 100-10³nM cell death was significantly increased in nPep-treated populations compared to controls. This was also accompanied by significant decreases in cell number at doses of 10-10³nM when compared to controls (Figure 5.8B[ii]).

Of the antimetabolite therapeutics, floxuridine displayed significantly increased efficacy when administered to nPep-treated MCF-7 cells (Figure 5.8C[i-ii]), increased cell death and decreased cell numbers were observed at all concentrations tested. Similarly, nPep-treated cells cultured with cytarabine displayed increased cell death at doses of 1µM and above, although cell numbers
were similar to that observed in control populations at all concentrations tested (Figure 5.8D[i-ii]).

**Figure 5.7: Exogenous inhibition of E-cadherin increases efficacy of mitotic inhibitors**
MCF-7 cells were treated with E-cadherin nPep or vehicle control in combination with small molecule mitotic inhibitors docetaxel (A), epothilone B (B), paclitaxel (C), vinblastine (D) and vincristine (E). Cell death was assessed by positive DRAQ7 staining and cell numbers were determined by nuclear counts after 3 days. Control and nPep data were statistically analysed using an un-paired Student’s t-test (n=4, p<0.05*, p<0.01**, p<0.001***).
Figure 5.8: Increased efficacy of folate cycle inhibitors and anti-metabolite therapeutics in n Pep-treated MCF-7 cells
MCF-7 cells were treated with E-cadherin nPep or vehicle control in combination with small molecule folate cycle inhibitors pralatrexate (A) and methotrexate (B), or anti-metabolite therapeutics floxuridine (C) and cytarabine(D). Cell death was assessed by positive DRAQ7 staining and cell numbers were determined by nuclear counts after 3 days. Control and nPep data were statistically analysed using an un-paired Student’s t-test (n=4, p<0.05*, p<0.01**, p<0.001***).
5.2.4 Reduced efficacy of small molecule therapeutics in nPep-treated MCF-7 cells

Generally, treatment of MCF-7 cells with E-cadherin nPep and small molecule cancer therapeutics enhanced drug efficacy, however, some exceptions were observed. Control-treated MCF-7 cells displayed increased cell death when cultured with vorinostat, a histone deacetylase inhibitor, at $10^{-10}$ nM and cell numbers were also decreased, however the percentage of dead cells were only significant at doses of $10^{-10}$-100 nM (Figure 5.9A[i-ii]). Mitoxantrone, a topoisomerase inhibitor, also induced a significant increase in cell death at concentrations of 100 nM and above in control populations, whereas little effect was observed in nPep-treated populations (Figure 5.9B). Interestingly a second topoisomerase inhibitor, Topotecan, displayed increased cell death when administered with nPep and resulted in a decrease in cell numbers in both control and nPep-treated populations (data CD) although cell counts generally remained significantly higher in nPep-treated populations. Azacytidine also induced a significant increase in cell death of control-treated cells and this was accompanied by a significant decrease in cell number (Figure 5.9C[i-ii]), however, cell death and decline in cell number was negligible in nPep-treated populations. Similar trends were also observed in population treated with bexarotene and fludarabine (Figure 5.9D[i-ii]-E[i-ii]). For complete results on all small molecules tested see data CD.
Figure 5.9: Treatment of MCF-7 cells with nPep decreases drug efficacy
Cell death and cell numbers were assessed after 3 days of treatment with nPep in combination with vorinostat (A), mitoxantrone (B), azacytidine (C), bexarotene (D) and fludarabine (E). Cell death was assessed by positive DRAQ7 staining and cell numbers were determined by nuclear counts after 3 days. Control and nPep data were statistically analysed using an un-paired Student’s t-test (n=4, p<0.05*, p<0.01**, p<0.001***).
5.2.5 Exogenous inhibition of E-cadherin increases sensitivity to VEGFR inhibitors and alters Akt phosphorylation

The most dramatic difference in drug efficacy was observed following application of Vandetanib, where concentrations of 10µM vandetanib induced cell death in 0.03% of control populations compared to 71.15% in nPep-treated populations (Figure 5.10A). Vandetanib is a small molecule inhibitor of VEGFR, a second VEGFR inhibitor Axitinib was tested as part of the drug screen and also demonstrated increased cell death in nPep-treated population but to a lesser extent (data CD). In parallel experiments the effects of SU5402, a VEGFR/FGFR inhibitor, were assessed on cell numbers in control and nPep-treated MCF-7 cells (Figure 5.10B). Treatment of MCF-7 cells with SU5402 significantly inhibited growth of nPep populations compared to controls, over a period of 9 days nPep+SU5402 cell numbers decreased to cell numbers observed in control+DMSO populations. Since application of SU5402 caused differential growth characteristics between control and nPep cells, phospho-westerns were performed to determine differences in endogenous cell signalling (Figure 5.10C[i]). Akt has been associated with cell survival and lies downstream of both VEGFR and FGFR (Downward, 2004), therefore, expression of phospho-Akt was assessed in MCF-7 populations treated with the E-cadherin neutralising peptide ± SU5402 or DMSO as a vehicle control. No significant differences were observed in the percentage of Akt across populations treated with H2O, cPep or nPep with DMSO, similarly no significant differences were observed in H2O or cPep-treated populations cultured with SU5402 (Figure 5.10C[ii]). However, application of nPep with SU5402 resulted in a significant decrease in phospho-Akt compared to DMSO-treated populations. These data suggests nPep-treated populations cultured with SU5402 experience decreased cell survival via Akt signalling, which may reflect the decreased proliferation observed in these cells.
Figure 5.10: Differential response of nPep-treated MCF-7 cells to VEGFR inhibitors
Application of Vandetanib, a VEGFR inhibitor, in combination with E-cadherin nPep significantly altered drug efficacy (A[i-ii]). Application of Vandetanib, a VEGFR inhibitor, in combination with E-cadherin nPep, or control-treated populations (C[i]), expression was significantly decreased proliferation of nPep-treated MCF-7 cells to control cell numbers (B). Expression of phospho-Akt was determined by western blot following application of SU5402 to nPep or control-treated populations (C[ii]), expression was quantified and normalised to expression of total Akt (C[iii]).
5.2.6 Exogenous inhibition of E-cadherin using nPep induces differences in the plasma membrane proteome

The cell surface proteome is crucial for cellular communication and transmission of extracellular signals within the internal cell environment. Given that differences in cell growth and response to small molecule inhibitors in MCF-7 cells treated with nPep were observed, experiments sought to identify alterations in the cell surface proteome. Exposed plasma membrane proteins of MCF-7 cells treated with nPep or vehicle control were biotinylated and isolated by streptavidin pulldown. Validation of plasma membrane protein isolation was performed by western blot analysis (Figure 5.11A), expression of EMP-1 was identified in membrane lysates with reduced expression observed in non-biotinylated membrane/intracellular lysates. Conversely, expression of the cytoplasmic protein Fas associated factor 2 (FAF2) was identified in the non-biotinylated membrane/intracellular lysates with reduced expression observed in the plasma membrane samples. Plasma membrane lysates were analysed by LC-MS/MS mass spectrometry, membrane proteins that were present in nPep-treated samples but absent in control samples, or vice versa, are listed in Figure 5.11B. Further analysis of these proteins by western blot using extracellular membrane lysates or flow cytometry were largely thwarted by a lack of specific antibodies. Western blot analysis did, however, confirm the increased expression of Eph receptor-B4 in nPep-treated samples (Figure 5.11C[i-ii]). In accordance with the earlier analysis of E-cadherin by flow cytometry, membrane expression levels of E-cadherin were similar between nPep and control-treated cells. Chapter 3 described decreased membrane expression of EMP-1 by flow cytometry following treatment of MCF-7 cells with E-cadherin SHE78.7 nAb, however, this was not corroborated by western blot analysis of MCF-7 cells treated with nPep. Expression of CD-9 antigen was undetectable by western blot analysis, however, flow cytometry did not reveal a decrease in CD-9 antigen expression in nPep-treated cells when compared to control populations (Figure 5.11D[i]). A small decrease in EMP-1 and small increase in Epithelial discoidin domain-containing...
receptor (DDR-1) expression were confirmed in nPep-treated MCF-7 cells when compared to controls by flow cytometry (Figure 5.11D[iii]).

