IDENTIFYING FUNCTIONAL DRIVERS OF EPITHELIAL- MESENCHYMAL TRANSITION (EMT) IN HUMAN BREAST CANCER: THE INTEGRIN/ILK AXIS

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ABSTRACT

Breast cancer is the leading cause of cancer in women worldwide, and over 90% of deaths caused by breast cancer are due to metastases, many of which are not responsive to current therapies. The ability for cells to acquire a metastatic phenotype includes epithelial mesenchymal transition (EMT), invoked as a critical component of the metastatic cascade. During the process of EMT, epithelial cells undergo a temporary conversion acquiring molecular and phenotypic changes that facilitate the loss of epithelial features, and the gain of mesenchymal phenotype. Such transformation promotes cancer cell migration and invasion. EMT is typically characterized as a loss of the epithelial cell adhesion proteins E-cadherin and cytokeratins, coupled with the gain of mesenchymal-associated molecules N-cadherin and vimentin. However, these proteins may not always be present in cancer systems. For example, one of the limitations in the use of vimentin as a prognostic marker in breast carcinomas is the likelihood that vimentin-positive cells may have migrated away from the primary mass, and become buried in the surrounding stroma, which is also vimentin-positive. Therefore, the identification of new markers which better represent EMT in breast carcinomas, and allow for a more specific detection of EMT-derived or EMT-prone breast cancer cells in the tumour vicinity, could have a dramatic impact on breast cancer prognosis.

The work presented in this thesis describes a comprehensive characterization of two human breast cancer EMT model systems: the in vitro PMC42 cell system and the in vivo EDW-01 patient derived xenograft system. Specifically, the focus of this project was to perform a sequence of studies to assess the regulation of α2 and β1 integrin (ITGα2, ITGβ1), and ILK. The functional role of the integrin/ILK axis in the mesenchymal state, and in the epithelial-to-mesenchymal transition is explored and assessed.
The thesis describes three specific aims:

1) To induce EMT in the PMC42 cell line using epidermal growth factor (EGF), and assess the expression of EMT markers over time (72hrs) under sparse and dense culture conditions, in order to determine the potential hierarchy of factors that regulate the EMT phenotype.

   - The results identified that in sparse conditions, resting epithelial cells were induced with EGF to undergo EMT, acquiring mesenchymal phenotypic changes. The cells showed upregulated integrin expression (ITGβ1 and ITGα2), as well as increased ILK. The timecourse of integrin expression regulation is indicative of early response genes in EGF-induced EMT. Interestingly, the ITGβ1 and ITGα2, as well as ILK were seen to be induced from 3hrs, and 1.5hrs, respectively, indicating that these genes are early response genes in EMT, and may indeed be required for the early manifestation of EMT.

2) To characterize the expression of EMT markers in the in vivo breast cancer model, EDW-01 xenograft, and determine whether expression patterns of integrins (ITGβ1 and ITGα2) and ILK correlate with the more progressive nature and mesenchymal properties of late passage tumours.

   - Results from this study identified altered expression of the classical EMT markers vimentin, E-cadherin, and N-cadherin. These changes were accompanied by up-regulated ITGβ1 and ITGα2, with a trend towards up-regulated ILK expression in this unique EDW-01 PDX model system. Hence, this investigation has shown the potential use of the early response molecular markers as possible targets for clinical breast cancer therapy.

3) To perform functional studies on ITGβ1, ITGα2 and ILK in the PMC42 EMT model system by employing siRNA abrogation approaches to reduce expression of the candidate effectors.
• Results presented in this chapter have identified for the first time that using RNAi, down regulated expression of ITGβ1, ITGα2, and ILK was observed in the PMC42 model system in varying degrees and translates into suppression of adhesion and migration. Despite these changes, no impact upon EMT was observed, indicating that these proteins do not play a functional role in the transition itself. However, results do demonstrate that ITGβ1, ITGα2, and ILK directly contribute to the EMT, enabling the PMC42 breast cancer cells to interact with the interstitial matrices, and highlighting a possibly crucial role in cross-talk between integrins and cadherins in EMT.

Overall, these findings highlight the possibilities that ITGβ1, ITGα2, and ILK have the potential to serve as biological EMT markers, and may be viable targets for the therapy of breast cancer, providing an important platform for further research in this field.
DECLARATION

According to the rules governing the submission of a thesis for the degree of Doctor of Philosophy at the University of Melbourne, I declare that:

(i) the thesis comprises only my original work towards the PhD,
(ii) due acknowledgement has been made in the text to all other material used,
(iii) the thesis is less than 100,000 words in length, exclusive of tables, bibliographies and appendices

Razan Wafai

Professor Erik W. Thompson  Dr Mark Waltham
Supervisor, Laboratory Head  Co-Supervisor, Laboratory Head

Department of Surgery (St. Vincent’s Hospital)
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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>µg</td>
<td>microgram</td>
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<tr>
<td>µl</td>
<td>microlitre</td>
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<tr>
<td>µm</td>
<td>micron</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>AE1/AE3</td>
<td>pan cytokeratin antibody</td>
</tr>
<tr>
<td>AES</td>
<td>amino-propyl-triethoxy-silane</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic Acid</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR-associated protein 9</td>
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</tr>
<tr>
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<td>neuronal cadherin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Ct</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>CTCs</td>
<td>circulating tumour cells</td>
</tr>
<tr>
<td>DAB</td>
<td>chromogen 3,3’ diaminobenzidine -tetrahydrochloride</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>dH₂O</td>
<td>milli Q ultra pure and deionised water</td>
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<tr>
<td>dATP</td>
<td>2’-deoxyadenosine-5’-triphosphate</td>
</tr>
<tr>
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<td>diethylpyrocarbonate</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>dNTPs</td>
<td>deoxynucleoside triphosphate</td>
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<td>dithiothreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>EGF receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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<td>fibroblast growth factor</td>
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<td>FFPE</td>
<td>formalin-fixed paraffin embedded</td>
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<td>g</td>
<td>gram</td>
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<tr>
<td>GEMMs</td>
<td>genetically engineered mouse models</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
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<td>GSK-3β</td>
<td>glycogen synthase kinase-3β</td>
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<tr>
<td>HA</td>
<td>hyaluronan</td>
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<tr>
<td>HBC</td>
<td>human breast cancer</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor 2</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>IFs</td>
<td>intermediate filaments</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
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<td>ILKAP</td>
<td>ILK-associated phosphatase</td>
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<tr>
<td>ITG</td>
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<td>integrin beta 1</td>
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<tr>
<td>kDa</td>
<td>kiloDalton</td>
</tr>
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<td>Description</td>
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</tr>
<tr>
<td>QRT-PCR</td>
<td>quantitative reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SF/HGF</td>
<td>scatter factor/hepatocyte growth factor</td>
</tr>
<tr>
<td>SFM</td>
<td>serum free medium</td>
</tr>
<tr>
<td>SiRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SMA</td>
<td>smooth muscle actin</td>
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<tr>
<td>SNAI-1</td>
<td>snail</td>
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<tr>
<td>SNAI-2</td>
<td>slug</td>
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<tr>
<td>SRB</td>
<td>sulforhodamine B</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′-tetramethyl-ethylenediamine</td>
</tr>
<tr>
<td>TFs</td>
<td>transcription factors</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TNBCs</td>
<td>triple negative breast cancers</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>v/v</td>
<td>volume/volume</td>
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<tr>
<td>WB</td>
<td>Western immunoblotting</td>
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<td>w/v</td>
<td>weight/volume</td>
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CHAPTER 1: LITERATURE REVIEW
Chapter 1: Literature Review

1.1 BREAST CANCER: A BRIEF OVERVIEW

Breast cancer is a major health issue worldwide; having a direct and indirect impact on the entire cross spectrum of the population, with an estimated 1,384,155 new cases worldwide each year (Tao et al., 2015), and nearly 459,000 related deaths (Youloden et al., 2012). It is estimated that over 508,000 women died in 2011 worldwide, due to breast cancer (Global Health Estimates, WHO 2013). According to 2012 GLOBOCAN statistics, nearly 1.7 million women were diagnosed with breast cancer with 522,000 related deaths—an increase in breast cancer incidence and related mortality by nearly 18% from 2008. It is by far the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers) (Ferlay et al., 2015). It has been predicted that the worldwide incidence of female breast cancer will reach approximately 3.2 million new cases per year by 2050 (Hortobagyi et al., 2005).

1.1.1 Breast Cancer Epidemiology- Incidence & Mortality

1.1.1.1 Incidence in Australian Women

Breast cancer is the most common invasive cancer amongst Australian women. In addition, the incidence is elevated with increasing age. From current demographic analyses, an average healthy Australian woman has a life expectancy of 84.5 years (AIHW, 2017a). In 2002, up to 24% of new breast cancer cases diagnosed were in women younger than 50 years, whilst 50% of cases presented in women aged between 50-69 years and, 26% in women aged 70 and over (AIHW, 2006). In 2008, more than 69% of breast cancers in females were diagnosed in those aged 40–69, while 25% were diagnosed in those aged 70 and over. The mean age at first diagnosis was 60 years (AIHW, 2012).

Recent investigations have shown that over the years, a woman’s risk of developing breast cancer has actually risen. For instance, first diagnosis of breast cancer before the age of 75 has increased from 1 in 16 in 1983, to 1 in 10 in 2013 (AIHW, 2017a). In women over the age of 85 years, this incidence rose from 1 in 12 in 1983, to 1 in 8 in 2013 (AIHW, 2017a).
Overall, the age-standardized incidence rate of breast cancer in Australian women, which was 81/100,000 females in 1983, has increased to 117.5/100,000 females in 2002, and 115.4/100,000 in 2008 ((AIHW), 2012) (Table 1.1). The number of new breast cancer cases per annum has risen, with 5,374 recorded cases in 1983, 12,093 recorded cases in 2002, and 13,567 recorded cases in 2008 ((AIHW), 2012).

Table 1.1: Incidence of breast cancer, females, Australia, 1982 to 2008

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of cases</th>
<th>Percentage of all cancers in females</th>
<th>Age-standardised rate (a)</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1982</td>
<td>5,310</td>
<td>24.1</td>
<td>81.1</td>
<td>78.9–83.3</td>
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<tr>
<td>1983</td>
<td>5,374</td>
<td>23.7</td>
<td>80.8</td>
<td>78.7–83.1</td>
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<tr>
<td>1984</td>
<td>5,731</td>
<td>24.1</td>
<td>83.9</td>
<td>81.7–86.1</td>
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<tr>
<td>1985</td>
<td>5,934</td>
<td>24.2</td>
<td>84.6</td>
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<td>6,098</td>
<td>24.2</td>
<td>85.3</td>
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<tr>
<td>1987</td>
<td>6,716</td>
<td>25.1</td>
<td>91.5</td>
<td>89.3–93.8</td>
</tr>
<tr>
<td>1988</td>
<td>6,764</td>
<td>24.8</td>
<td>90.0</td>
<td>87.9–92.2</td>
</tr>
<tr>
<td>1989</td>
<td>7,209</td>
<td>25.8</td>
<td>94.0</td>
<td>91.8–96.2</td>
</tr>
<tr>
<td>1990</td>
<td>7,457</td>
<td>25.8</td>
<td>95.3</td>
<td>93.2–97.5</td>
</tr>
<tr>
<td>1991</td>
<td>8,077</td>
<td>26.4</td>
<td>100.8</td>
<td>98.6–103.1</td>
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<tr>
<td>1992</td>
<td>8,059</td>
<td>25.6</td>
<td>98.6</td>
<td>96.4–100.8</td>
</tr>
<tr>
<td>1993</td>
<td>8,814</td>
<td>27.1</td>
<td>105.6</td>
<td>103.4–107.9</td>
</tr>
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<td>1994</td>
<td>9,782</td>
<td>28.5</td>
<td>114.6</td>
<td>112.3–116.9</td>
</tr>
<tr>
<td>1995</td>
<td>10,083</td>
<td>28.4</td>
<td>116.0</td>
<td>113.7–118.3</td>
</tr>
<tr>
<td>1996</td>
<td>9,783</td>
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<td>109.7</td>
<td>107.5–111.9</td>
</tr>
<tr>
<td>1997</td>
<td>10,223</td>
<td>27.7</td>
<td>111.8</td>
<td>109.6–114.0</td>
</tr>
<tr>
<td>1998</td>
<td>10,765</td>
<td>28.4</td>
<td>114.9</td>
<td>112.8–117.1</td>
</tr>
<tr>
<td>1999</td>
<td>10,673</td>
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<td>109.3–113.5</td>
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<tr>
<td>2000</td>
<td>11,401</td>
<td>28.4</td>
<td>116.1</td>
<td>114.0–118.3</td>
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<tr>
<td>2001</td>
<td>11,838</td>
<td>28.7</td>
<td>117.6</td>
<td>115.5–119.8</td>
</tr>
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<td>2002</td>
<td>12,093</td>
<td>28.2</td>
<td>117.5</td>
<td>115.4–119.6</td>
</tr>
<tr>
<td>2003</td>
<td>11,869</td>
<td>27.8</td>
<td>112.6</td>
<td>110.6–114.7</td>
</tr>
<tr>
<td>2004</td>
<td>12,208</td>
<td>27.7</td>
<td>113.5</td>
<td>111.5–115.5</td>
</tr>
<tr>
<td>2005</td>
<td>12,258</td>
<td>27.0</td>
<td>111.6</td>
<td>109.6–113.6</td>
</tr>
<tr>
<td>2006</td>
<td>12,681</td>
<td>27.5</td>
<td>112.9</td>
<td>111.0–114.9</td>
</tr>
<tr>
<td>2007</td>
<td>12,608</td>
<td>27.0</td>
<td>109.5</td>
<td>107.6–111.5</td>
</tr>
<tr>
<td>2008</td>
<td>13,567</td>
<td>28.2</td>
<td>115.4</td>
<td>113.5–117.4</td>
</tr>
</tbody>
</table>

(a) Standardised to the Australian population as at 30 June 2001 and expressed per 100,000 females.

Source: ((AIHW), 2012)
Overall, these analyses clearly show that breast cancer is an existing problem that will continue to escalate in Australian society. Given the ageing population, the number of females diagnosed with invasive breast cancer is expected to increase. Projections suggest that in 2020, the number of new breast cancer cases will be about 17,210 (Table 1.2). This would equate to 47 females being diagnosed with breast cancer every day in 2020 (AIHW, 2012).

Table 1.2: Projected\(^{(a)}\) breast cancer incidence, females, Australia, 2011 to 2020

<table>
<thead>
<tr>
<th>Year</th>
<th>Estimated number of new cases</th>
<th>Estimated age-standardised rate(^{(b)})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Lower 95% PI</td>
</tr>
<tr>
<td>2011</td>
<td>14,290</td>
<td>13,820</td>
</tr>
<tr>
<td>2012</td>
<td>14,610</td>
<td>14,130</td>
</tr>
<tr>
<td>2013</td>
<td>14,940</td>
<td>14,440</td>
</tr>
<tr>
<td>2014</td>
<td>15,270</td>
<td>14,740</td>
</tr>
<tr>
<td>2015</td>
<td>15,600</td>
<td>15,050</td>
</tr>
<tr>
<td>2016</td>
<td>15,930</td>
<td>15,360</td>
</tr>
<tr>
<td>2017</td>
<td>16,250</td>
<td>15,650</td>
</tr>
<tr>
<td>2018</td>
<td>16,570</td>
<td>15,950</td>
</tr>
<tr>
<td>2019</td>
<td>16,890</td>
<td>16,240</td>
</tr>
<tr>
<td>2020</td>
<td>17,210</td>
<td>16,530</td>
</tr>
</tbody>
</table>

The projections are based on breast cancer incidence data for females for 1995 to 2007. Standardised to the Australian population as at 30 June 2001 and expressed per 100,000 females.

Source: (AIHW, 2012)

1.1.1.2 Mortality in Australian Women

Breast cancer is the second most common cause of cancer-related death in women in Australia. The number of deaths due to breast cancer recorded in Australian women in 2004 was 2,665, and another 601 cases were associated, but were not the underlying cause, of death. From 1990 to 2004, mortality was shown to decrease from 31/100,000
females, to 23.4/100,000 females, respectively (Table 1.3). The median age of death due to breast cancer for women increased from 64 years in 1983, to 67 years in 2004 ((AIHW), 2006).

### Table 1.3: Mortality from breast cancer, females, Australia, 1982 to 2007

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of deaths</th>
<th>Percentage of all cancer deaths in females</th>
<th>Percentage of all deaths in females</th>
<th>Age-standardised rate(a)</th>
<th>95% confidence interval</th>
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</thead>
<tbody>
<tr>
<td>1982</td>
<td>1,987</td>
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<td>29.0–31.7</td>
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<tr>
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<td>4.1</td>
<td>30.2</td>
<td>28.9–31.6</td>
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<td>1984</td>
<td>2,166</td>
<td>18.6</td>
<td>4.2</td>
<td>31.6</td>
<td>30.3–33.0</td>
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<td>29.8–32.5</td>
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<td>4.1</td>
<td>29.9</td>
<td>28.7–31.2</td>
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<td>1987</td>
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<td>18.4</td>
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<td>29.9–32.5</td>
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<td>1988</td>
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<td>4.2</td>
<td>31.6</td>
<td>30.4–32.9</td>
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<td>1991</td>
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<td>25.0</td>
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<td>23.8–25.6</td>
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<td>15.5</td>
<td>4.0</td>
<td>22.1</td>
<td>21.2–22.9</td>
</tr>
</tbody>
</table>

(a) Standardised to the Australian population as at 30 June 2001 and expressed per 100,000 females.

Source: ((AIHW), 2017b)
The number of deaths from breast cancer increased from 1,987 cases in 1968, to 2,814 in 2014. It is estimated that this will increase to 3,128 deaths in 2018. Over the same period, the age–standardised mortality rate decreased from 17 deaths per 100,000 persons in 1968 to 11 deaths per 100,000 persons in 2014 (Figure 1.1). In 2017, it is estimated that the age–standardised mortality rate will be 11 deaths per 100,000 persons, and the estimated risk of an individual female dying from breast cancer by their 85th birthday will be 1 in 41 ((AIHW), 2017a).

![Figure 1.1 Age-standardised incidence rates for breast cancer 1982–2013 and age-standardised mortality rates for breast cancer 1968–2014, by sex. Figure taken from ((AIHW), 2017a)](image)

The number of deaths from breast cancer is expected to continue to marginally increase. In 2012, the number of deaths from breast cancer was expected to be 2,690; this number is expected to have increased to 2,730 by 2020 (Table 1.4). When expected changes in the age structure and size of the population are taken into account, the results suggest that the age-standardised mortality rate of breast cancer will decline from 19.5 deaths per 100,000 females in 2012 to 16.3 deaths per 100,000 females in 2020 (Table 1.4).
Table 1.4: Projected number of deaths\(^{(a)}\) and age-standardised rates with 95% prediction intervals, 2011–2020: breast cancer

<table>
<thead>
<tr>
<th>Year</th>
<th>Deaths</th>
<th>Lower 95% PI</th>
<th>Upper 95% PI</th>
<th>Estimated number of deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>2,690</td>
<td>2,580</td>
<td>2,790</td>
<td>20.0</td>
</tr>
<tr>
<td>2012</td>
<td>2,690</td>
<td>2,580</td>
<td>2,800</td>
<td>19.5</td>
</tr>
<tr>
<td>2013</td>
<td>2,700</td>
<td>2,580</td>
<td>2,820</td>
<td>19.1</td>
</tr>
<tr>
<td>2014</td>
<td>2,700</td>
<td>2,580</td>
<td>2,830</td>
<td>18.6</td>
</tr>
<tr>
<td>2015</td>
<td>2,710</td>
<td>2,580</td>
<td>2,840</td>
<td>18.2</td>
</tr>
<tr>
<td>2016</td>
<td>2,710</td>
<td>2,570</td>
<td>2,850</td>
<td>17.8</td>
</tr>
<tr>
<td>2017</td>
<td>2,720</td>
<td>2,570</td>
<td>2,870</td>
<td>17.4</td>
</tr>
<tr>
<td>2018</td>
<td>2,730</td>
<td>2,570</td>
<td>2,880</td>
<td>17.0</td>
</tr>
<tr>
<td>2019</td>
<td>2,730</td>
<td>2,570</td>
<td>2,890</td>
<td>16.7</td>
</tr>
<tr>
<td>2020</td>
<td>2,730</td>
<td>2,570</td>
<td>2,900</td>
<td>16.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Year</th>
<th>Lower 95% PI</th>
<th>Upper 95% PI</th>
<th>Estimated age-standardised rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>19.2</td>
<td>20.7</td>
<td>20.0</td>
</tr>
<tr>
<td>2012</td>
<td>18.7</td>
<td>20.3</td>
<td>19.5</td>
</tr>
<tr>
<td>2013</td>
<td>18.3</td>
<td>19.9</td>
<td>19.1</td>
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<tr>
<td>2014</td>
<td>17.8</td>
<td>19.5</td>
<td>18.6</td>
</tr>
<tr>
<td>2015</td>
<td>17.4</td>
<td>19.1</td>
<td>18.2</td>
</tr>
<tr>
<td>2016</td>
<td>17.0</td>
<td>18.7</td>
<td>17.8</td>
</tr>
<tr>
<td>2017</td>
<td>16.5</td>
<td>18.3</td>
<td>17.4</td>
</tr>
<tr>
<td>2018</td>
<td>16.1</td>
<td>18.0</td>
<td>17.0</td>
</tr>
<tr>
<td>2019</td>
<td>15.7</td>
<td>17.6</td>
<td>16.7</td>
</tr>
<tr>
<td>2020</td>
<td>15.3</td>
<td>17.3</td>
<td>16.3</td>
</tr>
</tbody>
</table>

(a) Projected estimates are based on national cancer mortality data.

Source: ([AIHW], 2017b)

Despite advances in detection and treatment of metastatic breast cancer, mortality from this disease remains high because current therapies are limited by the emergence of therapy-resistant cancer cells (Schultz and Weber, 1999, Stockler et al., 2000). As a result, metastatic breast cancer remains a largely incurable disease by current treatment strategies.

### 1.1.2 Breast Cancer: Anatomy

#### 1.1.2.1 Macroscopic Breast Anatomy

The breasts of an adult are supported by, and attached to, the front of the chest wall on either side of the breast bone or sternum by ligaments. They rest on the major chest muscle, the pectoralis major. Up until the onset of puberty, the breasts are much the same in the male and female, and their internal structure is similar - a collection of ducts emptying into the nipple.
In the female, during puberty, the breast tissue is responsive to a complex interplay of the female sex hormones estrogen and progesterone. These hormones stimulate the formation of additional ducts, and a system of milk secreting glands. There is also an increase in volume and elasticity of connective tissue, deposition of adipose tissue and increased vascularity (Ellis and Mahadevan, 2013).

Briefly, the breast is made up of milk-producing secretory glandular tissue and surrounding subcutaneous adipose tissue. The glandular tissue comprises between 15 to 20 lobes with varying numbers of ducts and lobules surrounded by connective tissue. The adipose tissue that covers the lobes gives the breast its size and shape. Each lobe connects to a lactiferous duct, several of which converge to form a lactiferous sinus or milk chamber. These sinuses empty into the nipple, where there are a number of duct openings. The nipple is surrounded by a pigmented area, the areola, which is lubricated by secretions from the sebaceous glands.

The cells making up the glandular tissue normally divide and grow throughout a woman’s life, in a regulated and orderly manner, controlled by her genes. However, occasionally a few cells lose this genetic control, and continue to divide and grow in an uncontrolled manner, causing a lump or tumour to be formed.

Most lumps found in the breast are benign, and they are usually left untreated. However, those found to be malignant tumours would, if left untreated, continue to grow in the breast and may spread (metastasise) to other parts of the body through the bloodstream or lymphatic system.

1.1.2.2 Microscopic Breast Anatomy
The normal human breast comprises 3 cellular lineages: the luminal epithelial lineage, the myoepithelial lineage, and the mesenchymal lineage (Bissell and Radisky, 2001). Originally, it was proposed that human breast cancer cells generally originated from the luminal epithelial lineage of terminal duct lobular units (Wellings et al., 1975), which are a basic mammary structure consisting of a branching ductal-alveolar system lined by an inner layer of luminal epithelial cells, and an outer layer of myoepithelial cells. Indeed,
there is evidence to suggest that the stem cells of the human and mouse mammary gland may be contained within the luminal epithelial compartment (Stingl et al., 1998, Pechoux et al., 1999).

1.1.3 Breast Cancer Pathology

Breast cancer is a heterogeneous disease that initiates as a localised neoplasm, also known as the primary tumour. The primary tumour may arise from any of the breast structures, but is not usually life-threatening when localised in the original site. However, when these tumours gain malignancy and invade neighbouring tissues and the vasculature, they pose a threat to patients’ health. Like other forms of cancer, breast cancer is classified as either non-invasive (in situ) or invasive.

1.1.3.1 Non-Invasive Breast Cancer

In-situ or non-invasive carcinomas of the breast are being increasingly recognised as ‘early lesions’, and usually occur in the mammary ducts, termed ductal carcinoma in situ (DCIS-Figure 1.2a), and lobules, termed lobular carcinoma in situ (LCIS- Figure 1.2b). The DCIS tumours appear heterogeneous and consist of malignant epithelial cells confined to the mammary ducts, without microscopic evidence of stromal invasion (Morrow, 1996). LCIS are less common than DCIS (Morrow, 1996), generally lack specific clinical or mammographic signs, and occur more frequently in premenopausal women. By definition, these cancer cells are confined to the mammary lobules without microscopic evidence of invasion. LCIS are characterised microscopically by a solid mass of small cells. The cells have a low proliferative rate, are typically estrogen receptor (ER)-positive, and rarely over-express the human epidermal growth factor 2 (HER2) oncogene.
Chapter 1: Literature Review

Figure 1.2 Non-Invasive Breast Cancer

a) Normal breast with non-invasive ductal carcinoma in situ (DCIS) in an enlarged cross-section of the duct.
A ducts; B lobules; C dilated section of duct to hold milk; D nipple; E fat; F pectoralis major muscle; G chest wall/rib cage. Enlargement: A normal duct cells; B ductal cancer cells; C basement membrane; D lumen (center of duct).

b) Normal breast with lobular carcinoma in situ (LCIS) in an enlarged cross-section of the lobule.
A ducts; B lobules; C dilated section of duct to hold milk; D nipple; E fat; F pectoralis major muscle; G chest wall/rib cage. Enlargement: A normal lobular cells; B lobular cancer cells; C basement membrane

(Taken from http://www.breastcancer.org)

1.1.3.2 Invasive Breast Cancer

The most common types of breast cancer are the epithelial tumours (carcinomas), and the most abundantly identified form of these epithelial carcinomas are the infiltrating ductal carcinomas, accounting for approximately 80% of all breast cancers and 75% of breast cancer deaths (Debies and Welch, 2001). The cells in this type of carcinoma can grow in either irregular or rounded sheets, or nests or cords of solid clusters of cells, frequently interspersed with isolated cells. The degree of differentiation of the tumour is reflected by the presence of poorly or well-formed gland lumens (Vincent-Salomon and Thiery,
2003a). The group of lobular carcinomas is the second largest category of invasive carcinomas, accounting for 10-15% of these invasive carcinomas. These tumours are composed of carcinoma cells, isolated or organized in single-file or narrow cords, usually with an abundant surrounding stroma. In addition to ductal and lobular types of carcinoma, other types, including medullary, tubular, and mucinous carcinomas have been described. Of these carcinomas, each has shown to obtain distinct aetiologies, tissues of origin, and metastatic behaviours (Vincent-Salomon and Thiery, 2003a). Medullary carcinomas are frequently well circumscribed, and exhibit lymphocytic infiltration. Mucinous tumours have a better prognosis than ductal or lobular carcinomas (Debies and Welch, 2001).

1.1.4 Breast Cancer Aetiology

The aetiology of breast cancer is still poorly understood, with known breast cancer risk factors explaining only a small proportion of cases. Well established and probable risk factors that modulate the development of breast cancer include: age, geographic location and socioeconomic status, reproductive events (menarche, menopause, pregnancy, breastfeeding), exogenous hormones (hormone replacement therapy, and oral contraceptives), lifestyle risk factors (alcohol, diet, obesity and physical activity), familial history of breast cancer, mammographic density, history of benign breast disease, ionizing radiation, bone density, height, insulin-like growth factor 1 (IGF-1) and prolactin levels, chemopreventive agents, as well as genetic factors (high and low-penetrance breast cancer susceptibility genes) (Dumitrescu and Cotarla, 2005).

1.1.5 Molecular Subtypes of Breast Cancer

Breast cancer is a diverse and complex disease which consists of distinct molecular subtypes, each with a unique gene-expression profile. Gene expression profiling of invasive breast cancer has led to a molecular classification of breast cancer characterised by a number of tumour subtypes with clinical relevance: Luminal A and B, Basal, human epidermal growth factor receptor 2 (HER2)-positive, and triple-negative (ER; Progesterone receptor (PR); HER2) (Perou et al., 2000, Sørlie et al., 2001). In their ground-breaking work, Perou and colleagues studied the gene expression profiles of 65
human breast tumour specimens from 42 individuals using complementary DNA microarrays representing 8,102 human genes (Perou et al., 2000). Of the 42 breast tumours, twenty of them were sampled twice, before and after chemotherapy. In addition, primary tumours from two patients were also paired with a lymph node metastasis from the same patient. The molecular profiles of breast tumours based on gene expression patterns revealed the similarities and differences among the tumours, and distinct functional gene clusters (Perou et al., 2000). Eight independent gene clusters appeared to reflect variation in specific cell types present within the tumours, such as endothelial cells, stromal cells, adipose-enriched cells, T-cells, B-cells, macrophages, luminal and basal epithelial cells.

Furthermore, the pioneer studies conducted by Sorlie et al. (2001) reported a distinctive “molecular portrait” of breast cancer using 456 cDNA clones, in which tumours were classified into intrinsic subtypes with distinct clinical outcomes (Sørlie et al., 2001). Patient outcome of these basic intrinsic subtypes are compared in Figure 1.3 (Dai et al., 2016).

The luminal breast subgroup is sub-divided into luminal A tumours, which show good prognosis, and luminal B tumours, which show poor prognosis. Both the basal/triple-negative tumours, and the HER2-enriched subtypes are ER-negative, and show poor outcomes (Perou et al., 2000, Sørlie et al., 2001). The rationale for such classification is that the differences underlying the gene expression patterns among cancer subtypes reflect the fundamental differences of the tumours at the molecular level (Sorlie et al., 2003). These subtypes have been repeated by other studies with varying numbers of genes included in the signature (Sotiriou et al., 2003, Fan et al., 2006, Hu et al., 2006, Parker et al., 2009, Lehmann et al., 2011, Reis-Filho and Pusztai, 2011, Gnant et al., 2013, Dai et al., 2016).
1.1.5.1 Luminal Tumours

Luminal tumours are the most common subtypes among breast cancer. Luminal-like tumours express hormone receptors, with expression profiles reminiscent of the luminal epithelial component of the breast (Perou et al., 2000). At least two subtypes exist within luminal-like tumours, i.e., luminal A and luminal B.

Luminal A tumours are hormone-receptor positive (ER- and/or PR- positive), HER2-negative, and have low levels of the protein Ki-67. Luminal A tumours are also low-grade (1 or 2), tend to grow slowly, and have the best prognosis, with fairly high survival rates, and fairly low recurrence rates. About 30-70 percent of breast cancers are luminal A tumours (Carey et al., 2006, Fan et al., 2006, Voduc et al., 2010, Howlader et al., 2014).

Luminal B tumours tend to be ER-positive, and may be HER2-negative, or HER2-positive. About 10-20% of breast cancers are luminal B tumours (Carey et al., 2006, Fan et al., 2006, Voduc et al., 2010).
Luminal B breast cancer has been reported to have lower expression of hormone receptors, higher expression of proliferation markers, and higher histologic grade than luminal A breast cancer (Goldhirsch et al., 2011). Luminal B breast cancer is defined by aggressive clinical behaviour, and has a prognosis similar to that of non-luminal cancers (including the HER2–enriched, and base-like subtypes).

1.1.5.2 Basal / Triple-Negative Tumours
Basal-like/triple-negative tumours are classified as hormone-receptor negative (ER- and PR- negative), and HER2-negative. They exhibit a high expression of genes characteristic of the basal epithelial cell layer, including expression of cytokeratins 5, 6, and 17 (Perou et al., 2000). There are several subsets of triple-negative breast cancer. Basal-like tumours represent one of these subsets, and have cells that look similar to those of the outer (basal) cells surrounding the mammary ducts. About 15-20% of breast cancers are triple-negative/basal-like (Carey et al., 2006, Fan et al., 2006, Voduc et al., 2010, Howlader et al., 2014). Triple-negative/basal-like tumours are often aggressive, and have a poorer prognosis compared to luminal tumours (Millar et al., 2009, Voduc et al., 2010, McGuire et al., 2017); however, they can be treated successfully.

1.1.5.3 HER2-Overexpressing Tumours
HER2-enriched tumours are hormone-receptor negative (ER- and PR-negative), and HER2-positive, and tend to grow faster than luminal cancers. They can have a worse prognosis, but they are often successfully treated with targeted therapies aimed at the HER2 protein (anti-HER2 drugs such as trastuzumab (Herceptin)). Approximately 5-15% of breast cancers are HER2 type (Carey et al., 2006, Voduc et al., 2010), and while most HER2-type tumours are HER2-positive, about 30 percent are HER2-negative (Foukakis and Bergh, 2016).

1.2 BREAST CANCER METASTASIS: THE METASTATIC CASCADE
Metastasis is the process by which cancer cells migrate from a primary tumour to distant locations of the body, and is a defining feature of cancer (Stetler-Stevenson et al., 1993, Valastyan and Weinberg, 2011, Scully et al., 2012). Metastasis is defined by end points,
i.e., metastatic lesions detected in specific organs distant from a primary tumour (Stetler-Stevenson et al., 1993), while steps by which metastases form have often been inferred, rather than directly observed. Some experimental and clinical evidence supports some of these steps, however, the process is complex and is still of major interest to researchers (Chambers and Matrisian, 1997, Scully et al., 2012).

Metastasis is a multi-step process requiring tumour cell progression through a series of distinct stages. This encompasses intravasation by tumour cells, their circulation in lymph and blood vascular systems, arrest in distant organs, extravasation, and growth into metastatic foci (Woodhouse et al., 1997, Valastyan and Weinberg, 2011, Scully et al., 2012). This process, termed the “metastatic cascade”, is a series of linked sequential steps leading to tumour metastasis ((Thompson and Newgreen, 2005, Valastyan and Weinberg, 2011); Figure 1.3). The order of these events can vary among different types of cancer (Pantel and Brakenhoff, 2004), e.g. in the mechanisms of invasion, and types of metastasis. Certain molecules have predicted roles in specific steps of the metastatic cascade, and are predicted to promote metastasis (Table 1.4).

1.2.1 Invasion
One of the first steps in the metastatic cascade is invasion (Scully et al., 2012). In order for tumour cells to invade the neighbouring environment, cells detach and escape from the site of the primary tumour (Liotta and Stetler-Stevenson, 1991), disturbing the epithelial integrity of the neighbouring tissue through a decrease in intercellular homotypic adhesion (Ghadimi and Schlag, 1998, Paris and Sesboüé, 2004).

Tumour invasion is accompanied by angiogenesis, the growth of new host blood vessels into the tumour. This process is induced at the post-capillary venule level by release of tumoural and stromal angiogenesis factors (Liotta et al., 1991).
Figure 1.4 Schematic Representation of the Metastatic Cascade (Adapted from Thiery, 2002).

The metastatic cascade is a multistep process during which cancer cells disseminate from the primary tumours and establish secondary tumours in distant organs. Tumour cells are believed to proceed through the sequential steps indicated to form clinically detectable metastases. Normal epithelium, which is lined by a basement membrane can proliferate locally to give rise to an adenoma. Still contained by an intact basement membrane, further transformation by epigenetic changes and genetic alterations leads to a carcinoma in situ. Further alterations can induce local dissemination of carcinoma cells, possibly through an Epithelial Mesenchymal Transition (EMT), where the basement membrane becomes fragmented. The tumour cells then intravasate into lymph or blood vessels, allowing their passive transport to distant organs. At secondary sites, solitary carcinoma cells extravasate and either remain solitary (micrometastasis) or form a new carcinoma through a Mesenchymal Epithelial Transition (MET).
Table 1.5 Steps in Metastasis and Examples of Proteins Involved at Each Stage of Progression (Modified from (Woodhouse et al., 1997)).

<table>
<thead>
<tr>
<th>Components of Metastasis</th>
<th>Examples of Proteins Involved in Progression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Angiogenesis</strong></td>
<td></td>
</tr>
<tr>
<td>Stimulation</td>
<td>Fibroblast Growth Factor Family, Transforming Growth Factor-α, Angiogenin, VPF, VEGF</td>
</tr>
<tr>
<td>Inhibition</td>
<td>CDI, TIMPs, PF4, Angiostatin</td>
</tr>
<tr>
<td><strong>Local Invasion</strong></td>
<td></td>
</tr>
<tr>
<td>Attachment (cell-cell)</td>
<td>Integrins, CD44, VLA-4, ICAM-1, OPN</td>
</tr>
<tr>
<td>Dissociation</td>
<td>Cadherins</td>
</tr>
<tr>
<td>Basement Membrane</td>
<td>Basement Membrane e.g. Collagens,</td>
</tr>
<tr>
<td>Disruption</td>
<td>Fibronectin, Elastin, Proteoglycans</td>
</tr>
<tr>
<td><strong>Proteolysis of Matrix</strong></td>
<td>Matrix Metalloproteinases, Interstitial</td>
</tr>
<tr>
<td></td>
<td>Collagenases, Stromelysins, Gelatinases</td>
</tr>
<tr>
<td>Antiproteolysis</td>
<td>TIMPs</td>
</tr>
<tr>
<td><strong>Motility</strong></td>
<td>HGF, IGF-II, ATX, Vitronecin, Fibronectin,</td>
</tr>
<tr>
<td></td>
<td>Laminin, Type I Collagen, Type IV Collagen,</td>
</tr>
<tr>
<td></td>
<td>Thrombospondin, IGF-I, IL-8, Histamine</td>
</tr>
<tr>
<td><strong>Metastasis Growth</strong></td>
<td>bFGF, IL-8, IGF-I, EGF, TGF-β, IL-6, IGF-II</td>
</tr>
</tbody>
</table>
1.2.2 Intravasation
The next phase of metastasis is termed ‘Intravasation’. Intravasation is when tumour cells enter the vasculature or lymphatic system, which tends to occur after these cells have invaded into adjacent tissues (Muraoka et al., 2002). Tumour cells entering the vasculature disseminate, and may travel either as individual cells, or as clusters (i.e. emboli). Furthermore, during intravasation, cancer cells evade immune recognition and physical stress (Khanna and Hunter, 2005a), as well as cell arrest in distant organs (Chambers et al., 2002, Wang et al., 2004, Valastyan and Weinberg, 2011). This characteristic may facilitate the longevity of cancer cells in the system, increasing their potential for growth.