**Figure 5.11: Exogenous inhibition of E-cadherin alters the cell surface proteome**

Plasma membrane proteins were isolated from MCF-7 cells treated with nPep or H₂O as a vehicle control for 3 days. Validation of the plasma membrane protein isolation was performed by western blot (A). Epithelial membrane protein-1 (EMP-1) was identified in membrane lysates (M) and reduced levels were detected in non-biotinylated membrane/intracellular (I) lysates. Conversely, intracellular FAS-associated factor 2 (FAF2) was identified in non-biotinylated membrane/intracellular lysates with reduced expression observed in the membrane lysates. Differences in plasma membrane protein expression between nPep and control-treated MCF-7 cells were determined by liquid chromatography-tandem mass spectrometry (B). Changes in protein expression were further investigated by western blot (Ci[ii]) and flow cytometry (D[iii]).
Chapter 5

5.3 Discussion

Chapter 3 described increased proliferation and decreased apoptosis of MCF-7 cells treated with an E-cadherin SHE78.7 neutralising antibody (nAb), which resulted in increased cell proliferation and resistance to apoptosis. This chapter describes treatment of MCF-7 cells with an E-cadherin neutralising peptide (nPep) to determine whether similar effects also occur. Increased MCF-7 cell numbers were observed following peptide treatment in both monolayer and 3-dimensional culture. These data are also in agreement with observations in E-cadherin\(^{-/-}\) mouse ES cells and wild-type D3 mouse ES cells treated with an E-cadherin neutralising peptide (Soncin et al., 2011). Similarly, in 3-dimensional culture an increase in proliferation of MCF-7 cells treated with an E-cadherin nAb has been described in Chapter 3 and in the literature (St. Croix et al., 1998). The authors attributed this increase in cell numbers to decreased contact inhibition, which reduced expression of the cell cycle inhibitor \(p27^{Kip1}\). In this chapter, increased proliferation of nPep-treated MCF-7 cells in 3-dimensional culture was evidenced by the increased percentage of cells in the S-phase of the cell cycle. Like St. Croix et al. (1998), this study failed to replicate these results in monolayer culture. For example, whilst increased cell numbers were observed in nPep-treated populations, no significant cell cycle differences were observed between control and nPep-treated populations in monolayer culture. Similarly, no significant differences were observed in expression of \(p27^{Kip1}\) or ki67 between control and nPep-treated MCF-7 cells, therefore this cannot explain the increased cell numbers observed in nPep-treated populations. Aggregation of cells in 3-dimensional culture restricts availability of nutrients and may enhance signals of contact inhibition due to the increased surface area of cell-cell contact, which may explain the increased cell numbers and lack of decreased \(p27^{Kip1}\) observed in monolayer culture. MCF-7 populations in this study were maintained at sub-confluence in all experiments, which may account for the lack of differences reported in expression of ki67, \(p27^{Kip1}\) or cell cycle analysis.
Increased numbers of viable cells were observed in nPep-treated cells culture in monolayer, which implies increased survival of these populations and may account for the increased cell numbers reported in monolayer culture. Unlike MCF-7 cells treated with the E-cadherin neutralising antibody SHE78.7, a lack of changes in apoptosis-related proteins was apparent in the protein arrays performed on nPep-treated MCF-7 cells. Conflicting results were observed between the expression of p53 following treatment with nAb or nPep, this suggests the increased cell numbers cannot be attributed to increased resistance to apoptosis and highlights a discrepancy between the two methods of exogenous E-cadherin inhibition. Interestingly MCF-7 cells have been reported to express wild-type p53 (p53 database, [http://p53.fr](http://p53.fr)), however, in agreement with results reported by Brouxhon et al. (2013) expression of p53 was not observed in control populations of MCF-7 cells in this study.

Increased drug efficacy in MCF-7 cells has been demonstrated for multiple cancer therapeutics following combined treatment with nPep. The nPep employed in this study is also reported to be an effective N-cadherin inhibitor (Devemy and Blaschuk, 2009), which could potentially improve drug delivery in heterogeneous epithelial tumours where an E-to-N-cadherin switch has occurred. In addition, this would create a somewhat homogenous population for additional therapeutics to target. The N-cadherin peptide antagonist (ADH-1), currently in clinical trials, has been shown promise for the treatment of gynaecological and pancreatic cancers (Perotti et al., 2009, Shintani et al., 2008). ADH-1 also enhances sensitivity to chemotherapeutics, such as melphalan, in treatment of melanoma (Augustine et al., 2008). Similarly, a recent patent application describes the use of E-cadherin inhibitors, active against cadherin regions EC2-EC5 but not EC-1, as a combination therapy (Brouxhon and Kyrkanides, 2013). Interestingly, the patent specified Vandetanib as a combination therapy that also induced a significant increase in cell death when applied in combination with nPep in this study. The authors have previously described suppression of growth and increased apoptosis following addition of DECMA-1 to MCF-7 cells (Brouxhon et al., 2013). This is in contrast to
observations with nPep in this study, which reports increased growth and survival of MCF-7 cells treated with nPep. Since increased growth and survival are advantageous traits for cancer cells, the use of nPep to improve drug delivery within a tumour could, therefore, be a cause for concern as it may exacerbate tumour cell growth. However, observations in this study show that some cancer therapeutics perform better in the presence of nPep. Therefore, knowledge of E-cadherin status in tumours may aid better prescription of therapeutics. Further drug screens using E-cadherin positive and E-cadherin negative cell lines and tumour biopsy analysis are required to confirm this hypothesis.

Differential cell signalling has been demonstrated between nPep and control-treated MCF-7 cells in response to the small molecule inhibitor SU5402. The data explains the decreased proliferation of nPep-treated MCF-7 cells cultured with SU5402, however, no significant differences were observed in phospho-Akt expression in H2O, cPep or nPep-treated cells cultured with DMSO. Therefore, the increased cell numbers observed in nPep-treated MCF-7 cells cannot be attributed to altered cell survival via Akt but an alternative mechanism that has yet to be determined.

Analysis of the plasma membrane proteome has revealed differences between the cell surface of E-cadherin neutralised and control-treated MCF-7 cells. Confirmation of many of the proteins has been hindered by a lack of specific antibodies against these proteins. Differences in expression of Eph receptor B4 by western blot and Epithelial discoidin domain-containing receptor 1 by flow cytometry have been confirmed, however, further confirmation of changes in the cell surface proteome is required. In accordance with these findings, genetic knock-out of E-cadherin (E-cadherin<sup>−/−</sup>) in embryonic stem cells has been associated with the upregulation of several Ephrin and Eph receptor gene transcripts, including Eph receptor B4, when compared to the wild-type cells (Orsulic and Kemler, 2000). The role of Ephrins and Eph receptors in cancer is complex, upregulation of Eph receptor B4 has been observed in breast cancer and associated with tumour progression, Eph receptor B4 knockdown decreased growth and survival in vivo and in vitro (Kumar et al., 2006).
On the other hand, both Ephrins and Ephrin receptors are cell membrane anchored so the loss of cell-cell contacts through inhibition of E-cadherin could reduce activation of receptors by ligands on adjacent cells and, therefore, promote tumour suppression. Recent comparisons of the cell surface proteome of E-cadherin\(-/\) and wild type mouse ES cells has revealed differences in protein expression (Ritson, personal communication), however, no similarities were observed with results obtained in this study. This may reflect the differences in cell types or differences between exogenous inhibition and genetic knockout of E-cadherin. In general analysis of cell surface protein expression is challenged by a number of factors, first by adequate separation of cell surface proteins from other cellular compartments. Sulfo-NHS-SS-Biotin, used in this thesis, is marketed as membrane impermeable and should only label amines, such as lysine residues of exposed cell membrane proteins (Elia, 2008). However, there is a risk of endogenous protein contamination from cells that have undergone lysis or proteins that have co-purified with plasma membrane proteins and this is shown by the detection of some cytoplasmic proteins in the mass spectrometry analysis. Second, many lysine residues are commonly located on the cytoplasmic side of the plasma membrane (Landolt-Marticorena et al., 1993), therefore, it is difficult to build a complete picture of the cell surface proteome. Despite this limitation, biotinylation has been demonstrated to detect an increased number of proteins compared to alternative methods such as glycoprotein affinity purification. A third limitation relates to the disaggregation of epithelial cells using nPep, it is possible that this increases the access of Biotin-NHS to the plasma membrane proteins and this may lead to increased tagging of nPep plasma membrane proteins compared to controls. This highlights the importance of confirming the mass spectrometry results using multiple methods, such as flow cytometry and staining of tissue sections where the status of E-cadherin is known. A fourth limitation is related to the analysis of the samples by mass spectrometry, only the most abundant peptides are detected using this method. Additional cell surface proteome differences are, therefore, likely to exist that were not detected by this method. Nonetheless, differences in the cell surface proteome were observed and confirmed by either western blot or flow cytometry,
although more analysis is required the results could lead to discovery of new biomarkers that are associated with aberrant expression of E-cadherin in cancer.
Chapter 6. General discussion
6.1 Introduction

Understanding the mechanisms that drive tumorigenesis and metastasis is the primary step for development of more effective cancer therapies. Dysregulation of E-cadherin in cancer is commonly described in the literature, with loss of E-cadherin described as a critical contributing factor, however, the effects of exogenous inhibition of E-cadherin are less well understood. In this thesis, dysregulation of E-cadherin was achieved by application of exogenous E-cadherin inhibitors, in the form of a neutralising antibody (nAb) or peptide (nPep), to induce disaggregation of mammary epithelial cells. This has extended our understanding of the effects of exogenous E-cadherin inhibition on tumorigenesis and metastasis in breast cancer and identified directions for future work.

6.2 Exogenous inhibition of E-cadherin increases cell numbers due to increased proliferation and reduced apoptosis

In accordance with the DENT hypothesis (Mohamet et al., 2011), increased MCF-7 cell numbers were observed in both monolayer and 3-dimensional culture following treatment with the two independent methods of exogenous E-cadherin inhibition. Experiments in this thesis sought to determine whether the increased cell numbers were due to increased proliferation or resistance to apoptosis. Cell cycle analysis corroborated previous observations, which demonstrated increased proliferation of MCF-7 cells in 3-dimensional culture following application of E-cadherin neutralising antibodies (St. Croix et al., 1998). However, this study has also demonstrated increased proliferation of nPep-treated MCF-7 cells in 3-dimensional culture, which further supports the notion that exogenous inhibition of E-cadherin increases proliferation. In contrast to results observed in 3-dimensional culture, cell cycle analysis failed to detect significant differences between the numbers of proliferating cells in monolayer culture. These findings are supported by similar experiments in mouse E-cadherin−/− ES cells (Soncin et al., 2011). Using two independent methods of E-cadherin inhibition, this study has shown increased viability of E-cadherin neutralised populations in monolayer but not 3-dimensional
culture, with increased resistance to apoptosis observed in nAb-treated MCF-7 cells. It remains a possibility that increased numbers of proliferating cells were not detected by cell cycle analysis in monolayer culture due to both control and E-cadherin neutralised populations being maintained at subconfluence and, therefore, actively growing.

Expression of phospho-Akt is commonly associated with increased cell survival (Downward, 2004), although no significant differences were identified between control and nPep-treated MCF-7 populations in this study. This suggests the increased cell numbers observed in E-cadherin neutralised populations cannot be attributed to increased survival by alterations in Akt signalling. This finding is in contrast to controversial reports in the literature that have described both activation and repression of Akt upon ligation of E-cadherin (Pece et al., 1999, Lau et al., 2011, De Santis et al., 2009). Nonetheless, the increased cell numbers observed suggest disaggregation of epithelial cells in vivo may induce increased growth and contribute to neoplasm formation. It would certainly be interesting to investigate alternative cell survival pathways in order to elucidate a mechanism for the increased survival observed nAb/nPep-treated cells. For example, application of E-cadherin neutralising antibodies in pancreatic and colon cancer cell lines has been shown to reduce apoptosis signalling via DR4/DR5 (Lu et al., 2014). In this study, microarray analysis revealed death receptor signalling was among the top pathways affected by nAb treatment of MCF-7 cells. It would be interesting to investigate how the downstream signalling components, such as Caspase 8, are affected. However, analysis of apoptosis-related proteins revealed no differences between cAb and nAb-treated MCF-7 cells in the expression of cleaved Caspase 3, which is required for commitment to apoptosis via this pathway (Gonzalvez and Ashkenazi, 2010). Alternatively, E-cadherin mediated contact inhibition of cell growth is thought to be regulated by the Hippo pathway with homophilic ligation of E-cadherin shown to be crucial for cytoplasmic retention of YAP in MCF-10A cells (Kim et al., 2011, Schlegelmilch et al., 2011, Yang et al., 2015). It remains a possibility that exogenous inhibition of E-cadherin induces nuclear accumulation of YAP in
MCF-7 cells used in this study, although this is yet to be experimentally tested. Additional avenues of investigation include energy metabolism, which is becoming increasingly recognised as a significant factor in sustaining proliferation of cancer cells (Hanahan and Weinberg, 2011). Upregulation of P-cadherin has been linked with high grade tumours and increased expression of the glucose transporter GLUT1 in breast cancer (Sousa et al., 2014). Disruption of E-cadherin has also been described in breast cancer cells that co-express P-cadherin (Ribeiro et al., 2013), therefore, it may be interesting to determine whether exogenous inhibition of E-cadherin alters expression of GLUT1 at the cell membrane.

6.3 **Prolonged exogenous inhibition of E-cadherin is insufficient to induce an EMT event or acquisition of a CSC phenotype**

Since the majority of cancer-related deaths are attributed to metastasis, understanding the mechanisms that contribute to metastatic spread and acquisition or maintenance of a CSC phenotype is crucial for development of more effective cancer therapies. Previous studies of EMT have invoked the use of growth and transcription factors that prevent E-cadherin mediated cell-cell contact but also orchestrate additional changes as a consequence of EMT (Mani et al., 2008). In this study, the use of an E-cadherin neutralising antibody has been used to study the loss of E-cadherin mediated cell-cell contact alone. Experiments determined the prolonged exogenous inhibition of E-cadherin mediated cell-cell contact was not a driver of EMT in MCF-7 cells within 50 days. Recent observations in HB2 mammary luminal epithelial cells also suggest loss of E-cadherin is not a causal factor in EMT since dominant negative expression of E-cadherin, which inhibited cell-cell contact, was unable to relieve repression of HER2 induced EMT at high cell densities (Nilsson et al., 2014). The authors also concluded loss of E-cadherin is not a prerequisite for activation of EMT since expression of E-cadherin was observed in the early stages of EMT.

Contrary to the DENT hypothesis, this study has demonstrated that loss of E-cadherin mediated cell-cell contact alone is insufficient to induce a characteristic
breast CSC phenotype within 50 days. MCF-7 cells failed to acquire a CD44\textsuperscript{high}/CD24\textsuperscript{low} expression profile. Similarly, an increased prevalence of a genuine stem cell side population was not detected. FOXC2, Twist, Snail and Slug have been described as breast CSC drivers in the literature (Hollier et al., 2013, Vesuna et al., 2009, Mani et al., 2008, Guo et al., 2012). Of these, only a modest +1.32 fold change of Slug (SNAI2) gene transcripts were observed in this study by microarray analysis and PCR. Emergence of additional signals, as well as loss of E-cadherin protein or further prolonged deprivation of E-cadherin mediated cell-cell contact, may be required to induce and EMT event and acquisition of CSC phenotype in MCF-7 cells. Support for this theory is evidenced by the observation that application of an E-cadherin neutralising antibody in combination with an EGFR agonist in epidermoid and colorectal epithelial cell lines induced an EMT-like state (Garnier et al., 2012). Similarly, short hairpin knockdown of E-cadherin in non-tumorigenic HMLE cells was sufficient to induce an EMT event and was associated with acquisition of a CD44\textsuperscript{high}/CD24\textsuperscript{low} CSC phenotype in 47.2% of the population (Gupta et al., 2009b, Onder et al., 2008). However, transfection of transformed HMLER cells that constitutively expressed the V12H-RAS oncogene increased the percentage of CD44\textsuperscript{high}/CD24\textsuperscript{low} cells to 90.3% (Gupta et al., 2009b), thus highlighting the significance of additional signals.

The acquisition of a gene transcript profile associated with CSCs following treatment with garcinol is intriguing. It is possible that garcinol exerts more efficient repression of p300 than exogenous inhibition of E-cadherin, which may explain the differences observed between the two treatments with regards to CD24 and CD44 gene transcript expression. Correlated expression of E-cadherin with p300 in breast tumour biopsies has been described here and it would be interesting to evaluate expression of p300 following short hairpin knockdown of E-cadherin to determine whether this causes more significant repression of p300 and acquisition of a CSC phenotype \textit{in vitro}. 
6.4 E-cadherin regulates gene transcription and cell surface protein expression

This study has further identified a role for E-cadherin mediated cell-cell contact in gene transcription. Much like results in HMLE and mouse embryonic stem cells, loss of E-cadherin cell-cell contact was associated with gene transcript changes of diverse functions not limited to cell adhesion (Hawkins et al., 2014, Soncin et al., 2011, Onder et al., 2008). Furthermore, network analysis in our lab revealed cross species correlation in the identification of E-cadherin as a positive regulator of p300 in MCF-7 and mouse ENPS, E-cadherin−/− and epiblast stem cells (Segal; personal communication). However, the mechanism of p300 regulation seems to differ between mouse and human cells (Segal; personal communication) since E-cadherin seems to regulate cellular localisation of p300 in mouse ES cells but not human ES or MCF-7 cells. This may also be due to the differences between loss of E-cadherin protein in mouse ES cells and treatment with exogenous inhibitors in human MCF-7 and ES cells. Although experiments confirmed inhibition of p300 drives gene transcription similar to E-cadherin nAb in MCF-7 cells, further ChIP analysis is required to confirm interaction of p300 with target DNA. However, given that increased expression of gene transcription was also observed it remains likely that inhibition of E-cadherin alters the conformation of transcriptional complexes.

Experiments in this thesis further support the DENT hypothesis by demonstrating that the exogenous inhibition of E-cadherin alters expression of plasma membrane proteins, including Eph receptor B4 and EMP-1. This will likely contribute to the altered response to exogenous stimuli, such as growth factors or xenobiotics, as evidenced by the increased efficacy of small molecule cancer therapeutics and reduced phosphorylation of Akt following SU5402 treatment. However, further validation of the mass spectrometry results are required. It would also be interesting to determine whether these results translate into nAb-treated MCF-7 cells but also tumour biopsies that demonstrate aberrant expression of E-cadherin.
This could aid discovery of new biomarkers associated with loss of E-cadherin in cancer.