The mechanism by which cells acquire this survival potential may be through interaction with specific molecules in particular organs or tissues via specific tumour cell surface receptors (Welch and Rinker-Schaeffer, 1999).

1.2.3 Extravasation
The third major stage of tumour progression is extravasation, which occurs when cells escape the circulation, and associate with the target organ interstitium and parenchyma (Scully et al., 2012). When cancer cells extravasate, they therefore are able to reach the secondary site, and are likely to follow through with growth (Valastyan and Weinberg, 2011).

1.2.4 Growth
Eventually, upon contact with distal target organs, tumour cells are stimulated to proliferate and grow from micro-metastases to form malignant secondary tumours (Fidler, 2003) in the new organ environment (Al-Mehdi et al., 2000). Growth of these small tumours requires the development of a new vascular supply through angiogenesis, and continuous evasion of host defence cells. Angiogenesis is, therefore, necessary at the beginning, and at the end of the metastatic cascade, providing the tumour cells with both nutritive agents, and growth factors. Tumour cells exhibit several characteristics such as
anchorage independent growth, high migratory capacity, the ability to produce high amounts of tumour proteases, and enhanced proliferation (Ossowski, 1992, Seynaeve et al., 1993, Malik and Parsons, 1996a, Malik and Parsons, 1996b).

Overall, tumour cell progression is a multi-stage process, whereby, tumour cells invade, intravasate, then extravasate and grow at secondary sites within the body. In addition, it is important to recognize that in order for these cells to establish this migratory and aggressive potential from a resting non-aggressive phenotype, certain changes are said to occur within the cell. Specifically, these changes resemble those required for the migration of epithelial cells during the physiological processes of tissue repair and embryogenesis. During tumour progression, the acquisition of migratory abilities in epithelial cells is accompanied by the loss of many epithelial features, and the gain of several mesenchymal characteristics, which has been referred to as epithelial mesenchymal transition (EMT).

1.3 EPITHELIAL MESENCHYMAL TRANSITION (EMT)

Epithelial cells are the most important cell types in the development of adult human malignancies. More than 90% of all malignant tumours are of epithelial origin (Birchmeier et al., 1996), which is why the transition of the epithelial nature to an aggressive potential is of great interest to many researchers.

Normal epithelia possess cell-to-cell contact that accounts for both the proper development of these tissues during embryonic life, and for the maintenance of homeostasis and architecture of epithelial structures during adult life. It is proposed that epithelial tumour cells lose this restriction during the last steps of tumourigenesis, concomitantly with the loss of epithelial characteristics, and the acquisition of motile behaviour (Iwatsuki et al., 2010). Such phenotypic conversions are reminiscent of the epithelial mesenchymal transitions (EMT) identified in developmental biology. These transitions are the hallmark of embryonic development, and are pivotal in cancer progression (Prindull and Zipori, 2004).
In cancer, EMT generally depicts a more aggressive behaviour of the tumour cells (Gilles and Thompson, 1996, Thiery, 2002b, Thompson and Newgreen, 2005). In fact, in breast cancer, EMT has been estimated to occur in as much as 18% of tumours \textit{in vivo} (Dandachi et al., 2001, Sørlie et al., 2001). Under these conditions EMT is defined as the occurrence of a variable proportion of tumour cells that express mesenchymal markers such as vimentin, tenascin, and stromelysin-3 (Ahmad et al., 1998, Dandachi et al., 2001).

EMT is an important process of cell remodelling that is critical during embryonic development and organogenesis. Based on the biological context under which it occurs (Kalluri and Neilson, 2003, Kalluri and Weinberg, 2009), EMT is classified into three types. Type 1 EMT is associated with embryonic development, whereby a transition of events occur that allow epithelial cells to become motile mesenchymal cells during implantation, embryo formation, gastrulation, and neural crest migration (Arias, 2001, Pérez-Pomares and Muñoz-Chápuli, 2002, Thiery, 2002b). Type 2 EMT is associated with wound healing, tissue regeneration, and organ fibrosis (Kalluri and Weinberg, 2009). During this process, tissue fibroblasts are generated from epithelial or endothelial cells during injury and chronic inflammation (Wang and Zhou, 2011). Type 3 EMT, which occurs in epithelial cancer cells, is associated with cancer progression and metastasis (Thiery, 2002b), whereby cancer cells at the invasive front of primary tumours undergo a phenotypic conversion to invade and metastasize through the circulation, and generate a metastatic lesion at distant tissues or organs by mesenchymal-epithelial transition (MET) (Wang and Zhou, 2011).

The process of EMT entails a range of cellular, phenotypic and functional changes. Specifically, EMT is described by a series of events typically involving changes in gene expression (loss of epithelial markers such as E-cadherin, and gain of mesenchymal markers such as vimentin), down-regulation of cell-cell adhesion, alteration in cell-matrix adhesion, changes in cell polarity and cytoskeletal organization, alterations in the ECM molecules synthesis and assembly, and up-regulation of synthesis and activity of extracellular proteases such as matrix metalloproteases (MMPs) (Iwatsuki et al., 2010). Overall, cells present with loss of epithelial features, changes to cell polarity and cell-cell
junctional linkage, the acquisition of fibroblast-like properties, as well as reduced intercellular adhesion, and increased motility, all of which are properties of mesenchymal cells (Birchmeier et al., 1996, Savagner, 2001, Thiery, 2002b, Ikenouchi et al., 2003, Christiansen and Rajasekaran, 2006, Planas-Silva et al., 2006). EMT profoundly affects major cell properties, which includes transcriptional down-regulation of epithelial cell markers such as Epithelial (E-)cadherin (Boyer et al., 2000, Savagner, 2001, Nieto, 2002, Thiery, 2002b, Zhou et al., 2004, Iwatsuki et al., 2010), and concomitant up-regulation of mesenchymal markers, including cell motility and mesenchymal molecules, such as vimentin (Barker and Clevers, 2001, Dandachi et al., 2001, Mellick et al., 2002, Iwatsuki et al., 2010), which are defining features of EMT (Huber et al., 2005). Given the importance of the EMT in carcinoma progression, there is considerable interest in understanding the mechanisms that contribute to this complex process (Bates and Mercurio, 2003).

The EMT program is understood to be activated by transcription factors (TFs), such as those from the SNAIL, TWIST, and ZEB families. Unlike cells in embryonic development, the activation of TFs in tumour cells rarely leads to a full EMT. Instead, tumour cells are believed to only partially proceed towards a complete mesenchymal state, resulting in an ‘incomplete’ or partial EMT (Yang and Weinberg, 2008, Kalluri and Weinberg, 2009, Micalizzi et al., 2010). The activation of a partial EMT by EMT transcription factors (EMT-TFs) is known to increase motility in cancer cells, and enhance their tumourigenicity (Brabletz et al., 2018).

Whilst there is a substantial amount of evidence supporting the role that EMT is important in carcinoma progression, conflicting research in recent years has suggested metastasis of carcinomas may occur without the activation of EMT (Tarin, 2005, Thompson and Newgreen, 2005, Fischer et al., 2015a, Zheng et al., 2015, Brabletz et al., 2018). This debate is largely due to an inability to monitor the EMT phenotype in vivo (Fischer et al., 2015a). In a prominent study by Zheng and colleagues (2015), genetically engineered mouse models (GEMMs) of pancreatic ductal adenocarcinoma (PDAC) were utilised to examine the role of EMT in metastasis. EMT was suppressed through the deletion of SNAIL1 or TWIST1. Interestingly, no impact was observed on the metastasis
of PDAC cells with either TF deletion, leading to a conclusion that EMT is unnecessary in the metastatic process (Zheng et al., 2015). Metastasis of tumour cells without the activation of EMT has also been reported in GEMMs of breast cancer (Fischer et al., 2015a). In an effort to suppress EMT in primary carcinoma cells, Fischer and colleagues (2015) utilised the overexpression of microRNA mir-200, which is capable of directly inhibiting the expression of the EMT-TF Zeb1. This study noted that despite no activation of the EMT program, the development of spontaneous breast-to-lung metastasis was not affected.

Recently, the studies by both Zheng et al. (2015) and Fischer et al. (2015) have been contested with claims of inadequate experimental design and insufficient evidence to demonstrate an absence of EMT in primary tumours (Aiello et al., 2017, Ye et al., 2017). The forced or endogenous absence of individual EMT-TFs does not provide evidence for an absent EMT (Aiello et al., 2017, Ye et al., 2017, Brabletz et al., 2018). An incomplete or partial EMT can be induced by the activation or suppression of individual EMT-TFs without being compensated by other EMT-inducing TFs (Yang et al., 2004, Korpal et al., 2008, Liu et al., 2008, Chakrabarti et al., 2012). Moreover, the manifestation of a partial EMT in cancer metastasis may also be dependent on the type of tumour, as the heterogeneous nature of cancer cells can result in different combinations of EMT-TF expression (Brabletz et al., 2018). Currently, whether metastatic processes can occur without the activation of EMT is debateable, and surely requires further investigation.

1.3.1 Morphological Features of Epithelial and Mesenchymal Cells during Embryogenesis

During early development, cells are categorised by their morphology and functional characteristics into two main structural types (Shook and Keller, 2003). These are the epithelial cells, and mesenchymal cells (Thiery and Sleeman, 2006). Regardless of the fact that these cells may originate from several germ layers such as ectoderm, endoderm, or mesoderm, overall, cells in early developing embryos display either an epithelial or mesenchymal morphology (Figure 1.5).
Chapter 1: Literature Review

Figure 1.5 Schematic Representations of Epithelial and Mesenchymal Cells Illustrating the Process of EMT (Modified from (Radisky, 2005)).

\[ \text{Epithelial Cells} \]

\[ \text{Mesenchymal Cells} \]

1.3.1.1 Epithelial Cells

Epithelial cells are closely packed cells forming the epithelium, appearing as a group of cobble-stoned, defined cells. Epithelial cells form layers of cells (clusters) in culture, that are closely joined to one another by specialised membrane structures, such as tight junctions, adherens junctions, desmosomal junctions, and gap junctions (Thiery and Sleeman, 2006). Epithelial cells also display apico-basal polarity manifested in the specific distribution of cell-surface molecules such as cadherins and certain integrins, organization of cell-cell junctions, polarized organization of the actin cytoskeleton and formation of a basal lamina extra-cellular matrix at the basal surface (Shook and Keller, 2003, Thiery and Sleeman, 2006). Within mature epithelial tissues, cell polarity regulates cellular morphology, intracellular signalling, asymmetric cell division, cell migration, cellular and tissue physiology, and complex organ morphogenesis. Epithelial cells are seen in tubular tissues, such as the neural tube and nephric duct, as well as in sheets such as the epiblast and epidermis. While remaining within the epithelial layer, epithelial cells can move away from their nearest cell population, as they may acquire the ability to
migrate (Schöck and Perrimon, 2002). Several proteins and genes are expressed in epithelial cells which are rendered as classic markers for the identification of this phenotype, including keratins and E-cadherin.

### 1.3.1.2 Mesenchymal Cells

Mesenchymal cells differ in both appearance and behaviour to epithelial cells (Figure 1.5). Mesenchymal cells are amorphous in shape, they do not form an organized cell layer, do not have apico–basso-lateral organisation, lack polarisation of the cell surface molecules, and have a disrupted, ill-defined actin cytoskeleton (Mackenzie and Raz, 2006). Mesenchymal cells form a relatively diffuse tissue network (Thompson and Newgreen, 2005), having only points on their surface which mediate their interaction with neighbouring cells (Vincent-Salomon and Thiery, 2003b). These cells usually reside in connective tissues as elongated cells, lack a basal lamina, and display front-back polarity (Hay, 1995), allowing them to move through the ECM as individual cells. In culture, mesenchymal cells have a spindle-shaped, fibroblast-like morphology and tend to be highly motile, although this is not necessarily the case *in vivo* (Thompson and Newgreen, 2005). In certain circumstances, mesenchymal cells exhibit motility and can migrate long distances in the body. Such mesenchymal cells include ingressing cells during amniote gastrulation, and migrating neural crest cells (Locascio and Nieto, 2001, Nieto, 2002, Shook and Keller, 2003).

Several proteins and genes expressed in mesenchymal cells are considered classic markers for the identification of this phenotype, including Neuronal (N)-cadherin, vimentin, smooth muscle actin (SMA), and Matrix Metalloproteinases (MMPs).

### 1.3.2 Epithelial Mesenchymal Transition (EMT) In Development

EMT occurs during critical phases of embryonic development of most metazoans (Vincent-Salomon and Thiery, 2003b). Originally defined by developmental cell biologists and cancer researchers as a mechanism of tissue morphogenesis in which cells within embryonic epithelia are rapidly remodelled and redistributed, giving rise to individual migratory cells, the EMT has since attracted increasing interest (Hay, 1995).
Developmental EMTs are multi-step processes and can be classified as either primary (early-stage) EMT, or secondary (late-stage) EMT. Primary developmental EMTs are one of the morphogenic mechanisms driving germ layer reorganisation of the initial primary embryonic epithelium during gastrulation (formation of mesoderm and endoderm from epiblast epithelium), (Hay, 1995, Viebahn, 1995, Tam and Behringer, 1997, Narasimha and Leptin, 2000, Ikenouchi et al., 2003, Lee et al., 2006, Thiery and Sleeman, 2006), neurulation and neural crest formation (Duband et al., 1995) from the neuro-epithelium. In contrast, secondary developmental EMT involves cells that have adopted an epithelial organisation, and then undergo an EMT during organogenesis. These secondary EMTs may not involve maintenance of epithelial integrity or ingress, as they occur largely within the embryonic environment (Shook and Keller, 2003). Examples include the formation of the sclerotome via ventral somite de-epithelialisation (Brand-Saberi et al., 1996), and formation of the endocardial cushions in the atrioventricular canal of the heart via EMT of cells in the endocardial endothelium (Markwald et al., 1996).

1.3.2.1 Events Comprising Developmental EMTs

Generally, there are several characteristic features or changes associated with early EMT (Shook and Keller, 2003):

i- Specification to differentiate into a type of cell that will go through EMT

ii- Temporal and spatial patterning of the progress of the EMT within the area destined to undergo EMT

iii- Move, or be moved, to the site of EMT, generally though epithelial morphogenesis

iv- Alteration or disruption of the basal lamina

v- Change in cell shape, generally by an apical actin-myosin contractile mechanism and/or changes in adhesion

vi- De-epithelialise (loss of apical junctions)

vii- Ingress

viii- Maintenance of epithelial integrity

ix- Differentiate cell behaviour and organisation characteristic of a mesenchymal phenotype
1.3.3 Molecular Mechanisms/Inducing Factors of Epithelial Mesenchymal Transition (EMT)

The molecular mechanisms contributing to EMT are diverse, and have been the subject of many reports and reviews (Savagner, 2001, Kalluri and Neilson, 2003, Thiery, 2003, Huber et al., 2005, Radisky, 2005, Thompson and Newgreen, 2005, Christiansen and Rajasekaran, 2006, Christofori, 2006, Thiery and Sleeman, 2006, Lamouille et al., 2014). EMT can be induced in in vivo occurrences by mechanisms that involve extra-cellular matrix components (ECM) such as collagens, laminin 5 (Grassi et al., 1999), hyaluronic acid (Zoltan-Jones et al., 2003), and growth factors which promote various signalling cascades through tyrosine kinase surface receptors (Figure 1.5).

These factors include epidermal growth factor (EGF), scatter factor/hepatocyte growth factor (SF/HGF), members of the fibroblast growth factors (FGF) (Valles et al., 1990) and transforming growth factor-β (TGFβ) families, and insulin-like growth factors (IGF) 1 and 2 (Morali et al., 2001, Thiery, 2002b). In vitro, these signals are not function-specific, and can trigger both EMT and proliferation, depending on the local environment (Savagner, 2001).

By binding epithelial receptors with ligand-inducible intrinsic kinase activity, EMT is facilitated by local expression of TGF-β, EGF, IGF-II, or FGF-2 (Okada et al., 1997, Fan et al., 1999, Morali et al., 2001, Strutz et al., 2002, Kalluri and Neilson, 2003). In response to these ligands, receptor mediated signalling triggers the activation of intracellular effector molecules, such as members of the small GTPase family (i.e. Ras, Rho and Rac), as well as members of the Src tyrosine-kinase family (Thiery and Sleeman, 2006).

Many studies have indicated a prominent role of EGF in driving breast cancer metastasis. Aberrant EGF and its receptor epidermal growth factor receptor (EGFR (ErbB-1)) signalling has been extensively described as a major cause of progression and metastasis of breast cancer (Navolanic et al., 2003, Lo et al., 2007). These factors can activate extracellular signal-regulated kinase 1/2 (ERK1/2) or phosphoinositide-3 kinase/Akt (PI3K/Akt) that can potently induce EMT in cancer cells (Kim et al., 2016).
TGF-β is one of the most prominent and potent inducers of EMT in cultured cells in vitro and in animal models in vivo (Thiery and Sleeman, 2006). The process of EMT induced by TGF-β is well-established as a critical mechanism of tumour progression (Zavadil and Böttinger, 2005, Moustakas and Heldin, 2007, Massagué, 2008, Kalluri and Weinberg, 2009, Buonato et al., 2015). TGF-β can activate a network of cellular signalling pathways, such as the TGF-β/Smads signal pathway which regulates the expression of several transcriptional factors, e.g., Snail (Snail1), TWIST1, Zeb1, and Slug (Snail2) to start an EMT process (Thiery et al., 2009), extracellular signal-regulated kinases 1 and 2 (also known as MAPK3 and MAPK1, respectively; ERK1/2), p38 proteins, and c-Jun N-terminal kinase (Zhang, 2009; Gonzales and Medici, 2014). Activity of ERK1/2 has been identified as particularly important for TGF-β-driven EMT (Zavadil et al., 2001, Grände et al., 2002, Buonato and Lazzara, 2014). EGF, which initiates its own signalling network including ERK, is often combined with TGF-β to enhance EMT outcomes in vitro (Grände et al., 2002, Docherty et al., 2006, Uttamsingh et al., 2008), and the augmented EMT that results has been attributed to ERK activation (Grände et al., 2002, Uttamsingh et al., 2008). In certain cells the addition of TGF-β alone to cells indirectly leads to tyrosine phosphorylation of EGFR, which augments ERK pathway activation (Murillo et al., 2005).

During EMT, a series of cascades and pathways are stimulated which can lead to the activation of gene transcription. Genes responsible for blocking epithelial markers, as well as factors ultimately involved in tumour progression, appear to be switched on or active, subsequently facilitating the migratory and malignant potential of mesenchymal cells.

1.4 MARKERS OF EMT

The EMT concept provides a new means of identifying genes that are important for the progression of carcinoma towards dedifferentiated and more malignant states, and studying the cellular and developmental biology of EMTs will provide insights into the mechanisms involved.
One group of proteins which have shown to be altered during EMT are intermediate filaments, and some of these proteins are considered to be putative markers for the EMT process. Specifically, keratins and vimentin have been shown to be significantly expressed during stages of EMT, and are currently used in pathology in some models of diagnosis. Apart from the “classical” EMT genes, vimentin, E-cadherin, and N-cadherin, there have been certain genes implicated in EMT which have been shown to have predicted roles in specific steps of the metastatic cascade, and are predicted to promote metastasis. Some of these genes include the integrins, and Integrin-Linked Kinase (ILK).

1.4.1 Intermediate Filament Proteins

Intermediate filaments (IFs) are one of the three major cytoskeletal systems of eukaryotic cells, and have been considered as providers of an intracellular scaffold that supplies cell strength and tissue integrity (Fuchs and Weber, 1994). Intermediate filament proteins are well recognised for their cell type specificity and their static structural role as components of the vertebrate cell cytoskeleton (Thomas et al., 1999). There is evidence for intermediate filament involvement in a variety of dynamic cellular functions, including intercellular and cell to extra-cellular matrix signal transduction, cellular motility, and tumour cell invasiveness (Steinert and Liem, 1990, Skalli and Goldman, 1991, Chu et al., 1993, Fuchs and Weber, 1994, Hendrix et al., 1996). However, a number of other more dynamic roles for intermediate filament proteins, e.g., vimentin, have been proposed (SundarRaj et al., 1992, Guarino, 1995, Hay, 1995, Gilles and Thompson, 1996).

Intermediate filaments compromise a heterogeneous family of 50 different proteins, which are expressed in a tissue-dependent, and differentiation-dependent manner (Duprey and Paulin, 1995). They are an abundant, and thus very convenient, indicator of cell lineage phenotype. Intermediate filaments can be divided into 6 sub-classes (Table 1.6) based on their biochemical properties, immunologic specificity, and tissue distribution. These include keratins in epithelial cells, vimentin in fibroblasts, leukocytes, and blood vessel endothelial cells, desmin in muscle cells, glial fibrillary acidic protein (GFAP) in astrocytes and other glia, peripherin in peripheral neurons, nestin, internexin, neurofilaments (NF) in neurons, and lamins (Steinert and Liem, 1990, Parry and Steinert,
In cells of epithelial origin, the major structural component of intermediate filaments is vimentin.

1.4.1.1 Vimentin

One of the most commonly used markers for EMT in carcinoma cells is the unusual expression of the 54kDa type III intermediate filament protein, vimentin, which is present in a range of cell types, including fibroblasts, endothelial cells, macrophages, neutrophils and lymphocytes (Luangdilok et al., 2007). Initially, vimentin is widely expressed in the embryo, but becomes progressively restricted to fewer cell types during embryonic development (Duprey and Paulin, 1995).

During development, vimentin is first expressed in the mouse at about embryonic day 8.5 in the cells of the parietal endoderm, and also in those cells that deliminate through the primitive streak to become primary mesoderm (Franke et al., 1982, Lane et al., 1983). In mature tissues, vimentin is normally restricted to cells of mesenchymal origin (Steinert and Roop, 1988), e.g. fibroblasts, endothelial cells, and cells of the haemopoetic lineages (Franke et al., 1987, Eckes et al., 2000).

Vimentin is present in all primitive cell types, but during differentiation it is co-expressed or replaced by other intermediate filaments (Hendrix et al., 1997, Dandachi et al., 2001). Expression of vimentin has been described in epithelial cells involved in physiological or pathological processes which require epithelial cell migration, as well as being described in epithelial cells involved in embryological and organogenesis processes (Guarino, 1995, Hay, 1995), placentation (Nawrocki et al., 1997), wound healing (SundarRaj et al., 1992), or tumour invasion (Ramaekers et al., 1983, Savagner et al., 1994, Guarino, 1995, Gilles et al., 1996, Gilles and Thompson, 1996).

Mutation analyses performed by Schietke and colleagues (2006) suggested that vimentin is the most widely expressed IF protein in mammals. In these epithelial cells, vimentin is believed to support cellular membranes, which is a similar role to keratins in epithelia.
Table 1.6 Primary Intermediate Filament Proteins in Mammals (Modified from (Galou et al., 1997)).

<table>
<thead>
<tr>
<th>Intermediate Filament</th>
<th>Tissue Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I</strong></td>
<td></td>
</tr>
<tr>
<td>Acidic Keratins</td>
<td>Epithelia</td>
</tr>
<tr>
<td><strong>Type II</strong></td>
<td></td>
</tr>
<tr>
<td>Basic Keratins</td>
<td>Epithelia</td>
</tr>
<tr>
<td><strong>Type III</strong></td>
<td></td>
</tr>
<tr>
<td>Vimentin</td>
<td>Mesenchyme</td>
</tr>
<tr>
<td>Desmin</td>
<td>Muscle</td>
</tr>
<tr>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Glial cells and astrocytes</td>
</tr>
<tr>
<td>Peripherin</td>
<td>Peripheral and central neurons</td>
</tr>
<tr>
<td><strong>Type IV</strong></td>
<td></td>
</tr>
<tr>
<td>Internexin</td>
<td>Developing central nervous system</td>
</tr>
<tr>
<td>Neurofilaments</td>
<td>Mature neurons</td>
</tr>
<tr>
<td><strong>Type V</strong></td>
<td></td>
</tr>
<tr>
<td>Laminin (A,B,C)</td>
<td>Nucleus of all cells</td>
</tr>
<tr>
<td><strong>Type VI</strong></td>
<td></td>
</tr>
<tr>
<td>Nestin</td>
<td>Nerve cells</td>
</tr>
</tbody>
</table>

A summary of vimentin expression in normal and developmental systems is outlined in Table 1.7 (Kokkinos et al., 2007).
Vimentin networks may also help keep the nucleus and other organelles in a defined place within the cell (Kokkinos et al., 2007).

Vimentin has also been detected in several human epithelial tumour cell lines. Nonetheless, comparative studies have revealed that expression of vimentin is restricted to invasive cells, suggesting that vimentin is related to high invasive abilities, rather than being non-specifically induced by culture conditions (Sommers et al., 1989, Sommers et al., 1992, Gilles and Thompson, 1996, Hendrix et al., 1996, Polette et al., 1998). Moreover, Hendrix and co-workers (Hendrix et al., 1997), and Gilles and co-workers (Gilles et al., 1999), showed that vimentin anti-sense transfection into invasive, vimentin-positive breast cell lines reduced their in vitro invasiveness or migration, emphasising a functional role of vimentin in epithelial cell invasion and migration.

**Table 1.7 Examples of Sites of Vimentin Expression in Normal and Developmental Processes (Modified from (Kokkinos et al., 2007)).**

<table>
<thead>
<tr>
<th>Vimentin Localization</th>
<th>Putative role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS</td>
<td>In adult mesenchymal cells</td>
<td>(Larsson et al., 2004)</td>
</tr>
<tr>
<td>Muscle</td>
<td>In adult mesenchymal cells</td>
<td>(Larsson et al., 2004)</td>
</tr>
<tr>
<td>Testes (monkey/rat)</td>
<td>Maturation of Sertoli cells</td>
<td>(Devkota et al., 2006)</td>
</tr>
<tr>
<td>Endothelial Cell Damage</td>
<td>Auto-immunity and tissue graft rejection shown when vimentin expression is at cell surface</td>
<td>(Carter et al., 2005)</td>
</tr>
<tr>
<td>lipids</td>
<td>Transport of cholesterol during steroidogenesis</td>
<td>(Sarria et al., 1990)</td>
</tr>
<tr>
<td>Placenta</td>
<td>At sites of growing placenta, facilitates placental growth</td>
<td>(Walter and Schönkypl, 2006)</td>
</tr>
<tr>
<td>Trophoblasts</td>
<td>Facilitates trophoblast invasion</td>
<td>(Haigh et al., 1999)</td>
</tr>
</tbody>
</table>
In the same way, impaired wound-healing has been observed in vimentin knock-out mice (Eckes et al., 2000).

During mouse development, vimentin expression has been associated with cell motility, a typical consequence of EMT, with neural crest (Cochard and Paulin, 1984, Page, 1989), parietal endoderm (Lane et al., 1983, Lehtonen et al., 1983), and mesenchymal cells (Franke et al., 1982). It has been observed that in response to growth factors (such as acidic fibroblast growth factor or hepatocyte growth factor), or several collagen types (Boyer et al., 1989, Zuk et al., 1989, Savagner et al., 1994), vimentin expression was induced in the NBT-II bladder carcinoma cells.

Expression of vimentin in invasive breast cancer is a repeatedly reported finding (Ackland et al., 2003, Gilles et al., 2003a, Korsching et al., 2005). Vimentin expression has been documented in approximately 15% of invasive breast cancers. The conventional criterion for scoring vimentin positivity is that ≥ 10% of tumour parenchyma are positive (Gilles and Thompson, 1996, Kokkinos et al., 2007). In normal breast, vimentin is expressed by myoepithelial breast cells (Guelstein et al., 1988), and in breast cancer its expression is associated to high histological grade, lack of ER expression, epidermal growth factor receptor (EGFR) positivity, and p53 expression (Raymond and Leong, 1989, Domagala et al., 1990, Santini et al., 1996, Tsuda et al., 2005, Umemura et al., 2005).

One of the limitations in the use of vimentin as a prognostic marker in breast carcinomas is the likelihood that vimentin-positive cells may have migrated away from the primary mass, and become buried in the surrounding stroma, which is also vimentin-positive. New markers which better represent EMT, and therefore allow for a more specific identification of EMT-derived breast cancer cells in the tumour vicinity, could have a dramatic impact on breast cancer prognosis.

In summary, the expression of vimentin in invasive breast cancers cannot be solely attributed to EMT or direct myoepithelial histogenesis (Savagner, 2001). Instead, breast cancers expressing vimentin may also derive from progenitor cells with a bilinear (glandular and myoepithelial) differentiation potential (Korsching et al., 2005).
1.4.1.2 Keratins

Among all intermediate filament proteins, keratins (also called cytokeratins) represent the largest and most complex subgroup. Keratins, existing as highly dynamic networks of cytoplasmic 10-12 nm wide intermediate filaments that are obligate heteropolymers, are expressed in a cell type-specific manner at various levels and compositions in all epithelial tumours, but rarely in other tissues.

There are 54 distinct keratins, subclassified as type I or type II, that form type I/type II heterodimers resulting in the formation of intermediate filaments that comprise the major structural units of the cytoskeleton of epithelial cells (Moll et al., 2008). Keratins, differentially expressed as pairs of type I (acidic keratins), and type II (neutral-basic keratins) intermediate filament proteins (Fuchs et al., 1981, Schiller et al., 1982, Hatzfeld and Franke, 1985, Eichner et al., 1986) in epithelial cells in vivo (Fuchs and Weber, 1994), consist of a) 17 human type I epithelial keratins (K9-K28), and b) 20 human type II epithelial keratins (K1-K8, K71-K80) (Schweizer et al., 2006). They are highly differentiation specific in their expression patterns, implying functional differences (Schweizer et al., 2006).

Keratins have important roles in maintaining epithelial structural integrity and have also been recognized as regulators of other normal cellular functions, including signaling, growth, and protein synthesis (DePianto and Coulombe, 2004). The immunohistochemical (IHC) localization of specific keratins, especially keratins 7 and 20, has been widely used as a diagnostic adjunct in surgical pathology and cytopathology (Moll et al., 2008). However, emerging evidence suggests that some keratins, including keratin 17 (K17), may also fundamentally impact hallmarks of cancer cell biology (Chung et al., 2012, Escobar-Hoyos et al., 2015, Hobbs et al., 2016).

Most single epithelia contain the type II keratin 8 (K8), and its type I partner keratin 18 (K18) (Oriolo et al., 2007), either alone, or with K7, K19, and K20 (Moll et al., 1982). In the early embryo, K8 and K18 are the main intermediate filaments (JACKSON et al., 1980, Kemler et al., 1981, Oshima et al., 1983, Oriolo et al., 2007).
The principal function of keratins is to act as rigid, yet adaptable scaffolds that prevent epithelial cells from mechanical, and non-mechanical stresses that result in cell death. They act as protein scaffolds with structural and regulatory functions in a cell-type-specific manner, as underscored by keratinopathies (Omary et al., 2004), and knockout mice (Hesse et al., 2004). The analysis of these models suggests that keratins have a major impact on cell architecture, cell size, and proliferation depending on cell context, but do not act as major regulators of epithelial differentiation (Magin et al., 2007). Other emerging functions include roles in cell signalling, translation control, proliferation, the stress response and apoptosis, malignant transformation, and unique roles that are keratin specific and tissue specific.

Numerous studies have sought to analyse differences in keratin expression between normal and tumour cells of various tissue origins (Schlegel et al., 1980, KREPLER et al., 1981, Winter and Schweizer, 1981, Moll et al., 1982, Winter et al., 1983, Blobel et al., 1984, Miettinen et al., 1984, Brawer et al., 1985, Lindberg and Rheinwald, 1989, Ring et al., 2004, Mikhitarian et al., 2005a, Mikhitarian et al., 2005b, Ring et al., 2005, Smirnov et al., 2005, Nissan et al., 2006, Zuk et al., 2017). Initially, studies by Moll and co-workers (1982) showed differences between normal and tumour tissues with keratins extracted from mammary glands (Moll et al., 1982). In a 1990 study, Trask and colleagues compared the full complements of keratins in normal, immortalised, and tumour cells, showing that the overall pattern of keratin protein expression is very different in normal and tumour cell lines. Dominant keratins produced in normal cells were K5, K6, K7, K14, and K17, while in tumour cells K8, K18, and K19 were the major intermediate filament proteins (Trask et al., 1990).

Previous reports evaluating prognostic markers for breast cancer aggressiveness in vitro have presented strong direct evidence correlating co-expression of keratin and vimentin IFs with an invasive and metastatic phenotype (Ramaekers et al., 1983, Hendrix et al., 1996, Hendrix et al., 1997). In an in vitro model of keratin and vimentin co-expression in breast cancer cells, developed as stable transfectants, Hendrix and co-workers (1997) revealed that these keratin/vimentin IF-positive cells display increased proliferation rates, invasive potential, clonogenicity, and tumourigenicity when compared with keratin-
positive/vimentin-negative controls displaying low invasive potential (Hendrix et al., 1997).

Keratins are differentially expressed among different breast cancer cell lines and are down-regulated during metastatic spread and progression in breast cancer (Joosse et al., 2012). In their study, Joose and co-workers demonstrated differential gene expression levels of distinct keratins among basal and luminal type breast cancer cell lines. Keratins 8 and 19 were significantly under-expressed in basal-like B as compared to basal-like A and luminal cell lines whereas, keratin 18 had significantly lower gene expression levels in all basal-like compared to luminal cell lines (Joosse et al., 2012). Polioudaki and colleagues (2015) investigated the correlation between keratin expression of Circulating Tumour Cells (CTCs) and patients’ outcome, and characterized the EMT status of CTCs via the establishment of a numerical “ratio” value of keratin and vimentin expression levels on a single cell basis. They found keratin expression was higher in the luminal breast cancer cell lines MCF-7 and T47D compared to the basal-like B, MDA-MB-231 and Hs578T cells (Polioudaki et al., 2015).

1.4.2 Cadherins

Cadherins are a large family of transmembrane glycoproteins, with over 80 members in both vertebrates, and invertebrates (Nollet et al., 2000, Yilmaz and Christofori, 2009). Cadherins include at least 6 subfamilies which can be distinguished on the basis of their structural or functional organisation, including their protein domain composition, genomic structure, and phylogenetic analysis of the protein sequences (Foty and Steinberg, 2004, Brasch et al., 2018). These sub-families comprise a) the classical (or type-I) cadherins, b) the atypical (or type-II) cadherins, which are mainly localised to the adherens junctions, c) the desmosomal cadherins, which form desmosomal junctions, d) the proto-cadherins, which are implicated in neural development, e) the flamingo cadherins which are seven transmembrane cadherins, and f) the FAT-like cadherins (Nollet et al., 2000, Heymann et al., 2002).

The classical cadherins, E-cadherin, N-cadherin, and Placental (P)-cadherin, were the first cadherins to be described (Takeichi et al., 1988, Takeichi et al., 1990, Yagi and
Takeichi, 2000), and currently represent the most well-characterised classical cadherins (Heymann et al., 2002, Brasch et al., 2018). They are the most widely studied and definitive group of the cadherin superfamily, and were originally identified in vertebrates (Hyafil et al., 1980, Marotta et al., 2001).

Classical cadherins are composed of three segments. These are a) an extra-cellular domain of 110 amino acids that mediates adhesion, b) a single pass transmembrane domain, and c) a highly conserved cytoplasmic domain that interacts with a group of proteins known as catenins to link the cadherins to the actin cytoskeleton (Islam et al., 1996, Banick et al., 1997).

Cadherins mediate calcium-dependent homotypic cell-cell adhesion proteins (Bogenrieder and Herlyn, 2003, Perez-Moreno et al., 2003), and have been shown to be involved in diverse biological processes such as cell adhesion, cell signalling (Perez-Moreno et al., 2003, Yap and Kovacs, 2003), cell recognition, control of cell division, inhibition of apoptosis (Alahari et al., 2002), migration, differentiation, morphogenesis (Banick et al., 1997, Heymann et al., 2002), implantation, tumour and invasion suppressors (Rowlands et al., 2000), and in cancer (Christofori and Semb, 1999). They also play significant roles in embryological development, and maintenance of normal tissue architecture and tumourigenesis (Takeichi, 1991, Takeichi et al., 1993, Ranscht, 1994, Kim et al., 2000b, Rowlands et al., 2000, Puch et al., 2001).

The members of the classical cadherin family show specific spatiotemporal expression patterns both during development, and in the adult tissues. Whereas E-cadherin and P-cadherin are often co-distributed, N-cadherin is mostly found on cell types devoid of E-cadherin and P-cadherin. E-cadherin and N-cadherin have been best characterised and studied. Briefly, the pioneering studies of Takeichi on E-cadherin and N-cadherin expression during mouse embryogenesis established a critical role for cadherins as regulators of morphogenesis (Takeichi et al., 1988, Takeichi, 1995). Both E-cadherin and N-cadherin are considered as prototypic cadherins in epithelial and mesenchymal tissues respectively, and they are involved in the regulation of morphological events such as gastrulation, neurulation, cardiogenesis, and somatogenesis (Peinado et al., 2004).
1.4.2.1 Epithelial (E)-Cadherin

The acquisition of an invasive and metastatic phenotype seems to be dependent on activation or suppression of molecules associated with cell adhesion. One such example, which has been the subject of extensive studies, is the homotypic cellular adhesion molecule, E-cadherin/CDH1. Reduced or absent E-cadherin expression and/or function is considered a hallmark of malignancy in a variety of cancers, including breast (Berx and Van Roy, 2001), lung (Bremnes et al., 2002), skin (McGary et al., 2002), pancreas (Joo et al., 2002b), cervical (Chen et al., 2003), endometrial (Saito et al., 2003), nasopharyngeal (Tsao et al., 2003), and gastric cancers (Joo et al., 2002a). E-cadherin loss has been associated with the induction of EMT, which is instrumental in carcinoma invasion (Christofori and Semb, 1999, Thiery, 2002a).

E-cadherin is a 120-kDa transmembrane glycoprotein (Frixen et al., 1991a, Ahmed et al., 2006) with a large extra-cellular domain comprising five cadherin-motif sub-domains, a single-pass transmembrane segment, and a short conserved cytoplasmic domain. E-cadherin is linked to the cytoskeleton via intracellular ligands termed catenins α, β, and γ, respectively (Dalseg et al., 1993, Behrens, 1994, MacCalman et al., 1998, Berx and Van Roy, 2001). E-cadherin, localised at the zonula adherens junction between epithelial cells (Rajasekaran et al., 1996, Tran et al., 1999), is responsible for calcium-dependent cell-to-cell adhesion (Takeichi, 1991, Takeichi, 1995, Vleminckx and Kemler, 1999), and has been implicated in maintenance of the normal phenotype of epithelial cells in various studies (Takeichi, 1995, Huber et al., 1996, El-Bahrawy and Pignatelli, 1998, Behrens, 1999, Yagi and Takeichi, 2000).


The presence of E-cadherin in normal cells is thought to stabilise epithelia architecture by restraining invasion, thereby regulating the differentiation of benign, non-invasive
phenotypes (MacCalman et al., 1998). Consequently, E-cadherin appears to be down-regulated following de-differentiation in metastatic malignant phenotypes (Siitonen et al., 1996, Charpin et al., 1997, Mori et al., 1998, Bankfalvi et al., 1999).