6.5 Manipulation of E-cadherin enhances the efficacy of cancer therapies

Loss of E-cadherin cell-cell contact is commonly associated with poor patient prognosis and loss of E-cadherin as part of an EMT event has been linked to chemoresistance (Gupta et al., 2009b, Hiscox et al., 2006). Data from this study demonstrate the loss of E-cadherin mediated cell-cell contact alone was insufficient to confer chemoresistance, in fact, loss of cell-cell contact more commonly rendered cells more vulnerable to cytotoxic therapies. The ADH-1 peptide has shown promise in clinical trials for cancer therapy in patients with N-cadherin positive tumours (Perotti et al., 2009, Beasley et al., 2009). Both ADH-1 and the neutralising peptide used in this study are effective inhibitors of E- and N-cadherin (Devemy and Blaschuk, 2009, Blaschuk et al., 2003). Cancerous tumours consist of heterogeneous cell populations likely to express a mixture of both E- and N-cadherin positive cells; by using the N-cadherin negative MCF-7 cell line for drug screening in this study, the value of E-cadherin inhibition in enhancing drug efficacy has been demonstrated.

Formation of adherens junctions bring epithelial cells into close contact and minimise intercellular space, the application of nPep results in disaggregation of the epithelial layer, which may allow greater access of therapeutics to cells. Therefore, this a potential cause for the increased efficacy observed. The heterogenic nature of tumours presents a challenge to eradicate all cells within the tumour. Treatment with E- and N-cadherin exogenous inhibitors as a combination therapy could potentially help to overcome this by creating a somewhat homogenous population of disaggregated tumour cells, which may improve efficacy of additional treatments. This study has also demonstrated changes in the cell surface proteome and reduced phosphorylation of Akt following treatment of MCF-7 cells with nPep and SU5402. Further interrogation of cell signalling pathways following treatment
with nPep and different small molecule therapeutics will undoubtedly identify additional signalling alterations and potential mechanisms for the enhanced drug efficacy observed. However, the use of peptides as therapeutics present challenges since they are less stable than small molecules and are susceptible to enzymatic degradation. The recent generation of E-cadherin peptidomimetics may help to circumvent this due to the enhanced stability and suitability for oral administration (Doro et al., 2015).

The suggested use of E-cadherin modulators as cancer therapeutics is being increasingly described in the literature (Brouxhon et al., 2013, Green et al., 2004, Devemy and Blaschuk, 2009). Suppression of carcinogenesis in MCF-7 cells has been demonstrated following application of the E-cadherin neutralising antibody DECMA-1, which led to the suggested use of DECMA-1 as a potential cancer therapeutic (Brouxhon et al., 2013). A patent application from the authors described the use of E-cadherin or sE-cad inhibitors that target EC2-5 but not EC1 in combination with additional treatments such as protein scaffolds targeting HER1-4 (Brouxhon and Kyrkanides, 2013). Vandetanib was also specifically named as a combination therapy, notably, combined application of E-cadherin neutralising peptide with Vandetanib significantly increased cell death in this study.

In contrast to observations by Brouxhon et al. (2013), using exogenous inhibition of E-cadherin increased proliferation and survival of MCF-7 cells in this study. These traits are advantageous for cancer growth and may preclude the use of E-cadherin exogenous inhibitors as potential therapeutics since they may exacerbate these cancer traits and contribute to neoplasm formation. Rather than prescribing exogenous E-cadherin inhibitors as a combination therapy, it may be more beneficial to characterise expression of E-cadherin in tumour biopsies prior to prescribing therapies.
6.6 Discrepancies between methods of E-cadherin inhibition

It is clear from the data that one method of exogenous E-cadherin inhibition is not equivalent to another. The differences observed between the data from this study and that of Brouxhon et al. (2013) may be dose dependant. MCF-7 cells were treated with E-cadherin SHE78.7 nAb at a concentration sufficient to induce disaggregation of the cells (2µg/ml), whereas, ten times the concentration of DECMA-1 was added in studies by Brouxhon et al. (2013). At doses of 40µg/ml DECMA-1 induced cell death in both normal and tumour cells but selectively killed tumour cells at lower doses (Brouxhon and Kyrkanides, 2013), this raises the question as to whether elevated doses of E-cadherin SHE78.7 nAb or nPep would selectively induce cell death in tumour cells. Furthermore, variations in concentration may also affect the ability to alter drug efficacy when employed as a combination therapy and this should also be investigated.

Alternatively, discrepancies between the results in this study and the literature are potentially due to the different extracellular cadherin epitopes recognised by E-cadherin SHE78.7 and DECMA-1, E-cadherin SHE78.7 nAb targets the EC1 domain of E-cadherin whereas DECMA-1 recognises an epitope within the extracellular membrane proximal domain (EC5) of E-cadherin (Laur et al., 2002, Ozawa et al., 1990). nPep is also believed to bind within the EC-1 domain since mutation of the Tryptophan amino acid in the peptide had the most significant adverse binding effect by Biacore interaction analysis (Segal, personal communication). However, discrepancies between E-cadherin nAb and nPep effects have been described in this study with regards to activation of p53 and other apoptosis-related proteins. In addition, microarray analysis of human ES cells in our lab revealed an increased number of altered gene transcripts when treated with E-cadherin SHE78.7 nAb compared to nPep, although significant similarities were also observed between the two methods (Mohamet, personal communication). This highlights the potential for nPep to fail to induce the same transcript alterations observed in this study with E-cadherin SHE78.7 nAb. Although E-cadherin SHE78.7 nAb and nPep are believed to
bind EC1, the 12-mer peptide represents a much smaller inhibitor of E-cadherin mediated cell adhesion when compared to the E-cadherin SHE78.7 nAb. Therefore, it is possible for the two methods to exert different effects. The potential for these exogenous inhibitors to enter the cell by endocytosis and modulate cell signalling should be explored. Maintenance of cell surface E-cadherin was described in both nAb and nPep-treated MCF-7 cells in this study. However, this does not negate the potential for these exogenous inhibitors to be internalised as part of the natural turnover of E-cadherin at the cell membrane. The conjugation of a fluorescent reporter to nAb and nPep may shed further light as to whether these can enter the cell.

This study has observed cross species correlation between the roles of E-cadherin in human MCF-7 and mouse embryonic stem cells. Corresponding gene transcript changes were observed between ENPS and E-cadherin\(^{-/-}\) mouse ES cells, which increases confidence in the deduction that E-cadherin regulates these gene transcripts. However, upon quantification of EMP-1 and IGFBP-3 by real-time PCR the fold changes in expression were much lower in nAb-treated MCF-7 cells, which suggest complete loss of E-cadherin protein stimulates more significant effects on these transcripts. This may be attributed to the displacement of E-cadherin intracellular binding proteins. Conflicting results have also been observed between different methods of E-cadherin inhibition in the literature, for example, dominant negative expression of E-cadherin (DN-Ecad) in HMLE cells induced repression of IGFBP-3 whereas short-hairpin knockdown increased expression (Onder et al. 2008). The repression of IGFBP-3 gene transcripts in DN-Ecad HMLE cells is in agreement with observations in DN-Ecad A431 cells but in contrast to results observed in nAb-treated MCF-7 cells presented here (Onder et al., 2008, Andersen et al., 2005). Interestingly, conflicting results were also observed in the proliferative effects of DN-Ecad in HMLE cells described by Onder et al. (2008) and nAb-treated MCF-7 cells in 3-dimensional culture. A decreased viable percentage of the initial seeding density was reported in DN-Ecad HMLE cells after 2 days whereas nAb-treatment of MCF-7 cells increased proliferation in this study. Although, these
differences may be attributed to the different cell lines employed. Fundamental differences have been described between the biology of luminal and basal derived mammary epithelial cells (Chaffer et al., 2013), which necessitates the need to explore the effects of exogenous inhibition in additional cell lines. The results also potentially reflect the differing modes of cell contact inhibition. The dominant negative form of E-cadherin used by Onder et al. (2008) was a truncated mutant; this facilitated the disaggregation of epithelial cells but precluded interaction with other proteins at the extracellular level, which may elicit alternative intracellular signals.

Most recently, peptidomimetic ligands that target the EC-1 domain of E-cadherin have been generated (Doro et al., 2015). It would certainly be interesting to test different E-cadherin peptidomimetic, nAbs, peptide and dominant negative mutants that inhibit exogenous E-cadherin mediated cell-cell contact in parallel to distinguish between the effects that are attributed to genuine loss of E-cadherin cell contact and those that are specific to the method of inhibition.

### 6.7 Final conclusions

In accordance with the DENT hypothesis, this thesis has established that dysregulation of E-cadherin, via exogenous inhibition, is sufficient to alter growth and survival of MCF-7 mammary epithelial cells in the absence of an EMT event. Contrary to the DENT hypothesis, exogenous inhibition of E-cadherin did not contribute to a characteristic breast CSC phenotype. Although exogenous inhibition of E-cadherin was insufficient to confer metastatic capability in MCF-7 cells, the increased proliferation observed may be sufficient to contribute to neoplasm formation in vivo. Re-establishment of E-cadherin mediated cell-cell contact might revert this increase and allow re-integration within the epithelium to result in a neoplasm of latent tumorigenicity or a benign tumour. Exogenous inhibition of E-cadherin was sufficient to induce gene transcript, cell surface proteome and response to small molecules within three days but tumour formation is a much
slower process. Prolonged inhibition of E-cadherin in vivo would likely result in the accumulation of additional alterations over time. Following these changes, emergence of additional signals may contribute to an EMT event and acquisition of a stem cell phenotype.

Experiments in this study have focussed on the exogenous inhibition of E-cadherin. In a wider context, these results may also apply to mutations of the E-cadherin extracellular domain in tumour cells that retain membrane expression of E-cadherin but prevent cell-cell adhesion. For example, such mutations would not be sufficient to induce metastatic spread via EMT but could potentially contribute to neoplasia through increased proliferation and survival, altered cell surface protein expression and cell signalling. The sensitivity of such cells to therapeutic agents may also be affected; therefore, characterisation of E-cadherin protein expression in tumour samples may be an important consideration for improved stratified healthcare.

6.8 Future directions

Although the increased cell numbers observed following exogenous inhibition of E-cadherin were attributed to increased cell survival in monolayer, a precise mechanism is yet to be elucidated. Increased expression of phosphorylated Akt has been eliminated as a potential mechanism in nPep-treated cells but additional survival pathways merit investigation.

Due to time constraints, it was not possible perform ChIP analysis to confirm interaction of p300 with target DNA. It would also be interesting to further evaluate the role of p300 inhibition in the acquisition of a CSC phenotype and whether this correlates with loss of E-cadherin expression. This could be achieved by short hairpin knockdown of E-cadherin.

Further verification of the nPep cell surface proteome is required. These results should be confirmed by flow cytometry and western blot. Subsequently, it would be interesting to investigate whether these confirmed changes correlate with loss of E-
cadherin cell-cell contact in tumour biopsies. This has the potential to identify novel biomarkers for classification of tumour grades.

Targeting E-cadherin with nPep has demonstrated the potential to enhance the cytotoxic effects of cancer treatments. However, further validation of nPep as a combination therapy using additional cell lines is required and experiments should be performed to establish whether the effects of nPep are dose dependant. It would also be interesting to determine whether nPep enhances drug efficacy in vivo and if this contributes to neoplasm formation.

This study has described discrepancies between the different methods of E-cadherin inhibition in the same cell line. Similarly, dominant negative forms of E-cadherin have demonstrated differential capacity to induce an EMT event in different cell lines (Andersen et al., 2005, Onder et al., 2008). It would be interesting to determine whether the effects of exogenous E-cadherin inhibition translate into additional cell lines and to compare the effects of different methods of inhibition in parallel. This would elucidate effects that truly relate to the loss of E-cadherin cell-cell contact and further identify discrepancies caused by the method of inhibition.
References


References


References


References


References


References


Appendix
A1: Supplementary data

Figure S3.1: MCF-10A and A549 cells express N-cadherin

MCF-10A and A549 cells were characterised for expression of N-cadherin by flow cytometry (A) and immunofluorescence (B). Both cell lines were excluded from further analysis due to expression of N-cadherin, typically associated with an epithelial-mesenchymal transition.
A2: List of companies

**Abcam**
330 Cambridge Science Park
Cambridge CB4 0FL
United Kingdom

**Agilent Technologies UK Limited**
Life Sciences & Chemical Analysis
Lakeside
Cheadle Royal Business Park
Stockport SK8 3GR
United Kingdom

**BD Biosciences**
Edmund Halley Road
Oxford Science Park
Oxford OX4 4DQ
United Kingdom

**Bioline Reagents Limited**
Unit 16 The Edge Business Centre
Humber Road
London NW2 6EW
United Kingdom

**Bio-Rad Laboratories Ltd.**
Bio-Rad House
Maxted Road
Hemel Hempstead
Hertfordshire HP2 7DX
United Kingdom

**BioTek**
6 Bull Street
Potton
Bedfordshire SG19 2NR
United Kingdom

**Cell Signaling Technology**
Schuttersveld 2
2316 ZA Leiden
The Netherlands

**Corning B.V. Life Sciences**
Fogostraat 12
1060 LJ Amsterdam
The Netherlands

**Dionex Corporation**
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
United States

**FUJIFILM UK Limited**
Unit 10A, St Martins Business Centre
St Martins Way
Bedford MK42 0LF
United Kingdom

**GE Healthcare Life Sciences**
Amersham Place
Little Chalfont
Buckinghamshire HP7 9NA
United Kingdom

**Greiner Bio-One Ltd.**
Brunel Way
Stroudwater Business Park
Stonehouse
Gloucestershire GL10 3SX
United Kingdom

**Imagen Biotech Ltd**
3F68, TheBiohub at Alderley Park
Mereside, Alderley Edge
Cheshire SK10 4TG
United Kingdom

**Jencons (a VWR division)**
Unit 15 The Birches
Willard Way
Imberhorne Industrial Estate
East Grinstead
West Sussex RH19 1XZ
United Kingdom
Leica Microsystems (UK) Ltd
Larch House
Woodlands Business Park
Breckland
Linford Wood
Milton Keynes MK14 6FG
United Kingdom

Life Technologies Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF
United Kingdom

Lonza Biologics plc
Granta Park
Great Abington
Cambridge CB21 6GS
United Kingdom

Matrix Science Ltd
64 Baker Street
London W1U 7GB
United Kingdom

Merck Millipore UK Ltd
Suite 3 & 5, Building 6
Croxley Green Business Park
Watford
Hertfordshire WD18 8YH
United Kingdom

NBS Biologicals Ltd.
14 Tower Square
Huntingdon
Cambridgeshire PE29 7DT
United Kingdom

New England Biolabs
75-77 Knowl Piece
Wilbury Way
Hitchin,
Hertfordshire SG4 0TY
United Kingdom

PAA Laboratories Ltd.
Termare Close
Houndstone Business Park
Yeovil
Somerset BA22 8YG
United Kingdom

Primerdesign Ltd
The Mill Yard
Nursling Street
Rowhams
Southampton SO16 0AJ
United Kingdom

PROMEGA
Delta House
Southampton Science Park
Southampton SO16 7NS
United Kingdom

QIAGEN
1700 Seaport Blvd, 3rd Floor
Redwood City CA 94063
USA

R&D Systems Europe Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon OX14 3NB
United Kingdom

Roche Ltd.
Hexagon Place
6 Falcon Way, Shire Park
Welwyn Garden City AL7 1TW
United Kingdom

Santa Cruz Biotechnology, Inc.
Bergheimer Str. 89-2
69115 Heidelberg
Germany

Sigma-Aldrich Company Ltd.
Brickfields Business Park
Gillingham
Dorset
United Kingdom
STARLAB (UK), Ltd
Unit 4 Tanners Drive
Blakelands
Milton Keynes MK14 5NA
United Kingdom

ThermoFisher Scientific Ltd
Bishop Meadow Road
Loughborough LE11 5RG
United Kingdom

Tocris Bioscience
Tocris House, IO Centre
Moorend Farm Avenue
Bristol BS11 0QL
United Kingdom

UVitec Limited
Unit 36, St John's Innovation Centre
Cowley Street
Cambridge CB4 0WS
United Kingdom

Vector Laboratories Ltd
3 Accent Park
Bakewell Road
Orton Southgate
Peterborough PE2 6XS
United Kingdom

Waters Limited
730-740 Centennial Court
Centennial Park
Elstree
Hertfordshire WD6 3SZ
A2: Publication

Familial Alzheimer’s disease modelling using induced pluripotent stem cell technology

Lisa Mohamet, Natalie J Miazga, Christopher M Ward

Abstract

Alzheimer’s disease (AD) is a progressive neurodegenerative disease in which patients exhibit gradual loss of memory that impairs their ability to learn or carry out daily tasks. Diagnosis of AD is difficult, particularly in early stages of the disease, and largely consists of cognitive assessments, with only one in four patients being correctly diagnosed. Development of novel therapeutics for the treatment of AD has proved to be a lengthy, costly and relatively unproductive process with attrition rates of > 90%. As a result, there are no cures for AD and few treatment options available for patients. Therefore, there is a pressing need for drug discovery platforms that can accurately and reproducibly mimic the AD phenotype and be amenable to high content screening applications. Here, we discuss the use of induced pluripotent stem cells (iPSCs), which can be derived from adult cells, as a method of recapitulation of AD phenotype in vitro. We assess their potential use in high content screening assays and the barriers that exist to realising their full potential in predictive efficacy, toxicology and disease modelling. At present, a number of limitations need to be addressed before the use of iPSC technology can be fully realised in AD therapeutic applications. However, whilst the use of AD-derived iPSCs in drug discovery remains a fledgling field, it is one with immense potential that is likely to reach fruition within the next few years.