Decreased E-cadherin is associated with elevated breast cancer invasion. It has been well established that E-cadherin plays a critical role as a potent suppressor of epithelial tumour cell invasion and metastasis (Frixen et al., 1991a, Schipper et al., 1991, Shiozaki et al., 1991, Vleminckx et al., 1991, Rasbridge et al., 1993, Takeichi, 1993, Birchmeier and Behrens, 1994, Berx et al., 1995, Islam et al., 1996, Christofori and Semb, 1999, Pasquet et al., 1999, Cavallaro et al., 2002, Conacci-Sorrell et al., 2002, Wheelock and Johnson, 2003a, Wheelock and Johnson, 2003b), largely because its expression is in some cases inversely correlated with tumour aggressiveness. Tumour cells lose or acquire invasive properties when E-cadherin-mediated adhesion is increased or inhibited, respectively (Behrens et al., 1989, Frixen et al., 1991a, Vleminckx et al., 1991). This is supported by the findings that most tumours derived from epithelia display down-regulated or inactivated E-cadherin, and several research groups have shown that regain of functional E-cadherin suppresses invasion in many tumour cell types (Hirohashi, 1998, Hsu et al., 2000b).

E-cadherin is notably absent or dysfunctional in most of the advanced, un-differentiated, and aggressive breast, and other epithelial, carcinomas (Perl et al., 1998b, Christofori and Semb, 1999). Consistent with this role in breast cancer progression, partial or complete loss of E-cadherin expression has been found to correlate with poor prognosis in breast cancer patients.

In cell culture conditions, several groups have demonstrated that reduced expression of E-cadherin correlates with the invasive phenotype of cancer cells (Behrens et al., 1989, Chen and Obrink, 1991, Frixen et al., 1991a, Mareel et al., 1992b, Mareel et al., 1992c, Takeichi, 1993, Oda et al., 1994, Mareel et al., 1997a, Mareel et al., 1997c). Invasive, fibroblast-like breast carcinoma cells (E-cadherin–negative cells) have shown to be converted to a non-invasive phenotype by transfection with a cDNA encoding E-cadherin (Frixen et al., 1991a, Luo et al., 1999, Kim et al., 2000a). When treated with function
blocking E-cadherin antibodies, the transfected cells returned to an invasive phenotype, thus implicating E-cadherin as an invasion suppressor (Frixen et al., 1991a). Likewise, forced expression of E-cadherin in rat astrocytoma cells suppressed motility (Banick et al., 1997). Furthermore, cells become less invasive following exogenous E-cadherin expression in epithelial tumours from various tissues (Frixen et al., 1991a, Vleminckx et al., 1991), including stomach, colon, head and neck, bladder, prostate, and breast (Schipper et al., 1991, Bringuier et al., 1993, Dorudi et al., 1993b, Mayer et al., 1993b, Oka et al., 1993b, Umbas et al., 1994). Additionally, down-regulation of E-cadherin expression by specific antisense RNA has been shown to render invasiveness to epithelial tumour cells of mouse mammary gland origin (Vleminckx et al., 1991). E-cadherin expression can also be modulated by growth factors and cytokines; for example, transforming growth factor (TGF) induces de-differentiation of the phenotype of normal mammary epithelial cells from epithelial to fibroblastic, which correlates with a reduction in the expression of E-cadherin (Miettinen et al., 1994, El-Hariry et al., 2001a, El-Hariry et al., 2001b).

1.4.2.2 Neuronal (N)-Cadherin

A hallmark of EMT is the switch from E-cadherin to N-cadherin, indicating that the altered expression of E-cadherin to N-cadherin in certain cells may be identified when changing from an epithelial to a migratory capacity. N-cadherin/CDH2, is another member of the cadherin gene family, originally detected in nervous tissue, is also a transmembrane protein which mediates calcium-dependent, homophilic, cell-cell adhesion (Reid and Hemperly, 1990). N-cadherin is associated with a heightened invasive potential in cancer, enhancing tumour cell motility and migration (Hazan et al., 2000, Lafleur et al., 2005a), thus showing an opposite effect in comparison to E-cadherin. It has been suggested that unlike E-cadherin, which functions as an invasion suppressor and is down-regulated in most carcinomas (Mareel et al., 1992c, Mareel et al., 1997c), N-cadherin functions as an invasion promoter, and can be frequently up-regulated during carcinoma progression (Kim et al., 2000b). Also unlike E-cadherin, N-cadherin seems to be required when cells start to migrate, as is the case in the process of gastrulation when epithelial cells ingress the primitive streak.
N-cadherin was originally described as a 130 kD glycoprotein in the chick neural retina that was protected by calcium from proteolysis (Grunwald et al., 1981). In 1984, N-cadherin was later described as a molecule that was localised at the adherens junctions (Volkov et al., 2006). N-cadherin consists of a large extra-cellular domain comprising five cadherin-motif sub-domains, a single pass transmembrane segment, and a short conserved cytoplasmic domain (Wallis et al., 1994).

In embryogenesis, during gastrulation, cells undergo an EMT leading to the expression of N-cadherin, and the down-regulation of E-cadherin in the mesoderm. In the embryo, N-cadherin is known to be expressed at different time points and tissues. For instance, in the adult, N-cadherin is not only expressed in neural tissues, but also in many non-neural tissues such as muscle tissue, retina, endothelial cells, fibroblasts, osteoblasts, mesothelium, myocytes, limb cartilage, oocytes, spermatids, and Sertoli cells (Takeichi et al., 1988, Knudsen, 1990, Knudsen et al., 1990, Wheelock and Knudsen, 1991, Salomon et al., 1992, Soler and Knudsen, 1994, Knudsen et al., 1995).

N-cadherin has been found to be up-regulated in invasive breast tumour cell lines, and promotes tumour-stroma interactions. Over-expression of N-cadherin in breast and squamous tumour cells has been found to stimulate cell motility, invasion, and metastasis (Islam et al., 1996, Nieman et al., 1999b, Hazan et al., 2000). Evidence has indicated that N-cadherin over-expression in breast carcinoma cells (e.g. BT549, MDA-MB-436, HS578T, HS578N, and MCF-7) correlated with invasiveness, which suggested that invasion was potentiated by N-cadherin–mediated interactions between the breast cancer cells and stromal cells, and that this induction appears to be correlated with the decrease in the expression of other cadherins, such as E-cadherin in squamous cell carcinoma lines. Breast cancer cells undergo de-differentiation from an epithelial to mesenchymal morphology as a result of N-cadherin transfection without a loss of E-cadherin expression (Hazan et al., 1997, Hazan et al., 2000).

A study by Islam et al. (1996), described the aberrant expression of N-cadherin in squamous cell carcinoma. In these squamous epithelial cells, N-cadherin expression was reported to have induced changes in morphology to a mesenchymal-scattered
(fibroblastic) phenotype with an EMT, in association with reduced E-cadherin and P-cadherin, rendering the cells more motile, and invasive. Thus, studies show that N-cadherin is important in invasion, and suggest that N-cadherin may play a more direct role in the process, and potentially even promotion, of invasion. Transfection with antisense N-cadherin has resulted in reversion to normal appearing squamous epithelial cell morphology, with increased expression of E-cadherin and P-cadherin. In addition, transfection of a normal squamous epithelial cell line with N-cadherin induced a scattered fibroblastic phenotype (Islam et al., 1996). Indeed, Nieman et al. (1999b) reported that in N-cadherin transfected breast cancer cells, N-cadherin promotes motility and invasion, however the reduction in the expression of E cadherin does not necessarily correlate with either motility or invasion (Nieman et al., 1999b). In another study, Nieman et al. (1999a) showed that N-cadherin expression by BT-20 human breast epithelial cells converted the cells to a motile and invasive phenotype. In this case, increased motility was not accompanied by decreased E-cadherin expression, suggesting that N-cadherin plays a direct role in epithelial cell motility (Nieman et al., 1999a). These findings were confirmed using the MCF7 human breast carcinoma cell line, where Hazan et al. (2000) showed N-cadherin expression increased the metastatic potential of transfected MCF7 cells in nude mice (Hazan et al., 2000). Growth factors such as EGF and HGF are able to induce a switch from E-cadherin to N-cadherin. An example is found in breast carcinoma cells (PMC42) co-expressing E-cadherin and N-cadherin. When treated with EGF, PMC42 cells undergo EMT-like changes, including down-regulation of E-cadherin, and up-regulation of vimentin and N-cadherin (Ackland et al., 2003).

Neo-expression of N-cadherin has also been observed in other tumour types, such as prostate (Yamaji et al., 2002), and intestinal gastric carcinomas (Rosivatz et al., 2002b), suggesting a functional role for this cadherin in cancer progression.

The detection of N-cadherin has been shown to occur in tumour biopsies, which raises the question of whether this protein may be a putative marker for tumour progression. In biopsies, N-cadherin was found in invasive carcinoma, but no correlation could be found with grade (Paredes et al., 2002), or patient survival (Peralta Soler et al., 1999). De novo expression of N-cadherin was found in prostate carcinoma: in one series, 60% was
positive in carcinomas (Yamaji et al., 2002). *In vitro* studies show that the expression of N-cadherin mediates an EMT, possibly improving the physical interaction with the surrounding stromal fibroblasts, and facilitating metastasis (Tran et al., 1999).

De-stabilization of cell-cell adhesion mediated by N-Cadherin, which allows the cells to detach from the primary tumour and associate selectively with the stroma and/or endothelium, could explain the increase in invasion and metastasis associated with N-cadherin expression (Peinado et al., 2004).

In summary, multiple *in vitro* and *in vivo* studies show that N-cadherin expression correlates with phenotypic changes, elevated motility, increased invasion and metastasis. Specifically, these features are characteristic of EMT. These findings emphasize a putative role for N-cadherin in metastasis and invasion.

1.4.3 Integrins

The integrins are a large family of heterodimeric cell surface receptors that play a prominent role in the adhesive interactions between cells and their surrounding extracellular matrix (ECM; (Hynes, 2002, Humphries et al., 2006, Naci et al., 2015)). Integrins mediate cell-to-cell and/or cell-to-ECM adhesion (Hynes, 1992, Giancotti and Mainiero, 1994), providing adhesion for stationary cells, as well as traction during cell movement. In addition, the interaction mediated by integrins promotes signalling pathways that regulate diverse physiological processes including inflammation, immunity, haemostasis, wound healing, angiogenesis, proliferation, migration, cell survival, tissue differentiation, apoptosis and stress response, tumour invasion, and metastasis (Hynes, 1992, Buckley et al., 1998, Ruoslahti, 1999, Kawano et al., 2001, Garrison and Kyprianou, 2004, Hwang and Varner, 2004, Askari et al., 2009, Naci et al., 2015). Integrin expression derangement may be responsible for a number of aberrant cellular activities during tumour onset, progression, and cell dissemination (Rudolph and Cheresh, 1990, Fawcett and Harris, 1992).

Integrins are composed of two non-covalently linked transmembrane subunits, α (150 to 180 kD), and β (~90 kD), except the β4 integrin, which differs in size from the other β
subunits (~ 200-210kD). These form distinct integrin sub-types linking ECM ligands, such as fibronectin, vitronectin, laminin, and collagen (Hynes, 1987, Sonnenberg et al., 1988, Bazzoni et al., 1998) to the intra-cellular actin cytoskeleton at focal adhesion sites, and provide bidirectional transmission of signals across the plasma membrane (Schoenwaelder and Burridge, 1999, Critchley, 2000, Hood and Cheresh, 2002, Hynes, 2002). Each subunit contains a large extra-cellular domain, a single-trans-membrane domain, and a carboxyl-terminal cytoplasmic tail (Gottschalk and Kessler, 2004, Nishiuchi et al., 2006). To date, 18 different integrin-α subunits, and eight different integrin-β subunits (Figure 1.8) have been identified in vertebrates (Hynes, 1992, Humphries, 2000), forming at least 24 different αβ hetero-dimers which act as receptors for connective tissue components, and also bind to counter receptors on other cells (Käpylä et al., 2000), perhaps making the integrins the most structurally and functionally diverse family of cell adhesion molecules.

These integrins differ with respect to which cell surface or ECM ligands they bind, the mechanisms by which their binding activity for ligands is activated, the cytoskeletal components to which they bind, and the types of signalling pathways that they activate within cells. There are two major families of integrins. Those in which the β1-chain combines with any one of nine α-chains, and the αv group, in which the αv associates with either β1-, β3-, β5-, β6-, or β8-subunits (Ahmed et al., 2002).

Although most integrin subunits have been found in a broad variety of different cell types, some have a more restricted distribution. In many cases, integrin expression patterns in vivo differ from those in the corresponding cultured cell types (Albelda and Buck, 1990, Damjanovich et al., 1992).

During development, integrins, as a family, are ubiquitously expressed. Analysis of integrin function has revealed that they contribute to the formation of virtually every cell and tissue studied (Virtanen et al., 1990, De Arcangelis and Georges-Labouesse, 2000, Bökel and Brown, 2002). They are involved in regulating morphogenetic cell movements and migration, gastrulation, neurulation, and histogenesis (Albelda and Buck, 1990).
However, other likely functions of integrins during development appear to be more variable between experiments and model organisms.

For instance, in *C. elegans*, *Drosophila*, mice and humans, integrin function in mediating adhesion between tissue layers via the intervening ECM is well documented. In mammalian cells, integrins function in culture in establishing cell polarity, and providing anchorage-dependent growth (Juliano, 1996, Zegers et al., 2003), however, in genetic analysis of integrin function in *Drosophila* or *C. elegans*, similar functions have not been apparent.

Although there are many integrin family members with different functions that can be related to EMT, their specific role in “driving” EMT during cancer progression is unclear. Co-ordinated changes in integrin expression have been found to occur during embryonic development, and tumour progression (Rosen et al., 1992, Damsky et al., 1994). Many integrins have been reported to show increased and/or have protumorigenic/pro-metastatic functions in various cancer cells, including the laminin-binding integrins α3β1, α6β4 and α6β1 (Chung and Mercurio, 2004, Yang et al., 2008, Mitchell et al., 2010, Stipp, 2010, Subbaram and DiPersio, 2011, Ramovs et al., 2017) as well as α2β1, α1β1, α5β1, and αv integrins (Desgroisellier and Cheresh, 2010, Subbaram and DiPersio, 2011). Altered expression of these receptors is typical in breast tumours (Zutter et al., 1990, d'Ardenne et al., 1991, Koukoulis et al., 1991, Pignatelli et al., 1991, Jones et al., 1992, Natali et al., 1992, Pignatelli et al., 1992, Berdichevsky et al., 1994), indicating that integrins may be critical to the development of malignancy by altering ECM-induced differentiation. Their functional contribution to cancer progression, including breast cancer, and/or their association with EMT has been demonstrated (Subbaram and DiPersio, 2011).

Expression of the α1β1, α2β1, α3β1, α6β1, and α6β4 integrins has been reported for mammary tumour cell lines and tissue sections, and was shown to be associated with tissue disorganisation, loss of polarity, increased tumour aggressiveness, and metastasis (Natali et al., 1992, Berdichevsky et al., 1994, Rosen et al., 1994, Gui et al., 1995, Zutter et al., 1995). In particular, α3β1, α6β1 and α6β4 integrins mediate the migration and
invasion of breast tumour cells and/or promote their survival and proliferation at metastatic sites. Significant evidence implicates α3β1 integrin in breast cancer tumourigenesis and metastasis-associated cell behaviours in vitro and in vivo (Zhou et al., 2014). Also, it has been shown that when cells from human squamous cell lung cancer are inoculated into severe combined immuno-deficient (SCID) mice, increased expression of α1β1 and α2β1 integrin is shown, which correlates with increased metastatic ability (Chen et al., 1994).

During EMT, as epithelial cells de-differentiate into mesenchymal cells, the cells downregulate some epithelial integrins, and activate the expression of other integrins; some of which have key roles in EMT progression (Yilmaz and Christofori, 2009, Lamouille et al., 2014). For example, EMT downregulates the expression of the epithelial α6β4 integrins, that mediate contacts with the basement membrane through epigenetically silencing the gene encoding β4 (Yang et al., 2009). Additionally, epithelial α3β1 integrin, which binds laminin but also associates with E-cadherin, is required for progression through EMT and integrates β-catenin and transforming growth factor-β (TGF-β)–SMAD signalling (Kim et al., 2009). Increased α5β1 integrin expression, during EMT, increases cell adhesion to fibronectin, the expression of which is also activated during EMT and promotes cell migration (Maschler et al., 2005, Mise et al., 2012). The increased expression of α1β1 or α2β1 integrins and their interactions with type I collagen facilitate the disruption of E-cadherin complexes and the nuclear translocation of β-catenin (Koenig et al., 2006).

It is widely appreciated that carcinoma cells exhibiting certain mesenchymal traits are enriched for cancer stem cells (CSCs) and can give rise to tumours with aggressive features (Bierie et al., 2017). Increasing evidence suggests that CSCs represent an important subset of tumour cells that are biologically distinct from the others and have stem cell-like features (Hanahan and Weinberg, 2011), and these are responsible for tumour metastasis, tumour recurrence and chemoresistance (ElShamy and Duhé, 2013, Adorno-Cruz et al., 2015). CSCs have now been shown to exist in different tumour types including breast cancer (Al-Hajj et al., 2003).
Recent findings have identified specific integrins that are enriched in epithelial stem cells and critical for their behaviour, and have demonstrated that integrins participate in the regulation of stem-cell and cancer stem-cell biology and are required for cancer progression and drug resistance (Bierie et al., 2017). These include integrin subunits β1, α6, and β3 (Medema, 2013). Among these, α6 is the most widely observed, enriching for CSCs in breast (Martin and Jiang, 2014), prostate (Hoogland et al., 2014), squamous cell carcinoma (Schober and Fuchs, 2011), and colorectal (Haraguchi et al., 2013) cancers. Integrin β1 is highly expressed in normal stem cells and regulates their biology in various organs. Increasing evidence demonstrates that integrin β1 maintains the stem cell niche, preserves a stable stem cell population, and controls the balance between stem cell renewal and differentiation in mammary gland (Taddei et al., 2008). Recent studies have also characterized integrin β4 as a CSC marker in lung cancer, where it is involved in self-renewal, tumour propagation, and chemotherapy resistance (Zheng et al., 2013). In particular, the utility of integrin-β4 in segregating these cells into distinct sub-populations with differing tumour-initiating abilities and pathological behaviours has been demonstrated (Bierie et al., 2017). Integrins αv (CD51) and β3 (CD61) were also recently shown to define partial-EMT subpopulations that had differential metastatic capacity in a squamous cell carcinoma model (Pastushenko et al., 2018).

Integrin-mediated adhesion to ECM components, as well as integrin expression, has been shown to alter the expression of several gene products through a variety of transcriptional, translational, and post-translational mechanisms. Plantefaber and Hynes (1989) were the first to describe an altered integrin expression pattern during malignant transformation (Plantefaber and Hynes, 1989). Since then, several studies have demonstrated that acquisition of an invasive and metastatic phenotype is accompanied by changes in integrin-dependent cell adhesion.
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Figure 1.6 Diagrammatic Representation of the Integrin Superfamily (Taken from Hynes, 2002).

18α and eight β subunits have been identified combining to form at least 24 heterodimeric integrins which can be considered in several subfamilies based on ligand specificity and, in the case of β2 and β7 integrins, restricted expression on leucocytes. The different subunits show selectivity in their binding partners: for example the αv subunit can pair with multiple β subunits (β1, β3, β5, β6 and β8) but the β6 subunit can only bind with αv.

Changes in expression or modification of the integrins have been noted during malignant transformation in various cells (Albelda et al., 1990, Damjanovich et al., 1992). However, few studies have reported a clear correlation between the degree of transformation or malignancy, and the pattern of integrin expression in melanomas (Albelda et al., 1990). In a study by Fujita and co-workers (1992), the authors showed that β1-integrins played an essential role in the invasion and metastasis of human carcinoma cells (Fujita et al., 1992). These findings are supported by other research suggesting a correlation between increased β1-integrin expression, and metastasis in a range of carcinomas, including melanoma (Hangan et al., 1997, Tsuji et al., 2002), breast (Tawil et al., 1996), colon (Andrews et al., 2001), and bladder cancer (Saito et al., 1996).
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1.4.3.1 α2β1 Integrin

One of the most studied integrins with respect to both structure and function is the α2β1 (VLA-2) integrin, a founding member of the integrin family. The α2β1 integrin, one of the first integrins to be identified and characterised, is universally expressed in numerous types of tissues (Zutter and Santoro, 1990). These include the epithelial cells, endothelial cells, fibroblasts, and cells of hematopoietic origin (Wu and Santoro, 1994). Immunocytochemical staining show α2β1 integrin localised at cell-cell contacts in endothelial cells, and some epithelial cells (Larjava et al., 1990, Lampugnani et al., 1991), suggesting that the roles of this integrin may also be to facilitate cell attachment.

The α2β1 integrin was originally reported to be a collagen receptor (Staatz et al., 1989). This integrin also possesses the ability to function as a dual collagen/laminin receptor on some cell types such as epithelial, fibroblast, endothelial cells, and on immune cells, and it is found on many types of cancer cells (Madamanchi et al., 2014). For example, in fibroblasts and platelets, α2β1 integrin binds to collagen, but not to laminin (Elices and Hemler, 1989, Kirchhofer et al., 1990). However, in endothelial cells, it serves as a receptor for both collagen and laminin (Languino et al., 1989, Kirchhofer et al., 1990). Numerous studies have shown evidence for the α2β1 integrin in a range of biological, and patho-biological functions (Zutter et al., 1998, Santoro, 1999, Mercurio, 2002, Leabu et al., 2005). α2β1 integrin is widely expressed on epithelial cells, and its levels are increased in several carcinoma cells from the epithelial origin (Gardner, 2014, Madamanchi et al., 2014). Early studies have shown that expression of α2β1 integrin can modulate cell adhesion, spreading, post-extravasation migration, collagen gene transcription, matrix metalloproteinase production, and collagen matrix contraction (Chan et al., 1991, Schiro et al., 1991, Chan et al., 1992, Langholz et al., 1995, Hangan et al., 1996, Ivaska et al., 1999a, Ivaska et al., 1999b, Lochter et al., 1999). Although α2β1 integrin is normally expressed in a regulated manner in normal development, its expression has often shown to be altered in malignancies (Pignatelli et al., 1990, Bonkhoff et al., 1993, Nigam et al., 1993).

Growing evidence indicates that α2β1 integrin can be a key pathway in cancer pathogenesis (Luo et al., 2007, Heino et al., 2009, Heino, 2014). In mammary epithelial
cells, α2β1 integrin mediates collagen-induced morphogenesis (Berdichevsky et al., 1992), suggesting that in the mammary gland, α2β1 integrin appears to play a vital role in development and maintenance of regulatory tissue architecture. In breast cancer, expression of α2β1 integrin is numerously reported to be down-regulated in contrast with the normal mammary gland (Zutter et al., 1990, Pignatelli et al., 1992, Arihiro et al., 1993, Mecktersheimer et al., 1993). Pignatelli and Co-workers (1991) found weak or absent expression of α2β1 integrin in the majority of invasive breast carcinomas compared to the normal breast epithelium and pre-invasive carcinomas (Pignatelli et al., 1991). Expression of the α2β1 integrin correlates highly with differentiation and ER expression (Zutter et al., 1990, Pignatelli et al., 1992, Mecktersheimer et al., 1993). In contrast to these findings, a study by D’Ardenne and colleagues (1991), found that the α2 subunit was, in fact, not down-regulated (d’Ardenne et al., 1991). Furthermore, α2β1 is highly expressed on the epithelium of ductules of normal breast tissue and benign fibroadenomas (Koukoulis et al., 1993, Howlett et al., 1995).

The metastatic potential of melanoma, osteosarcoma, rhabdomyosarcoma, and lung tumours has been shown to correlate with increases in α2β1 expression, whereas decreases in α2β1 expression has been associated with epithelial malignant transformation (Dedhar and Saulnier, 1990, Chan et al., 1991, Mortarini et al., 1991, Danen et al., 1993, Chen et al., 1994, Santala et al., 1994, Zutter et al., 1995).

Overall, these findings all support that α2β1 expression is associated with the metastatic activities of tumour cells. Interestingly, there is both a direct correlation (Dedhar and Saulnier, 1990, Chan et al., 1991, Mortarini et al., 1991, Danen et al., 1993, Chen et al., 1994, Santala et al., 1994), and an inverse correlation (Pignatelli et al., 1990, Zutter and Santoro, 1990, Pignatelli et al., 1991, Zutter et al., 1995) of α2β1 expression with tumour metastasis.

Since integrins have no enzymatic activity of their own, accessory proteins are required for downstream cytoplasmic signalling (Schaller et al., 1995), one such example being Integrin-Linked Kinase (ILK).
1.4.4 Integrin-Linked Kinase (ILK)

ILK, ubiquitously expressed in mammalian cells, was first isolated and described by Hannigan et al. (1996) in a yeast two-hybrid genetic screen for β1 integrin cytoplasmic tail-binding proteins (Hannigan et al., 1996a). ILK is a 59kDa intracellular serine-threonine kinase that interacts with β1, β2, and β3 integrin cytoplasmic tail-binding proteins (Hannigan et al., 1996a, Li et al., 1997, Attwell et al., 2003, Persad and Dedhar, 2003, Nho et al., 2005a), and numerous cytoskeleton-associated proteins (Dedhar, 1999, Dedhar et al., 1999, Dedhar, 2000a, Wu, 2001, Wu and Dedhar, 2001).

ILK is structurally comprised of three distinct domains. These are a) the N-terminus, which contains four ankyrin repeats that have been demonstrated to interact with the LIM (Lin-11, Isl-1 and Mec-3)-only adapter protein PINCH (Li et al., 1999, Tu et al., 1999); b) a central pleckstrin homology (PH)-like domain, that partially overlaps with the N-terminal region of the kinase domain (Hannigan et al., 1996a), and is reported to bind to phosphatidylinositol 3,4,5-triphosphate (PIP3) (Delcommenne et al., 1998); and c), the carboxyl terminus, which contains a protein kinase catalytic domain and the binding site for β1 integrin (Hannigan et al., 1996a).

1.4.4.1 ILK Interactions

It has been suggested that ILK mediates protein–protein interactions, and a number of proteins that interact with ILK have been identified (Wu and Dedhar, 2001). ILK has been shown to act downstream, and independently of the phosphatidylinositol-3-kinase (PI3K) pathway to phosphorylate target proteins (Figure 1.9). These include integrins (β1/β3), Akt/protein kinase B (PKB), glycogen synthase kinase-3β (GSK-3β) (Attwell et al., 2003), the adaptor protein paxillin, the LIM domain proteins PINCH 1 and 2, the actin-binding proteins CH-ILKBP/actopaxin/α-πarvin and b-parvin/affixin (Wu and Dedhar, 2001), and the FERM domain protein UNC-112/mig-2 (Mackinnon et al., 2002). In addition, ILK associates with a serine/threonine protein phosphatase of the PP2C family, referred to as ILKAP (ILK-associated phosphatase; (Leung-Hagesteijn et al., 2001)). The existence of multiple diverse ILK partners suggests that many regulatory
mechanisms converge at the level of ILK to mediate integrin signal transduction (Vouret-Craviari et al., 2004).

ILK is capable of phosphorylating the cytoplasmic tail of the β1 integrin subunit (Hannigan et al., 1996b). ILK is also capable of undergoing autophosphorylation, and synthetic peptides imitating the β1 integrin subunit cytoplasmic domain are able to selectively inhibit autophosphorylation of ILK (Hannigan et al., 1996b, Mulrooney et al., 2000).

1.4.4.2 ILK Functions
Numerous publications have described the role of ILK in many tissues in cancer biology (Hannigan et al., 2005), and in several developmental systems. Since its identification, ILK has been found to be crucial in the regulation of many integrin-mediated and biological processes, resulting in cytoskeletal reorganization, and changes in gene expression that affect adhesion and migration, proliferation, differentiation, and survival of cells (Giancotti and Ruoslahti, 1999, Huang et al., 2000, Ishii et al., 2001, Wu and Dedhar, 2001, Brakebusch et al., 2002, Sakai et al., 2003, Vespa et al., 2003, Barker et al., 2005, Vespa et al., 2005, Belvindrah et al., 2006, Boulter et al., 2006, Friedrich et al., 2006, Mills et al., 2006) by transducing signals from ECM components and growth factors to downstream signalling components.
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Figure 1.7 Schematic Representation of the Domain Structure of ILK and Interactions Involving ILK and Other Related Proteins and the Downstream Signals these Elicit.

This figure depicts the domain structure of ILK, and the interaction of ILK with various proteins, many of which, including ILK, are found at sites of integrin connections to the actin cytoskeleton.

1) ILK binds to PINCH, comprising five LIM domains.
2) This ILK-PINCH binding is mediated by the N-terminal ankyrin (ANK) repeat domain of ILK and the second Zn finger located within the N-terminal- most LIM domain (LIM1) of PINCH.
3) ILK also interacts with CH-ILKBP, an actin-binding focal adhesion protein containing two calponin homology (CH) domains. Human CH-ILKBP is structurally closely related to rat actopaxin, a paxillin- and actin binding protein.
4) In addition, ILK can bind to the paxillin LD1 motif. The interaction between ILK and CH-ILKBP is mediated by the C-terminal domain of ILK, and the CH2 domain of CH-ILKBP. Simultaneous interactions mediated by the two separate (the N- and C-terminal) domains of ILK result in the formation of a stable PINCH–ILK–CH-ILKBP ternary complex in cells. The formation of this complex facilitates the localisation of PINCH, ILK and CHILKBP to cell-ECM contact sites, at which they provide a crucial physical connection between transmembrane receptors such as integrins, and the actin filaments.

(Modified from (Wu, 2001))
ILK has also been crucial for embryonic development (Hynes, 2002, Grashoff et al., 2003, Sakai et al., 2003, Terpstra et al., 2003), brain development (Niewmierzycka et al., 2005, Mills et al., 2006), and tumour angiogenesis (Friedrich et al., 2004b, Tan et al., 2004, Koul et al., 2005b, Watanabe et al., 2005). ILK dysregulation has also been implicated in the development of several chronic glomerular diseases (Guo et al., 2001, Kagami et al., 2006).

ILK is implicated in the regulation of anchorage-dependent cell growth and survival, cell-cycle progression (Wu and Dedhar, 2001, Persad and Dedhar, 2003), EMT, invasion and migration (Oloumi et al., 2004), cell motility and contraction (Deng et al., 2001), vascular development (Friedrich et al., 2004a, Vouret-Craviari et al., 2004), and tumour angiogenesis (Kaneko et al., 2004, Tan et al., 2004). The expression and activity of ILK are increased in a range of cancers, and small-molecule inhibitors of ILK activity have been identified (Persad et al., 2001) and shown to inhibit tumour growth (Tan et al., 2004), invasion and angiogenesis (Tan et al., 2004).

Numerous studies have shown that ILK is aberrantly overexpressed and activated in diversified types of human malignancies (Li et al., 2010, Schaeffer et al., 2010, Watzka et al., 2010, Yu et al., 2011, Zhao et al., 2012, Li et al., 2013b). Furthermore, ILK has been reported to promote cancer cell migration and invasion by inducing the EMT process (Becker-Santos et al., 2012, Chen et al., 2013, Serrano et al., 2013).

1.4.4.3 ILK and EMT

It has been demonstrated that ILK can induce a complete EMT in various epithelial cell lines, and thus be involved in the initiation of EMT in vivo, and the maintenance of the mesenchymal phenotype and disease progression (Wu and Dedhar, 2001, Becker-Santos et al., 2012, Chen et al., 2013, Serrano et al., 2013). ILK can modulate the expression of not only E-cadherin, but also other epithelial markers such as cytokeratin 18 (Somasiri et al., 2001) and MUC1 (Guaita et al., 2002), as well as mesenchymal markers such as LEF1 (Novak et al., 1998b) and vimentin (Novak et al., 1998b, Guaita et al., 2002). Therefore, ILK is able to initiate an EMT. Gain and loss of function strategies have shown that over-expression, and/or constitutive activation of ILK results in oncogenic
transformation and progression to invasive and metastatic phenotypes (Persad and Dedhar, 2003).

1.5 EXPERIMENTAL MODELS OF EMT

The role of EMT in breast cancer has been demonstrated via numerous in vitro studies in normal and malignant mammary epithelial cells, and via in vivo studies using mouse models of breast cancer (Blick et al., 2008, Trimboli et al., 2008). The plasticity of EMT program during tumour metastasis is difficult to address using tumour cell lines, as they are usually fixed in an epithelial or mesenchymal state, partially due to in vitro culture conditions (Prat et al., 2010, Francia et al., 2011), and significant importance of the hybrid state / partial-EMT is emerging (Pastushenko et al., 2018, Thompson and Nagaraj, 2018).

Syngeneic and xenogeneic mouse models of breast cancer cell lines have been extensively used to establish a relationship between EMT and organ-specific metastasis by several rounds of transplantation/metastasis formation and the selection of metastatic cell lines in vivo, by interfering with critical mediators of EMT, including EMT-inducing growth factor signaling or transcription factor activities (Thiery et al., 2009).

One such model is the 4T1 mouse mammary carcinoma model (originally derived from a spontaneous mouse mammary tumour of a BALB/C mouse), which is a transplantable tumour cell line that is highly tumorigenic and invasive (Tao et al., 2008, Bailey-Downs et al., 2014). Unlike most breast cancer models, the 4T1 mouse mammary tumour cell line model can spontaneously metastasise efficiently from the primary tumour in the mammary gland to multiple distant sites including lymph nodes, blood, liver, lung, brain, and bone (Pulaski and Ostrand-Rosenberg, 1998, Pulaski and Ostrand-Rosenberg, 2000, Yoneda et al., 2000, Eckhardt et al., 2005, Bailey-Downs et al., 2014, Fischer et al., 2015b, Zhao et al., 2016, Fischer et al., 2017). The behavior of this tumour line reflects the sequence of multistep metastasis progression. The 4T1 tumour is a suitable experimental animal model for human mammary cancer, as the tumour cells are easily transplanted into the mammary gland so that the primary tumour grows in the
anatomically correct site (Pulaski and Ostrand-Rosenberg, 2000). Also, as in human breast cancer, 4T1 metastatic disease develops spontaneously from the primary tumour, and the progressive spread of 4T1 metastases to the draining lymph nodes and other organs is very similar to that of human mammary cancer. A study by Lou and coworkers (2008) analysed the clinically relevant mouse 4T1 breast cancer model to determine whether EMT can be linked to metastatic ability. They found that the highly metastatic 4T1 cells appear very epithelial, and express robust amounts of E-cadherin and catenin, suggesting that these cells maintain expression of epithelial proteins (Lou et al., 2008). E-cadherin and ZO-1 are localized to cell–cell junctions, and these cells do not express N-cadherin and express very little vimentin. Thus, these cells would be defined as being largely “epithelial. However, the 4T1 cells are highly migratory and invasive in vitro, clearly showing that EMT is not a prerequisite for invasiveness. The findings are in contrast with those of Yang et al. (2004), in which differential gene expression between 4T1 cells and the variant 67NR cells resulted in the identification of Twist, a transcriptional repressor of E-cadherin (Yang et al., 2004). Twist was found to induce EMT and promote a metastatic phenotype. The reasons for the differences in results in study by Lou et al. (2008), as compared to those reported by Yang et al. (2004) are not clear, but could be attributed to clonal differences between cell lines.

Another model suitable for studying EMT, is the MMTV-PyMT transgenic model of mammary tumour formation, in which MMTV-LTR is used to drive the expression of mammary gland specific polyomavirus middle T-antigen, leading to a rapid development of highly metastatic tumours (Guy et al., 1992). The MMTV-PyMT transgenic model emulates the multi-step progression of human breast cancers beginning from hyperplastic lesions to high-grade carcinomas that spontaneously metastasise to the lungs (Lin et al., 2003, Ye et al., 2015, Ye et al., 2017). It is the most commonly used model for the study of mammary tumour progression and metastasis due to its close similarity to human breast cancer. This is exemplified by the fact that in these mice, a gradual loss of steroid hormone receptors (estrogen and progesterone) and β1-integrin is associated with overexpression of ErbB2 and cyclin D1 in late-stage metastatic cancer (Maglione et al., 2001). The MMTV-PyMT mouse model of breast cancer is furthermore characterized by
short latency, high penetrance, and a high incidence of lung metastasis occurring independently of pregnancy and with a reproducible kinetics of progression (Fantozzi and Christofori, 2006).

The human mammary epithelial cell (HMLE) model, which is described in several studies (Mani et al., 2008, Taube et al., 2010), is also another useful model the study of EMT. HMLE cells line the ducts and lobules of the mammary gland, and most of the breast cancers arise from this epithelial cell layer. One disadvantage of using these HMLE cells is that they have a limited life span which makes it difficult to study them in vitro for most purposes (Berthon et al., 1992, Joshi et al., 2017). These cells undergo senescence eventually thereby suppressing tumourigenesis and also promoting tissue repair (Joshi et al., 2017). HMLE immortalization (Rao et al., 2006) is one of the key steps in malignant transformation and involves the alterations of a number of genes that are involved in the checkpoints of the cell cycle (Dimri et al., 2005). Induction of an EMT in HMLEs results in the acquisition of mesenchymal traits, the expression of stem-cell markers, and an increased ability to form mammospheres, a property associated with mammary epithelial stem cells (Mani et al., 2008). Transcription factors (TFs), such as Snail and Twist, as well as TGF-β1, all inducers of EMT, are capable of inducing an EMT in the HMLE line. Overexpression of each of the above EMT inducers up-regulates a subset of other EMT-inducing TFs, with Twist, Zeb1, Zeb2, TGF-β1, and FOXC2 being referred to as the ‘core interactome’ (Taube et al., 2010).

Most of the existing in vivo models for preclinical assays of anticancer drugs are based on a limited number of cell lines previously isolated from human tumours and selected through culture before their implantation into immunodeficient mice. Cancer cell xenografts have been extensively used and have yielded highly valuable and informative data, and remain very important and valuable models for molecular genetics and biochemistry studies, due to their well-characterized features, the possibility of inter-laboratory comparison, and reproducibility (Kao et al., 2009). However, as these cancer cell lines only partially recapitulate the biology of the original tumours, they do not reflect breast cancer heterogeneity, and their predictive value may be weak. For these
reasons, there has recently been renewed interest in preclinical assays based on tumour xenografts acquired by engraftment of human tumour samples directly transplanted into immunocompromised mice, such as athymic nude or severe combined immunodeficient (SCID) mice, and more recently “NSG” mice. Contrasting with cell line-derived xenografts, such tumour xenografts may provide a more accurate reflection of human tumour biological characteristics (i.e. maintain the cell differentiation and morphology, the architecture, and molecular signatures of the original patient tumours) (Marangoni and Poupon, 2014). Numerous studies have demonstrated that during sequential passage, patient-derived xenograft (PDX) models retained their similarity to the corresponding original donor tumours in histologic presentation and biologic behaviour, such as protein expression, tumour biomarker status and genomic and genetic status (Visonneau et al., 1997, Beckhove et al., 2003, Wang et al., 2005, Cutz et al., 2006, Huynh et al., 2006, Rubio-Viqueira et al., 2006, Marangoni et al., 2007, Whiteford et al., 2007, Fichtner et al., 2008, DeRose et al., 2011, Zhang et al., 2013a, McAuliffe et al., 2015). Furthermore, breast cancer PDXs produce tumour pathology, growth and metastasis of breast cancer (DeRose et al., 2011), and also maintain the genetic characteristics of their original tumours (Petrillo et al., 2012, Reyal et al., 2012, Zhang et al., 2013a). Together, these studies demonstrate the power of PDX models for cancer research.

1.5.1 In Vitro Model of EMT: The PMC42 Cell System

The human breast PMC42 cell line (Whitehead et al., 1983a) is a unique human breast cancer cell line originally established from a pleural effusion of a female patient with disseminated breast cancer (Whitehead et al., 1983a). These cells are of particular significance due to the fact that they are thought to represent a mammary stem cell, and have proved to be a useful tool for defining EMT in human breast carcinoma (Ackland et al., 2003).

Original cytological studies have shown that the PMC42 cell line exhibits eight morphological subtypes in vitro, as defined by phase contrast and electron microscopy (Whitehead et al., 1983a, Whitehead et al., 1983b, Whitehead et al., 1984, Monaghan et al., 1985). In addition, these cells grow both as a monolayer, and as floating cords in suspension (Whitehead et al., 1983a, Whitehead et al., 1983b). When grown in specific
three-dimensional collagen or matrigel matrices, the PMC42 cells may also form breast-like structures, including large secretory vacuoles, as well as features of myo-epithelial cells, including parallel actin cables (Whitehead et al., 1983a, Whitehead et al., 1983b). They possess mitogenic responses to estrogen, progesterone (Whitehead et al., 1984), as well as responding to EGF with increased cloning efficiency in semisolid agar (Whitehead et al., 1983b). EGF normally plays a role in regulating signalling pathways in cells through its interaction with tyrosine kinase receptors, which leads to the induction of cell processes such as migration, apoptosis, and proliferation. EGF has been found to stimulate increased adherence of cultured PMC42 cell-organoid structures, together with increased membrane expression of the cell-adhesive proteins laminin and fibronectin (Keskanokwong et al., 2007). In a previous study by Ackland et al. (2001), the authors showed that PMC42 cells, which normally grow as a monolayer culture, can be induced to form organoids, reminiscent of secretory alveoli found in the lactating breast, which express β-casein, a milk specific protein, in the presence of lactogenic hormones and interactions with the extracellular matrix (Ackland et al., 2001).

Subsequently, PMC42-LA, a stable variant of the parental PMC42 human breast cancer cell line (PMC42-ET), was characterised by Ackland and co-workers (2003). PMC42-LA cells exhibit a predominantly epithelial phenotype when cultured on plastic, where the cells assemble into pavement epithelial sheets in which E-cadherin and beta-catenin are localised at cell–cell junctions. Approximately 10% of the PMC42-LA cells express vimentin, while also abundantly expressing cytokeratins, compared with 100% vimentin expression in the PMC42-ET (Ackland et al., 2003). These features are characteristic of epithelial cells. When stimulated with EGF, both the PMC42-ET and PMC42-LA cells display enhanced migratory ability, however, a dramatic morphological transition is only seen in the parental line, where the cells become spindle-shaped and elongated (mesenchymal-like). The PMC42-LA cells, on the other hand, transform to a ‘relaxed’ epithelial phenotype, and acquire vimentin. Thus phenotypically, PMC42-LA cells may be induced to become elongated and less attached upon stimulation with EGF, indicating that cells lose their attached epithelial potential, and develop a more migratory capacity. Furthermore, since EGF appears to push these cells to changes in morphology, factors
involved in this growth-factor pathway may be important aspects of EMT in PMC42-LA cells.

Stimulation of the PMC42-LA cells with EGF in two-dimensional culture leads to EMT-like changes at a molecular level, including down regulation of epithelial markers, i.e. E-cadherin, and elevated expression of mesenchymal proteins i.e. vimentin. Vimentin expression is seen in virtually all cells, and this increase is abrogated by treatment of cells with an EGF receptor antagonist. Ackland and colleagues demonstrated that in response to EGF, beta catenin persisted at the cell periphery, even though E-cadherin staining at cell-cell junctions disappeared. Further analyses also showed N-cadherin at the cell-cell junctions of untreated cells, and that expression was increased after EGF treatment (Ackland et al., 2003). Due to these observations, the PMC42 breast cancer cell line was defined as a model of EGF-induced EMT (Ackland et al., 2003). This model system is currently in use in the Thompson laboratory (St. Vincent’s Institute, Melbourne, Australia) to determine factors that regulate the EMT phenotype, and reveal molecules that drive this phenomenon in human breast cancer.

These observations demonstrate that the PMC42 cells can behave like normal breast cells and can be induced to change in morphology, making them an exemplary model system of EMT in breast cancer.

1.5.2 In Vivo Model of EMT: EDW-01 Xenograft System

The EDW-01 xenograft is a novel human breast cancer xenograft model in SCID mice, derived from the bone metastasis (Lafleur et al., 2005a) of a 44 year old woman who succumbed to her disease one year later. She had been diagnosed with invasive breast cancer 10 years earlier. Xenografts have the advantage that their biology may more accurately reflect the human system. The bone metastasis and EDW-01 xenograft share glandular histology (adenocarcinoma), and although both are ER-positive, EDW-01 lacks PR. The EDW-01 xenograft showed focal vimentin expression, which increased in area through five subsequent serial passages in SCID mice. The EDW-01 xenograft system provides a novel opportunity to assess whether vimentin expression in human breast
cancer results from EMT, and to assess the molecular and cellular context of vimentin expression.

1.6 HYPOTHESIS

The central hypothesis of this thesis is that integrins, and ILK, contribute to the mesenchymal phenotype, and are functionally required for the transition from an epithelial to a mesenchymal state. Herein is described a sequence of studies to assess the regulation of a range of integrins, and ILK, in two human breast cancer model systems: the \textit{in vitro} PMC42 cell system, and the \textit{in vivo} EDW-01 xenograft system. The functional role of the integrin/ILK axis in the mesenchymal state, and in epithelial-to-mesenchymal transition is explored.

1.7 AIMS AND SCOPE OF THESIS

The research work performed and described in this thesis has three major components.

1) To perform expression time-course analysis of the PMC42 EMT model system in order to determine the potential hierarchy of factors that regulate the EMT phenotype.

2) To characterise the EDW-01 xenograft model system with a focus on expression patterns of integrins and ILK in order to determine whether they correlate with the more progressive nature and mesenchymal properties of late passage tumours.

3) To perform functional studies on the integrins and ILK in the PMC42 EMT model system by employing siRNA abrogation approaches to reduce expression of the candidate effectors and determine them as being either functional “drivers” or “passengers” of EMT.
CHAPTER 2: MATERIALS AND METHODS
This chapter describes the experimental materials and methods used throughout the course of this work. Unless otherwise stated, all reagents were of analytical grade. Solutions were autoclaved when necessary, at 121°C for 20-30 min.

2.1 MATERIALS AND REAGENTS:

2.1.1 General Molecular and Cell Biology Reagents

The following materials and reagents were used throughout the course of this work for the different experimental techniques used (Table 2.1).

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Bovine serum albumin (BSA)</td>
<td>Sigma-Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>- Crystal Violet- powder form</td>
<td></td>
</tr>
<tr>
<td>- Polyoxyethylene-Sorbitan-Mono Laurate (Tween-20)</td>
<td></td>
</tr>
<tr>
<td>- Reagents for Phosphate Buffered Saline (PBS)</td>
<td></td>
</tr>
<tr>
<td>- Reagents for Tris-Buffered Saline (TBS)</td>
<td></td>
</tr>
<tr>
<td>- Aerosol barrier pipette tips</td>
<td>Axygen Scientific Inc (Union City, CA, USA)</td>
</tr>
<tr>
<td>- Plastic pipette tips</td>
<td></td>
</tr>
<tr>
<td>- Microtubes</td>
<td></td>
</tr>
</tbody>
</table>

2.1.2 Tissue Culture Materials and Reagents

The following materials and reagents were used throughout the course of this work for tissue culture-related experiments (Table 2.2).
<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Rosewell Park Memorial Institute Medium 1640 (RPMI)</td>
<td>Invitrogen Australia (Mount Waverley, Vic, Australia).</td>
</tr>
<tr>
<td>- Foetal Bovine Serum (FBS 216)</td>
<td></td>
</tr>
<tr>
<td>- Dimethyl sulfoxide (DMSO)</td>
<td>Sigma-Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>- Reagents for Phosphate Buffered Saline (PBS)</td>
<td></td>
</tr>
<tr>
<td>- Reagents for Versene (ethylene diaminetetra-acetic acid)</td>
<td></td>
</tr>
<tr>
<td>- Trypsin</td>
<td>CSL (Melbourne, Vic, Australia)</td>
</tr>
<tr>
<td>- Tissue Culture Flasks</td>
<td>Greiner Bio-One (Germany)</td>
</tr>
<tr>
<td>- Tissue Culture Plates</td>
<td></td>
</tr>
<tr>
<td>- Filtrona Tubes</td>
<td></td>
</tr>
<tr>
<td>- Petri Dishes</td>
<td>Becton Dickinson Labware (Franklin Lakes, NJ, USA)</td>
</tr>
<tr>
<td>- 50ml Falcon Tubes</td>
<td></td>
</tr>
<tr>
<td>- Disposable Pipettes</td>
<td></td>
</tr>
<tr>
<td>- Syringes</td>
<td></td>
</tr>
<tr>
<td>- Needles</td>
<td></td>
</tr>
<tr>
<td>- Minisart Filter Units</td>
<td>Sartorius (Goettingen, Germany)</td>
</tr>
<tr>
<td>- Cryotubes</td>
<td>Nunc (Rochester, NY, USA)</td>
</tr>
<tr>
<td>- Plastic Cell Scrapers</td>
<td>Costar (Corning Inc. Acton, MA, USA)</td>
</tr>
<tr>
<td>- Haemocytometer</td>
<td>Baxter Healthcare Corporation (NSW, Australia)</td>
</tr>
<tr>
<td>- DharmaFECT 4 transfection reagent</td>
<td>Dharmacon (GE Healthcare, Australia)</td>
</tr>
<tr>
<td>- siRNAs</td>
<td>Proligo Sigma-Aldrich (St. Louis, MO, USA)</td>
</tr>
</tbody>
</table>
2.1.3 Growth Factors and Extracellular Matrices
The following growth factors and extracellular matrices were used throughout the course of this work (Table 2.3).

Table 2.3 Growth Factors and Extracellular Matrices Reagents

<table>
<thead>
<tr>
<th>Item</th>
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</tr>
</thead>
<tbody>
<tr>
<td>- Recombinant Human EGF</td>
<td>BD Biosciences, (Bedford, MA, USA)</td>
</tr>
<tr>
<td>- Collagen I</td>
<td></td>
</tr>
<tr>
<td>- Collagen IV</td>
<td></td>
</tr>
<tr>
<td>- Laminin (from Engelbreth-Holm Swarm murine sarcoma)</td>
<td>Sigma-Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>- Fibronectin (from bovine plasma)</td>
<td></td>
</tr>
</tbody>
</table>

2.1.4 RNA Purification and Q-RT-PCR Materials and Reagents
The following materials and reagents were used throughout the course of this work for RNA purification and Q-RT-PCR experiments (Table 2.4). Primers (oligonucleotides) were designed using Primer-Blast software available free through the National Centre for Biotechnology Information (NCBI) web site.

Table 2.4 RNA Purification and Q-RT-PCR/MT-PCR Materials and Reagents

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Qiagen RNeasy Mini prep Kit</td>
<td>QIAGEN Australia (Doncaster, Vic, Australia)</td>
</tr>
<tr>
<td>- β-mercaptoethanol (β-ME)</td>
<td>Bio-Rad Laboratories (Hercules, CA, USA)</td>
</tr>
<tr>
<td>- Gene Specific Primers</td>
<td>GeneWorks (Adelaide, SA, Australia) and Proligo (Lismore, NSW, Australia)</td>
</tr>
<tr>
<td>- SYBR Green I dye</td>
<td>Molecular Probes (Oregon USA)</td>
</tr>
<tr>
<td>- AmpliTaq Gold polymerase.</td>
<td>Applied Biosystems (Foster City, CA, USA).</td>
</tr>
<tr>
<td>- Q-RT-PCR Plates (MicroAmp, Optical 96-well Reaction Plates)</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 2: Materials and Methods

- Ultra Clear Cap Strips
  ABgene Biotechnologies Ltd. (Epson, United Kingdom).
- Thermoscript™ Reverse Transcriptase Kit
  Invitrogen (CA, USA)
- SUPERaseIn
  Ambion (Austin, TX, USA)
- MT-PCR MasterMix Kit
  Corbett Research, Australia

2.1.5 Oligonucleotides

The following oligonucleotides were used throughout the course of this work for Q-RT-PCR.

Table 2.5 Oligonucleotides used for Q-RT-PCR

<table>
<thead>
<tr>
<th>Oligonucleotide Name</th>
<th>Species</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ Hs L32</td>
<td>Human</td>
<td>CAGGGTTTCGTAAGAGATTCAAGGG</td>
</tr>
<tr>
<td>3’ Hs L32</td>
<td>Human</td>
<td>CTTGGAGGAAACATTGTGAGCGATC</td>
</tr>
<tr>
<td>Hs L32 RT</td>
<td>Human</td>
<td>CAGAAAACGTGCACATGAGCTGC</td>
</tr>
<tr>
<td>5’ Mm L32</td>
<td>Mouse</td>
<td>CAGGGTGCGGAAGAAGATTTCAAGGG</td>
</tr>
<tr>
<td>3’ Mm L32</td>
<td>Mouse</td>
<td>CTTAGAGGACACATGTGAGCAATC</td>
</tr>
<tr>
<td>Mm L32 RT</td>
<td>Mouse</td>
<td>GCTGTGCTGCTCTTTCTACAATGGCTTTT</td>
</tr>
<tr>
<td>5’ Hs Vimentin</td>
<td>Human</td>
<td>CAGGCGATATATTACCACGCAAGAA</td>
</tr>
<tr>
<td>3’ Hs Vimentin</td>
<td>Human</td>
<td>CTTGATGGAGTGTCGGTTTAAAGAA</td>
</tr>
<tr>
<td>Hs Vimentin RT</td>
<td>Human</td>
<td>CTTGGAGAGGTGTCGGTTTAAAGAA</td>
</tr>
<tr>
<td>5’ Hs E-cadherin</td>
<td>Human</td>
<td>GGCACATGGGTGTGATTACAGTCAAAA</td>
</tr>
<tr>
<td>3’ Hs E-cadherin</td>
<td>Human</td>
<td>GTCCCAGGCGTAGACCAAGAAA</td>
</tr>
<tr>
<td>5’ Hs N-cadherin</td>
<td>Human</td>
<td>GAAAGCACAAGTCGCGCACCTACAAAA</td>
</tr>
<tr>
<td>3’ Hs N-cadherin</td>
<td>Human</td>
<td>CCAGGTACACTGTACCGGACAGTGA AA</td>
</tr>
<tr>
<td>5’ Hs ILK</td>
<td>Human</td>
<td>GATGCAAGCAAGGTAGGACTGGA AA</td>
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<tr>
<td>3’ Hs ILK</td>
<td>Human</td>
<td>CAACCAGAGGGCTGCTGCTTT</td>
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<tr>
<td>Gene</td>
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<td>Sequence</td>
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</tr>
<tr>
<td>Hs ILK RT</td>
<td>Human</td>
<td>GCCCCTCTTTGCGCACAGT</td>
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<tr>
<td>5’ Hs SNAIL</td>
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<tr>
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<td>Human</td>
<td>CATCGCAGTGAGCCTGTATGTTT</td>
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<tr>
<td>5’ Hs ITG α1</td>
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<tr>
<td>3’ Hs ITG α1</td>
<td>Human</td>
<td>CTCCAGAAGAGCAGTACAGAGGT</td>
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<tr>
<td>Hs ITG α1 RT</td>
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<td>GTACCGAGGGCTGCTCCAGCAATATA</td>
</tr>
<tr>
<td>5’ Hs ITG α2</td>
<td>Human</td>
<td>GACCTATCCACTGCACATGTGAAAAAA</td>
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<tr>
<td>3’ Hs ITG α2</td>
<td>Human</td>
<td>CCACAGAGGACACATGTGAGAAAA</td>
</tr>
<tr>
<td>Hs ITG α2 RT</td>
<td>Human</td>
<td>GTCAAGACACACACCCGTTGTGTAATA</td>
</tr>
<tr>
<td>5’ Hs ITG α2 siCUT _5</td>
<td>Human</td>
<td>GGTGCCTGCAGAGGAATATGAGTAGTAAA</td>
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<tr>
<td>3’ Hs ITG α2 siCUT _5</td>
<td>Human</td>
<td>CCAGACTGAGTGCCACACGCAAA</td>
</tr>
<tr>
<td>Hs ITG α2 siCUT _5 RT</td>
<td>Human</td>
<td>GATGCAGGGACCTGAGCATTTA</td>
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<tr>
<td>5’ Hs ITG α2 siCUT _6</td>
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<td>GATCAATGGCAACCAGACATATACCTGAGTTTT</td>
</tr>
<tr>
<td>3’ Hs ITG α2 siCUT _6</td>
<td>Human</td>
<td>CACTGCACCCAGCATCAGAAATATCATTTTT</td>
</tr>
<tr>
<td>Hs ITG α2 siCUT _6 RT</td>
<td>Human</td>
<td>GCAGCCACAGAGTAACCTAAATATAGACT</td>
</tr>
<tr>
<td>5’ Hs ITG α3</td>
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<td>CCAGCGCTGTGGAGCCTTACAA</td>
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<tr>
<td>3’ Hs ITG α3</td>
<td>Human</td>
<td>GAGGGCTTCACAGAGATGCAGGA</td>
</tr>
<tr>
<td>Hs ITG α3 RT</td>
<td>Human</td>
<td>CTCCCCAATCTGTCTGTCCAGAATT</td>
</tr>
<tr>
<td>5’ Hs ITG α6</td>
<td>Human</td>
<td>CACGTTAGGTGCACCATCACAA</td>
</tr>
<tr>
<td>3’ Hs ITG α6</td>
<td>Human</td>
<td>GGCAACATCACCCTCTATTTGACATTTGTTTT</td>
</tr>
<tr>
<td>Hs ITG α6 RT</td>
<td>Human</td>
<td>CCTGAAAGGGTAAACTTGGAGACTCTTT</td>
</tr>
</tbody>
</table>
### 2.1.6 Protein Purification and Western Blot Materials and Reagents

The following materials and reagents were used throughout the course of this work for protein purification and Western blot experiments (Table 2.6).

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<thead>
<tr>
<th>5’ Hs ITG α10</th>
<th>Human</th>
<th>CAGAGGACTTCTCATACTGGACAGAAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’ Hs ITG α10</td>
<td>Human</td>
<td>CGTGGGTGATGTCCATCCAGGTTAAA</td>
</tr>
<tr>
<td>5’ Hs ITG αv</td>
<td>Human</td>
<td>GCCAGTGCCATAGCTCCTTT</td>
</tr>
<tr>
<td>3’ Hs ITG αv</td>
<td>Human</td>
<td>CCCACTGCCTTTCAAGGGATTT</td>
</tr>
<tr>
<td>5’ Hs ITG β1</td>
<td>Human</td>
<td>GACTGATCAGTTCTGCTGTGTGTTT</td>
</tr>
<tr>
<td>3’ Hs ITG β1</td>
<td>Human</td>
<td>CCCTGCTTGATACATTCTCCACATGATTT</td>
</tr>
<tr>
<td>5’ Hs ITG β1 siCUT</td>
<td>Human</td>
<td>GCGTCTGCGGAGCTGTGTGTTT</td>
</tr>
<tr>
<td>3’ Hs ITG β1 siCUT</td>
<td>Human</td>
<td>CCCGTGTTCCATTTGGCATTCTATT</td>
</tr>
<tr>
<td>5’ Hs ITG β3</td>
<td>Human</td>
<td>GCCCCCATGCTGCACTTT</td>
</tr>
<tr>
<td>3’ Hs ITG β3</td>
<td>Human</td>
<td>CGGCAGAAACGGGTGCAGGTATTTTT</td>
</tr>
<tr>
<td>Hs ITG β3 RT</td>
<td>Human</td>
<td>CTGCATCCTTGCCAGTGCTCTTAA</td>
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<tr>
<td>5’ Hs ITG β4</td>
<td>Human</td>
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<td>3’ Hs ITG β4</td>
<td>Human</td>
<td>GGAGCTTCACCTGCAGCTCTTT</td>
</tr>
<tr>
<td>Hs ITG β4 RT</td>
<td>Human</td>
<td>GGGTGGCTGTGAGCAACACTCT</td>
</tr>
<tr>
<td>5’ Hs ITG β6</td>
<td>Human</td>
<td>GGTGTCCATTGAATTGTGACAGCAAAAA</td>
</tr>
<tr>
<td>3’ Hs ITG β6</td>
<td>Human</td>
<td>CACTAGCCCTGTACTCTGTAAAA</td>
</tr>
<tr>
<td>Hs ITG β6 RT</td>
<td>Human</td>
<td>GTTCTGCGCATCAGTCAGGAACATAAA</td>
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</table>
Table 2.6 Protein Purification and Western Immunoblotting Materials and Reagents

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Ponceau S Solution</td>
<td>Sigma-Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>- Dithiothreitol (DTT)</td>
<td></td>
</tr>
<tr>
<td>- Reagents for RIPA</td>
<td>Sigma-Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>- Complete Mini Protease Inhibitor</td>
<td>Roche Diagnostics Australia Pty. Ltd. (Castle Hill, NSW, Australia).</td>
</tr>
<tr>
<td>Cocktail tablets</td>
<td></td>
</tr>
<tr>
<td>- Bicinchronic Acid (BCA) Assay</td>
<td>Pierce Biotechnology (IL, USA)</td>
</tr>
<tr>
<td>- Super Signal West Pico</td>
<td></td>
</tr>
<tr>
<td>Chemiluminescent Substrate</td>
<td></td>
</tr>
<tr>
<td>- See Blue Plus2 Pre-Stained Protein</td>
<td>Invitrogen (CA, USA)</td>
</tr>
<tr>
<td>Standards</td>
<td></td>
</tr>
<tr>
<td>- Ammonium Persulfate (APS)</td>
<td>Bio-Rad Laboratories (Hercules, CA, USA)</td>
</tr>
<tr>
<td>- 30% Acrylamide/Bis Solution</td>
<td></td>
</tr>
<tr>
<td>- N, N', N'-tetramethyl-ethylenediamine</td>
<td></td>
</tr>
<tr>
<td>(TEMED)</td>
<td></td>
</tr>
<tr>
<td>- Bromophenol Blue</td>
<td></td>
</tr>
<tr>
<td>- Coomassie Brilliant Blue R250</td>
<td></td>
</tr>
<tr>
<td>- Sodium Dodecyl Sulfate (SDS)</td>
<td>BDH (Merck, Pty. Ltd. Australia)</td>
</tr>
<tr>
<td>- Whatman Gel Blot Paper</td>
<td>Bioscience (USA)</td>
</tr>
<tr>
<td>- Skim Milk Powder</td>
<td>Carnation (Sydney, NSW, Australia)</td>
</tr>
<tr>
<td>- Hyperfilm</td>
<td>Amersham Biosciences (Castle Hill, NSW, Australia)</td>
</tr>
<tr>
<td>- Immobilon-P Transfer Membrane</td>
<td>Millipore Corporation (Bedford, MA, USA).</td>
</tr>
</tbody>
</table>

2.1.7 Immunohistochemical Reagents

The following materials and reagents were used throughout the course of this work for immunohistochemistry (IHC) experiments (Table 2.7).


## Table 2.7 Immunohistochemical Reagents

<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraffin Wax</td>
<td>Sigma-Aldrich (St. Louis, MO, USA)</td>
</tr>
<tr>
<td>Chromogen 3,3’ Diaminobenzidine</td>
<td></td>
</tr>
<tr>
<td>Tetrahydrochloride (DAB)</td>
<td></td>
</tr>
<tr>
<td>Amino-Propyl-Triethoxy-Silane (AES)</td>
<td></td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)- powder</td>
<td></td>
</tr>
<tr>
<td>Triton X-100</td>
<td></td>
</tr>
<tr>
<td>10% Buffered formalin</td>
<td>Orion Laboratories (Welshpool, WA, Australia).</td>
</tr>
<tr>
<td>Serum-Free Protein Block/ Pap Pen</td>
<td>Dako, Australia</td>
</tr>
<tr>
<td>Slides</td>
<td>SuperFrost® Plus, Menzel-Glaser, Braunschweig, Germany</td>
</tr>
<tr>
<td>Cover Slips</td>
<td>HD scientific supplies Pty Ltd, Australia</td>
</tr>
<tr>
<td>Histosol, Ethanol, Methanol</td>
<td>BDH (Merck, Pty. Ltd. Australia)</td>
</tr>
<tr>
<td>Hydrogen Peroxide</td>
<td></td>
</tr>
<tr>
<td>Sodium Citrate</td>
<td></td>
</tr>
<tr>
<td>Sodium Hydrogen Carbonate</td>
<td></td>
</tr>
<tr>
<td>DePex Mounting Medium</td>
<td></td>
</tr>
<tr>
<td>Extran detergent</td>
<td></td>
</tr>
<tr>
<td>Xylene cyanol FF</td>
<td>Amersham Biosciences (Castle Hill, NSW, Australia)</td>
</tr>
<tr>
<td>Eosin</td>
<td>Histo Labs (Australia)</td>
</tr>
<tr>
<td>Mayer’s Hematoxylin</td>
<td>Amber Scientific (Australia)</td>
</tr>
<tr>
<td>Tris</td>
<td>Bio-Rad Laboratories (Hercules, CA, USA)</td>
</tr>
<tr>
<td>Microtome Blades</td>
<td>ThermoFisher Scientific</td>
</tr>
</tbody>
</table>

### 2.1.8 Antibodies

The following antibodies were used throughout the course of this work for Western Immunoblotting (WB) (Table 2.8) and immunohistochemistry (IHC) experiments (Table
2.9). Any antibodies used in the thesis which are not listed here, were from experiments performed by others, in collaboration with/for this work.

Table 2.8 Primary and Secondary Antibodies used for Western Immunoblotting

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin</td>
<td>Mouse Monoclonal IgG (V9)</td>
<td>1:750 (WB)</td>
<td>Dako, Australia</td>
</tr>
<tr>
<td>N-cadherin (A-CAM)</td>
<td>Mouse Monoclonal IgG (GC-4)</td>
<td>1:2000 (WB)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Mouse Monoclonal IgG (36)</td>
<td>1:12500 (WB)</td>
<td>BD Transduction Laboratories</td>
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<tr>
<td>β1 Integrin</td>
<td>Mouse Monoclonal IgG</td>
<td>1:5000 (WB)</td>
<td>Chemicon</td>
</tr>
<tr>
<td>α2 Integrin</td>
<td>Rabbit Polyclonal</td>
<td>1:10000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Integrin-Linked Kinase (ILK)</td>
<td>Rabbit monoclonal IgG</td>
<td>1:4500 (WB)</td>
<td>Cell Signalling Technologies (Danvers, MA, USA)</td>
</tr>
<tr>
<td>Pan-Actin, Ab-5</td>
<td>Mouse Monoclonal IgG</td>
<td>1:10000</td>
<td>Neomarkers</td>
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</table>

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG-HRP</td>
<td>Goat Anti-Mouse</td>
<td>1:20000</td>
<td>Dako, Australia</td>
</tr>
<tr>
<td>IgG-HRP</td>
<td>Goat Anti-Rabbit</td>
<td>1:20000</td>
<td>Dako, Australia</td>
</tr>
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</table>
Table 2.9 Primary and Secondary Antibodies used for IHC

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimentin (V9)</td>
<td>Mouse Monoclonal IgG1κ</td>
<td>1:40 (IHC)</td>
<td>Dako, Australia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:800 (ICC)</td>
<td></td>
</tr>
<tr>
<td>N-cadherin (3B9)</td>
<td>Mouse Monoclonal IgG1κ</td>
<td>1:100-1:1000</td>
<td>Zymed Laboratories</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Mouse Monoclonal Human IgG1</td>
<td>1:50</td>
<td>Novo Castra Laboratories (UK)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotinylated Immunoglobulin</td>
<td>Polyclonal Rabbit Anti-Mouse</td>
<td>1:200</td>
<td>Dako, Australia</td>
</tr>
</tbody>
</table>

2.1.9 Equipment

Protean II Minigel system, Mini Trans-Blot Electrophoretic Transfer Cell and power packs were from Bio-Rad laboratories Pty. Ltd. (Hercules, CA, USA). The ultraviolet (UV) transilluminator was from Fisher Biotech (WA, Australia), and Speedvac Concentrator was from Savant, (Ramsey, Minnesota, USA). Tissue culture microscopes used included the Olympus CK30 tissue culture microscope Olympus (Victoria, Australia). Images of cells were taken using the MCID-M2 version 3.0 imaging system (Imaging Research Inc., Ontario, Canada). Centrifuges used included Biofuge Primo (Heraeus Spatech, Germany), Beckman Centrifuge GS-15R (Beckman Coulter, Fullerton, CA, USA) and Eppendorf bench-top centrifuge 5415D (Eppendorf, Germany). The spectrophotometer used to measure RNA concentrations was the NanoDrop UV-Vis Spectrophotometer (Nanodrop, Delaware, USA). Polymerase Chain Reaction (PCR) and reverse transcription (RT) reactions were performed using a GeneAmp PCR system 9700 from Applied Biosystems (Australia). Q-RT-PCR was performed on the ABI Prism 5700 Sequence Detection System from Perkin-Elmer Applied Biosystems (Foster City, CA, USA) and Q-RT-PCR data was analysed using the GeneAmp 5700 SDS software (Perkin-Elmer Corporation, Foster City, CA, USA). The MT-PCR was carried out on the RotorGene Thermal Cycler, RG3000 (Corbett Life Sciences, Australia). Absorbance
readings of 96-well plates were performed utilising the Powerwave RPRWI96 Microplate Spectrophotometer and analysed using the KC4 program, both of which were from Biotek (Winooski, VT, USA).

Formalin-fixed paraffin embedded (FFPE) tissues were processed on the Shandon Citadel (ThermoFisher Scientific – Waltham, Massachusetts, USA) tissue processor, and embedded in paraffin utilizing the Shandon Embedding Centre (ThermoFisher Scientific – Waltham, Massachusetts, USA). Formalin-fixed tissues were sectioned on the Microm HM330 microtome. AES coated slides were placed to dry on a Solid State Control warming tray (Ratek Instruments, Boronia, Victoria, Australia). All animal manipulations were performed in a Class 2 Biosafety laminar flow hood.

2.2 MAMMALIAN TISSUE CULTURE:

All tissue culture work was performed in a Class 2 Biosafety Cabinet within the confines of a physical containment level 2 (PC2) facility (Department of Surgery, University of Melbourne). Unless otherwise stated, general microscopy for the visual monitoring of cells grown in culture was performed within the same PC2 facility as mentioned above, on an Olympus CK30 inverted microscope equipped with CK40-SLP phase contrast slider (Olympus Corporation – Shinjuku, Tokyo, Japan). Images of cells were taken, when necessary, using the MCID-M2 version 2.0 imaging system.

2.2.1 Cell Lines and Maintenance

The following cell lines were used throughout the course of this work (Table 2.10). PMC42-ET cells were derived from a breast cancer pleural effusion by Dr. Robert Whitehead, Ludwig Institute for Cancer Research, Melbourne, Australia. The PMC42-LA subline was derived further from the parental PMC42-ET cells by Dr Leigh Ackland, Deakin University, Melbourne, Australia. PMC42-ET cells were provided by A/Professor Erik Thompson (SVI, Fitzroy, Australia). PMC42-LA cells were kindly provided by Dr. Leigh Ackland (Deakin University, Melbourne, Victoria, Australia). The relationship between these cells has been reported (Hugo et al., 2011, Hugo et al., 2013, Cursons et al., 2015).
Table 2.10 Cell lines Used, Their Species, Tissue of Origin and Media Used to Maintain.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Details</th>
<th>Media</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMC42-ET</td>
<td>Cell line derived from a pleural effusion from a patient with metastatic breast cancer.</td>
<td>RPMI/10% FBS</td>
<td>(Ackland et al., 2001)</td>
</tr>
<tr>
<td>(Parental)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMC42-LA</td>
<td>Cell line derived from a pleural effusion from a patient with metastatic breast cancer.</td>
<td>RPMI/10% FBS</td>
<td>(Ackland et al., 2001)</td>
</tr>
<tr>
<td>(Subline)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.1.1 Cell Line Maintenance:

PMC42-ET and PMC42-LA cell lines were maintained in RPMI 1640 medium and supplemented with 10% FBS (complete media) as indicated above in Table 2.10. All tissue culture media were prepared by Ms. Virginia Leopold (SVI, Fitzroy, Victoria, Australia).

Both PMC42-ET and PMC42-LA lines were cultured as described in Section 2.2.1 and passaged in a culture flask at 1x10^6 cells per 75 cm² surface area once per week. If cells were thawed they were passaged at least once prior to use for any given experiment, and only used when in log-phase growth. Any cells grown in culture that reached ten passages post-thawing were discarded and a new vial of cells thawed in order to avoid biological variation that may result from extensive time in culture.

Cells were grown as monolayers in tissue culture flasks and maintained at 37°C in a humidified atmosphere with 5% CO₂. Near-confluent (70-80%) cell lines were passaged by decanting the media, washing the cells twice with sterile tissue culture grade phosphate buffer saline (1× PBS; pH 7.4), then exposing to a minimal volume (~2ml) of trypsin/versene solution to cover cells, for approximately 5-15 min at 37°C. The flask was gently tapped to loosen the cells, and complete media was added to the flask to inactivate trypsinisation. The cell suspension was transferred to a 20ml Filtrona tube and centrifuged at 300g for 5 min. The supernatant was decanted and the pelleted cells were
gently resuspended in 10 ml of fresh complete media (RPMI + 10% FBS) by pipetting. The appropriate number of cells were either seeded into vessels containing pre-warmed media (RPMI + 10% FBS), or utilized in experiments, where they were resuspended in an appropriate volume following centrifugation and counted using a haemocytometer. Cells were used within 10 passages of the initial experiment.

STR profiling (Promega, Stem Cell Elite TM ID) was used to confirm derivation and relatedness of PMC42-LA and ET cell lines. They were also confirmed human and free from significant cross contamination (including mouse). Data is presented in Appendix X. Additionally, cells were periodically checked and confirmed free of mycoplasma contamination using the MycoAlert kit (Lonza, Allendale, NJ, USA).

i) 1x Phosphate buffered saline (PBS):
140nM NaCl, 2mM KCl, 1mM KH$_2$PO$_4$, 8mM Na$_2$HPO$_4$

ii) 1x Versene:
1x PBS and 0.05mM EDTA

iii) Trypsin/Versene:
0.025% (w/v) Trypsin in 1x Versene

2.2.2 Cryopreservation of Cell Lines
Sub-confluent cells in log-phase growth were lifted with trypsin, resuspended in appropriate complete media (RPMI + 10% FBS) and then centrifuged for 5min at 300xg. The resultant pellet was resuspended into a chilled solution containing 10% DMSO and 90% FBS, and 1 ml aliquots were added to the cryotubes, which were immediately placed in a rack within a container suspended over room temperature methanol and allowed to freeze slowly overnight in the -80ºC freezer. The following day the cryotubes were transferred to liquid nitrogen for long term storage of the cells.
2.2.3 **Revival of Cell Lines**

For propagation of cells, frozen vials were rapidly thawed at 37°C and slowly resuspended in 5ml appropriate pre-warmed complete media (RPMI + 10% FBS) and then centrifuged for 5min at 300g. The medium was removed; the cell pellet resuspended in the appropriate pre-warmed complete media (RPMI + 10% FBS), and then transferred into a tissue culture flask. The following day, the medium was replaced with fresh complete media (RPMI + 10% FBS) and cells were passaged as described previously in section 2.2.1.

---

2.3 **IN VITRO CELL CULTURE ASSAYS:**

2.3.1 **EMT Assay (Growth Factor Treatment)**

Unless otherwise specified, Epidermal Growth Factor (EGF) was added to cultures at final concentrations of 10ng/ml. In addition, PMC42-ET and PMC42-LA cells were either untreated or treated with EGF i) under sparse and dense conditions for a period of 0hrs, 24hrs, 72hrs, and ii) under sparse conditions for an extensive time-course (0hrs, 1.5hrs, 3hrs, 6hrs, 24hrs, 48hrs, and 72hrs) over a period of 3 days. For observations of cell morphology, PMC42-ET and PMC42-LA cells were cultured in plates/flasks and treated with or without EGF for up to 3 days and viewed with Olympus CK30 microscope and photographed, when necessary, by MCID-M2 (version 3.0) imaging system.

2.3.2 **Transient Transfection of PMC42 Cells:**

Transient transfections of PMC42 cells were performed using the delivery agent DharmaFECT 4. DharmaFECT 4 transfection reagent was utilized to knock-down the gene and protein expression using siRNA. Briefly, PMC42 cells were plated at a concentration of 6x10^4 cells per well in complete media (RPMI + 10% FBS), in a 6 well plate (9.4 cm²) and allowed to adhere to the plastic for 2 days. Cells approximately 30-40% confluent were then transfected according to the manufacturer’s method.

Briefly, in separate tubes, the appropriate volume (**Table 2.11**) of 2µM siRNA (Tube 1) and the appropriate volume (**Table 2.11**) of DharmaFECT 4 transfection reagent (Tube 2)
were diluted with serum-free medium (RPMI). The contents of each tube were mixed gently by careful pipetting and incubated for 5 min at room temperature. The contents of tube 1 was added to tube 2 and mixed by pipetting, followed by 20 min incubation at room temperature. Sufficient antibiotic-free complete medium (RPMI + 10% FBS) was added to the mix for the desired volume of transfection medium. The culture medium was removed from the cells, siRNA/DharmaFECT mix added to the wells and the plate returned to the incubator for 24 hrs. The transfection medium was removed and replaced with fresh serum containing medium (RPMI + 10% FBS) and maintained at 37°C in a humidified atmosphere with 5% CO₂ until ready to harvest for protein and/or RNA.

For EGF treatment of transfected cells, where an EMT model was utilised, the transfection medium was removed and replaced with fresh serum-containing medium (RPMI + 10% FBS) 6-8 hr post transfection followed by a 10 ng/ml EGF treatment. Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ until ready to harvest for protein and/or RNA.

Each experiment was performed in triplicate and 3 or more independent experiments were performed, unless otherwise stated.

Table 2.11 Volumes for Transfecting 100nM siRNA in a 6 Well Plate.