© 2014 Baishideng Publishing Group Co., Limited. All rights reserved.

Key words: Human induced pluripotent stem cells; Alzheimer’s disease; Neurodegenerative diseases; High-throughput screening assays; Cholinergic neurons; Drug discovery; Stratified medicine

Core tip: Alzheimer’s disease (AD) affects 36 million people worldwide and is set to double by 2030. Progress in understanding AD has been hindered by a lack of suitable in vitro and in vivo models reflected in > 90% drug attrition rates. Induced pluripotent stem cells are an alternative source of neural cells that can be derived from patients’ somatic cells and exhibit AD pathophysiological phenotypes. These cells are amenable to HTS formats required for drug discovery applications. Harnessing this combined potential would provide an unprecedented opportunity to significantly reduce timeframes and costs associated with developing novel therapeutics, ultimately improving patient outcomes.


INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegen-

© 2014 Baishideng Publishing Group Co., Limited. All rights reserved.

Key words: Human induced pluripotent stem cells; Alzheimer’s disease; Neurodegenerative diseases; High-throughput screening assays; Cholinergic neurons; Drug discovery; Stratified medicine

Core tip: Alzheimer’s disease (AD) affects 36 million people worldwide and is set to double by 2030. Progress in understanding AD has been hindered by a lack of suitable in vitro and in vivo models reflected in > 90% drug attrition rates. Induced pluripotent stem cells are an alternative source of neural cells that can be derived from patients’ somatic cells and exhibit AD pathophysiological phenotypes. These cells are amenable to HTS formats required for drug discovery applications. Harnessing this combined potential would provide an unprecedented opportunity to significantly reduce timeframes and costs associated with developing novel therapeutics, ultimately improving patient outcomes.


INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegen-

© 2014 Baishideng Publishing Group Co., Limited. All rights reserved.

Key words: Human induced pluripotent stem cells; Alzheimer’s disease; Neurodegenerative diseases; High-throughput screening assays; Cholinergic neurons; Drug discovery; Stratified medicine

Core tip: Alzheimer’s disease (AD) affects 36 million people worldwide and is set to double by 2030. Progress in understanding AD has been hindered by a lack of suitable in vitro and in vivo models reflected in > 90% drug attrition rates. Induced pluripotent stem cells are an alternative source of neural cells that can be derived from patients’ somatic cells and exhibit AD pathophysiological phenotypes. These cells are amenable to HTS formats required for drug discovery applications. Harnessing this combined potential would provide an unprecedented opportunity to significantly reduce timeframes and costs associated with developing novel therapeutics, ultimately improving patient outcomes.


INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegen-

© 2014 Baishideng Publishing Group Co., Limited. All rights reserved.

Key words: Human induced pluripotent stem cells; Alzheimer’s disease; Neurodegenerative diseases; High-throughput screening assays; Cholinergic neurons; Drug discovery; Stratified medicine

Core tip: Alzheimer’s disease (AD) affects 36 million people worldwide and is set to double by 2030. Progress in understanding AD has been hindered by a lack of suitable in vitro and in vivo models reflected in > 90% drug attrition rates. Induced pluripotent stem cells are an alternative source of neural cells that can be derived from patients’ somatic cells and exhibit AD pathophysiological phenotypes. These cells are amenable to HTS formats required for drug discovery applications. Harnessing this combined potential would provide an unprecedented opportunity to significantly reduce timeframes and costs associated with developing novel therapeutics, ultimately improving patient outcomes.


INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegen-

© 2014 Baishideng Publishing Group Co., Limited. All rights reserved.

Key words: Human induced pluripotent stem cells; Alzheimer’s disease; Neurodegenerative diseases; High-throughput screening assays; Cholinergic neurons; Drug discovery; Stratified medicine

Core tip: Alzheimer’s disease (AD) affects 36 million people worldwide and is set to double by 2030. Progress in understanding AD has been hindered by a lack of suitable in vitro and in vivo models reflected in > 90% drug attrition rates. Induced pluripotent stem cells are an alternative source of neural cells that can be derived from patients’ somatic cells and exhibit AD pathophysiological phenotypes. These cells are amenable to HTS formats required for drug discovery applications. Harnessing this combined potential would provide an unprecedented opportunity to significantly reduce timeframes and costs associated with developing novel therapeutics, ultimately improving patient outcomes.


INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegen-
erative disease in which patients exhibit gradual loss of memory that impairs their ability to learn or carry out daily tasks. The classic, post-mortem neuropathology exhibited in AD largely consists of amyloid plaques and neurofibrillary tangles\cite{1}, however, there is significant controversy within the field as to the causative mechanism(s). Worldwide nearly 36 million people have AD or related dementia, with a reported 7.7 million new dementia sufferers worldwide per year. The global cost of neurodegenerative diseases was over United States $600 billion in 2010 and affects people in all countries, with 58% living in low- and middle-income countries\cite{2}. In the United Kingdom alone, specific neurodegenerative diseases (including AD and Parkinson’s disease), have a combined patient population in excess of 800000 and the cost for provision of care was an estimated £23bn in 2012\cite{3}.

Diagnosis of AD is difficult, particularly in early stages of the disease, and largely consists of cognitive assessments, with only one in four patients being correctly diagnosed\cite{4}. Lack of knowledge of disease pathology is a major disadvantage in diagnosis and prescribing treatments since drug regimens are not the same for all dementias or patients. Moreover, development of a successful drug for the treatment of AD has, as yet, eluded pharmaceutical companies as current medicines treat only symptoms and not the cause(s) of AD. For example, in just over a decade there have been over 100 failed medicines for treatment of AD, including recent late stage failures of solanezumab and bapineuzumab with just five approved medications available to treat the symptoms of various stages of AD (three in United Kingdom). Therefore, a failure in pre-clinical to clinical development exists and can be attributed to several key factors; existing animal models or cellular models are inadequate, insufficient knowledge of drug action on human physiology and a lack of pharmacologically relevant biomarkers. Consequently, there is a pressing need for technologies that can provide definitive assays that can confirm disease pathology as well as predict novel or optimal drug regimens.

Since the creation of induced pluripotent stem cells (iPSCs) from human adult somatic cells in 2007\cite{5}, the potential applications of stem cells in regenerative medicine are considerable. Human pluripotent stem cells (that include iPSCs and embryonic stem cells) are self-renewing, which permits them to be grown indefinitely, and retain the potential to give rise to all cell types of the body. iPSCs are an ideal alternative cell source as they can be derived (reprogrammed) from somatic cells from any individual and are genetically identical to the donor, making them invaluable for use in cell-based models for human disease (Figure 1). Reprogramming of somatic cells is a highly inefficient and lengthy methodology and, as such, certain parameters should be considered when making disease specific iPSCs. These include; source of somatic cells (e.g., dermal fibroblast, blood cells), method of cellular reprogramming (e.g., retroviral, episomal) and the robustness of differentiation protocols for mature cell types. Here, we focus on AD-specific iPSCs and their derivatives to illustrate how they might be used in various applications in regenerative medicine. For a detailed overview of reprogramming, we refer the reader to another review\cite{6}.

Crucially, previous research demonstrates that iPSC-derived neural cells harvested from individuals suffering from a range of neurodegenerative disorders exhibit similar abnormal disease characteristics \textit{in vitro}\cite{2-9}. This observation presents an invaluable opportunity for the use of diseased cell lines in \textit{in vitro} studies to further our understanding of disease modelling, early toxicity screening and in the development of novel therapeutics. Performance of a literature search using the NCBI database, PubMed, under specific search terms [disease modelling AND ips cells NOT “review” (Publication Type)] in original research publications reveals that the field of disease modelling using iPSCs has increased at a substantial rate since the creation of iPSCs in 2007 (Figure 2). A year-on-year increase in the number of publications from 2009 (n = 20) to 2011 (n = 114) is observed, however, in 2012 this trend appeared to slow. In 2013, a reduction in papers is recorded (n = 52) which could indicate that the field is maturing, whereby the initial raft of papers reflected high impact method-based publications (\textit{i.e.}, the production of diseased iPSCs), whereas current work is focussed on disease modelling and drug discovery, which are lengthy studies. The number of original research articles containing iPSCs for disease modelling of AD patients was very small and there are only 8 research papers that have utilised AD-derived iPSCs between 2011-2013. This demonstrates that the use of iPSCs to model AD is still in its infancy and may reflect the difficulty of isolation of these cells and identification of appropriate donor patients. This review will discuss the pathology and cellular targets of AD, how we can utilise iPSCs as a model to investigate AD, applications and limitations of these cells in high throughput analyses and future opportunities in personalised medicine.