<table>
<thead>
<tr>
<th>Tube 1 Volumes per well</th>
<th>Tube 2 Volumes per well</th>
<th>Plating volume (µL/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2uM siRNA (µL)</td>
<td>Serum-free Medium (µL)</td>
<td>DharmaFECT (µL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Serum-free Medium (µL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Transfection Medium</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>195.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2000</td>
</tr>
</tbody>
</table>

2.3.3 Functional Assays

2.3.3.1 Adhesion Assay

Attachment assays were performed as follows. In brief, 24-well plates (polystyrene, non-tissue culture treated; Nunc Inc., Naperville, IL) were coated with 100 µg/ml Collagen-I,
100µg/ml Collagen-IV, 20µg/ml Fibronectin or 50µg/ml Laminin. Proteins were allowed to bind overnight at room temperature in the hood, before the wells were rinsed and non-specific interactions were blocked for 1hr at 37°C with 3 % BSA in PBS, pH 7.4. Cells were detached from culture with PBS containing 0.05mM EDTA and 0.025% (w/v) Trypsin and washed twice with serum-free medium (SFM). To allow surface re-exposure of integrin receptors, cells were incubated on a rotating platform for 1hr at room temperature in SFM containing 0.1% BSA. Cell suspensions were prepared in SFM then added in triplicate to the wells at a cell density of $1.7 \times 10^5$ cells/well of the same medium. After 1hr incubation at 37°C, non-adherent cells were washed away. Attached cells were trypsinised and adhesion was estimated by the average of cell counts from five random high-power fields under light microscopy.

Results were analysed using Two-way ANOVA, with Dunnett’s multiple comparison test, to correct for multiple comparisons, with P values adjusted for multiple comparisons. Results presented are the average (±SD) from 4 biological replicates. Nonspecific cell adhesion as measured on BSA-coated wells has been subtracted.

### 2.3.3.2 Monolayer Wound Healing Assay

The in vitro wound-healing assay was performed with specific siRNA-transduced PMC42-ET and PMC42-LA breast cancer cells, which were either untreated or treated with 10ng/ml EGF for 48 hours. At this time-point, cells had grown to confluence. The assay was performed on 6-well polystyrene plates. Before plating the cells onto the plates, 2 perpendicular lines were drawn on the underside of the wells using a needle tip, which served as fiducial marks/points for the wound areas to be analysed. At the day of analysis, when the monolayer had reached absolute confluence, linear mechanical “wounds” were made using a sterile plastic pipette tip, by scoring the wells perpendicular to the lines on the bottom of the well. (The width of the wound was maintained as consistent as possible, since narrow wounds tend to close faster than wider wounds. If more than 2 scratches are made, wounds with similar widths were chosen for analysis). The medium containing dislodged cells was aspirated and each well was gently washed 3 times with fresh medium. Cells were further cultured in 2ml of fresh medium with or without 10ng/ml EGF (100µg; BD Biosciences, USA) and 10mM Thymidine (Sigma
Aldrich, USA; to rule out possible effect of EGF on cell proliferation), and incubated in a humidified incubator at 37°C with 5% CO₂ for a further 24hrs. The wounds were observed at multiple random sites for each time point using phase contrast microscopy on an inverted microscope. Representative images of the wound were taken at the time (t) of wounding (t=0), and at 24hr post wounding of both areas flanking the intersections of the wound and the marker lines. The position of the camera was marked on the plate at the 0hr time point to ensure consistency.

Using the lines on the bottom of the wells as reference points, the size of the wound was measured at predetermined locations at each timepoint. The distance of wound closure (compared with control at t=0) was measured in 4 independent wound sites per group. The mean percentage of wound closure for each time point and each treatment was calculated relative to baseline values (t=0). Relative cell motility was calculated as the wound width at t=0 minus the wound width at t=24, as indicated. Data analysis was done using Image J software. A 2-way ANOVA followed by Holm-Sidak's multiple comparisons test was conducted to examine the effect of siRNA transfection of PMC42-ET cells (without and with EGF treatment) on their wound healing migration ability.

2.3.3.3 Boyden Chamber Migration Assay

Transmigration assays were performed using 8um-pore Transwell chambers (Corning, Corning, NY). Briefly, polycarbonate membranes (8-µM pore size) of the upper compartment of 24-well Transwell culture chambers were coated with 100µg/ml Collagen-I in SFM (RPMI). siRNA-transduced PMC42-ET cells (-/+/EGF) harvested by trypsinisation were re-suspended in RPMI medium supplemented with 0.2% BSA, and the cell suspension (2.5x10⁵ cells suspended in 250µl of SFM; RPMI) was applied in triplicate wells to the upper compartment. The lower compartment was filled with 650µl of chemoattractant (RPMI containing 10% FBS). After 24hrs of incubation, the Transwells were rinsed in PBS to eliminate non-adherent cells and remaining non-migrated cells on the upper surface of the filter were removed carefully with a cotton swab. Migrated cells on the lower side of the filter were stained with 0.5% crystal violet for 15 minutes. The crystal violet dye retained on the filters was extracted with 10% acetic acid and cell migration was measured by reading the absorbance at 560nM on a
Chapter 2: Materials and Methods

micro-titre plate reader (PolarStar Optima). Results were analysed with the two way ANOVA and Dunnett’s multiple comparison test, which corrects for multiple comparisons and reports the 95% confidence intervals of the difference between sample and control siRNA. Results represent the number of migrated cells ±SD from 3 biological experiments.

2.3.3.4 Cell Invasion Assay

Sub-confluent monolayers of the PMC42-ET cells were either left untreated or treated with the ILK inhibitor QLT0267 (QLT, Inc., Vancouver, Canada) at a final concentration of 6.25µM for 24hrs, and the invasion of PMC42-ET cells in vitro was assessed by the invasion of the cells through Collagen-I-coated Transwell inserts. The inhibitor was dissolved in DMSO, which was used as the vehicle control for all the experiments.

Briefly, polycarbonate membranes (8-µM pore size) of the upper compartment of 24-well Transwell culture chambers were coated with 100µg/ml Collagen-I in SFM (RPMI). Cells which were untreated and treated with the ILK inhibitor were trypsinised, and cell suspensions (2.5x10^5 cells suspended in 250µl of serum-free medium), were applied to the upper compartment of triplicate wells, and the lower compartment was filled with 650µl of chemoattractant (RPMI containing 10% fetal bovine serum). After 24hrs of incubation, the Transwells were rinsed in PBS to eliminate non-adherent cells and remaining non-invaded cells on the upper surface of the filter were removed carefully with a cotton swab. Invading cells on the lower side of the filter were stained with 0.5% crystal violet for 15 minutes. The crystal violet dye retained on the filters was extracted with 10% acetic acid and cell invasion was measured by reading the absorbance at 560nM on a micro-titre plate reader. Unpaired two-tailed t test was used with Welch's correction (P<0.05) based on results of f-test between groups; i.e. control and tests are unlikely to have equal variances. Experiments were performed in triplicate from 3 independent experiments.
2.4 PROTEIN ANALYSIS: EXTRACTION, ASSAYS AND QUANTIFICATION

2.4.1 Protein Isolation from Cells

Radioimmunoprecipitation Buffer (RIPA):
1.58g Tris base and 1.8g Sodium Chloride (NaCl) was dissolved in 150ml of dH₂O and the pH adjusted to 7.4 with HCl. 20ml of 10% NP40 (Igepal) and 5ml of 10% Na-deoxycholate (deoxycholic acid) was added and stirred until the mixture was clear. To this, 2ml of 100mM EDTA was added and the total volume adjusted to 200ml with dH₂O. One protease inhibitor cocktail tablet (Roche) was added to 10ml of RIPA immediately prior to use.

For an EGF-induced EMT model, PMC42-ET and PMC42-LA cells were grown in complete media (RPMI + 10% FBS). Cells were then either left untreated ‘0hr’ or treated with 10ng/ml EGF at a range of time points and harvested at the same time such that cells were exposed to EGF for 1.5hr, 3hr, 6hr, 12hr, 24hr, 48hr and 72hrs.

For siRNA transduced experiments, PMC42-ET and PMC42-LA cells, untreated or treated with 10ng/ml EGF for 72hrs, were transfected with the specific siRNA as in section 2.3.2.

Monolayers of cells were washed twice with cold 1x PBS and then harvested in the appropriate volume of chilled RIPA (250µl per well of a 6 well dish, 150µl per well of a 12 well dish, or 100µl per well of a 24 well dish) containing protease inhibitors. Cells were then scraped from the well using a rubber policeman, and cell lysates were transferred to Eppendorf tubes and sonicated on ice for 3 x 10 seconds then transferred to fresh Eppendorf tubes. The lysates were incubated at 4ºC with gentle agitation for 15min, followed by centrifugation at 13,000xg for 10min. The supernatant were aspirated to fresh Eppendorf tubes, and pellets discarded. An aliquot of supernatant was removed for protein assay using the bicinchoninic acid (BCA) protein assay kit (Pierce) and the remainder was stored at either -20ºC or -80ºC.
2.4.2 Bicinchoninic Acid (BCA) Protein Quantification Assay
Protein concentrations were determined using the BCA assay (Pierce, USA) according to the manufacturer’s instructions. Briefly, a standard curve was created using BSA at final concentrations of 0, 1, 5, 10, 25, 50 and 100µg/ml of protein in a total volume of 10µl. 5 µl of each sample (neat) was added to each well. All samples and standards were aliquoted into 96 well plates in triplicates, and 200µl of working reagent was added to each well, which consisted of 50 parts of manufacturer’s Solution A (e.g. 50ml) mixed with 1 part of manufacturer’s Solution B (e.g. 1ml). The plate was then incubated at 37ºC for 30mins, and read on a microplate spectrophotometer at an absorbance of 562nm. Protein concentrations were determined by extrapolation from the standard curve by the spectrophotometer software, and corrected by the appropriate dilution factor.

2.4.3 Polyacrylamide Gel Electrophoresis (PAGE)

i) 4× Resolving Gel Buffer (1.5M Tris, pH 8.8):
91g of Tris base was dissolved in 300ml of dH₂O and the pH adjusted to 8.8 with 10M HCl. The volume was adjusted to 500ml with dH₂O, and stored at 4ºC.

ii) 4× Stacking Gel Buffer (1.0M Tris, pH 6.8):
60.6g of Tris base was dissolved in 300ml of dH₂O and the pH adjusted to 6.8 with 10M HCl. The volume was adjusted to 500ml with dH₂O, and stored at 4ºC.

iii) 10% SDS:
50g of SDS powder was dissolved in 500ml of dH₂O and stored at room temperature.

iv) 10% APS:
0.1g of APS powder was dissolved in 1ml of dH₂O and stored at -20ºC in 100µl aliquots.

v) 1M DTT:
0.77 g of DTT powder was dissolved in 5 ml dH₂O. Aliquots of 100 µl were stored at -20ºC.
vi) **10% Resolving Gel:**
The volumes of each reagent used to prepare 10% resolving gels are provided in table 2.12 below.

vii) **5% Stacking Gel:**
The volumes of each reagent used to prepare 5% stacking gels are provided in table 2.13 below.

i) **1M Tris Base pH 6.8:**
12.1g of Tris base was dissolved in 90ml of dH$_2$O and the pH was adjusted to 6.8. The volume was adjusted to 100ml with dH$_2$O.

ii) **4× Protein Gel Loading Buffer:**
2ml of 1M Tris pH 6.8, 4ml of 10% SDS, and 4ml of 100% glycerol was mixed. 0.01g Bromophenol blue was added, and the volume adjusted to 10ml. The loading buffer was stored at 4ºC. 100µl of 1 M DTT was added per 900µl of loading buffer prior to use for reducing gels.

iii) **10× SDS-PAGE Running Buffer:**
121.14g (0.25M) Tris, 576g (1.92M) glycine and 40g (1%) SDS were dissolved in dH$_2$O and made up to 4L.

The Protean II Minigel apparatus (Bio-Rad) was used according to the method of Laemmli (Laemmli, 1970). Briefly, the resolving gel mixture was poured into the gel forming apparatus, overlayed with dH$_2$O and allowed to polymerise for at least 30 min. The dH$_2$O was poured off the set resolving gel, the stacking gel mix was poured over the set resolving gel, and an appropriate comb inserted and left to polymerise for at least 20 min. Samples containing identical amounts of protein (15-20µg) were diluted in 4x gel loading buffer and dH$_2$O, boiled for 10min and cooled on ice for 5min prior to loading onto the gel. The samples were separated in 1× SDS-PAGE running buffer at a constant
current of 40mA/100 volts per gel for approximately 1hr. SeeBlue molecular weight markers were loaded on each gel to track the protein separation.

Table 2.12 Volumes for 10% Resolving Gel

<table>
<thead>
<tr>
<th></th>
<th>5ml Gel</th>
<th>10ml Gel</th>
<th>15ml Gel</th>
<th>20ml Gel</th>
<th>30ml Gel</th>
<th>50ml Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>1.9ml</td>
<td>4.0ml</td>
<td>5.9ml</td>
<td>7.9ml</td>
<td>11.9ml</td>
<td>19.8ml</td>
</tr>
<tr>
<td>30% Acryl. Mix</td>
<td>1.7ml</td>
<td>3.3ml</td>
<td>5.0ml</td>
<td>6.7ml</td>
<td>10.0ml</td>
<td>16.7ml</td>
</tr>
<tr>
<td>1.5M Tris pH 8.8</td>
<td>1.3ml</td>
<td>2.5ml</td>
<td>3.8ml</td>
<td>5.0ml</td>
<td>7.5ml</td>
<td>12.5ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50ul</td>
<td>100ul</td>
<td>150ul</td>
<td>200ul</td>
<td>300ul</td>
<td>500ul</td>
</tr>
<tr>
<td>10% APS</td>
<td>50ul</td>
<td>100ul</td>
<td>150ul</td>
<td>200ul</td>
<td>300ul</td>
<td>500ul</td>
</tr>
<tr>
<td>TEMED</td>
<td>2ul</td>
<td>4ul</td>
<td>6ul</td>
<td>8ul</td>
<td>12ul</td>
<td>20ul</td>
</tr>
</tbody>
</table>

Table 2.13 Volumes for 5% Stacking Gel

<table>
<thead>
<tr>
<th></th>
<th>2ml Gel</th>
<th>4ml Gel</th>
<th>6ml Gel</th>
<th>8ml Gel</th>
<th>10ml Gel</th>
<th>12ml Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>1.4ml</td>
<td>2.7ml</td>
<td>4.1ml</td>
<td>5.5ml</td>
<td>6.8ml</td>
<td>8.2ml</td>
</tr>
<tr>
<td>30% Acryl. Mix</td>
<td>0.33ml</td>
<td>0.67ml</td>
<td>1.0ml</td>
<td>1.3ml</td>
<td>1.7ml</td>
<td>2ml</td>
</tr>
<tr>
<td>1M Tris pH 6.8</td>
<td>0.25ml</td>
<td>0.5ml</td>
<td>0.75ml</td>
<td>1.0ml</td>
<td>1.25ml</td>
<td>1.5ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>20ul</td>
<td>40ul</td>
<td>60ul</td>
<td>80ul</td>
<td>100ul</td>
<td>120ul</td>
</tr>
<tr>
<td>10% APS</td>
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<td>40ul</td>
<td>60ul</td>
<td>80ul</td>
<td>100ul</td>
<td>120ul</td>
</tr>
<tr>
<td>TEMED</td>
<td>2ul</td>
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<td>6ul</td>
<td>8ul</td>
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<td>12ul</td>
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</tbody>
</table>
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Following electrophoresis, gels were transferred to nitrocellulose membranes as described below.

2.4.4 Western Blot Analysis: Transfer and Detection

i) 10x Transfer Buffer:
0.25M Tris, 1.92M glycine, 20% (v/v) methanol.

ii) 10×TrisBuffered Saline - 1% Tween (TBS-T):
Dissolved 24.2g tris base and 80g NaCl in 900ml of dH₂O and the pH was adjusted to 7.6. 10ml of Tween-20 was added, and the volume adjusted to 1L.

iii) Blocking Solution (5%):
5% (w/v) skimmed milk powder dissolved in 1× TBS-T.

iv) Wash buffer:
1 x TBS containing 0.05% (v/v) Tween-20.

v) Primary Antibody Dilution Buffer:
5% blocking solution

Using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad), proteins were transferred from the SDS-PAGE gel to PVDF (Immobilon-P for detection by chemoluminescence) membrane by electroblotting at 100V for 2h at 4°C. All washes and blocks were performed on a rocking table. All antibody incubations were performed on a rotating wheel.

2.4.5 Chemiluminescence Detection
The PVDF membranes were incubated in blocking buffer for 1h at room temperature on a rocking platform to reduce the non-specific background antibody binding. Membranes were washed in TBS-T (3×10min), then incubated in the appropriate primary antibody (dilution specific for each antibody, ranging from 1:1000 to 1:10,000, refer to Table 2.8)
diluted in 5ml blocking buffer, overnight at 4°C with agitation. The next day, membranes were washed in TBS-T (3×10min), and incubated in the Horseradish peroxidase (HRP)-conjugated secondary antibody diluted to the appropriate dilution in TBS-T for 90 min at room temperature with gentle agitation. Blots were again washed in TBS-T (3×10min) and immunoreactive proteins were detected by incubating the membrane with either the SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA) for 5 minutes or SuperSignal® West Femto Maximum Chemiluminescent Substrate (Thermo Scientific, USA) for 7 minutes at room temperature. Membranes were then drained, wrapped in cling wrap and exposed to autoradiography film in an automated developer (Fuji X-Ray Film Processor RII). The intensity of the signal on the x-ray film was quantified by densitometry. The relative signal of the protein of interest was normalized with the ‘housekeeping’ control protein, actin, using a pan actin antibody recognising all forms.

2.5 GENE EXPRESSION ANALYSIS: RNA EXTRACTION AND QUANTIFICATION

2.5.1 RNA Isolation

i. **Buffer RLT (Lysis Buffer), Buffer RW1 (Wash Buffer), Buffer RPE (Wash Buffer):**

Confidential QIAGEN composition.

2.5.1.1 Total RNA Isolation from Mammalian Cells

RNA from cultured cell lines was isolated using RNeasy mini columns (Qiagen, Clifton Hill, Australia) as per the manufacturer’s instructions. Briefly, cells for RNA isolation, grown in a monolayer in culture vessels, were disrupted (lysed) by addition of buffer RLT/β-Mercaptoethanol (β-ME). Cell lysates were collected with a rubber policeman, pipetted into microcentrifuge tubes, and vortexed at high speed for 5 seconds, to ensure no clumps were visible, before being homogenized. Cell lysates were passed 5 times through a 20-gauge needle fitted to an RNase-free syringe. An equal volume of 70% ethanol was added to the homogenized lysates and mixed by tube inversion. The samples
were then loaded onto Qiagen RNeasy mini columns and centrifuged at 8,000xg for 15 seconds. Flow-through was discarded; 700μl of Qiagen’s RW1 buffer was added to the RNeasy columns, and centrifuged at 8,000xg for 15 seconds. Flow-through and collection tubes were discarded, and the RNeasy mini columns were transferred to new collection tubes. The columns were washed twice with 500μl of Qiagen’s RPE buffer. RNA was eluted by addition of 50μl of nuclease-free water to the columns, and centrifugation for 1 min at 8,000xg.

2.5.1.2 Total RNA Isolation from Mammary Fat Pad Tumours: (EDW-01 Breast Cancer Xenograft)

Total RNA from tumours was extracted using RNeasy mini columns (Qiagen, Clifton Hill, Australia) as per the manufacturer’s instructions. Briefly, tumour segments of up to 30mg were pulverized whilst frozen and lysed in 600μl of buffer RLT containing β-mercaptoethanol (10μl β-ME per 1ml buffer RLT). The lysed tumours were transferred into microfuge tubes, and homogenized by passing approximately 5-10 times through a 21-gauge needle fitted to an RNase-free syringe. The samples were subsequently centrifuged at 13,000rpm for 3min and the supernatant transferred to fresh microfuge tubes. 600μl of 70% ethanol was added to the homogenized lysates and mixed by gentle tube inversion and pipetting. The samples were then loaded onto Qiagen RNeasy mini columns and centrifuged at 8,000xg for 15 seconds. Flow-through was discarded and any remaining mixture was then applied to the column and spun again so that all starting material had passed through the column. The flow-through was discarded and 700μl of Qiagen’s RW1 buffer was added to the RNeasy columns, and centrifuged at 8,000xg for 15 seconds. Flow-through and collection tubes were discarded, and the RNeasy mini columns were transferred to new collection tubes. The columns were washed twice with 500μl of Qiagen’s RPE buffer. RNA was eluted by addition of 50μl of nuclease-free water to the columns, and centrifugation for 1min at 8,000xg.

2.5.2 RNA Quantitation

RNA preparations were analysed by absorbance to determine concentration ($A_{260}$) using NanoDrop UV-Vis Spectrophotometer (Nanodrop, Delaware, USA). An $OD_{260}$ of 1 was
taken as 40μg/ml. All preparations had $A_{260/280}$ ratios greater than 1.8 indicating negligible protein contaminants. A portion of sample was retained to determine the yield and quality of RNA, while the remainder was transferred to Eppendorf tubes and stored at -80°C until use.

2.6 QUANTITATIVE RT-PCR ANALYSIS (Q-RT-PCR)

2.6.1 Oligonucleotide Synthesis
Quantitative RT-PCR primers (oligonucleotides) were designed by Mr. Tony Blick (SVI, Victoria, Australia) using the primer-Blast software available freely through the National center for Biotechnology Information (NCBI) web site. Where feasible, PCR primers were selected, such that amplicons spanned exon/exon boundaries, to minimize the risk of detecting genomic DNA and to avoid the problems encountered with possible genomic DNA contamination of RNA samples. The oligonucleotide primers were designed to be of approximately 18-22 base pairs (bp) long and the length of the corresponding PCR amplicons (PCR products) were in the range of 50-550 bp. To enhance the efficiency and the specificity of primers, the 5’ end was designed with a higher bonding potential (GC rich) and the 3’ end rich in A and T. Additionally, primers were designed to have a melt temperature (Tm) of approximately 70 -72°C.

The primers used in this study, are listed in Table 2.15. Primers were purchased from GeneWorks (Adelaide, SA, Australia) and were solubilized in nuclease-free water and stored at -20°C.

2.6.2 cDNA Synthesis (Gene Specific Priming)
The complementary DNA (cDNA) was produced from total RNA using ThermoScript TM reverse transcriptase kit (Invitrogen, USA), according to the manufacturer’s instructions, using gene-specific primers. Each cDNA synthesis reaction contained the following mixture 1 (Table 2.14), and mixture 2 (Table 2.15):
Samples were heated to 65°C for 5min in a PCR machine to remove secondary structure, before cooling to 55°C. While samples were heating, a second mixture, as shown below, was prepared and preheated to 55°C.

Four μl of the enzyme mixture was rapidly transferred to each reaction without cooling and the reaction incubated at 55°C for 1hr. Samples were heated to 85°C for 5 min before cooling to 4°C. The cDNA was stored at -20°C.

The lyophilized oligonucleotide pellets were resuspended in 100μl of dH₂O water. Working dilutions (10μM) were prepared from the initial stocks.

Table 2.14 Contents and Volumes of cDNA Synthesis Reaction (Mixture 1)

<table>
<thead>
<tr>
<th></th>
<th>μl per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA (100 ηg/μl)</td>
<td>1</td>
</tr>
<tr>
<td>Primer pool (4mM)</td>
<td>0.5</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>1</td>
</tr>
<tr>
<td>Super RNase In</td>
<td>0.5</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>3</td>
</tr>
<tr>
<td>Total volume</td>
<td>6</td>
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</tbody>
</table>

Table 2.15 Contents and Volumes of cDNA Synthesis Reaction (Mixture 2)

<table>
<thead>
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<th></th>
<th>μl per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase-free water</td>
<td>0.5</td>
</tr>
<tr>
<td>5× Thermoscript buffer (250mM Tris acetate [pH 8.4], 375mM potassium acetate, 40mM magnesium acetate)</td>
<td>2</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>0.5</td>
</tr>
<tr>
<td>RNase out</td>
<td>0.5</td>
</tr>
<tr>
<td>Thermoscript</td>
<td>0.5</td>
</tr>
<tr>
<td>Total volume</td>
<td>4</td>
</tr>
</tbody>
</table>
Quantitative RT-PCR was performed on an ABI Prism 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Australia) on cDNA generated from an equivalent of 100ng of RNA in 10mM Tris-HCl (pH 8.0), 2.5mM Mg (C$_2$H$_3$O$_2$)$_2$, 50mM KCl, 200μM deoxynucleoside triphosphates (dNTPs), 1:40,000 dilution of SYBR Green I (Molecular Probes, OR, USA), 1μg/ml 6-carboxy-X-rhodamine (6-ROX) (Molecular Probes, OR, USA), 8% DMSO, 200 nM primers and 0.625U AmpliTaq Gold polymerase (Applied Biosystems, Australia) per 25μl reaction. Specific sequences were amplified by 55 cycles (10min of denaturation at 95°C, 15 seconds annealing at 95°C, and a 1 min extension at 65°C). Melt curve analysis from 65°C to 75°C was performed at the end of each run as a quality control step. A ‘no template control’ (NTC) reaction was incorporated as a negative control which consisted of the same reaction mix as above, minus the cDNA template. In addition to the desired genes, Human ribosomal protein L32 mRNA was used as a housekeeping gene to normalize all samples. Cycle threshold (Ct) for each run was set to ensure that the curve crossed at a linear point. The difference in average cycle threshold (δCt) between L32 (housekeeping) and the gene of interest was determined by quadruplicate readings for each sample and the mean and standard deviation determined. A difference in δCt between the expression of two genes (n) is the equivalent of a $2^n$ fold change in mRNA expression of that gene in that tissue, assuming 100% PCR efficiency.

Relative expression of mRNA was determined by the expression of fluorescent dyes. SYBR Green I dye was employed with its mechanism of action through fluorescence whilst bound to the minor groove of double-stranded DNA (Zipper et al., 2004), allowing quantification of total PCR product in real-time throughout the reaction. During the exponential phase of the PCR reaction a fluorescence signal threshold is determined. This is calculated as a function of the amount of background fluorescence and is plotted at a point where the signal from the sample is greater than background. The increase in fluorescence was monitored during the complete gene amplification process (real-time) which is an indicator of amplicon production during the PCR cycle. Therefore, the threshold cycle (Ct) is defined as the fractional number of PCR cycles required to generate a fluorescent signal to reach an arbitrary threshold during the exponential phase of the PCR. The slope of the amplification plot on a log scale during the exponential
phase is a reflection of the PCR efficiency. The Ct values are directly proportional to the amount of starting template which is used to calculate mRNA expression levels (Ginzinger, 2002). The PCR products were analysed by gel electrophoresis to verify their molecular size and melt curve analysis was performed at the end of each run from 60°C to 95°C to confirm their specific melting temperatures.

The ‘No Template Control’ (NTC) (negative control) reaction mix, which contained all reagents except for the cDNA template, was added to all the plates first to avoid contamination with the cDNA template, and was run with every assay to assess the overall specificity. For all primer pairs, each template was maintained in the same well positions to compensate for potential well-to-well variation across the plate. Normalization to human ribosomal protein L32 mRNA, the “housekeeping” gene, was used to correct for minor differences due to variations in RNA amount or in efficiencies of reverse transcription. Quantitation of the L32 housekeeping gene was performed for every RT-PCR analysis. The PCR was performed in a 96-well setup with 25 μl of reaction mix per well. Each template was analysed in quadruplicate wells together with a housekeeping gene and a no template control.

2.6.3 Comparative Calculations
Specificity of the assay and PCR product verification was achieved by plotting fluorescence as a function of temperature to generate a melt curve of the amplicon (Ririe et al., 1997). The melting temperature (T_m) of the amplicon depends on the nucleotide length and G/C composition therefore it is possible to identify the specific C_t value generated from the correct gene product. For each gene the characteristic melting curve at the T_m of the amplicon was used to distinguish it from other artefacts such as primer-dimers and non-specific products that may melt at other temperatures. The melt curve was analysed and the data was disregarded if the dissociation temperature (observed melting temperature) of the product was not correct. An amplification plot for each gene was determined and this displays normalized fluorescent values for each well plotted against the cycle number.
Results of the Q-RT-PCR data were represented as $C_t$ values and the average and standard deviation of the quadruplicate values for each sample was calculated. The parameter $C_t$ was defined as the threshold cycle number of PCR at which amplified product was first detected. Relative quantitation was performed by normalizing the $C_t$ values of each sample gene with $C_t$ values of L32 “housekeeping” gene. $\delta C_t$ corresponds to the difference in the $C_t$ values derived from the specific gene being assayed and the L32 control, while $\delta \delta C_t$ reflects the difference between $\delta C_t$ of the control sample and the $\delta C_t$ of the sample of interest. Data was presented as the linearised fold-change difference relative to an arbitrarily chosen sample in each case, which was set to 1. The range for the linearised fold-change of the genes was calculated from $2^{-(\delta \delta C_t + s)}$ and $2^{(\delta \delta C_t - s)}$ for lower and higher values respectively and where ‘s’ is the standard deviation of the $\delta \delta C_t$ value.

2.7 MULTIPLEXED TANDEM POLYMERASE CHAIN REACTION (MT-PCR)

Multiplexed tandem polymerase chain reaction (MT-PCR) was carried out using MT-PCR MasterMix Kit for Corbett Rotor-Gene™ Gene-Disc (Quantace Ltd, London) according to manufacturer’s instructions (Corbett Research). MT-PCR is a technique that enables simultaneous profiling of a large number of genes in a sample with considerable precision (Stanley and Szewczuk, 2005). The technique was developed by Corbett Research Pty Ltd (NSW, Australia) and is based on two PCR steps in tandem (Stanley and Szewczuk, 2005). In the first step, multiplexed amplicons of interest were amplified with a limited number of PCR cycles. In the second step, the multiplexed PCR product from the first step was diluted and used as a template for a number of corresponding single-gene PCRs, using EvaGreen as the reporter. The MT-PCR primers were designed in such a way that the primers used in the second step were nested inside the corresponding primers of the first step. The multiplexed ‘outer’ primers used in the first step produced amplicons of <150 base pairs (bp), whiles the amplicons of the corresponding nested ‘inner’ primers were about 70-90 bp. All primer pairs spanned a 34 intron-exon boundary. The primers were designed by the manufacturer (AusDiagnostics, NSW, Australia) and supplied in lyophilized state. The multiplexed ‘outer’ primers were
supplied in a single tube whereas the corresponding ‘inner’ primers were distributed in a 72-well ‘gene disc’. The gene discs used in this study were already validated in the lab for the PMC-42 breast cancer cell system and also had been used on clinical samples (Thompson et al., 2010).

In general each disc comprised at least three housekeeping genes, L32, GUSB and NONO, and 33 specific primer pairs in duplicate wells. The MT-PCR was carried out on the RotorGene Thermal Cycler, RG3000 (Corbett Life Sciences, Australia) using the RotoGene 6 software. The reagents from the MT-PCR kit for Corbett RotorGene Gene disc (Quantance, Australia) were used for the reaction. Briefly, RNA was extracted from cultured cell lines using RNeasy mini columns (Qiagen, Clifton Hill, Australia), as outlined in section 2.5.1 above, and MT-PCR was performed.

2.7.1 First Round Multiplexed Amplification

An RT-PCR reaction mix was made up in 0.2ml PCR tubes provided with the Corbett gene-disc, containing all of the lyophilised primer sets. Each MT-PCR first round amplification reaction contained the following (Table 2.16):

| Table 2.16 Contents and Volumes of First Round Multiplexed Amplification Reaction |
|--------------------------------|-----|
| μl per tube:                  |     |
| Total RNA (100ng) + sterile water | 8   |
| Quantace Step One Mastermix    | 10  |
| Quantace Step One Additive Mix | 2   |
| Total volume                  | 20  |

Each tube was placed in a RotorGene thermal cycler (RG3000, Corbett Research) and heat treated as follows: 1 min at 55°C (reverse transcription), 5 min at 95°C (RT denaturation) followed by 15 cycles of 10 s at 95°C, 20 s at 60°C and 20 s at 72°C. This completes the reverse transcription reaction and multiplexed amplification.
2.7.2 Second Round Gene Quantification Amplification

Following first round multiplexed amplification, each MT-PCR second round gene amplification reaction was set up and contained the following (Table 2.17). An aliquot of 20 µl of PCR mixture (Table 2.17) was then added to each position within the gene disk containing the lyophilized inner primers. The disc was sealed in a disc sealer, fitted into a Gene-disc rotor and placed in the Rotor-Gene, where PCR was performed for 35 cycles of 1s at 95°C, 10 s at 60°C and 10 s at 72°C. Fluorescence was measured at the end of each 72°C extension step. All the important parameters for the reaction were set up automatically by the software of the machine.

Table 2.17 Contents and Volumes of Second Round Multiplexed Amplification Reaction

<table>
<thead>
<tr>
<th></th>
<th>µl per tube:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product from multiplexed amplification</td>
<td>20</td>
</tr>
<tr>
<td>Quantace Mastermix</td>
<td>750</td>
</tr>
<tr>
<td>Step 2 additives</td>
<td>20</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>710</td>
</tr>
<tr>
<td>Total volume</td>
<td>1500</td>
</tr>
</tbody>
</table>

2.7.3 Comparative Analysis of Gene Expression

An amplification plot for each gene represented the fluorescence values for each well plotted against the number of cycles. In the amplification plot, an arbitrary threshold fluorescence was set at 0.0325 in the exponential phase to obtain the representative CT values corresponding to each well of the PCR assay. Relative expression of the gene of interest was quantified by averaging the CT values of duplicates and normalizing it with the average CT value of the “housekeeping genes”. GUSB (glucuronidase beta), NONO (non-POU domain containing, octamer-binding), NONO-L (long amplification for NONO) and RPL32 were used as the housekeeping genes. The relative expression (δCT) corresponds to the difference in the CT values of the gene of interest being assayed and the CT values of the average of the housekeeping genes. The δCT was calculated as described in section 2.6.3.
2.8 IN VIVO EXPERIMENTATION:

2.8.1 Animals & Animal Maintenance
Animal studies were conducted with ethical approval from the St. Vincent’s Hospital Animal Ethics Committee (SVH AEC) and in accordance with the Australian National Health and Medical Research Councils Guidelines for care and use of laboratory animals. All animal work was performed with the guidance and help of Dr. Mark Waltham, Mr. Tony Blick, and Ms Emma Walker (St. Vincent’s Institute, Fitzroy).

Severe combined immunodeficient (SCID) female mice (Animal Resource Centre, Perth, WA) were purchased at 3–4 weeks old and housed in the Thomaïy Research Facility (Department of Surgery, St Vincent’s Hospital, Fitzroy, Australia). Animals were housed (maximum of 10 mice/cage) in sterile, individually ventilated cages under filtered air (Techniplast, Italy) bedded with sterilized soft wood granulate. All manipulations were performed in a PC2 laminar flow hood.

2.8.2 Anaesthesia of Mice
Before inoculation of tumours, mice were anaesthetized with an intraperitoneal injection mixture of ketamine (40µg/g mouse) and xylazine (16µg/g mouse) using a 26g needle. Once reflex to toe pinch was absent, mice were positioned for the procedure. For euthanasia, animals were given a lethal dose of ketamine and xylazine prior to dislocation of the neck.

2.8.3 Mammary Fat Pad (MFP) Inoculation of Tumor Xenograft (EDW-01) From Human Breast Cancer (HBC) Tissue
One transplantable primary HBC xenograft (EDW-01) was used in the current study. It was derived from bone metastasis deposits harvested at the time of orthopaedic surgery for fracture. Human tissue samples were obtained in accordance with the standards of the St. Vincent’s Hospital Human Research Ethics Committee.

SCID mice were anaesthetized with an intraperitoneal injection mixture. Once reflex to toe pinch was absent, mice were positioned for the procedure. Following skin preparation
with ethanol, a 5mm incision was made lateral to the most caudal nipple and the underlying fat pad exposed. In each case, the tumor tissue for immediate re-implantation was chopped into chunks of 1-2 mm³ and 2-4 chunks were mixed with 50-100µl Matrigel (a mouse tumour extract rich in basement membrane components that allows localization of the tumor cells in the mammary fat pad and stimulates their take rate). The tumor chunks were implanted in and around the MFP of the anaesthetized SCID mice and the skin was closed with a metal clip. The procedure was repeated bilaterally. Following the procedure, mice were allowed to recover from anaesthetic in a box on a heat pad and monitored every 15 minutes during recovery, before being returned to the cages. The skin clip was removed 1 week later. Mice were monitored daily for any signs of distress, and weighed twice weekly to monitor their general health. Mice with a weight loss of 10% body weight or more were sacrificed immediately in accordance with SVH AEC SOP.07. Tumours were allowed to grow and measured twice weekly using callipers. Tumours implanted in the mammary fat pads were expected to take a long time to establish and thus the endpoint was set at 36 weeks. On the day of harvest, mice were anaesthetized and tumour harvested for analysis when the tumours reached approximately 15mm in one dimension, and mice subsequently sacrificed by anaesthetic overdose using ketamine (200µg/g)/ xylazine (80µg/g) through an intra-peritoneal injection, prior to dislocation of the neck, as described above. Where appropriate, material was snap frozen at -80°C for RNA analysis, frozen down for freeze-thaw, and prepared for histological as well as IHC evaluation.

2.9 HISTOLOGY OF XENOGRAFT SPECIMENS

2.9.1 Amino-propyl-tri-Ethoxy-Silane (AES) Treatment of Slides
Glass slides were decontaminated in 2% (v/v) Extran detergent (BDH) and incubated overnight at 37°C prior to three 5min washes in distilled H₂O. Slides were subsequently incubated in absolute ethanol at room temperature for 2hr and air-dried. Slides were treated in amino-propyl-tri-ethoxy-silane (AES)/acetone mixture (1:50 dilution) for 1min, followed by two 5min rinses in distilled H₂O and air-dried prior to sectioning.
2.9.2 Tissue Processing, Wax Embedding and Sectioning

All tissues collected for histology were placed within small plastic embedding cassettes (Techno-Plas – St. Marys, South Australia, Australia) and then into neutral buffered formalin (Amber Scientific – Midvale, Western Australia, Australia) overnight at room temperature, and on the following day transferred into 10% neutral buffered formalin in PBS and subsequently placed at 4°C until being run through the tissue processor. Tissues were processed using the Shandon Citadel (ThermoFisher Scientific–Waltham, Massachusetts, USA) tissue processor, and put through a standard programmed overnight run. Briefly, the tissues were dehydrated through a series of gradually increasing concentrations of ethanol (70-100%), and then put through a number of clearing steps to remove the dehydrant, in this case using xylene. Finally, the post-dehydration tissues were embedded in paraffin wax. Following the overnight tissue processing run, the wax-coated tissues were removed from their respective cassettes and embedded into paraffin wax blocks for sectioning using the Shandon Embedding Center (ThermoFisher Scientific). In doing so, the wax baths were pre-heated and the cold plates cooled, once the equipment was ready, the tissue of interest was placed within a suitable metallic mould and covered in a small volume of wax, the mould containing the tissue was briefly placed on the cold plate to allow for the lower portion of wax to set and hold the tissue in place, the lid from the plastic cassette was placed on top of the tissue/wax in the metallic mould, and the maximum amount of wax was further added without overflowing out of the mould. Immediately the mould containing the lid-covered tissue was placed onto the cold plate to set. Once the embedding was complete, the moulds were left to further set on the cold plate for at least 30 minutes or overnight at room temperature. Embedded tissues were removed from moulds and stored at room temperature prior to sectioning.

Sectioning of formalin fixed paraffin embedded (FFPE) tissues was performed on the Microm HM330 microtome (Zeiss, Australia, a ThermoFisher Scientific subsidiary). MX35 Premier+ (ThermoFisher Scientific) blades were used on the microtome for cutting sections, and all tissues were kept in icy water prior to cutting. Further, all the forceps used to manipulate sections were also kept on ice to minimise sticking of sections to equipment. FFPE sections were sectioned at 5 micron (μm) thickness, as thicker sections make morphological assessment much more difficult. and transferred/floated
into a pre-warmed water bath (45°C) containing dH2O to allow the sections to flatten out. AES-coated Superfrost Plus slides were used to ‘pick up’ sections from the water bath and then placed on a Solid State Control Warming Tray (Ratek Instruments–Boronia, Victoria, Australia). Once dry, slides with sections were collected into slide holder boxes and stored at room temperature away from moisture prior to use for routine histology (either H&E or IHC).

2.9.3 Haematoxylin & Eosin (H&E) Staining of Xenograft Sections
Haematoxylin and eosin (H&E) staining was performed on 5 micron tissue sections of FFPE material, for nuclear and cytoplasmic staining, respectively, according to standard protocols. Briefly, paraffin-embedded sections were deparaffinized in two changes of Histosol for 7 min each, and then gradually rehydrated by rinsing in a series of down-graded ethanol (2x100%, 90%, 80%, and 70%) for 1 min each, and finally 1 course of rehydration with double-distilled water for 1 min. Sections were then stained in Mayer haematoxylin for 1 min, and then rinsed with tap water until residual haematoxylin was removed. Sections were then counterstained with eosin for 90sec, before the sections were dehydrate in absolute ethanol, followed by two subsequent changes of Histosol for 5-10 min, then cleared in Xylene for 5min each and mounted under a cover-slip using clear DePeX mounting medium (BDH, England).

2.9.4 Immunohistochemistry (IHC) of Xenograft Specimens
For analysis of EMT-relevant proteins, (vimentin, E-cadherin, N-cadherin) in FFPE EDW-01 xenograft tumour specimens, antibodies to differentially expressed genes were obtained commercially, where available. Briefly, paraffin embedded tissues were cut at a thickness of 5µm and then deparaffinised as described above. Some antibodies require an antigen retrieval step before immunohistochemical staining due to the formation of methylene bridges during fixation, which cross link proteins and therefore can mask antigenic sites. Antigen retrieval on the deparaffinized sections was performed by immersing the samples in 10 mM citrate buffer (pH 6.0), boiling the samples in a hot water bath for 20 min at 95°C, followed by subsequently cooling down at room temperature for 20 min. Endogenous peroxidase activity was blocked by immersing the
samples in methanol containing 30% (w/w) hydrogen peroxide for 30 min, followed by a rinse in double-distilled water. Next, the sections were encircled using a hydrophobic marker (PAP-pen, Dako, Australia) to conserve reagents, and blocked in a serum-free protein block (DAKO, USA) for 30 min (to inhibit the nonspecific staining during the IHC detection of antigens), prior to the application of the primary antibody. All antibodies were diluted in 0.1% BSA/PBS. After overnight incubation at 4°C in a moist chamber, the primary antibody was drained off, one slide at a time, and the sections washed thoroughly three times for 5 mins each in 0.1% Tween20/PBS wash buffer before the application of the biotinylated secondary rabbit-anti mouse antibody (DAKO, Denmark) and incubation for a further 1 hr at room temperature. The sections were once again thoroughly washed twice for 5 min each with 0.1% Tween20/PBS wash buffer, followed by one 5 min PBS wash. Streptavidin conjugated peroxidase (DAKO, Denmark) was added for a 30 min incubation period. The Streptavidin conjugated peroxidase was then drained off and the sections washed twice in PBS for 5 min each, followed by one 5 min wash with TBS, before the 2 min application of the chromogen 3, 3’–Diaminobenzidine -tetrahydrochloride (DAB; SIGMA, St. Louis, MO) in TBS containing 150 µl of 30% (w/w) H₂O₂ as the substrate. The sections were then washed once in TBS for 5 min, followed by a 5 min wash in double-distilled water, before a nuclear counterstain (Hematoxylin) was applied for 4 min. This was followed by a further 2 min rinse in double-distilled water until clear, then further rinsed in double-distilled water containing sodium hydrogen carbonate, before the sections were dehydrated in ascending grades of ethanol (70%, 80%, 90% and 100%), followed by two changes of histosol for 5–10 min, then cleared in Xylene for 5 min each and mounted under a cover slip using clear depex mounting medium (BDH, England). Negative controls (tissues that were not incubated with the primary antibody) were used in all the immunohistochemical reactions performed in this study, to ensure the specificity of the primary antibody. Negative controls were performed with goat IgG antibody (DAKO, Denmark) and with no primary antibody to confirm the specificity of the results and none of them showed any immunoreaction.
For the EDW-01 studies, staining of sections for certain genes (ER, PR, HER2), was performed in collaboration by the staff in the Department of Pathology, St. Vincent’s Hospital, Melbourne, using the BenchMark® ULTRA automated slide stainer (Ventana Medical Systems, Inc., USA).

2.9.5 *Image Analysis of Xenograft Specimens*

Following IHC staining, tissues were examined using the Zeiss Axioskop 2 microscope (Zeiss, Australia). Images were captured using the Zeiss ZxioCam MRC5 mounted-camera and analysed using the AxioVision software package (version 4.2.0.0). Unless otherwise stated, all images were captured at 20x magnification. Professor Jane Armes from St. Vincent’s Hospital Pathology Department performed the IHC staining for ER, PR, and HER2, described in section 4.2.4.

2.9.6 *IHC Staining Assessment of Xenograft Specimens*

Five randomly chosen fields of view were taken at the same magnification from each section. The immuno-localization of the stains was noted and assessed for expression intensity/score. In addition, percentage of cells with positive staining was also determined.

The staining score was rated as 0 (no staining), 1 (weak), 2 (moderate), or 3 (strong) based on the intensity of the staining pattern. Staining score 0 was defined as no staining visible. Staining score 1 was defined as light staining requiring high power (x200) to identify the staining pattern. Staining score 2 was defined as moderate staining requiring medium power (x100) to identify the staining pattern. Staining score 3 was defined as dark staining visible on low power (x40). For percentage assessment, 0% was defined as 0, 1-30% positives were defined as 1, 30-70% defined as 2, and >70% defined as 3.

2.10 **COMPUTER PROGRAMS**

The data collected for Q-RT-PCR, adhesion assays, migration assays and invasion assays were analysed and graphed using Microsoft Excel (Redmond, WA, USA) and GraphPad Prism 5 (San Diego, CA, USA) software.
Chapter 2: Materials and Methods

The BLAST search engine (http://www.ncbi.nlm.nih.gov/BLAST) was used to search databases for homologous sequences.

Pubmed was used for literature review: http://www.pubmed.org.

Primer-Blast software available free through the National Centre for Biotechnology Information (NCBI) web site was used to design primers for Q-RT-PCR (Refer to Chapter 2.5).

The Burnet Institute M-Fold Server was used to predict the secondary RNA folding patterns from combined primer extension joining PCR (http://mfold.burnet.edu.au).

2.11 STATISTICAL ANALYSIS

Unless otherwise stated, results are presented as the mean ± the standard deviation (SD) and the data were subjected to statistical tests as stated below. Statistical programs were sourced from Graphpad Prism. P<0.05 was considered statistically significant.

CHAPTER 4:

- Data presented in section 4.2.8 (Figures 4.7-4.8) was analysed using Two-tailed Mann-Whitney test (Non-parametric).

CHAPTER 5:

- Data presented in section 5.2.2 (Figures 5.8 to 5.9) was analysed using Repeated Measures One-way ANOVA with the “Dunnett’s multiple comparison test”, which corrects for multiple comparisons.

- Data shown in section 5.2.4 (Figures 5.11 to 5.14) were subjected to Two-way ANOVA analysis, with Dunnett’s multiple comparison test, which corrects for multiple comparisons and reports the 95% confidence intervals of the difference between sample and control siRNA.

- Data shown in section 5.2.5 (Figure 5.15) were subjected to a Two-way ANOVA analysis, followed by Holm-Sidak's multiple comparisons test.
- Data presented in section 5.2.5 (Figure 5.16) was analysed with the Two-way ANOVA, with Dunnett’s multiple comparison test, which corrects for multiple comparisons.

- Data presented in section 5.2.6 (Figure 5.17) was subjected to an unpaired Two-tailed t test analysis, with Welch's correction (P<0.05) based on results of f-test between groups (i.e. control and tests are unlikely to have equal variances).
CHAPTER 3: EFFECTS OF CULTURE-DENSITY AND TIME-COURSE ON CANDIDATE EPITHELIAL MESENCHYMAL TRANSITION (EMT) MARKERS/EFFECOTORS IN THE PMC42 HUMAN BREAST CANCER MODEL SYSTEM.
Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System

3.1 INTRODUCTION

The PMC42 human breast cancer cell line is an epithelial cell line originally established from a pleural effusion of a female patient with disseminated breast cancer (Whitehead et al., 1983a). Originally, in vitro studies using phase contrast and electron microscopy described this cell line as being heterogeneous, displaying features of myoepithelial cells, as well as being epithelial in nature (Whitehead et al., 1983a, Whitehead et al., 1983b, Monaghan et al., 1985). PMC42 human breast cancer cells were thought to behave like a mammary stem cell, and were shown to differentiate into eight morphological subtypes after cloning, and grow as a monolayer with floating cords of cells present in suspension (Whitehead et al., 1983a, Whitehead et al., 1983b). PMC42 cells exhibited proliferative responses to estrogen and progesterone in monolayer culture (Whitehead et al., 1984), in addition to responding to epidermal growth factor (EGF) with increased cloning efficiency in semi-solid agar (Whitehead et al., 1983b).

Cell culture analyses revealed that the PMC42 line was comprised of distinct morphological and behavioural subtypes (Whitehead et al., 1983a, Whitehead et al., 1983b, Monaghan et al., 1985), and a stable, relatively epithelial subline was subsequently derived (Ackland et al., 2001). These cells have been studied further and are classified as a) the parental (PMC42-ET) cells and b) the epithelial subline (PMC42-LA) (Hugo et al., 2011, Hugo et al., 2013, Cursons et al., 2015).

a) PMC42-ET cell line: The PMC42 parental cells are a stable cell line with enhanced migratory ability and a clear morphological transition when stimulated with EGF (Hugo et al., 2007, Blick et al., 2008, Hugo et al., 2011, Hugo et al., 2013, Cursons et al., 2015). Morphologically, these cells exhibit a slightly elongated and stellate appearance and are adherent to plastic, but may easily be removed from this surface. In addition, these cells are 100% vimentin positive, further supporting the lineage as a mesenchymal-like breast cell line. The PMC42 cells have recently been classified as relatively ‘normal-like’ in terms of microRNA profile (Git et al., 2008), which was previously suggested by their activin receptor status (Liu et al., 1996). Since the
mesenchymal / basal B subgroup of breast cancer cell lines also comprises normal mammary cell lines such as MCF10A, MCF12A and HBL100 (Neve et al., 2006), it is unclear whether PMC42 parental cells are more normal-like or mesenchymal-like. The generation of LA cells with such pronounced epithelial nature also suggests in favor of ‘normal-like’. Preliminary assignment based Affymetrix gene array indeed places these cells in the Basal B subgroup (Tony Blick, Eva Tomascovic-Crook; Personal Communications). The PMC42-ET cells, although already 100% vimentin-positive, show further EMT-like changes after EGF treatment with increased vimentin expression and reduced E-cadherin expression (Blick et al., 2008, Hugo et al., 2011, Cursons et al., 2015).

b) PMC42-LA cell line: The stable subline of the parental PMC42 human breast cancer cell line, PMC42-LA, was originally derived by Ackland and co-workers (Ackland et al., 2001, Ackland et al., 2003). These cells present with an epithelial phenotype, have a distinct cobblestone appearance and adhere well to the cell culture plastic during growth. Hence, PMC42-LA cells show clear phenotypic differences to PMC42-ET cells. PMC42-LA cells also differ from the PMC42-ET line with their markedly lower expression of vimentin (i.e. approximately 10-15% vimentin positivity in culture (Hugo et al., 2007, Blick et al., 2008, Hugo et al., 2011, Cursons et al., 2015). These cells may be induced to undergo epithelial-mesenchymal transition (EMT) processes. For instance, PMC42-LA cells may be stimulated by EGF, leading to a transformation to a ‘relaxed’ epithelial phenotype, and a partial EMT (Ackland et al., 2003, Hugo et al., 2011, Cursons et al., 2015). Furthermore, Lebret and co-workers showed that the PMC42-LA cells can also be induced to undergo an EMT by carcinoma-associated fibroblast secreted factors (CAFs) (Lebret et al., 2006, Lebret et al., 2007). Hence, the PMC42-LA subline is as an epithelial variant of this breast cell line, which presents as a useful model for investigating the process of breast cancer EMT.

In previous studies conducted in this facility, gene expression profiling of the PMC42 human breast cancer cell system was performed using >5000-element breast specific complimentary DNA (cDNA) filter arrays (Isotope filter array; I and Fluorescent cDNA
This was undertaken to identify differentially expressed genes in the PMC42-ET and PMC42-LA cells, with or without EGF exposure. Briefly, PMC42-ET and PMC42-LA cells were treated or untreated with 10ng/μl EGF for either 3hrs or 3 days. Total RNA was harvested from the cells and gene array analysis using cDNA microarrays were subsequently performed to identify candidate genes expressed as a result of this treatment. A list of the array-identified differentially expressed candidate genes in the PMC42-ET and PMC42-LA cells is shown in Table 3.1 (Arvanitis, 2005).

Assessment of the gene expression profiling of the PMC42 cell line resulted in identification of candidate genes differentially expressed in the PMC42-ET and PMC42-LA cells and/or regulated by EGF in either or both cell lines. Many of the changes were validated by RT-PCR and/or Western blot. It is believed that these genes are relevant in promoting or resulting from an EMT. Induction of EMT signalling changes were identified as early as 3hrs post stimulation with EGF, whilst EGF-induced gene expression changes as a result of EMT were monitored 3 days post stimulation with EGF. These time-points were based upon EGF-induced morphological changes in the PMC42 cells.

However, whilst these PMC42 cells can be induced to undergo an EMT, the conditions used to grow and monitor these cells are rather stringent. Factors such as cell confluence have to be monitored, as cell confluency can have a big impact on the expression of EMT like genes.

The questions which remained from this study were whether a reduced cell density would lead to a greater proportion of cells undergoing an EMT in the presence of EGF. Furthermore, would cells grown in less dense (sparse) populations be influenced to undergo mesenchymalisation/late stage EMT changes in the presence of EGF? Thus the hypothesis of this investigation was that culture density and treatment time will affect the degree to which PMC42 cells will undergo EMT with EGF stimulation.
Table 3.1 Summary of Array-Identified Differentially Expressed Genes for the PMC42 Model of EMT (Taken from (Arvanitis, 2005))

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Title</th>
<th>Untreated cell lines</th>
<th>3 hour EGF arrays</th>
<th>3 day EGF arrays</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABLIM1</td>
<td>Actin-binding LIM protein 1</td>
<td></td>
<td>↓ ET</td>
<td></td>
</tr>
<tr>
<td>ACTN1</td>
<td>Actinin alpha 1</td>
<td>ET</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTR1A</td>
<td>ARPI1 (actin-related protein 1, yeast)</td>
<td></td>
<td>↑ ET</td>
<td></td>
</tr>
<tr>
<td>ADRA1B</td>
<td>Adrenergic, alpha-1B-, receptor</td>
<td>LA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKAP12</td>
<td>A kinase (PRKA) anchor protein (gravin) 12</td>
<td></td>
<td>↑ LA</td>
<td>↑ ET</td>
</tr>
<tr>
<td>AKRIB1</td>
<td>Aldo-keto reductase family 1, Member B1</td>
<td></td>
<td>↑ LA</td>
<td></td>
</tr>
<tr>
<td>ANXA1</td>
<td>Annexin A1</td>
<td>LA</td>
<td>↑ ET</td>
<td></td>
</tr>
<tr>
<td>ANXA8</td>
<td>Annexin VIII</td>
<td></td>
<td>↑ ET</td>
<td></td>
</tr>
<tr>
<td>AQP3</td>
<td>Aquaporin 3</td>
<td>LA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRF1</td>
<td>Butyrate response factor (EGF-response factor)</td>
<td></td>
<td>↑ ET</td>
<td></td>
</tr>
<tr>
<td>CACNA2D2</td>
<td>Calcium channel, voltage-dependent, alpha2/delta2 subunit</td>
<td></td>
<td>↑ ET</td>
<td></td>
</tr>
<tr>
<td>CALD1</td>
<td>Caldesmon 1</td>
<td>ET</td>
<td>↓ ET</td>
<td></td>
</tr>
<tr>
<td>CCL2</td>
<td>Chemokine (C-C motif) ligand 2</td>
<td>ET</td>
<td>↓ ET</td>
<td></td>
</tr>
<tr>
<td>CCL8</td>
<td>Chemokine (C-C motif) ligand 8</td>
<td>ET</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1 (PRAD1: parathyroid adenomatosis 1)</td>
<td></td>
<td>↑ ET</td>
<td></td>
</tr>
<tr>
<td>CDH6</td>
<td>Cadherin 6, type 2, K-cadherin</td>
<td>ET</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDH13</td>
<td>Cadherin 13, H-cadherin (heart)</td>
<td>LA</td>
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<td>Coagulation factor VII</td>
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<td>MAX binding protein</td>
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<td>Neimann-Pick disease type C2 gene</td>
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<td>Metalloprotease 1 (pitrilysin family)</td>
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<td>Polo-like kinase 2</td>
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<td>Proteolipid protein 2 (colonic)</td>
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### Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System

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<th>Gene</th>
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<td>PLS3</td>
<td>Plastin 3 (t isoform)</td>
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<td>PROL3</td>
<td>Salivary proline-rich 3</td>
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<td>PTPRN</td>
<td>Protein tyrosine phosphatase, receptor type N</td>
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<td>PTPN13</td>
<td>Protein tyrosine phosphatase, non receptor type 13</td>
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<td>IG2R</td>
<td>Putative Insulin-like growth factor 2 associated protein</td>
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<td>RAB7, member RAS oncogenes family</td>
<td>ET</td>
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<td>REST corepressor 1, KIAA0071 protein</td>
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<td>Ras homolog gene family, member B</td>
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<td>S100 calcium-binding protein A2</td>
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<td>TFPI2</td>
<td>Tissue factor pathway inhibitor 2</td>
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<td>TRIP10</td>
<td>Thyroid receptor interacting protein 10 (CDC42-interacting protein)</td>
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<td>Ezrin (villin 2)</td>
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<td>Vimentin</td>
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<tr>
<td>WFDC2</td>
<td>WAP four disulfide core domain 2 (epididymis specific)</td>
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**ET:** Expressed higher in the PMC42 ET (Parental) cells, **LA:** Expressed higher in the PMC42 LA cells (both untreated).

↑,↓: Arrows indicate up- or down-regulation of genes with EGF in the PMC42 cells.

Therefore, the aims of this part of the thesis were to:

a) Evaluate the morphological and molecular aspects of the PMC42-LA and -ET cells under both sparse and dense conditions, to determine whether cell density affects the extent of EMT in these cells,

b) Establish whether increasing the EGF treatment time in the presence of fewer cells would facilitate the extent of EMT in PMC42-LA and -ET cells.
Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System

3.2 RESULTS

3.2.1 The PMC42 Cell System - A model of EGF-driven EMT: EGF-Induced Morphological Changes under Sparse and Dense Culture Conditions.

As discussed in section 3.1, the PMC42 cell system is represented by the ‘Parental’ cell line, referred to as the PMC42-ET line, and a derived sub-line known as PMC42-LA. To characterize this in vitro model of EMT and obtain further insights into the regulation of EMT by the growth factor EGF, primary cultures of PMC42-ET and PMC42-LA were examined for their response to EGF in either sparse (30-40% confluence) or dense cultures (70-80% confluence).

Under phase contrast microscopy, untreated PMC42-ET cells plated at high density displayed a mixed epithelial compact morphology (Figure 3.1) with frequent cell-cell contact. When seeded at low density on plastic, the sub-confluent PMC42-ET cells showed less cell-cell contact (Figure 3.1). In addition, the PMC42-ET cells formed distinct colonies with rigid edges, designated as “relaxed epithelial” morphology.

Exposure to EGF altered the morphology of the PMC42-ET cells, presenting an epithelial-like compact morphology in dense cultures by 24hrs (Figure 3.1), and a more elongated and spindle-shaped morphology in some of the cells after 72hrs (Figure 3.1). In sparse cultures, the mesenchymal features of the cells were increased and more prominent following EGF treatment, causing the cells to become dramatically more elongated and spindle-shaped at 24hrs and 72hrs (Figure 3.1). EGF-induced morphological changes occurred after 3hr stimulation in the sparse cultures (data not shown), but were more prominent by 72hr EGF stimulation.

In contrast, under sparse conditions, unstimulated PMC42-LA cells grew as patches of high density rather than low density overall and displayed a typical epithelial phenotype in which cells had a uniform cuboidal shape arranged in a pavement pattern (Figure 3.1). When confluent, PMC42-LA cells appeared as a sheet of tightly associated, epithelial-like colonies (Figure 3.1). The results showed subtle morphological changes in dense and sparse cultures when exposed to EGF, whereby the cells became less regular, lost some
of their epithelial morphology and detached from one another, with a halo-like appearance at the cell edge.

EGF-induced morphological changes for both the PMC42-ET and the PMC42-LA were reproducible and distinct after 72hrs, but noticeable within 3 - 6hr treatment (data not shown). Overall, these results show that the EGF-induced PMC42 morphologic changes were more evident under sparse culture conditions.

3.2.2  The PMC42 Cell System-A model of EGF-driven EMT: The impact of Cell Culture Density on EGF-Induced Gene Expression.

Based on the morphological observations described in section 3.2.1, it was likely that the sparse and dense conditions could influence gene expression responses in the PMC42 cell system. The next part of the aim was to establish the expression of certain genes of interest at the mRNA level under both sparse and dense culture conditions.

Results from the previous gene array study of PMC42-ET and PMC42-LA cells (Table 1 above) were examined in conjunction with published reports and other breast cancer cell line array databases (Neve et al., 2006), to identify candidate markers/gene products which may better reflect EMT in human breast cancer systems. For validation of the system, and to better characterise the PMC42 cells, the “classical” EMT genes were pursued for further study. These included vimentin, E-cadherin, and N-cadherin, and the transcription factors: SNAI1 (Snail), SNAI2 (Slug) and TWIST1.
Figure 3.1 EGF-Driven EMT-Like Morphological Changes in Primary Cultures of PMC42 Cells, Under Sparse and Dense Conditions.

Phase contrast images of the major morphological features of PMC42-ET and PMC42-LA cell cultures grown in RPMI medium with 10% FBS when untreated or treated with EGF (10ng/ml) for 24hrs or 72hrs on plastic surfaces under sparse and dense conditions.

a) PMC42-ET cells
b) PMC42-LA cells
Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System
While the gain of vimentin and N-cadherin, and the loss of E-cadherin may be among the initial events representative of EMT, alterations in the expression of other adhesion molecules are also reported to occur (Hanahan and Weinberg, 2000, Gloushankova et al., 2017, Hagen, 2017, Nieberler et al., 2017, Boesch et al., 2018, Pal et al., 2018) to generate a mesenchymal phenotype. As such, the integrin family of adhesion molecules, which represent the major receptors that mediate attachment to the ECM, have been shown to play critical roles in the capability of tumour cells for tissue invasion and metastasis (Hanahan and Weinberg, 2000, Gloushankova et al., 2017, Hagen, 2017, Nieberler et al., 2017, Boesch et al., 2018, Pal et al., 2018).

Derangement of integrin expression has been studied and shown to be responsible for a number of aberrant cellular activities during tumour onset, progression, and metastatic regulated during EMT. As such, integrin dynamics were also analysed as a function of the EMT in the PMC42 model of human breast cancer. Specifically, the integrins (α- and β-chains) and ILK were investigated. As ITGa3 was regulated co-ordinately with EMT on the gene array data of the PMC42 system, this marker was investigated, along with a panel of other α- and β-integrin markers (α1, α2, α3, α6, α10, α11, β1, β3, β4 and β6).

At the end of morphological studies (section 3.2.1), a primary screen on the above chosen targets was performed in order to prioritize the number of candidate genes assessed. Quantitative reverse transcriptase polymerase chain reaction (Q-RT-PCR) was performed on the total mRNA extracts from the PMC42-ET and PMC42-LA cells grown under either sparse or dense conditions, with or without EGF-induced EMT.

The expression levels of these chosen targets revealed mixed modulation of the genes. Results are of one experiment, representative of two independent experiments. These results are described in detail in section 3.2.3 below. The levels of mRNA are represented as δCt, representative of the differences between the expressions of each of the genes of interest normalised to ribosomal protein L32 (RPL32) mRNA. δCt represents expression on a log2 scale relative to the highly abundant RPL32 mRNA.
Q-RT-PCR analyses of PMC42-ET and PMC42-LA cultures, seeded at different cell densities, manifested density-dependent regulation of the “classical” EMT markers (vimentin, E-cadherin, and N-cadherin) at the RNA level (Figure 3.2). In both the sparse and dense conditions, the baseline levels of vimentin message (Figure 3.2), is higher in the PMC42-ET cells than in the PMC42-LA cells. The increase in vimentin mRNA expression in the sparse EGF-stimulated PMC42-LA cells was more marked than it was in the EGF-stimulated PMC42-ET cells. The PMC42-ET cells exhibit high vimentin expression and do not appear to express more with EGF stimulation.

In the sparse PMC42-LA cells, the increase in vimentin mRNA with 72hr EGF treatment was ~8.5-fold ($\delta C_t = -4.8, -EGF; \delta C_t = -1.7, 72\text{hr EGF}$), and ~2-fold ($\delta C_t = -1.4, -EGF; \delta C_t = -0.3, 72\text{hr EGF}$) in the sparse PMC42-ET cells. In the dense EGF-treated PMC42-LA cells, the increase in vimentin mRNA expression was ~5.7-fold ($\delta C_t = -3.2, -EGF; \delta C_t = -0.7, 72\text{hr EGF}$) and by ~2-fold ($\delta C_t = -1.8, -EGF; \delta C_t = -0.7, 72\text{hr EGF}$) in the PMC42-ET cells.

Under sparse conditions, N-cadherin mRNA levels in the PMC42-LA cells was increased with 72hr EGF treatment, with the increase being ~2-fold ($\delta C_t = -4.1, -EGF; \delta C_t = -3.1, 72\text{hr EGF}$). In the sparse PMC42-ET cells, treatment with EGF for 72hr did not exhibit a difference in N-cadherin mRNA levels ($\delta C_t = -4.2, -EGF; \delta C_t = -4.3, 72\text{hr EGF}$). In the dense conditions, neither EGF-treated PMC42-LA nor PMC42-ET cells exhibited a change in N-cadherin mRNA levels (Figure 3.2).

In both the sparse and dense conditions, the baseline levels of E-cadherin message, is higher in the PMC42-LA cells than in the PMC42-ET cells. EGF treated PMC42-LA cells did not exhibit a difference in E-cadherin mRNA expression levels in either sparse or dense conditions (Figure 3.2). On the other hand, EGF-treated PMC42-ET
Figure 3.2 Vimentin, N-cadherin and E-cadherin mRNA Expression in EGF-Induced PMC42 Cells- Sparse vs. Dense.

PMC42-ET and PMC42-LA cells were grown in sparse and dense cultures and either left un-stimulated (0hr) or stimulated with 10ng/ml EGF for 24 and 72hrs. RNA was harvested from these cells utilizing the QIAGEN RNeasy kit. 100ng of RNA was reverse transcribed using gene-specific primers, and probed with primers specific for human vimentin, N-cadherin, E-cadherin and L32 ribosomal housekeeping gene.

Q-RT-PCR analyses of vimentin, N-cadherin and E-cadherin mRNA was performed in quadruplicate on total RNA extracted from the un-stimulated or stimulated PMC42-ET and PMC42-LA cells.

Expression levels of vimentin, N-cadherin and E-cadherin mRNA were normalized to L32 ribosomal protein mRNA levels. Relative quantitation was performed by normalizing the Ct values of each sample gene with Ct values of L32.

The average δCt values for each sample are shown with standard deviation, representing the relative amounts of human vimentin, N-cadherin and E-cadherin mRNA present in relation to L32. Results are from one experiment, representative of two independent experiments. Error bars represent standard deviation.
Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System

Vimentin mRNA Expression

N-cadherin mRNA Expression

E-cadherin mRNA Expression
cells exhibited a ~79-fold (ΔCt= -9.5, –EGF; ΔCt= -15.8, 72hr EGF) decrease in E-cadherin mRNA in sparse conditions and a ~12-fold (ΔCt= -9.5, –EGF; ΔCt= -13.1, 72hr EGF) decrease in E-cadherin mRNA levels in dense conditions.

Additionally, expression of transcriptional repressors that regulate E-cadherin expression (TWIST1, SNAI1, and SNAI2) were also assessed in the EGF-treated sparse and dense PMC42 cells (Figure 3.3). Under sparse conditions, the increase in TWIST1 mRNA (Figure 3.3) was ~2-fold (ΔCt= -10.2, –EGF; ΔCt= -9.2, 72hr EGF) in the 72hr EGF-treated PMC42-LA cells and ~4-fold (ΔCt= -11.7, –EGF; ΔCt= -9.6, 72hr EGF) in the 72hr EGF-treated PMC42-ET cells. In the dense conditions, regulation of TWIST1 mRNA levels in both PMC42-LA and PMC42-ET cells was not detected with 72hrs of EGF treatment.

Similarly, increases in SNAI1 mRNA expression levels were measured in sparse conditions following 72hr EGF-treatment, with the increase being ~3.5-fold (ΔCt= -10.8, –EGF; ΔCt= -9.0, 72hr EGF) in the PMC42-LA cells and by ~3-fold (ΔCt= -10.2, –EGF; ΔCt= -8.6, 72hr EGF) in the PMC42-ET cells (Figure 3.3). In the dense conditions, regulation of SNAI1 mRNA in both PMC42-LA and PMC42-ET cells was not detected with 72hr EGF treatment.

SNAI2 mRNA levels were only detected in the sparse conditions, where there was an ~5-fold (ΔCt= -9.8, –EGF; ΔCt= -7.5, 72hr EGF) increase in the 72hr EGF-treated PMC42-LA cells and ~6-fold (ΔCt= -10, –EGF; ΔCt= -7.4, 72hr EGF) increase in the 72hr EGF-treated PMC42-ET cells (Figure 3.3).

This study revealed that integrins were affected by cell density in the PMC42 cellular model treated with EGF (Figure 3.4). Integrins specifically shown to be regulated with EGF exhibited stronger increases in mRNA expression in sparse cultures. In contrast, expression changes were much lower / undetected in more dense cultures. EGF-treated PMC42-LA and PMC42-ET cells in either sparse or dense conditions, did not exhibit a measured change in ITGα1, ITGα10, ITGα11, or ITG β6 mRNA expression levels (Figure 3.4).
Figure 3.3 TWIST1, SNAI1 and SNAI2 mRNA Expression in EGF-Induced PMC42 Cells- Sparse vs. Dense.

PMC42-ET and PMC42-LA cells were grown in sparse and dense cultures and either left un-stimulated (0hr) or stimulated with 10ng/ml EGF for 24 and 72hrs. RNA was harvested from these cells utilizing the QIAGEN RNeasy kit. 100ng of RNA was reverse transcribed using gene-specific primers, and probed with primers specific for the E-cadherin transcriptional repressors human TWIST1, SNAI1, SNAI2 and L32 ribosomal housekeeping gene.

Q-RT-PCR analyses of TWIST1, SNAI1, and SNAI2 mRNA was performed in quadruplicate on total RNA extracted from the un-stimulated or stimulated PMC42-ET and PMC42-LA cells.

Expression levels of TWIST1, SNAI1, and SNAI2 mRNA were normalized to L32 ribosomal protein mRNA levels. Relative quantitation was performed by normalizing the Ct values of each sample gene with Ct values of L32.

The average δCt values for each sample are shown with standard deviation, representing the relative amounts of human TWIST1, SNAI1, and SNAI2 mRNA present in relation to L32. Results are from one experiment, representative of two independent experiments. Error bars represent standard deviation.
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**TWIST1 mRNA Expression**

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**SNAI1 mRNA Expression**

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**SNAI2 mRNA Expression**

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In both the sparse and dense conditions, the baseline levels of ITGα2 message (Figure 3.4) are higher in the PMC42-ET cells than in the PMC42-LA cells. Under sparse conditions, the expression of ITGα2 increased in both the EGF-stimulated PMC42-ET and PMC42-LA cell lines. In PMC42-LA cells, there was an ~9.8-fold (δCt= -7.7, –EGF; δCt= -4.4, 72hr EGF) increase in mRNA levels by 72hr EGF treatment, where as in PMC42-ET cells, there was an ~7-fold (δCt= -7.1, –EGF; δCt= -4.3, 72hr EGF) increase by 72hrs. In the dense conditions, neither EGF-treated PMC42-LA nor PMC42-ET cells exhibited a difference in ITGα2 mRNA levels (Figure 3.4).

In both the sparse and dense conditions, the baseline levels of ITGα3 message (Figure 3.4), is higher in the PMC42-ET cells than in the PMC42-LA cells. EGF-treated PMC42-LA cells exhibited a ~4-fold (δCt= -4.3, –EGF; δCt= -2.2, 72hr EGF) increase in ITGα3 mRNA levels; whereas EGF treated PMC42-ET cells exhibited a ~3-fold (δCt= -3.1, –EGF; δCt= -1.6, 72hr EGF) increase in ITGα3 mRNA expression levels under sparse conditions. In the EGF-treated PMC42-LA and PMC42-ET cells grown under dense conditions, there was no detectable change in ITGα3 mRNA levels (Figure 3.4).

The expression of ITGα6 mRNA (Figure 3.4) increased by ~6.5-fold (δCt= -7.5, –EGF; δCt= -4.8, 72hr EGF) with 72hr EGF treatment in the sparse PMC42-LA cells, and by ~5.6-fold (δCt= -6.8, –EGF; δCt= -4.3, 72hr EGF) in the sparse PMC42-ET cells. In the dense PMC42-LA cells, the difference in ITGα6 mRNA expression was an increase of ~1.8-fold (δCt= -7.0, –EGF; δCt= -6.2, 72hr EGF) with 72hr EGF treatment. In the dense PMC42-ET cells, expression of ITGα6 mRNA increased by ~1.5-fold (δCt= -7.3, –EGF; δCt= -6.7, 72hr EGF) with 72hr EGF treatment (Figure 3.4).

In both the sparse and dense conditions, the baseline levels of ITGβ1 message (Figure 3.4), is higher in the PMC42-ET cells than in the PMC42-LA cells. ITGβ1 was clearly expressed in the PMC42 cells under both sparse and dense conditions.
Figure 3.4 Integrin mRNA Expressions in EGF-Induced PMC42 Cells- Sparse vs. Dense.

PMC42-ET and PMC42-LA cells were grown in sparse and dense cultures and either left un-stimulated (0hr) or stimulated with 10ng/ml EGF for 24 and 72 hrs. RNA was harvested from these cells utilizing the QIAGEN RNeasy kit. 100ng of RNA was reverse transcribed using gene-specific primers, and probed with primers specific for human α- and β-integrins and L32 ribosomal housekeeping gene.

Q-RT-PCR analyses of α- and β-integrins and mRNA was performed in quadruplicate on total RNA extracted from the un-stimulated or stimulated PMC42-ET and PMC42-LA cells.

Expression levels of α- and β-integrins mRNA were normalized to L32 ribosomal protein mRNA levels. Relative quantitation was performed by normalizing the Ct values of each sample gene with Ct values of L32.

The average δCt values for each sample are shown with standard deviation, representing the relative amounts of human α- and β-integrins and mRNA present in relation to L32. Results are from one experiment, representative of two independent experiments. Error bars represent standard deviation.
Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System

### α1 Integrin mRNA Expression

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### α2 Integrin mRNA Expression

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### α3 Integrin mRNA Expression

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Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System

\[ \delta Ct \]

\[ \alpha_6 \text{ Integrin mRNA Expression} \]

\[ \delta Ct \]

\[ \alpha_{10} \text{ Integrin mRNA Expression} \]

\[ \delta Ct \]

\[ \alpha_{11} \text{ Integrin mRNA Expression} \]

\[ \delta Ct \]
Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System
Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System

**β4 Integrin mRNA Expression**

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**β6 Integrin mRNA Expression**

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<td>PMC 42 ET Dense</td>
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In sparse PMC42-LA cells, there was an increase of ~4.6-fold ($\delta Ct = -6.1$, -EGF; $\delta Ct = -3.9$, 72hr EGF) in ITG$\beta$1 expression, and an ~3.5-fold ($\delta Ct = -4.3$, -EGF; $\delta Ct = -2.5$, 72hr EGF) increase in ITG$\beta$1 expression in the PMC42-ET cells with 72hr EGF stimulation. In the dense conditions, the increase in ITG$\beta$1 mRNA levels was ~1.9-fold ($\delta Ct = -6.0$, -EGF; $\delta Ct = -5.1$, 72hr EGF) in the PMC42-LA cells and ~2.3-fold ($\delta Ct = -5.0$, -EGF; $\delta Ct = -3.8$, 72hr EGF) in the PMC42-ET cells with 72hr EGF stimulation (Figure 3.4).

In both the sparse and dense conditions, the baseline levels of ITG$\beta$3 message (Figure 3.4), was higher in the PMC42-ET cells than in the PMC42-LA cells. In the sparse PMC42-LA cells, there was an increase in ITG$\beta$3 mRNA expression with 72hr EGF treatment of ~4.3-fold ($\delta Ct = -13.6$, -EGF; $\delta Ct = -11.5$, 72hr EGF), and an increase of ~2.5-fold ($\delta Ct = -11.2$, -EGF; $\delta Ct = -9.9$, 72hr EGF) in the PMC42-ET cells. In the EGF-treated PMC42-LA and PMC42-ET cells grown under dense conditions, there was no detectable change in ITG$\beta$3 mRNA levels (Figure 3.4).

Under sparse conditions, there was a measured ~2-fold ($\delta Ct = -5.2$, -EGF; $\delta Ct = -4.2$, 72hr EGF) increase in ITG$\beta$4 mRNA levels (Figure 3.4) in the PMC42-LA cells with 72hr EGF treatment, whilst in the PMC42-ET cells, there was a measured ~4-fold ($\delta Ct = -7.2$, -EGF; $\delta Ct = -5.2$, 72hr EGF) increase in ITG$\beta$4 mRNA levels. In both the EGF-treated PMC42-LA and PMC42-ET cells grown under dense conditions, there was no detectable change in ITG$\beta$4 mRNA levels (Figure 3.4).