### DISEASE PATHOLOGY

AD can be divided into familial or sporadic genetic events with early- or late-onset. Whilst the majority of AD cases manifest as late-onset sporadic form, familial cases present a unique opportunity to investigate the inheritance of genes contributing a higher risk of AD. The familial form of AD is associated with mutations in amyloid precursor protein (APP), presenilin-1 and presenilin-2. Risk of AD is also observed to be increased where mutations in apolipoprotein E4 (APOE4) or triggering receptor expressed on myeloid cells 2 (TREM2) are present. Genes associated with the pathology of AD include APP, which results in β-amyloid plaques (Aβ), and microtubule associated protein Tau (MAPT),
which results in hyperphosphorylated tau aggregates (tau tangles) within neurons of AD patients\(^\text{[10]}\). Despite tau tangles being identified as a pathological feature of AD, mutations in this gene are unusual in such patients. AD is characterized by extracellular amyloid deposition, intracellular neurofibrillary tangle formation, and neuronal loss. Below, we discuss the contribution of these genes to the pathology of AD. Other confounding factors in AD include oxidative stress, mitochondrial function, inflammation and microglia function.

**Amyloid precursor protein**

A significant pathological feature of AD is the presence of extracellular plaques in the brain comprised of $\beta$-amyloid ($A\beta$) peptides derived from the amyloid precursor protein\(^\text{[11,12]}\). APP is located on chromosome 21 in humans and is associated with dementia in Down syndrome patients, who exhibit a triplication of this chromosome (trisomy 21). Whilst APP in AD has been studied in significant detail, the events leading to $A\beta$ deposition are less well defined and likely to involve stimulation of APP expression via the neuroinflammation-promoting cytokines IL-1 and S100B\(^\text{[12]}\). Drugs developed to target $A\beta$ deposits for the treatment of AD have proved relatively unsuccessful. This may be due to the fact that overexpression of APP is associated with other events, such as glial activation, suggesting that the deposition of $A\beta$ is associated with, rather than being a causal factor of, AD. As such, APP is now generally disdained as a drug target for AD treatment with hyperphosphorylated tau aggregates now being a major focus.

**Microtubule Associated Protein Tau**

The Microtubule Associated Protein Tau (MAPT; Tau) functions to assemble and stabilize microtubules within neurons, playing an important part in regulation of neuronal polarity, axonal transport and neurite outgrowth\(^\text{[10]}\). Phosphorylation of Tau allows regulation of binding and stability within neurons and aberrant phosphorylation or dephosphorylation in specific residues of the Tau protein lead to pathology, collectively known as tauopathies. The main component of the protein aggregates found in tauopathies is hyperphosphorylated tau protein within neurons. Although the exact mechanisms are unclear, the neurofibrillary tangles (NFT) associated with tau protein lead to pathology, collectively known as tauopathies. The main component of the protein aggregates found in tauopathies is hyperphosphorylated tau protein within neurons. Although the exact mechanisms are unclear, the neurofibrillary tangles (NFT) associated with tauopathies may also involve conformational changes in Tau protein. Whilst tau in NFT forms the basis for pathology of tauopathies it has been suggested that tau oligomers act as a toxic species by providing a template for the misfolding of native tau and spreading from cell to cell leading to propagation of the disease\(^\text{[13]}\). Research is now focused on the targeting of Tau oligomers for drug therapies for the treatment of AD.

**Apolipoprotein E4**

Apolipoprotein E consists of 3 isoforms of which apoE4 is a genetic risk factor for late-onset familial and sporadic forms of AD and is also associated with dementia in DS, Huntington’s disease, vascular dementia and cerebrovascular disease\(^\text{[14]}\). APOE4 exhibits multifunctionality in lipid and lipoprotein transport systems, mainly in the metabolism of dietary lipids\(^\text{[15]}\). Carriers
of polymorphic variants of APOE4 are between 4- and 10-times more likely to exhibit late onset AD. In the CNS, APOE4 is produced by glial cells and interacts with receptors of the low-density lipoprotein family. APOE4 binds to Aβ peptide and onset of AD is likely to reflect the inability of APOE4 to aggregate and clear Aβ in the brain, although other factors such as the effect of APOE4 on synaptic plasticity, lipid transport, neuroinflammation may also account for this. Since the APOE4 isoform can be assessed prior to onset of neurodegeneration it is considered a promising target for drug therapy.

**Presenilin-1 and -2**

Presenilin-1 (PSEN1) and PSEN2 are major components of the atypical aspartyl protease complex that is required for γ-secretase complex activity and cleavage of APP. Mutations in PSEN1 are the major cause of early onset AD and also account for the most severe forms of the disease. Early onset AD in PSEN1 mutation carriers can occur as early as 30 years of age, although the mean age of onset is over 58 years. More than 180 mutations have been described in PSEN1, of which the majority are missense mutations. PSEN2 mutations are less common and 14 specific mutations have been associated with AD. Mutations within the PSEN proteins affect APP synthesis and proteolysis leading to an increase in the ratio of Aβ42 peptide compared to Aβ40, the former a more toxic form of Aβ peptide that is more prone to oligomerisation and fibril formation. Drug treatments have focussed on γ-secretase modulators capable of decreasing the ratio of Aβ42 to Aβ40 peptides.

**Triggering Receptor Expressed on Myeloid Cells 2**

Variants in Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) have been identified that triple the risk of developing late onset AD. TREM2 is a cell surface receptor, which triggers activation of the immune response in association with DAP12. In the CNS, TREM2 is expressed by microglial cells and functions to activate phagocytosis in these cells and to suppress neuroinflammation and cytokine production. Several functions of TREM2 include aiding clearance of Aβ and synapse remodelling. Whilst the exact mechanism of TREM2 in late onset AD is unclear it is likely that mutations in this gene contribute to disease pathogenesis via insufficient clearance of Aβ and increased localised inflammation.

**AD MODELLING USING HUMAN iPSCS**

The single most important factor in the utility of iPSCs in AD modelling, is that mature cell type(s) affected by the disease, e.g., neurons, exhibit phenotypic characteristics of the disease. Numerous studies have demonstrated that iPSCs can be used to model genetic diseases by showing that cells affected by the disease recapitulate these traits in vitro. iPSC AD modeling is still in its infancy and only a few studies have demonstrated successful generation and characterization of AD patient-derived neurons (Figure 2). Five out of eight publications reported isolation of iPSC-derived neurons from patients with familial AD, however a key development in the field showed that reprogramming could similarly be used to recapitulate patient specific phenotypes in vitro of sporadic forms of the disease. iPSC-derived neurons generated from familial AD patients with mutation of the APP gene and sporadic AD showed, relative to non-demented controls, elevated levels of Aβ, phosphorylated tau and glycogen synthase kinase 3B.

A known pathology of AD progression is significant neurodegeneration in the cortical regions, with all regions of the brain registering degenerative changes as the disease progresses. Initial reports using iPSC-derived neurons from patients with familial AD utilised heterogeneous neuron populations. Although results demonstrated an increase in Aβ42 secretion from mutant PSEN1, PSEN2 and APP iPSC-derived neurons compared to control cells both studies observed inconsistencies in Tau expression. For example, no Tau expression or tangles were observed in the Yagi et al. study, whereas increased levels of phosphorylated Tau were observed in both familial AD-derived neurons and one of the two sporadic AD-derived neurons compared to non-demented control neurons in the Israel et al. study. In addition, a recent paper reported increased levels of intracellular neuron specific amyloid aggregates in cells derived from familial APP-E693Q and one of two sporadic AD derived neurons. These disparities may reflect the disparate differentiation periods used in the studies and differences in the proportion of cholinergic neurons within the populations. However, it is also possible that these differences reflect inherent variability of iPSCs, which is discussed further below.

In a seminal study, iPSCs derived from patients with Downs Syndrome (a model for early onset AD) were used to generate, highly enriched populations of cholinergic neurons in significant numbers. Following differentiation times of 28-100 d following neural induction of iPSCs, analysis of these cells showed production of neuron specific Aβ secretion, amyloid aggregate formation and altered Tau protein localisation and phosphorylation. Another key finding from this report (and others) demonstrates that early AD pathologies, such as the formation of Aβ42 aggregates, occur in relatively short culture periods in vitro opposed to years in vivo. Furthermore, iPSC-derived neurons are able to respond functionally to various modulators highlighting their potential use in validation and identification in drug discovery.

**LIMITATIONS OF iPSCS AS MODELS OF DISEASE**

At present, a number of limitations need to be ad-
dressed before the full potential of iPSC technology in predictive efficacy, toxicology and disease modelling can be realised. Human iPSCs are effectively man-made cells that are similar to embryonic stem cells, which themselves only exist in vivo for a matter of days. These nuances may be reflected in the challenges faced in the differentiation of pluripotent stem cells into mature cell derivatives, despite a good understanding of the molecular mechanisms that occur during development. In order to fully exploit opportunities in disease modelling, but in particular in HTS formats, robust, efficient and cost-effective methods are fundamental. Differentiation protocols that require cocktails of growth factors are costly and are susceptible to significant batch-batch variation, however, alternative methods to acquire differentiated phenotypes are being explored, such as the use of more cost effective small molecules.

A significant research focus in the pluripotent stem cell field has been the development of robust differentiation protocols to enrich for specific mature cell types and populations. However, homogenous cell populations are difficult to obtain in practice and are unlikely to reflect the true pathophysiology of the disease. In addition, modelling complex, idiopathic diseases such as AD, likely requires exposing the cells to biological, chemical or environmental factors to reveal pathophysiological phenotypes. For example, Israel et al demonstrated a favorably enriched neuron population (90%), however since neurons and synapses are largely dependent upon endocytic activity they found it necessary to co-culture with astrocytes.