As shown for the morphological responses to EGF, sparse cultures were also more highly responsive in terms of gene expression responses.

3.2.3 Expression of known “classical” EMT markers in the PMC42 cell system:

Changes at the RNA and protein levels:

To confirm whether the morphological and gene expression changes identified under sparse conditions correlated with the loss of epithelial and the gain of mesenchymal marker expression, the time-course used to investigate these responses in each cell line under sparse conditions was expanded. This was to determine whether the genes that
were regulated with EGF treatment could be considered “early onset” genes, and thus may be critical to driving the EMT or “late onset” genes more likely to mediate the EMT phenotype.

PMC42-ET and PMC42-LA cells were grown sparsely (30-40%) in culture for the same period of time and either left un-stimulated (0hr) or stimulated with 10ng/ml EGF for 1.5, 3, 6, 24, 48, and 72hrs, in reverse addition. Total RNA and protein were extracted as described in Chapter 2.7, and Q-RT-PCR and Western Immunoblotting performed.

The measure of mRNA levels of these genes was represented as fold-induction. The fold induction is representative of the differences between the expressions of each of the gene of interest (i.e. vimentin, E-cadherin, N-cadherin) compared to the human L32 ribosomal protein housekeeping gene, relative to untreated cells (See Chapter 2.8.3).

For all experiments, equivalent concentrations of protein from cell lysates were resolved by SDS-PAGE electrophoresis, transferred to nitrocellulose, and immunoblotted with antibodies specific for vimentin, E-cadherin or N-cadherin. Pan-actin was used as an internal control for estimation of protein loading.

**3.2.3.1 Vimentin mRNA and Protein Expression in PMC42-ET and LA cells Following EGF Treatment.**

The expression of vimentin is characteristic of mesenchymal cells and is absent or weakly expressed in epithelial cell lineages. The PMC42 cell line clearly expressed vimentin (Figure 3.5). In PMC42-ET cells, there was ~2.6-fold increase (δCt= -1.2, –EGF; δCt= 0.2, 72hr EGF) in vimentin mRNA levels (Figure 3.5a) with EGF treatment in the timecourse, reaching plateau by 24hrs. In PMC42-LA cells, there was approximately ~9-fold increase (δCt= -4.3, –EGF; δCt= -1.2, 72hr EGF) in vimentin mRNA levels with EGF treatment in the time-course, where the maximal increase in mRNA levels was again seen by 24hrs.

Immunoblotting of protein extracts from PMC42 cells revealed prominent bands of vimentin at approximately 55kDa (Figure 3.5b). Increased vimentin protein levels were
Figure 3.5 Expression Levels of Vimentin in the PMC42 Human Breast Cancer Cell Line System.

PMC42-ET and PMC42-LA cells were grown in culture and either left untreated or stimulated with 10ng/ml EGF for 1.5, 3, 6, 24, 48, and 72 hrs.

a) RNA was harvested from these cells utilizing the QIAGEN RNaseasy kit. Following quantitation of RNA, 100ng of RNA was reverse transcribed using gene-specific primers, and probed with primers specific for human vimentin and L32. Q-RT-PCR analysis of vimentin mRNA was performed in quadruplicate on total RNA extracted from the un-stimulated or EGF-stimulated PMC42-ET and PMC42-LA cells. Expression levels of vimentin mRNA were normalized to L32 ribosomal protein mRNA levels. Relative quantitation was performed by normalizing the Ct values of each sample gene with Ct values of L32. Data is presented as the linearised fold-change relative to untreated cells, and set to 1. Results are representative of two independent experiments.

b) Total protein was extracted from the cells, and 20µg of protein were subjected to SDS-PAGE electrophoresis. Protein was transferred to a nitrocellulose membrane and probed with Vimentin and Pan-actin antibodies. Results are of one Western blot representative of two individual experiments.
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a)

b)
seen in both PMC42-ET and PMC42-LA cells after EGF treatment, consistent with the Q-RT-PCR results. Vimentin protein increased co-ordinately with mRNA. Both mRNA and protein levels of vimentin were elevated markedly in the PMC42-ET line compared to the more epithelial subline, PMC42-LA.

Furthermore, when assessing the optimal time point by which vimentin expression was most affected upon stimulation with EGF, more pronounced expression in the EGF-stimulated PMC42-ET cells was observed after 24hrs. Therefore, not only was there altered mRNA but this change correlated with the same trend in protein expression for vimentin. Upon EGF stimulation, vimentin expression remained close to baseline expression between 48hrs (δCt= -1.5) and 72hrs (δCt= -1.2), where there was a marked increase in expression levels in PMC42-LA cells.

3.2.3.2 E-cadherin mRNA and Protein Expression in PMC42-ET and LA Cells Following EGF Treatment.

Confirmation that expression of E-cadherin was altered in the PMC42 cells as a result of EGF stimulation was carried out. As the PMC42 cells acquired a more elongated and less attached phenotype, it was expected that E-cadherin function in maintaining cell-cell contact was changed in these cells. Specifically, analysis of E-cadherin mRNA levels (Figure 3.6a) in the PMC42-LA cells showed no significant changes after 72hrs of EGF stimulation (δCt= -5.1, -EGF; δCt= -5.2, 72hr EGF). In PMC42-ET cells, there was a ~12 fold (δCt= -8.5, -EGF; δCt= -12.1, 72hr EGF) reduction in E-cadherin expression with EGF-treatment, with maximal decrease evident by 24hr stimulation with EGF (δCt= -11.2).

E-cadherin, as detected by Western immunoblotting, ran as a 120kDa protein (Figure 3.6b). Prior to EGF stimulation, there was high expression of E-cadherin protein in the PMC42-LA cells. Following 72hr stimulation with EGF, protein levels were markedly reduced, with maximal reduction seen at the end timepoint (72hrs). In contrast, a weak signal was observed prior to incubation with EGF for E- cadherin in the PMC42-ET cells.
Figure 3.6 Expression Levels of E-cadherin in the PMC42 Human Breast Cancer Cell Line System.

PMC42-ET and PMC42-LA cells were grown in culture and either left untreated or stimulated with 10ng/ml EGF for 1.5, 3, 6, 24, 48, and 72 hrs.

a) RNA was harvested from these cells utilizing the QIAGEN RNeasy kit. Following quantitation of RNA, 100ng of RNA was reverse transcribed using gene-specific primers, and probed with primers specific for human E-cadherin and L32. Reaction Q-RT-PCR analysis of E-cadherin mRNA was performed in quadruplicate on total RNA extracted from the un-stimulated or EGF-stimulated PMC42-ET and PMC42-LA cells. Expression levels of E-cadherin mRNA were normalized to L32 ribosomal protein mRNA levels. Relative quantitation was performed by normalizing the Ct values of each sample gene with Ct values of L32. Data is presented as the linearised fold-change relative to untreated cells, and set to 1. Results are from one experiment, representative of two independent experiments.

b) Total protein was extracted from the cells, and 20µg of protein was subjected to SDS-PAGE electrophoresis. Protein was transferred to a nitrocellulose membrane and probed with E-cadherin and Pan-actin antibodies. Results are of one Western blot, representative of two individual experiments.
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a)

![Graph showing fold induction (relative to untreated cells) over time for PMC42 LA and PMC42 ET cultures.]

b)

![Image showing Western blot analysis for E-cadherin and Pan actin with EGF-stimulation at different time points.]

E-cadherin (120kDa)

Pan actin (42kDa)
Exposure to EGF resulted in the diminishing of E-cadherin in a time-dependent manner by 72hrs.

3.2.3.3 N-cadherin mRNA and Protein Expression in PMC42-ET and LA Cells Following EGF Treatment.

N-cadherin, normally present mainly in neuronal and muscle tissues, is aberrantly expressed in epithelial tumours. It was therefore of interest to investigate if N-cadherin might be up-regulated in the PMC42-ET and LA cell system, specifically to address whether its expression is altered in normal epithelial cells undergoing EMT.

N-cadherin mRNA expression (Figure 3.7a) was assessed in both PMC42-ET and LA cells. An ~2.6-fold increase ($\delta$Ct= -4.9, –EGF; $\delta$Ct= -3.5, 72hr EGF) in N-cadherin mRNA expression levels was detected in the PMC42-LA cells with EGF stimulation, with higher levels of N-cadherin being detected at 24hrs ($\delta$Ct= -3.6), and remaining steady throughout until 72hrs. In the PMC42-ET cells, there is no detectable change in N-cadherin with EGF stimulation ($\delta$Ct= -4.9, –EGF; $\delta$Ct= -4.6, 72hr EGF).

Immunoblotting of protein extracts from PMC42 cells revealed prominent bands of N-cadherin expression at 135kDa (Figure 3.7b). N-cadherin protein levels were not detected prior to EGF stimulation. However, with EGF stimulation, N-cadherin protein was increased in a time-dependent manner, with detection occurring at 3hrs and increasing at 72hrs. Conversely, PMC42-ET cells showed a minor increase in N-cadherin expression in response to EGF stimulation, with maximal expression occurring by 6hrs and maintained throughout.

Therefore, this investigation has shown that stimulation of sparse PMC42-ET and PMC42-LA cells with EGF satisfy the most basic criteria for EMT, therefore validating these samples as being appropriate for further analysis of other potential markers or mechanisms of EMT.
Figure 3.7 Expression Levels of N-cadherin in the PMC42 Human Breast Cancer Cell Line System.

PMC42-ET and PMC42-LA cells were grown in culture and either left untreated or stimulated with 10ng/ml EGF for 1.5, 3, 6, 24, 48, and 72 hrs.

a) RNA was harvested from these cells utilizing the QIAGEN RNeasy kit. Following quantitation of RNA, 100ng of RNA was reverse transcribed using gene-specific primers, and probed with primers specific for human N-cadherin and L32. Q-RT-PCR analysis of N-cadherin mRNA was performed in quadruplicate on total RNA extracted from the un-stimulated or EGF-stimulated PMC42-ET and PMC42-LA cells. Expression levels of N-cadherin mRNA were normalized to L32 ribosomal protein mRNA levels. Relative quantitation was performed by normalizing the Ct values of each sample gene with Ct values of L32. Data is presented as the linearised fold-change relative to untreated cells, and set to 1. Results are from one experiment, representative of two independent experiments.

b) Total protein was extracted from the cells, and 20µg of protein was subjected to SDS-PAGE electrophoresis. Protein was transferred to a nitrocellulose membrane and probed with N-cadherin and Pan-actin antibodies. Results are of one Western blot representative of two individual experiments.
Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System

a)

![Graph showing fold induction of PMC42 LA and PMC42 ET over time](image)

b)

![Western blot images showing N-cadherin and Pan actin](image)
3.2.4 **ITGβ1, ITGa2, and ILK as Markers of EGF-Driven EMT in the PMC42 Cell System.**

Since both ITGa2 and ITGβ1 subunits were shown to be regulated the most amongst the panels of integrins investigated under sparse conditions in section 3.2.2, the transcriptional regulation of ITGa2 and ITGβ1 in breast cancer cells under EGF stimulus was assessed. Furthermore, as the serine/threonine kinase ILK, interacts with the cytoplasmic domains of ITGβ1, this marker was also examined under EGF stimulus.

The changes in the classic EMT markers across this timecourse (Figures 3.5-3.7) was accompanied by an up-regulation of ITGa2 (Figure 3.8) and ITGβ1 (Figure 3.9) mRNA as well as ILK (Figure 3.10) mRNA in the PMC42 cells.

Q-RT-PCR analysis revealed an increase in ITGa2 mRNA expression level by ~4.6-fold in the 72hr EGF treated PMC42-LA cells (ΔCt = -6.7, –EGF; ΔCt = -4.5, 72hr EGF), (Figure 3.8), with maximal increase shown at 3-6hrs, reaching plateau by the 24hr mark. In PMC42-ET cells, ITGa2 mRNA expression was upregulated by 3hrs, with maximal expression seen at 6hrs, at which it reached a plateau. There was ~5.7-fold increase in mRNA expression (ΔCt = -7.0, –EGF; ΔCt = -4.5, 72hr EGF).

Expression levels of ITGβ1 mRNA increased by ~2.3-fold in PMC42-LA cells (Figure 3.9) with EGF treatment (ΔCt = -2.7, –EGF; ΔCt = -1.5, 72hr EGF). In PMC42-ET cells, there was an indication of a modest up-regulation of ITGβ1 following EGF treatment (ΔCt = -2.5, –EGF; ΔCt = -1.8, 72hr EGF).

By Q-RT-PCR, the ILK mRNA expression was upregulated ~3.2-fold in the 72hr EGF treated PMC42-LA cells (ΔCt = -6.7, –EGF; ΔCt = -5.0, 72hr EGF) (Figure 3.10), with an increase seen at 1.5hrs EGF stimulation and reaching maximum by 72hr EGF stimulation. In PMC42-ET cells, ILK mRNA expression was upregulated by 1.5-3hrs, with maximal expression seen at 72hrs. There was an increase in ILK mRNA expression of up to ~4-fold (ΔCt = -7.4, –EGF; ΔCt = -5.5, 72hr EGF).
Figure 3.8 mRNA Expression Levels of ITGα2 in the PMC42 Human Breast Cancer Cell Line System.

PMC42-ET and PMC42-LA cells were grown in culture and either left untreated or stimulated with 10ng/ml EGF for 1.5, 3, 6, 24, 48, and 72 hrs. RNA was harvested from these cells utilizing the QIAGEN RNaseasy kit. One-hundred nanograms (100ng) of RNA was reverse transcribed using gene-specific primers, and probed with primers specific for human ITGα2 and L32.

Quantitative Reverse Transcription Polymerase Chain Reaction (Q-RT-PCR) analysis of ITGα2 mRNA was performed in quadruplicate on total RNA extracted from the un-stimulated or EGF-stimulated PMC42-ET and PMC42-LA cells. Expression levels of ITGα2 mRNA were normalized to L32 ribosomal protein mRNA levels. Relative quantitation was performed by normalizing the Ct values of each sample gene with Ct values of L32. Data is presented as the linearized fold-change relative to untreated cells, and set to 1. Results are representative of two independent experiments.
Figure 3.9 mRNA Expression Levels of ITGβ1 in the PMC42 Human Breast Cancer Cell Line System.

PMC42-ET and PMC42-LA cells were grown in culture and either left untreated or stimulated with 10ng/ml EGF for 1.5, 3, 6, 24, 48, and 72 hrs. RNA was harvested from these cells utilizing the QIAGEN RNeasy kit. 100ng of RNA was reverse transcribed using gene-specific primers, and probed with primers specific for human ITGβ1 and L32. Quantitative Reverse Transcription Polymerase Chain Reaction (Q-RT-PCR) analysis of ITGβ1 mRNA was performed in quadruplicate on total RNA extracted from the unstimulated or EGF-stimulated PMC42-ET and PMC42-LA cells. Expression levels of ITGβ1 mRNA were normalized to L32 ribosomal protein mRNA levels. Relative quantitation was performed by normalizing the Ct values of each sample gene with Ct values of L32. Data is presented as the linearized fold-change relative to untreated cells, and set to 1. Results are representative of two independent experiments.
PMC42-ET and PMC42-LA cells were grown in culture and either left untreated or stimulated with 10ng/ml EGF for 1.5, 3, 6, 24, 48, and 72hrs. RNA was harvested from these cells utilizing the QIAGEN RNeasy kit. 100ng of RNA was reverse transcribed using gene-specific primers, and probed with primers specific for human ILK and L32. Quantitative Reverse Transcription Polymerase Chain Reaction (Q-RT-PCR) analysis of ILK mRNA was performed in quadruplicate on total RNA extracted from the unstimulated or EGF-stimulated PMC42-ET and PMC42-LA cells. Expression levels of ILK mRNA were normalized to L32 ribosomal protein mRNA levels. Relative quantitation was performed by normalizing the Ct values of each sample gene with Ct values of L32. Data is presented as the linearized fold-change relative to untreated cells, and set to 1. Results are representative of two independent experiments.
Therefore, upon assessment of the data derived in this part of the thesis, it can be concluded that the EMT phenotype acquired following EGF incubation was accompanied by an up-regulation of ITGα2 and ITGβ1 as well as ILK mRNA in the PMC42 cells, which were seen to be induced from as early as 3hr-6hrs and 1.5hrs, respectively. These data show that ITGα2 and ITGβ1 as well as ILK are specifically altered in early EMT, and may in fact be relevant for early induction of EMT events upon stimulation with EGF.

3.2.5 **MT-PCR Analysis of EGF-Induced PMC42 Cells:**

The next part of the project used a multiplex tandem Q-RT-PCR (MT-PCR) approach to further screen for and identify genes involved in the EMT response to EGF in the PMC42 cell system.

MT-PCR is a cost-effective two-step PCR process for highly multiplexed gene expression profiling. It enables the expression of multiple genes to be analysed simultaneously with PCR accuracy. In step one, the RNA is converted into cDNA and amplified using multiplexed gene-specific primers. In step two, expression of each individual gene is quantitated by PCR using a pre-fabricated Gene-Disc (Corbett Research, Australia). One advantage of MT-PCR is that it can be configured to profile the gene expression of up to 72 or more genes on a disc from a sample as small as 10 pg of RNA in <90 min.

In this study, two commercially designed MT-PCR discs comprising genes believed to be involved in the EMT process, including the “classical” EMT genes, as well as a selection of integrins and ILK, were quantitated. The MT-PCR format used investigated approximately 66 genes and identified gene expression changes over an extensive timecourse in both cell types (PMC42-ET and PMC42-LA), consisting of RNA from untreated sparse cultures as well RNA from sparse cultures treated with EGF for periods of time ranging from 1.5 to 72hrs. This RNA was the same as the RNA used in section 3.2.4.
A supervised uncentered correlation hierarchical clustering (centroid linkage) method was used to group genes based on the similarity of their expression pattern. The results of the hierarchical clustering are presented as dendogram trees displayed next to the expression matrix (heat map) (Figure 3.11), in which the distance along the tree from each element represents their relative degree of similarity.

The expression value of each gene is represented by the colour of the corresponding cell in the matrix, and creates a visual representation of the qualitative and quantitative gene expression patterns across the timecourse experiment in each of the PMC42-LA (Figure 3.11a) and PMC42-ET (Figure 3.11b) cell lines. A striking property of the clustered images in Figure 3.11 is the presence of large contiguous patches of colour that represent groups of genes that share similar expression patterns with EGF treatment over a period of 72hrs. In these matrixes, the genes are arranged on the vertical axis and the EGF-treated time-points are presented on the horizontal axis. The colour of each square represents the measured gene expression ratio where red indicates high gene expression (upregulated), green indicates low gene expression (downregulated), and black indicates no difference in gene expression with EGF treatment over a period of 72hrs in the PMC42-ET and PMC42-LA cells. Figure 3.12 further presents a more comprehensive interpretation of the level of gene expression changes for all changes examined with MT-PCR. Detection and identification of these genes by MT-PCR demonstrated correlation with the RT-PCR results, where the expression levels of the classical EMT markers vimentin, and N-cadherin were upregulated, and E-cadherin was downregulated, and ITGa2, and ILK were also upregulated.
Figure 3.11 Cluster Analysis of MT-PCR Gene Expression in the Novel Human Breast Cancer Cell Line System, PMC42.

Clustered display of data from the timecourse of EGF stimulated PMC42 cells. Briefly, PMC42 cells were grown in culture and either left un-stimulated (0hr) or stimulated with 10ng/ml EGF for 1.5, 3, 6, 24, 48, and 72hrs. Total RNA was extracted from the PMC42-LA cell lines utilizing the QIAGEN RNeasy kit, and 100ng of each was subjected to MT-PCR using 15 cycles of multiplex pre-amplification.

Results of the hierarchical clustering are presented as dendogram trees displayed next to the expression matrix. The distance along the tree from each element represents groups of genes that share similar expression patterns with EGF treatment over a period of 72hrs. In these matrixes, the genes are arranged on the vertical axis and the EGF-treated time-points are presented on the horizontal axis. The colour of each square represents the measured gene expression ratio where red indicates high gene expression (upregulated), green indicates low gene expression (downregulated), and black indicates no difference in gene expression with EGF treatment over a period of 72hrs in the PMC42 cells.

a) PMC42-LA cells  
b) PMC42-ET cells
Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System
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Figure 3.12 MT-PCR Gene Expression in the Novel Human Breast Cancer Cell Line System, PMC42.

Briefly, PMC42 cells were grown in culture and either left un-stimulated (0hr) or stimulated with 10ng/ml EGF for 1.5, 3, 6, 24, 48, and 72hrs. Total RNA was extracted from the PMC42-LA cell lines utilizing the QIAGEN RNeasy kit, and 100ng of each was subjected to MT-PCR using 15 cycles of multiplex pre-amplification.

Relative quantitation was performed by normalizing the Ct values of each sample gene with Ct values of NONO. The average δCt values for each sample are shown in relation to NONO. Results are from one experiment.

a) PMC42-ET  
b) PMC42-LA
Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System

a)

![Graph showing gene expression changes](image-url)
Chapter 3: Effects of Culture-Density and Time-Course on Candidate Epithelial Mesenchymal Transition (EMT) Markers/Effectors in the PMC42 Human Breast Cancer Model System

b)
3.3 DISCUSSION

The current data derived in this chapter gives an insight into the effects of cell culture density on EGF-induced EMT in the PMC42 human breast cancer cell system. It expands from previous analyses conducted in this facility, where EGF was shown to effectively induce EMT-like events in PMC42 cells without cell density being closely monitored (Ackland et al., 2003).

The PMC42 ET parental cell line was compared to the PMC42 LA subline with respect to morphological and molecular aspects under sparse and dense culture conditions. Further to that, an extensive time-course of the PMC42 cell line was further studied to determine and confirm factors that regulate the EMT phenotype, in an attempt to reveal molecules that drive this process in human breast cancer. Specifically, the findings from this part of the analysis show clear phenotypic changes, as well as changes to key EMT genes involved in this EGF-induced EMT system, which may be important in helping our understanding of breast cancer development and progression.

The molecular mechanisms and signals involved in EMT have previously been studied, with particular interest in the implication of several growth factors, such as EGF, FGF and TGF-β family factors (Grände et al., 2002, Strutz et al., 2002, Lu et al., 2003, Zhen et al., 2003, Forino et al., 2006).

To gain insights into how cell density affects EGF-induced PMC42 cells, time-course experiments were performed with EGF treatment of the PMC42 cells at two densities (sparse and dense). In the first part of this study, the morphological effects that cell density had upon the EGF-induced PMC42 cells were investigated (Figure 3.1). Following exposure to EGF at 24 and 72hrs, the study confirmed that the PMC42 cells developed morphological changes depending upon cell confluence, being more evident under sparse culture conditions. The PMC42-ET cells displayed a mixed epithelial morphology, and when cultured on plastic, grew to form distinct colonies with rigid edges. Stimulation of PMC42-ET cells with EGF for 72hrs altered the morphology of the PMC42-ET cells, showing an epithelial-like compact morphology in dense cultures. The
development of a dramatically more elongated spindle-shaped, fibroblastoid morphology in the PMC42-ET cells was promoted with EGF stimulation under sparse conditions. The EGF-induced morphological changes appeared to occur after 3hrs, but were more prominent by 3 days stimulation with EGF.

In contrast, in response to low cell density, un-stimulated PMC42-LA cells grew as an adherent monolayer and displayed a typical epithelial phenotype in which cells had a uniform cuboidal shape arranged in a cobblestone pavement pattern. When confluent, PMC42-LA cells appeared as a sheet of tightly associated, epithelial-like colonies. Exposure of PMC42-LA cells, which display a typical epithelial phenotype, to EGF for a period of 72hrs showed only subtle morphological changes whereby the cells became less regular, lost some of their epithelial morphology and detached from one another, thus displaying a “relaxed epithelial” morphology.

As exemplified by these studies, the EGF-induced morphological changes for both the PMC42-ET and the PMC42-LA cell lines were shown to be time-dependent, as well as reproducible and distinct after 72hrs with EGF in the sparse cultures. Therefore, it is likely that these cells may have a particular phenotypic plasticity that makes them especially prone to undergoing EMT under the sparse culture conditions. It is well known that modulation of growth factor receptors may play a significant role in determining the response of a cell to a given growth factor. Under the confluent conditions used in this study, it is possible that the PMC42 cells manifest relatively few EGF receptors (EGFR) and therefore exhibit little response to EGF, whereas these cells at a lower density manifest more EGF receptors and exhibit a more pronounced response to EGF.

The findings in this part of the thesis are supported by published data describing the effects of EGF on cell density. Previous early findings have shown that EGF binding to some cells is affected by cell density, but these findings were not characterised in depth (Pratt and Pastan, 1978, Hollenberg et al., 1979). One prominent exception was the finding that increasing the cell density of BSC-1 cells reduced their number of EGF receptors by approximately 9-fold, and this correlated with a decreased response of these
cells to EGF (Holley et al., 1977). Findings by Rizzino and coworkers (1988) indicate that increasing cell density exerts an effect on at least four different growth factor receptors (TGF-B, EGF, PDGF and FGF) and this effect occurs in a number of different cell lines (three non-transformed cell lines and two human tumour cell lines) (Rizzino et al., 1988).

Overall, these results show that treatment of the PMC42 breast cancer cells with EGF produces EMT phenotypic changes, being more evident under sparse culture conditions. As EGF acts by binding with high affinity to EGFR on the cell surface, it stimulates the intrinsic protein-tyrosine kinase activity, and in turn, initiates a signal transduction cascade that results in a variety of biochemical changes to the cell such as increases in the expression of certain genes. As EGF had a profound impact on cellular morphology in the PMC42 cells under sparse and dense culture conditions in this study (Figure 3.1), it was of interest to compare the expression of certain genes in low and dense culture conditions since cell density may have an effect on the expression of epithelial and mesenchymal marker proteins (Strutz et al., 2002).

Thus, when assessing the impact of cell culture density in the EGF-induced PMC42 cellular model, gene expression of a number of key EMT markers ((Figure 3.2, vimentin, N-cadherin, E-cadherin); (Figure 3.3, Twist-1, Snail, Slug); and target genes (Figure 3.4, integrins)) were significantly changed in the sparse-cultured cells relative to confluent cells. Whilst the expression of E-cadherin was decreased in the EGF-induced PMC42 system under the sparse conditions, the expression of vimentin and N-cadherin were increased with EGF under sparse conditions over time. Likewise, the transcription factors Slug, Snail, and Twist-1, were all increased with EGF over time under the sparse conditions in comparison to the dense conditions. Slug and Snail have been described to be direct repressors of E-cadherin in epithelial cells in vitro and in vivo through an interaction of their COOH-terminal region with a 5’-CACCTG-3’ sequence in the E-cadherin promoter. Studies have shown that over-expression of Snail and Slug leads to a reduction of E-cadherin expression and that E-cadherin is inversely correlated with Snail levels in breast carcinoma (Blanco et al., 2002). In bladder cancer, an over-expression of
Twist-1 resulted in a further decrease of E-cadherin expression (Yu et al., 2010). Recently it has also been postulated that Twist-1 may be involved in the tumoural invasion step (Hugo et al., 2011).

This is the first study to report how cell density and subsequent gene changes in PMC42 cells are affected by EGF. The data from this part of the investigation are supported by an in vitro study which found that in response to low cell density, the immortalised breast MCF10A cells suffered spontaneous morphologic and phenotypic EMT-like changes, including cytoskeletal reorganisation, vimentin- and Slug-upregulation, cadherin switching (E-cadherin to N-cadherin), and diffuse cytosolic relocalisation of the catenins (Sarrió et al., 2008).

Therefore, this inducible PMC42 cell line is a useful model for studying EMT-like changes, specifically for the key EMT makers (vimentin, E-cadherin, N-cadherin) and regulators (Twist-1, Snail, Slug), would be under sparse conditions; where the specific changes identified in the PMC42 system, are in support of the notion that during breast cancer progression, these are likely to be key changes occurring upon the initiation of EMT events and subsequent progression of the tumour cells from epithelial to mesenchymal phenotype. Thus, under these carefully defined sparse conditions, a more extensive timecourse of EGF treatment was used to further investigate the key markers of EMT.

A requirement for acquisition of a migratory phenotype to define EMT is somewhat controversial. Investigation of EMT requires the use of panels of markers that describe an EMT profile. Loss of the epithelial phenotype can be clearly defined by loss of expression of specific epithelial proteins, in particular, E-cadherin, which is a universal feature of EMT. The acquisition of a mesenchymal phenotype, however, has been more difficult to define due to the lack of specificity of many available phenotypic markers (Zavadil and Böttinger, 2005). The mesenchymal markers most widely used to define the mesenchymal phenotype include vimentin and N-cadherin. However, as vimentin is expressed in endothelial cells as well as myofibroblasts, macrophages, neutrophils and
lymphocytes (Franke et al., 1987, Evans, 1998, Eckes et al., 2000), it is not entirely specific for fibroblastoid cells.

In the PMC42 cell system used in this study, besides the change in morphology (Figure 3.1), the EGF-induced phenotype transition in the cells was characterised by a markedly up-regulated expression of the “classical” mesenchymal markers, vimentin (Figure 3.5) and N-cadherin (Figure 3.6) with a parallel decline in the expression of the “classical” epithelial marker E-cadherin (Figure 3.7), as measured both by Q-RTPCR and Western immunoblotting analyses over an extensive time-course period of 72 hrs under sparse conditions.

Vimentin, the mesenchymal marker, was assessed in this investigation to further characterise the potential of PMC42 cells as inducible EMT breast cancer cells. Vimentin is a well reported aggressive marker in breast cancer cell lines (Thompson et al., 1992b, Gilles et al., 2003b), and is often used as a marker for invasiveness and poor prognosis in pathological analyses, reviewed in (Kokkinos et al., 2007). In the native state, PMC42-ET cells present with almost 100% vimentin positivity, whilst PMC42-LA cells are predominantly negative for vimentin unless located at the cell cluster periphery. In this investigation, vimentin was clearly expressed in the EGF-induced PMC42 system. In both the PMC42-ET and PMC42-LA cells, vimentin mRNA and protein was increased further with EGF treatment. This data indicates that the PMC42-LA cells have the potential to gain a mesenchymal potential and the PMC42-ET cells can acquire further EMT. The fact that PMC42-LA cells, which are primarily epithelial in morphology express vimentin, could be explained by the fact that there is a small population of cells that are vimentin positive and also the transcription of vimentin is very sensitive to serum factors (Lenferink et al., 2004).

However, numerous data have now shown that vimentin can also be expressed in epithelial cells involved in physiological or pathological processes. These include epithelial cell migration (Gilles et al., 2003a), embryological and organogenesis processes (Guarino, 1995, Hay, 1995), placentation (Nawrocki et al., 1997), wound
healing (SundarRaj et al., 1992), or tumour invasion (Ramaekers et al., 1983, Savagner et al., 1994, Guarino, 1995, Gilles et al., 1996, Gilles and Thompson, 1996). In normal breast, vimentin is expressed in a regulated manner by myoepithelial breast cells (Guelstein et al., 1988). In contrast, in breast cancer, its expression is associated with high histological grade, lack of estrogen receptor (ER) expression, EGFR positivity and p53 expression (Raymond and Leong, 1989, Domagala et al., 1990, Koutselini et al., 1995, Santini et al., 1996, Tsuda et al., 2005, Umemura et al., 2005, Kokkinos et al., 2007).

As shown by Q-RT-PCR, vimentin levels in un-induced PMC42-LA cells were significantly lower than that of vimentin levels in un-induced PMC42-ET cells. Thus the findings from this thesis showing that vimentin is weakly expressed in PMC42-LA cells may be explained by the role of vimentin in normal cellular processes; however its elevated expression with EGF is indicative of EMT.

The higher mRNA reading in the absence of EGF treatment in PMC42-ET cells compared to PMC42-LA cells corresponds well with the higher levels of protein seen in Western immunoblotting. Of note, vimentin message was increased by 24hrs in the PMC42-LA cells, whereas the vimentin protein was increased by 1.5hrs. Also in the PMC4-ET cells, vimentin message is upregulated by 6-24hrs, whereas the vimentin protein was upregulated by 3hrs EGF stimulation. This could suggest that in these cells, vimentin regulation is more than transcriptional, but rather, that there is some translational or post translational regulation mechanism that occurs faster than transcriptional mechanism, and this may be explained by the increased time needed for EGF-EGFR signaling and subsequent transcription of genes responsible for mesenchymal transition.

In EGF-stimulated PMC42-ET cells, E-cadherin mRNA was markedly reduced, with maximal decrease evident by 24hrs. Western immunoblotting analysis revealed that EGF stimulated PMC42-ET cells caused a dramatic decrease in E-cadherin protein expression by 3hrs, and was not detected by 72hrs, indicating that a post-translational regulation
mechanism is occurring faster than the transcriptional regulation in these cells. This contention is supported by published data (Masterson and O’Dea, 2007, Bonazzi et al., 2008, Pinho et al., 2009) showing that E-cadherin downregulation may occur through post translational modification.

In contrast, PMC42-LA cells, presented with no significant down-regulation in E-cadherin mRNA at early stages of incubation, however further stimulation with EGF for increased time points caused a reduction in E-cadherin protein expression by 72hrs. These findings may be due to the fact that the cells are epithelial and require more time for E-cadherin gene expression changes to occur. Specifically, it is likely that EGF ligand bound to its receptor EGFR resulted in signalling events which subsequently stimulated E-cadherin regulators (i.e. transcription factors, Twist-1, Snail, Slug) to be upregulated, which in turn led to downregulation of E-cadherin.

Furthermore, since a complete loss of E-cadherin did not occur at 72hrs, this indicates that a partial EMT had occurred. Partial EMT, also known as a hybrid EMT state, or metastable state (Thompson and Nagaraj, 2018), has often been observed in carcinoma (Jolly et al., 2016), developmental processes and wound healing (Nieto et al., 2016). In this hybrid state, individual cells express both epithelial and mesenchymal markers, Whilst a number of in vitro data (mainly co-expression of epithelial and mesenchymal markers within the same cells) have revealed that EMT may advance in a step-wise manner through the generation of subpopulations that represent different intermediate states between the epithelial and mesenchymal states (Huang et al., 2013, Zhang et al., 2014b, Hong et al., 2015, Jolly et al., 2016, Bierie et al., 2017), it remained unclear whether EMT proceeds through these intermediate states in vivo. Pastushenko and coworkers (2018), in their most recent study, used genetically engineered mice to investigate whether stable EMT states in tumours could be identified in vivo. They demonstrated that spontaneous EMT in primary tumour cells in vivo proceeds through distinct intermediate states with different invasive, metastatic and differentiation characteristics (Pastushenko et al., 2018).
Hence, using EGF is a useful way in initiating EMT in epithelial breast cancer cells and this may allow for the identification of the early changes that occur in breast cancer formation.

These results are supported by findings in other tissue culture conditions, where decreased E-cadherin expression has been shown to correlate with the invasive phenotype of cancer cells (Behrens et al., 1989, Frixen et al., 1991b, Oda et al., 1994, Siitonen et al., 1996, Boyer et al., 2000, Thiery, 2002b, Sarrió et al., 2008). In most epithelial tumours from various tissues, including stomach, colon, head and neck, bladder, prostate, and breast (Schipper et al., 1991, Bringuier et al., 1993, Dorudi et al., 1993a, Mayer et al., 1993a, Oka et al., 1993a, Umbas et al., 1994), E-cadherin expression is down-regulated or lost and research groups have shown that regain of functional E-cadherin suppresses invasion in many tumour cell types (Hirohashi, 1998, Hsu et al., 2000a). The loss of expression of the E-cadherin molecule is considered as a critical step and an indicator of EMT in both normal and pathological situations, and the downregulation of which can be achieved by transcriptional factors Twist-1, Slug, and Snail (Hajra et al., 2002, Vernon and LaBonne, 2004, Yang et al., 2004, Hugo et al., 2011), all of which were upregulated with EGF in the PMC42 cells in this study under sparse conditions. Recent work from our laboratory has shown that E-Cadherin suppression dramatically reduced proliferation of MDA-MB-468 human breast carcinoma cells (Hugo et al., 2017).

N-cadherin, normally present in neuronal and muscle tissues, is aberrantly expressed in epithelial tumours and has recently gained increasing interest in its role in migration and invasion in carcinoma cells. Many invasive breast cancer cell lines express N-cadherin, while the less invasive epithelial lines lack it, supporting its possible role in invasive breast cancer and accelerated EMT. As such this molecule was assessed in this part of the thesis. Untreated PMC42-LA cells presented with minimal basal levels of N-cadherin, indicating that in breast cancers, N-cadherin expression is not associated with the non-invasive phenotype. Upon incubation with EGF, N-cadherin expression was increased within the first 72hrs. Hence, these findings support that N-cadherin up-regulation is an
indicator of the initiation of EMT events, and may be coupled with the reduction of E-cadherin, also seen at 72hrs in EGF-stimulated PMC42-LA cells.

These findings are supported by published data showing that N-cadherin is associated with a heightened invasive potential in cancer, enhancing tumour cell motility and migration (Hazan et al., 2000, Grände et al., 2002). As such, there have been suggestions that unlike E-cadherin, which functions as an invasion suppressor and is down-regulated in most carcinomas (Mareel et al., 1992a, Mareel et al., 1997b), N-cadherin functions as an invasion promoter, and can be frequently up-regulated during carcinoma progression (Kim et al., 2000a).

As shown in this investigation, cells exhibiting initiation of EMT resulted in a) down-regulation of E-cadherin and b) upregulation of N-cadherin. The present findings in this study show that the loss of E-cadherin in favour of N-cadherin expression is synchronous, indicating the switch from E- to N-cadherin as a possible phenomenon in EMT. Here these findings may also be explained by cadherin switching which is often reported in normal and pathologic conditions, a now increasingly an accepted phenomenon for EMT regulation (Cavallaro et al., 2002, Christofori, 2003), whereby the presence of E-cadherin in epithelial cells allows for greater cell–cell adhesive strength compared with that of N-cadherin-expressing cells. Moreover, the minimal adhesive interaction between E− and N-cadherin would be predicted to allow N-cadherin expressing cells to migrate through a layer of E-cadherin-expressing cells (Gil et al., 2011). In addition, published findings have reported an inverse correlation in some human cancers such as breast and prostate (Hazan et al., 1997, Tomita et al., 2000), between the expression of E-cadherin and N-cadherin, in what is called “the cadherin switch” (Wheelock et al., 2008). In a study by Nieman and co-workers (1999b), a switch from E-to N-cadherin in breast cancer cells correlated with invasion and motility (Nieman et al., 1999b). Therefore, it is likely that PMC42 cells represent a cell line with the potential for inducible EMT, with down-regulation of E-cadherin correlating with elevated mesenchymal marker expression upon growth factor stimulation.
Not only can EMT be induced by various intrinsic (e.g. gene mutations) as well as extrinsic (e.g. growth factors) signals, alterations in the composition of the ECM are also able to induce EMT (Shintani et al., 2008). The communication between the extracellular matrix and cells is based on integrins, whereby integrins, upon binding extracellular ligand(s), causes the stimulation of intracellular signaling processes involving molecules such as focal adhesion kinase (FAK) or integrin linked kinase (ILK). The functions of integrins during EMT are diverse and dynamic and being involved in signal transduction, they are able to initiate and enforce EMT and invasion (Koenig et al., 2006, Shintani et al., 2008, Yilmaz and Christofori, 2009).