In addition, it has been shown by hierarchical cluster analysis that AD-derived neurons are akin to fetal neurons and, therefore, not fully mature. Although, this is considered one of the major hurdles to overcome in modelling degenerative diseases, the recapitulation of a fetal phenotype presents an opportunity to isolate specific progenitors, which can be used to study developmental aberrations in congenital/developmental disorders. Conversely, for the study of late-stage onset diseases, such as sporadic AD, adult disease phenotypes might not be exhibited under standard differentiation conditions. As such, further work is necessary to identify appropriate differentiation methods for the derivation of adult neurons in vitro.

An advantage with the use of patient specific iPSCs means that each iPSC-derived cell reflects this genetic variation. Despite this being a clear advantage in the toxicological evaluation of patient populations to novel therapeutics, conclusions from studies using iPSCs from donors with different genetic backgrounds may be problematic. For example, are any phenotypic differences observed due to the mutation of interest or the genetic background of the patients? At present, parameters such as gender-, age- and ethnicity-matching are used in the selection of control donors, however, genome-wide studies show that each person has single nucleotide polymorphisms that may have disease relevance. Therefore, a fundamental feature in the use of iPSCs in regenerative applications is careful consideration of appropriate control patients. A further aspect to consider is the reprogramming event required to derive iPSCs from donors. It is well known that epigenetic variations can, and often do, occur during the reprogramming stage of iPSC derivation. Therefore, iPSC clones must be fully characterised prior to use in therapeutic analysis.

HIGH THROUGHPUT SCREENING OF NOVEL THERAPEUTICS FOR AD: IN VITRO CLINICAL TRIALS

Development of novel therapeutics for treatment of disease is a lengthy and costly process with extremely high attrition rates of > 90%, in particular, CNS therapeutics exhibit one of the lowest success rates. Current practices involve evaluation of the safety and efficacy of new drugs in animal and in vitro models of relevant tissues and biological processes. Existing in vitro cell models attempt to recapitulate core pathologies or targets of AD. For example, Georgievsk et al recently described inhibition of Tau phosphorylation in response to AZD1080, an inhibitor of Glycogen synthase kinase-3β, using a mouse 3T3 fibroblast cell line transfected with human Tau. Stable over expression of Tau has also been achieved in the human SH-SY5Y neuroblastoma cell line, similarly, over expression of APP695wt in the SH-SY5Y cell line was used to determine Aβ40 secretion in response to AZD3839 in pre-clinical studies. The use of animal cells, however, lacks human context and the cancer-derived SH-SY5Y cell line may not accurately reflect the cellular processes associated with AD. A recent paper highlighted the importance of the endoplasmic reticulum (ER) in protein catalysis and correlated the presence of amyloid-β plaques with age-related diminished ER function. The author went on to call for better drug discovery cell models which enable enhancement of ER function to be detected through embedding fluorescent reporter proteins within an exon of a target gene. In short, these methods of target validation focus on the recapitulation of only one key feature of AD in an often-irrelevant cell line, failing to account for other components of the signalling pathway. Primary neurons offer more relevant pre-clinical cell models and are capable of synapse formation, but are costly, difficult to transfect and are typically animal derived. Transgenic animal models and cell lines have undoubtedly aided our knowledge of AD mechanisms and predictive pharmacology, however, these are hindered by inter-species differences and lack of clinical relevance and genetic heterogeneity, which has resulted in poor clinical translation.

The derivation of iPSCs from patients with AD would, however, enable the applicable recapitulation of AD phenotype in a dish, since iPSCs retain the patient’s genotype. Circumventing cross species differences and
negating any ethical constraints associated with the use of human embryonic stem cells would create increased translational value. Indeed, neurons derived from disease specific iPSCs have been used to validate the potency of candidate drugs in the treatment of neurological pathologies. Of further importance, studies have shown treatment of AD iPSC-derived neurons with β-secretase inhibitors, but not γ-secretase inhibitors, causes significant reductions in phosphorylated Tau expression and GSK-3β levels. The accessibility of iPSCs allows many compounds to be tested simultaneously, reflecting a real-life scenario of patients taking a variety of prescription and non-prescription drugs.

Harnessing this potential could provide an unprecedented opportunity to improve preclinical predictions by allowing therapeutics to be tested in multiple cell lines derived from a cohort of patients. This may also allow the repositioning, repurposing or repurposing of old, failed and existing drugs. The use of patient-derived iPSCs could be highly amenable to high throughput screening (HTS) practices using multi-well formats to enable rapid analysis of thousands of compounds. Early identification of toxic or efficacious compounds would, thus, prevent expensive animal studies and subsequent clinical failures. Traditional HTS techniques have focussed on biochemical assays measuring enzyme activity and protein interactions using absorbance, luminescence or fluorescence based readings. For example, Haugabook et al describe the use of a sandwich ELISA (in 96-well formats) to detect aggregation of amyloid plaques, a key contributor to the formation of senile plaques in AD. HTS assays have also been developed to enable detection of Aβ42 aggregation using a GFP fusion construct expressed in E. Coli, in which compound inhibition of Aβ42 aggregation resulted in the emission of a fluorescent signal. As a result of these methods often lacking cellular context, high content screening (HCS) in whole cells has been recognised as a powerful tool for drug discovery and has been adopted largely by the pharmaceutical industry due to the large volume of multiparametric data that can be obtained. HCS encompasses the automated acquisition of fluorescent images and image analysis using mathematical algorithms to extract and quantify phenotypic information, including signal shape, intensity and cellular localisation, which can be statistically analysed. To increase throughput and reduce human error, additional processes such as compound storage, dosing and immunofluorescent staining can also be automated. The principle of HCS in neuronal cultures has already been demonstrated. Neurite loss is one of the core pathologies of AD and application of HCS to quantify neuronal outgrowth has already been achieved and proven to be faster than traditional manual tracing methods. Assessment of chemical toxicity has also been demonstrated by HCS in three neuronal cell lines, whereby proliferation was detected by BrdU incorporation (an indicator of actively proliferating cells) and cell counts were obtained with Hoechst 33342 nuclear dye in a 96-well plate format. HCS has applications in additional areas of neuroscience including neurogenesis, cell signalling and inclusion formation as reviewed by Dragunow. An example of HCS applications in AD therapeutics is shown in Figure 3.

Overall, powerful high-throughput and -content screening assays are in place that can be applied to multiple areas of drug discovery, but clinical success is hindered by a lack of relevant cell models in the preclinical stages. High throughput toxicity screening using human iPSC-derived cardiomyocytes has been reported.
using electrode sensors to acquire oscillating impedance measurements to detect the contraction and relaxation or beating of iPSC-derived cardiomyocytes in a 96-well plate format\textsuperscript{[58]}. Arrhythmia data obtained from iPSC-derived cardiomyocytes treated with cardiac modulators was qualitatively comparable to results obtained from more traditional, low throughput microelectrode arrays in parallel experiments. Therefore, the potential use of iPSC technology in high throughput drug discovery has been demonstrated but to date has not been described in the literature for iPSC-derived neurons. The UK Government and pharmaceutical industry have recognised the potential for iPSC AD models in HCS and by late 2013 several calls for funding such technology have been announced. As a result, we expect to see significant reasons. In short, the use of disease specific iPSC AD models in HCS and by implementing genomic testing of large cohorts of patients, representing different ethnic/genetic backgrounds may allow scientists and clinicians to model, in vitro, the progression of AD (or other degenerative diseases) for each individual patient, perform “customised” pharmacologic screening to determine the optimal therapeutic regimes and implement genomic testing of large cohorts of patients, representing different ethnic/genetic backgrounds in order to inform pharma of susceptible populations. There is a clear unmet drug need for the treatment of AD and the utility of iPSC technology will provide a more efficacious model to reassert (or rescue) former drug candidates that either have been withdrawn from use or aborted at a late stage of development for safety reasons. In short, the use of disease specific iPSC derived neural cells, in conjunction with high throughput/content screening methods, offer improved clinically relevant cell models that will significantly reduce timelines and costs associated with the development of novel therapeutics, ultimately improving the number of new medicines to the market to treat patients with neurodegenerative diseases.

**REFERENCES**


Mohamet L et al. Alzheimer's disease modeling using iPSC cells


32 Han SS, Williams LA, Eggan KC. Constructing and deconstructing stem cell models of neurological disease. Neuron 2011; 70: 626-644. DOI: 10.1016/j.neuron.2011.05.003


40 Hu M, Schurad ME, Puttfarcken PS, El Kouhen R, Gopalakrishnan M, Li J. High content screen microscopy analysis of Aβ 1-42-induced neurite outgrowth reduction in rat...


P- Reviewers: Freter R, Perron M  S- Editor: Ma YJ  L- Editor: AE  E- Editor: Zhang DN