An increasing number of reports demonstrate functional and physical interaction between cadherins and integrins, suggesting a connection between both integrin and cadherin signalling pathways (Alexander et al., 2006, Koenig et al., 2006, Shintani et al., 2008). Therefore cadherins and integrins must function in a coordinated manner to effectively mediate the cellular interaction essential for migration and tumour metastasis, with ILK being the most likely candidate to coordinate this integrin/cadherin expression or functions.

Due to this, and since a direct or indirect interaction of vimentin with integrins has been described (Homan et al., 1998), the next part of the study investigated EMT regulation processes resulting from EGF stimulation, using ITGα2 (Figure 3.8), ITGβ1 (Figure 3.9) as well as the ILK (Figure 3.10) genes, as these showed to be elevated in the sparse cultured PMC42 cells.

Validation by Q-RT-PCR in a more extensive timecourse of EGF-induced PMC42 cells grown under sparse conditions showed that EMT induced a particularly appreciable transcriptional up-regulation of ITGα2 and ITGβ1, as well as ILK within 1.5-3/6hrs. Identification of these early onset changes in the genes indicates that these are important initiating factors representative of EMT in breast cancer cells.

Changes in expression or modification of the integrins have been discussed in early studies during malignant transformation in various cells (Plantefaber and Hynes, 1989,
Albelda et al., 1990, Damjanovich et al., 1992). α2β1 integrin is widely expressed on epithelial cells and its levels are increased in several carcinoma cells from the epithelial origin (Madamanchi et al., 2014). Growing evidence indicates that α2β1 integrin can be a key pathway in cancer pathogenesis (Koenig et al., 2006, Shintani et al., 2008, Naci et al., 2015). Furthermore, Chen et al (1994) showed that increased expression of α2β1 integrin is positively correlated with increased metastatic ability in human squamous cell lung cancer cells when i.v. inoculated in severe combined immunodeficiency (SCID) mice (Chen et al., 1994). Therefore support for the results of this part of the thesis exist in the literature, indicating that in early breast cancer formation, key changes occur in ITGα2 and ITGβ1 as well as ILK. Whilst the focus of this study was ITGα2 and ITGβ1, as well as ILK, a number of other integrin subunits were found to be transcriptionally upregulated, in particular the α3 and α6 integrins. The laminin-binding integrins α3β1 and α6β1 have been reported to show increased expression and/or have pro-tumorigenic/pro-metastatic functions in various cancer cells (Ramovs et al., 2017). Therefore, their potential role in EGF-driven EMT should definitely be looked into for future studies.

Furthermore, in this thesis it was shown that this upregulation in ITGα2 and ITGβ1 integrins, as well as ILK, may be directly affected by EGF. For instance, it is well established that EGF has roles in diverse essential cell functions (Wells, 1999, Wells et al., 1999, Jorissen et al., 2003). An over-expression of the EGF receptor (EGFR) has been associated with tumour malignancy (Libermann et al., 1985, Pavelic et al., 1993, Lu et al., 2001). Not only does EGFR transduce the signalling effects of EGF binding, it is also able to influence the downstream signalling function of integrins and a variety of other cell surface receptors (Miyamoto et al., 1996, Moro et al., 1998, Prenzel et al., 1999, Lee and Juliano, 2002, Moro et al., 2002, Marcoux and Vuori, 2003).

Various studies have demonstrated cooperation between EGF-mediated signalling pathways and integrins (Yu et al., 2000, Reginato et al., 2003, Cabodi et al., 2004), whereby EGFR activation modulates integrin function by means of regulating the expression and/or activity of various integrins, leading to altered adhesion, motility, and
invasive capacity (Krensel and Lichtner, 1999, Mariotti et al., 2001). Since α2β1 integrin serves predominantly as a collagen type I receptor, ITGα2 expression is selectively modulated by EGFR activation in numerous epithelial cell types (Chen et al., 1993, Krensel and Lichtner, 1999). Furthermore, co-localization and direct interaction between integrin α2β1 and the EGFR has been reported in human epithelial A431 cells (Yu et al., 2000), suggesting that aberrant EGFR activity may alter ITGα2 expression and/or function.

Hence it is likely that in this part of the thesis, up-regulated integrin α2β1 resulting from EGF stimulation may be directly associated with EGFR signalling. Thus, not only has integrin expression been achieved in breast cancer EMT, but the mechanisms by which integrins, key indicators of EMT, are up-regulated appear to be associated with EGFR signalling in early EMT events in breast cancer.

It is well accepted that EMT is regulated by multiple regulators such as growth factors, cytokines, hormones, adhesion molecules, ECM and intracellular signalling molecules including ILK (Bates and Mercurio, 2003, Grünert et al., 2003, Masszi et al., 2003). In particular, the growth factor TGF-β is a factor that can solely initiate and complete the EMT. Recently, TGF-β1 has been shown to enhance the expression of ILK in many cell types (Li et al., 2003, Xu et al., 2003, Lee and Juliano, 2004). This is indeed the case in the studies in this chapter, using another growth factor, EGF, where increased ILK expression was observed with EGF stimulation. ILK is stimulated by extracellular matrix interactions and by certain growth factors (Dedhar, 2000b). Several reports have described that ILK is a critical mediator for induction of EMT (Somasiri et al., 2001, Kalluri and Neilson, 2003, Li et al., 2003, Oloumi et al., 2004, Lee et al., 2006, Gil et al., 2011, Gil et al., 2016). One study by Shimizu and colleagues (2006), showed that ILK expression and activity are likely involved in the acquisition of the mesenchymal phenotype and represent an early event in EMT necessary for the development of tissue fibrosis (Shimizu et al., 2006).
Hence, the fact that ILK was significantly upregulated with EGF treatment in the PMC42 cells suggests that the mechanism by which ILK regulation occurs in breast cancer is also through EGFR signalling pathways. These results indicate that these genes may be critical in driving the EMT, and thus may facilitate early detection methods in early breast tumour formation.

Whilst the cell density-dependent phenotypic changes seen in this study involved the modulation of a number of classical EMT and candidate genes (described in section 3.2.4), the identification of new markers of the EMT in the sparse PMC42 cell system were explored in a PCR array approach (MT-PCR) (Figure 3.11). The MT-PCR format used investigated ~66 genes and looked at a comprehensive analysis of gene expression changes over the extensive timecourse in both cell types (PMC42-ET and PMC42-LA), consisting of untreated RNA as well as RNA treated with EGF for a period of time ranging from 1.5 to 72hrs. A major benefit of the MT-PCR was to extend on what was seen with the Q-RT-PCR. We found MT-PCR to be comparable to the target-specific RT-PCR, and results were congruent in the classical EMT genes. The MT-PCR assay was able to provide rapid, sensitive, and specific simultaneous detection and identification of the individual genes, vimentin, E-cadherin, N-cadherin, ITGα2 and ILK studied in QRT-PCR. ITGβ1 was unfortunately not investigated in the MT-PCR assay as it was unavailable on the pre-made commercial discs used. Future MT-PCR assays would incorporate ITGβ1. Not only does this data confirm that the EMT genes vimentin and N-cadherin were upregulated, and E-cadherin was downregulated with EGF treatment over the timecourse, but it also represents coordinated changes amongst quite a few integrins, as well as ILK which occurred relatively early in the EGF-treated cells in the time frame. A comparison between the level of gene expression identified with QRT-PCR and MT-PCR also indicated that MT-PCR may be a more sensitive method of detecting gene expression, as the detected level of the individual gene expressions were generally higher in the MT-PCR assay. A number of other genes screened and identified in this part of the project will need to be investigated in future work, as time limitations proved too difficult to allow further study. For example, trends towards increased CD44 in both PMC42 cell
lines, and reduced CD24 in PMC42-ET cells are consistent with an increased breast cancer stem cell-like phenotype, that has been linked to EMT. Increases in CLDN4 (ET only), COL1A1 (ET only), FGFR1 (LA, ET), LGALS1 (ET only), PLAU (ET), SNAI2 (LA only), and decreased levels of FGFR2, FGFR4 and FGF2 (ET only) specify some subline-specific, EMT-associated effects. In future studies, it would be beneficial to further examine these gene changes.

Overall, in this study, the EMT in the EGF-induced PMC42 cell system was density dependent, where evidence of an EMT was most apparent under sparse culture conditions. In addition, the EGF-induced EMT was time dependent and morphological changes became evident after 1.5-3/6hrs of EGF stimulation and became maximal after 72hrs. The EGF-induced EMT promoted the development of a spindle-shaped, fibroblastoid morphology, accompanied by elevated levels of vimentin and N-cadherin, and reduced expression levels of E-cadherin. Furthermore, while the expression of vimentin has been traditionally used as a marker of EMT, additional early EMT markers are required since vimentin can also be expressed in other cellular contexts not associated with EMT. Interestingly, ITGα2 and ITGβ1 as well as ILK, examined in this study, were seen to be induced from 3hrs, and 1.5hrs, respectively, indicating that these genes are early response genes in EMT, and may indeed be required for the early manifestation or EMT, all of which are modulated by EGFR signalling.

Hence, this investigation has shown the potential use of the early response molecular markers not only as predictive factors in breast cancer, but also as targets for clinical therapy, where identification of these markers could be a useful step to developing early detection methods.

Chapter 5 of this thesis will further explore ITGα2 and ITGβ1 as well as ILK, using functional studies, to determine whether they are indeed required for the early manifestation of EMT.
CHAPTER 4: PROPAGATION AND CHARACTERIZATION OF THE EDW-01 HUMAN BREAST CANCER XENOGRAFT: CANDIDATE EMT MARKERS/EFFECTORS.
Chapter 4: Propagation and Characterization of the EDW-01 Human Breast Cancer Xenograft: Candidate EMT Markers/Effectors

4.1 INTRODUCTION

The use of preclinical models to study tumour biology as well as response to pharmacological treatment is a core component of translational cancer research. Previous models used for drug evaluation generally consist of implantation into immunodeficient mice of xenografts generated from well-established human cancer cell lines, which have already been adapted to in vitro growth. These models have been extensively used for decades, and have proven useful in identifying cellular and molecular mechanisms underlying metastasis, and for developing new therapies. However, they are limited in their effectiveness for sufficiently representing clinical cancer characteristics, as they often fail to recapitulate the heterogeneity of tumours in patients (Ellis and Fidler, 2010, Gillet et al., 2011), hence hampering the progress in oncology drug development (Whittle et al., 2015).

Over recent years, there has been increasing interest in the development and characterisation of patient-derived xenograft (PDX) models from different tumour types. These models represent a major advancement in cancer research, and have emerged as important tools for translational research (Hidalgo et al., 2014, Whittle et al., 2015, Davies et al., 2017, Kanaya et al., 2017, Williams, 2018). They are increasingly being used to evaluate anti-cancer drug activity instead of human tumour xenografts derived from established cultured cell lines (Tentler et al., 2012, Calles et al., 2013, Siolas and Hannon, 2013).

PDXs are surgically resected tumour samples that are engrafted subcutaneously into immunocompromised mice and propagated through multiple generations. The development of PDXs offers several advantages over standard cell-line xenograft models and represents a major advance in cancer research (Jin et al., 2010, Siolas and Hannon, 2013), as they can be sustained and expanded by successive passages with minimal change to the genetic and biological characteristics of the tumour grown at the initial passage. Moreover, they more accurately reflect tumour heterogeneity, histopathology, gene expression, genetic mutations, and therapeutic response than xenografts derived from human cancer cell lines (Marangoni et al., 2007, DeRose et
al., 2011, Garrido-Laguna et al., 2011, Cottu et al., 2012, Kabos et al., 2012, Lum et al., 2012, Charafe-Jauffret et al., 2013, Zhang et al., 2013a, Hidalgo et al., 2014, Hanna et al., 2016, Davies et al., 2017, Kawaguchi et al., 2017). These models are now comprehensively used as a platform for personalising cancer therapeutics (Landis et al., 2013, Norum et al., 2014), as a preclinical platform in drug development (Lum et al., 2012, Tentler et al., 2012), and as an experimental tool for studying cancer biology (Kabos et al., 2012, Zhang et al., 2014a). The development of PDXs offers several advantages over standard cell-line xenograft models and represents a major advance in cancer research (Jin et al., 2010, Siolas and Hannon, 2013) as they may provide a more accurate reflection of a patients tumour biological characteristics than tumour cell lines and allow an invaluable assessment of tumour evolution and adaptive response to therapy (Hidalgo et al., 2014, Marangoni and Poupon, 2014, Gao et al., 2015, Lodhia et al., 2015).

Human breast tumour PDXs have been of substantial value in studying numerous aspects of breast cancer biology. Despite the lack of immune elements, since xenografts require immunocompromised hosts, their reproducibility, stability, and reflection of their human origin are clearly considerable strengths. Thus, the application of human xenograft models that better replicate components of the metastatic process has much to offer both for increasing our understanding of malignant progression, and for the development and screening of novel therapies.

Breast cancer is a diverse and complex disease which consists of distinct molecular subtypes, each with a unique gene-expression profile. Gene expression profiling of invasive breast cancer has defined a number of tumour subtypes with clinical relevance: Luminal A and B, Basal, human epidermal growth factor receptor 2 (HER2)-positive, and triple-negative (Estrogen receptor (ER); Progesterone receptor (PR); HER2) (Perou et al., 2000). These protein biomarkers are used clinically to assess breast cancer patients at presentation, and if possible, at progression of the disease. Since the expression of these protein biomarkers dictates the treatment course for breast cancer patients, in which patients with hormone receptor positive tumours receive endocrine therapy, and HER2-positive patients receive anti-HER2 therapy, the
concordance of these markers in PDXs derived from the patient being treated is often associated as a measure of reliability (Evans et al., 2017). Further stratification of these subgroups has also occurred, with several additional subgroups of so-called ‘triple negative breast cancers / TNBCs that lack ER, PR and HER2 (Lehmann et al., 2011, Network, 2012, Burstein et al., 2014). These molecular subgroups will likely influence responses to standard and/or novel therapies (Carey et al., 2006), and present a unique opportunity to individualize therapies tailored specifically to a patient’s tumour subtype (Reyal et al., 2012).

Multiple groups have established cohorts of breast cancer PDXs (Al-Hajj et al., 2003, Beckhove et al., 2003, Fiebig et al., 2004, Marangoni et al., 2007, De Plater et al., 2010, DeRose et al., 2011, Petrillo et al., 2012, Reyal et al., 2012, Landis et al., 2013, Zhang et al., 2013a, Marangoni and Poupon, 2014, McAuliffe et al., 2015, Whittle et al., 2015, Kanaya et al., 2017). Among the breast cancer PDX models, the vast majority of breast PDXs generated are from triple-negative (ER-negative, PR-negative, and HER2-negative) tumours, owing to their higher rates of engraftment. ER-positive PDX models have been difficult to establish (Marangoni et al., 2007, Cottu et al., 2012, Charafe-Jauffret et al., 2013, Landis et al., 2013), and those that engraft represent luminal B (rather than luminal A) tumours that are characterised by high Ki67 scores (Whittle et al., 2015) and poor prognosis (Inic et al., 2014). These ER-positive tumours retain their hormone receptor status over multiple passages, and their dependence on estrogen for growth. According to the 2013 St Gallen Consensus, the luminal B subtype accounted for nearly 40% of all breast cancers (Metzger-Filho et al., 2013). The luminal-B subtype is a clinically important classification of breast cancer with prognostic and potential predictive implications.

In our laboratory, several novel human transplantable breast carcinoma xenograft models were established in SCID mice by Assoc. Prof. Elizabeth Williams, and Dr. Emma De Sousa (Lafleur et al., 2005b). Three were derived from bone metastasis deposits harvested at the time of orthopaedic surgery for fracture (ED02, ED03 and EDW01), and one from a primary breast cancer (ED09). ED02 was derived from a pelvic breast carcinoma metastasis in a 73-year-old woman; ED03 was derived from
metastatic breast carcinoma in the left proximal femur of a 40-year-old woman; ED09 was derived from a primary breast carcinoma removed from an 83-year-old woman (Lafleur et al., 2005b). The EDW-01 xenograft was derived from the bone metastasis of a 44-year-old woman who succumbed to her disease one year later. She had been diagnosed with invasive breast cancer 10 years earlier.

In the course of earlier studies in the laboratory (De Sousa, Walker, Williams, Thompson) it was noted that the EDW-01 xenograft became incrementally more vimentin-positive with each successive passage. As detailed in the general introduction, vimentin is the mesenchymal marker most commonly associated with EMT (Gilles et al., 2005). Its aberrant expression correlates with up-regulated migration and invasion by cancer cells, coupled with a reduced epithelial marker expression that is expected with EMT (Gilles and Thompson, 1996, Hugo et al., 2007, Kokkinos et al., 2007, Sarrió et al., 2008, Yang and Weinberg, 2008, Kalluri and Weinberg, 2009, Polyak and Weinberg, 2009, Bonnomet et al., 2012, Gunasinghe et al., 2012).

In association with the EMT changes seen with each increasing passage, it was hypothesised that the EDW-01 xenograft would also exhibit altered expression of ITGβ1 and ITGα2, and ILK, which could potentially contribute towards the mesenchymal phenotype that develops. These genes might potentially be useful as prognostic markers or molecular targets for novel therapies, and may have clinical implications for the management of breast cancer.

This chapter therefore explores the EDW-01 xenograft as an *in vivo* EMT PDX model system. The research presented in this chapter aimed to:

1) Examine the successive passages of this EDW-01 xenograft model system for any evidence of EMT changes

2) Assess the steady-state levels of ITGβ1, ITGα2 and ILK mRNA.
Chapter 4: Propagation and Characterization of the EDW-01 Human Breast Cancer Xenograft: Candidate EMT Markers/Effectors

4.2 RESULTS

4.2.1 Further Xenografting of the EDW-01 Human Breast Cancer Xenograft Model.

The EDW-01 xenograft model of human breast cancer, derived from bone metastasis, was generated in the Thompson Lab by Dr Elizabeth D Williams, from bone metastasis deposits harvested at the time of orthopaedic surgery for fracture (abstracts: Wafai et al., 2004; combio; (Lafleur et al., 2005b) Figure 4.1). It is important to note that the bone metastasis from which the EDW-01 was derived was clinically overt “macro-metastatic” deposits (Kokkinos et al., 2007).

Earlier studies on EDW-01 were taken to 5 passages, with material either stored frozen at -80ºC for molecular assessment, or stored viably in DMSO and foetal calf serum under liquid nitrogen for propagation, since tests at that stage showed that the material could be revived from the freezer when re-implanted into mice as chunks (see Figure 4.2; M1, M2, M23, M24). It is important to note that the laboratory freezer which contained some of the EDW-01 material failed and unfortunately the samples could no longer be used for experimental work. To further propagate this material, and re-obtain earlier passage material to replace that which was lost to freezer failure, viable propagation of the EDW-01 xenograft in SCID mice was re-initiated as shown in Figure 4.2. The frozen EDW-01 xenograft was successfully regrown from the passage 1 (P1) / #11 (Figure 4.2), in the same manner as the original EDW-01 propagation.

4.2.2 General Characterization of the EDW-01 Xenograft Model:

The first part of this study aimed to investigate whether the events associated with in vitro EMT processes occurred in the specific biological context of breast tumour progression in vivo. An initial screen of candidate effector molecules implicated previously in the EMT process (see Chapter 3) was conducted across a number of passages. A series of early and late passage samples of the EDW-01 xenograft were studied immunohistochemically for the expression of the mesenchymal markers vimentin and N-cadherin, and epithelial markers E-cadherin and cytokeratins, together
with markers currently used for the identification of specific subgroups of breast tumours with biological relevance (ER, PR, and HER2).

Figure 4.1 Faxitron X-ray at Harvest of the First Mouse (Mouse 2), that was Propagated in the EDW-01 Xenograft.

This image represents growth of the EDW-01 xenograft. Shown here are the micro-calcifications in the tumour arising on the RHS (red arrows), but lack of tumour growth on the LHS (green circle). Figure kindly provided by Associate Professor Elizabeth D Williams.
The tumour tissue derived initially from the bone metastasis deposits were diced into 1mm pieces, mixed with Matrigel and implanted bilaterally in the mammary fat pad region of 3 SCID mice. The tumour material grew bilaterally in only one of these mice (mouse “2”) over a 5 month period and was passaged from there into 3 separate mice (mice “11, 12 & 13”). These tumours grew in 5-7 months, and were passaged serially through 5 passages (P0 – P5). For each passage, the tumour tissue was again chopped into chunks, mixed with Matrigel, and implanted into fresh mice. Once tumour volume for each mouse reached 2000mm3, mice were euthanized, and the tumours were removed, snap frozen for RNA and immunohistochemical analyses, and also viably stored in liquid nitrogen.

**Legend:** Original: original patient material, P0: Passage 0, P1: Passage 1, P2: Passage 2, P3: Passage 3, P4: Passage 4, P5: Passage 5
4.2.3 **Histological Comparison of the EDW-01 Xenograft Model System.**
To determine the morphology of the EDW-01 xenograft, and confirm the status of the tumours, archival formalin-fixed, paraffin embedded sections of the EDW-01 xenograft material, representative of early and late passages, were stained with H&E. H&E staining of EDW-01 sections of original patient material and 2 subsequent serial passages, representing early (P1 “11R”) and late (P5 “M98”) passages in SCID mice, revealed histology consistent with adenocarcinoma (i.e. evidence of glandular formation) in all 3 specimens *(Figure 4.3).* The late xenograft (M98) showed local invasion into surrounding tissues.

4.2.4 **Assessment of Predictive Markers of Breast Cancer in the EDW-01 Xenograft Model System.**
Predictive molecular markers investigated in the EDW-01 xenograft system included ER, PR, and HER2. The location of the immunohistochemically staining was predominantly nuclear for ER and PR, and membranous for HER2 *(Figure 4.4).* Immuno-reactivity to ER was seen in less than 10% of positive cells in the original patient material in the EDW-01 xenograft. This positivity was gradually increased with each passage, showing 40% ER-positivity at late passage. Consistent with the original patient material, PR and HER2 expression was weak to negative throughout the EDW-01 xenograft, regardless of passage.

4.2.5 **IHC Localisation of the Mesenchymal Markers Vimentin and N-cadherin, in the EDW-01 Xenograft.**
To determine the expression of vimentin as a guide to the EMT status in the EDW-01 xenograft, immuno-histochemical staining for vimentin was assessed semi-quantitatively. When seen, vimentin expression was intense (2+ to 3+), with vimentin being localised to the cytoplasmic region and no observable staining on the cellular membrane. A gradual increase in the vimentin status with each passage was observed, such that passage 5 (P5) materials showed up to 30% vimentin positivity, in comparison to the original patient material. This illustrates the heterogeneous expression of vimentin *(Figure. 4.5).* This pattern of vimentin expression is typical of
those seen in ~15% of invasive human breast cancer specimens (Gilles and Thompson, 1996, Willipinski-Stapelfeldt et al., 2005, Sarrió et al., 2008).

Whilst N-cadherin staining was attempted on the EDW-01 xenograft, this study was unable to reproducibly obtain any staining patterns, and in most instances, N-cadherin was not appreciably higher than the negative controls.

4.2.6 **IHC Staining of the Epithelial Markers E-cadherin and Pan Cytokeratin in the EDW-01 Xenograft:**

To explore whether the vimentin positivity (section 4.2.5) correlated with a loss of epithelial markers, E-cadherin and cytokeratin (Pan Cytokeratin antibody AE1/AE3) were further examined in sections of the original patient material and those representative of early and late passage EDW-01 xenografts (Figure. 4.6). IHC analyses of tumour specimens using an antibody specific for E-cadherin showed intense staining in 30% of the tumour cells in the original patient material. Early passage material (also vimentin negative) showed reduced E-cadherin staining, whereas the late passage material (10% vimentin positive) showed no E-cadherin staining.

Immuno-histochemical analysis of the tumour for cytokeratin showed positive overall staining, and regions of intense focal staining in the original patient material. Early passage material showed similar staining. In the late passage material, weak Pan Cytokeratin staining was evident in ~60% cells (Figure. 4.6).

4.2.7 **Integrin Marker Analysis by IHC of the EDW-01 Xenograft.**

In the previous results chapter (Chapter 3), coordinated changes were observed amongst quite a few integrins, and in ILK, supporting the hypothesis that integrins and ILK may play a role in the EMT phenotype in the model systems being investigated. It was therefore of interest to explore these integrin markers in the EDW-01 xenograft system. However, technical complications hindered the successful staining for
Figure 4.3 Histological Comparison of the EDW-01 Xenograft Model System.

Formalin-fixed, paraffin-embedded archival sections of the EDW-01 xenograft material taken from the original patient material, and 2 subsequent serial passages, representing early and late passages in SCID mice, were counterstained with Hematoxylin and eosin (H&E). Scale bars (100µm).
Immunohistochemical analysis of tumour specimens for ER, PR and HER2. In the original patient material, there was less than 10% positive ER staining. However, a gradual increase in the ER status with each passage was observed, such that the late passage material showed up to 40% intense ER-positivity localized to the nuclear region, as compared to the original patient material. PR and HER2 were negative throughout. Scale bars (100µm).

Figure 4.4 IHC Analysis of Predictive Markers of Breast Cancer in the EDW-01 Xenograft Model System: ER, PR, HER2.
Figure 4.5 Mesenchymal Marker Analysis by IHC of the EDW-01 Xenograft: Vimentin. Immunohistochemical analysis of tumour specimens for vimentin. In the original patient material, there was no observable vimentin staining. However, a gradual increase in the vimentin status with each passage was observed, such that the late passage material showed up to 30% intense vimentin positivity localized to the cytoplasmic region. Scale bars (100µm).
Figure 4.6 Epithelial Marker Analysis by IHC of the EDW-01 Xenograft: E-cadherin, Pan Cytokeratin (AE1/AE3).
Immunohistochemical analyses of tumour specimens for E-cadherin and Pan Cytokeratin. E-cadherin showed intense staining in 30% of the tumour cells or the tumour cell ECM in the original patient material. Early passage material showed reduced E-cadherin staining compared to original patient material, with the late passage material showing no E-cadherin staining. Positive Pan Cytokeratin staining was seen in focal cells in the original patient material. Early passage material showed similar Pan Cytokeratin staining. In the late passage material however, Pan Cytokeratin was actually increased, with weak Pan Cytokeratin staining in ~60% cells. Scale bars (100µm).
these markers. More study is required to determine the specificity and sensitivity of the antibodies used in the study. Due to time constraints, the IHC approach was abandoned in favour of species-specific RT-PCR (see section 4.2.8 below).

4.2.8 RNA Analysis of the EDW-01 Xenograft.
Xenograft mRNA expression of a number of gene products (epithelial or mesenchymal) was further examined using human-specific Q-RT-PCR analysis of RNA extracted from initial EDW-01 xenograft tumours, as well as those propagated further (Figure 4.2). RNA extraction from all available frozen xenograft samples (P3 through to P7) was performed. Due to freezer failure, and loss of stored early passage material, extraction was limited to only a few samples from the initial propagation for P3 and P4.

The products detected for each gene were specific to human mRNA respectively. Expression levels of the genes of interest were normalised to ribosomal protein L32 mRNA, a high abundance mRNA species. Values are presented as \( \delta C_t \), which are representative of mRNA expression levels relative to L32 ribosomal protein mRNA expression levels. Negative \( \delta C_t \) values represent lower mRNA abundance than for the RPL32 mRNA to which it is normalized.

4.2.8.1 RNA Analysis of the Classic EMT Markers Vimentin, N-cadherin and E-cadherin in the EDW-01 Xenograft.
The classical EMT markers vimentin, N-cadherin, and E-cadherin were investigated in the EDW-01 xenograft model system as an indication of their EMT status. Q-RT-PCR was performed with oligonucleotide primer sets specific for human L32, vimentin, N-cadherin, and E-cadherin.

Q-RT-PCR analysis of the EDW-01 xenograft demonstrated that vimentin mRNA expression progressively increased with each successive passage, such that the passage 6 material displayed an approximate 8-fold increase (\( P=0.008 \)), and the passage 7 material displayed an approximate 12-fold increase (\( P=0.024 \)) in comparison to the passage 3 material (Figure 4.7). A similar up-regulation of mRNA was also observed for N-
cadherin, with an approximate 18-fold increase (P=0.014) in N-cadherin mRNA levels seen for passage 6 material when compared with passage 3 material (Figure 4.7). Whilst passage 7 material exhibited approximately a 20-fold increase in N-cadherin mRNA, results did not reach statistical significance. In contrast, E-cadherin mRNA levels were reduced with increasing passage (Figure 4.7), reaching statistical significance at passage 5 (P=0.004) and passage 6 (P=0.001) material, both with a 0.1 fold reduction.

The pattern of mRNA expression seen for vimentin, N-cadherin, and E-cadherin, satisfy the most basic criteria for an epithelial to mesenchymal transition.

4.2.8.2 RNA Analysis of ITGβ1, ITGα2, and ILK in the EDW-01 Xenograft.

Similar to the increased vimentin and N-cadherin seen with each passage, the measured expression levels of ITGβ1 mRNA (Figure 4.8) in the xenograft increased with successive passage, demonstrating an approximately 26-fold increase (P=0.026) at passage 6 in comparison to the passage 3. Whilst passage 7 material showed approximately a 38-fold increase in ITGβ1 mRNA levels when compared to passage 3 material, this did not reach significance, due to large variance.

ITGα2 mRNA expression (Figure 4.8) was significantly upregulated by approximately 33-fold in the passage 7 material when compared to passage 3 material (P=0.024).

A trend was observed towards upregulation of ILK mRNA expression in the xenograft samples tested with increasing passage, exhibiting approximately 5-fold induction in the passage 7 material when compared to passage 3 material (Figure 4.8), however results did not reach statistical significance (P=0.393).

In summary, the presented co-induction of the mRNA levels of ITGβ1 and ITGα2, and a suggested trend for ILK, imply that these may be important for the EMT process because they track with the indices of EMT. These findings illustrate that EMT may be accruing in EDW-01 progression with serial passage.
RNA was harvested from the available frozen EDW-01 xenograft tumour samples (P3 through to P7) using the QIAGEN RNeasy mini kit. 100ng RNA was reverse transcribed using gene specific primers (Hs L32, Hs vimentin, Hs N-cadherin, and Hs E-cadherin), and probed with primers specific for Hs L32, Hs vimentin Hs N-cadherin, and Hs E-cadherin.

Data is presented as δCt, representing the expression levels of each, vimentin mRNA, N-cadherin mRNA, and E-cadherin mRNA, relative to the expression of the L32 ribosomal protein housekeeping gene.

Box and whiskers plots were utilised to depict the levels of each, vimentin, N-cadherin, and E-cadherin mRNA in the tumours. The upper and lower ends of the boxer represent the upper and lower quartiles respectively, and the median is marked by a line within the box. The whiskers extend to the highest and lowest observations. Statistical significance was calculated using two-tailed Mann-Whitney test (Non-parametric). * P<0.05
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**Figure 4.8 Levels of Human ITGβ1, ITGα2, and ILK mRNA in the EDW-01 Xenograft System.**

RNA was harvested from the available frozen EDW-01 xenograft tumour samples (P3 through to P7) using the QIAGEN RNeasy mini kit. 100ng RNA was reverse transcribed using gene specific primers (Hs L32, Hs ITG β1), and probed with primers specific for Hs L32, Hs ITGβ1, Hs ITGα2, and Hs ILK. Data is presented as δCt, representing the expression levels of ITGβ1, ITGα2, and ILK mRNA relative to the expression of the L32 ribosomal protein housekeeping gene.

Box and whiskers plots were utilised to depict the levels of ITG β1, ITG α2, and ILK mRNA in the tumours. The upper and lower ends of the boxer represent the upper and lower quartiles respectively, and the median is marked by a line within the box. The whiskers extend to the highest and lowest observations. Statistical significance was calculated using two-tailed Mann-Whitney test (Non-parametric). *P<0.05
4.3 DISCUSSION

The in vitro results presented in chapter 3 have been extended in this chapter by the use of an in vivo xenograft model of human breast cancer. This chapter examined and described the unique in vivo EDW-01 human breast cancer xenograft model system for any evidence of EMT changes, based on putative EMT markers. The regulation of ITGβ1, ITGα2, and ILK was also assessed to determine whether these gene products may contribute to the mesenchymal phenotype.

In accordance with the literature, the findings presented in this chapter demonstrate a successful propagation of the EDW-01 xenograft, which was reproducibly regenerated from frozen tissues. Morphologically, the EDW-01 xenograft showed histology consistent with adenocarcinoma (i.e. evidence of glandular formation), with local invasion into the surrounding tissues with increased passage. These findings indicate an incremental onset of EMT and invasive phenotype, suggesting that the EDW-01 xenograft provides an effective system to examine the progression of invasive breast cancer.

Numerous studies have demonstrated that during sequential passage, PDX models retain their similarity to the corresponding original donor tumours in histologic presentation and biologic behaviour, such as protein expression, tumour biomarker status and genomic and genetic status (Visonneau et al., 1997, Beckhove et al., 2003, Wang et al., 2005, Cutz et al., 2006, Huynh et al., 2006, Rubio-Viqueira et al., 2006, Marangoni et al., 2007, Whiteford et al., 2007, Fichtner et al., 2008, DeRose et al., 2011, Zhang et al., 2013a, McAuliffe et al., 2015). Furthermore, breast cancer PDXs produce tumour pathology, growth and metastasis of breast cancer (DeRose et al., 2011), and also maintain the genetic characteristics of their original tumours (Petrillo et al., 2012, Reyal et al., 2012, Zhang et al., 2013a).

An excellent conservation of the histological features of the tumour of origin in breast cancer PDXs was first shown by Marangoni and colleagues (Marangoni et al., 2007), and then repeatedly observed by others (DeRose et al., 2011, Landis et al., 2013, Du Manoir
et al., 2014). The EDW-01 xenograft results presented in this chapter are in agreement with these observations, thus confirming that PDXs strongly recapitulate the morphologic characteristics of primary tumours.

The most widely used predictive factors guiding treatment in breast cancer, and determining the utility of endocrine and anti-HER2 therapy, are the ER and the PR (both used as a basis for hormonal therapy), both of which are ligand-activated transcription factors belonging to the family of nuclear hormone receptors (Duffy, 2005). Another is the HER2 protein (essential in selecting patients for treatment with Herceptin and newer HER2-directed therapies). As HER2 is a major target for the development of new cancer therapies, its greatest value as a predictive marker lies in the prediction of response to therapies that target HER2, such as trastuzumab (Herceptin) (Finn et al., 2009). In breast cell lines and model tumour systems, overexpression of the HER2 gene has been associated with increased mitogenesis, malignant transformation, increased cell motility, invasion, and metastasis (Olayioye et al., 2000). In human breast cancer, amplification of the HER2 gene is found in 15-30% of primary invasive tumours. A recent study by Manoir and colleagues, 2014, established a collection of 20 breast PDXs that resulted from engraftment of 130 primary breast tumours, and characterised their biological and clinical features, as well as their genetic stability, to investigate what differentiates tumours that produce PDXs from tumours that do not. While 70% of the PDXs originated from triple negative breast cancers, which lack expression of all 3 targetable receptors (ER, PR and HER2), 25% of the PDXs comprised of ER-positive cases (Du Manoir et al., 2014). They showed that breast PDXs retain transcriptomic and genomic stability over sequential passages, with gradual selection of a dominant genetic clone. They also showed that breast tumours producing stabilised PDXs derive from a subset of aggressive cancers associated with poor clinical outcome, and that despite an excellent conservation of original features, they remain genetically dynamic elements.

Whilst sporadic loss of ER expression in PDXs established from ER-positive breast tumours has been reported (Bergamaschi et al., 2009), in the EDW-01 xenograft studied in this chapter, ER-positivity was successfully detected throughout the xenograft and positivity increased with accumulated EMT. The tumour cells are ER-positive, strong in
40% of cells and negative for PR and HER2 throughout the EDW-01 xenograft, regardless of passage, hence classifying the EDW-01 as **ER-positive PR-negative HER2-negative subtype (ER+PR-HER2-)**. This result is interesting because estrogen supplementation was not used to establish or propagate the EDW-01 xenograft, and PR is a target gene for estrogen-activated ER. Lacking PR expression in ER positive tumours may suggest aberrant growth factor signalling that, in turn, contributes to tamoxifen resistance of such tumours (Dai et al., 2016).

Several clinical reports have suggested that decreased PR levels in breast cancer may be associated with high growth factor signalling (Balleine et al., 1999, Bamberger et al., 2000, Dowsett et al., 2001, Konecny et al., 2003, Arpino et al., 2004). In fact, growth factors that activate the PI3K-Akt-mTOR pathway have been shown to decrease PR transcription (Cui et al., 2003, Petz et al., 2004). The cumulative data raise the possibility that PR loss is a surrogate marker for excessive growth factor receptor activation, which translates into reduced tamoxifen benefit.

ER-positive lines have been difficult to establish among breast cancer PDX models, with a low average intake rate of engraftment reported (2-15%) (Marangoni et al., 2009, Cottu et al., 2012, Charafe-Jauffret et al., 2013, Landis et al., 2013), thus these established ER-positive PDXs may not represent the overall tumour environment population. Hence, characterisation of ER-positive PDXs is crucial in order to properly use them as preclinical models to examine treatment outcome. Based on HER2 expression, ER-positive tumours can be further classified as ER-positive HER2-negative and ER-positive HER2-positive. Women with ER-positive HER2-positive breast cancers have a worse 5 year overall survival, and disease-free survival, than women with ER-positive HER2-negative cancers (Onitilo et al., 2009, Parker et al., 2009), and require different treatment strategies selective to their subtype (Wu et al., 2015a), due to their differing biology.

Although HER2-negative disease has a more favorable prognosis than HER2-positive disease, it lacks the benefit of using these targeted therapies. Previous studies have reported that HER2 status usually shows an inverse association with ER expression (Konecny et al., 2003, Dowsett et al., 2006).
Among the various molecular subtypes of breast cancer, ER-positive cancer comprises ~75% of all breast cancers (Perou et al., 2000). The ER status is therefore a critical discriminator of breast cancer molecular subtypes (Sotiriou et al., 2003). The effectiveness of treatment has been confirmed by observations that ER-positive tumours were associated with hormonal responsiveness, longer relapse-free survival, and overall survival in comparison to the ER-negative subtypes (Hoch et al., 1999). However, only about two-thirds of patients with ER positive breast tumours will respond favourably to endocrine therapy, while only 10% of ER-negative tumours respond to such treatments (Lacroix et al., 2001). The reason for failed responses to endocrine therapy in the ER-positive and ER-negative tumours is that, with a low intake rate, established ER-positive PDX lines might not be representative of the overall tumour population. Although outside the scope of the current study, it would be interesting to determine whether the EDW-01 xenograft responds to endocrine therapy.

As a guide to the EMT status of the EDW-01 xenograft, the classical EMT markers vimentin, N-cadherin, and E-cadherin were investigated.

Increased vimentin expression has been reported in various tumour cell lines and tissues, such as prostate cancer, breast cancer, endometrial cancer, tumours of the central nervous system (CNS), malignant melanoma, lung cancer, and gastrointestinal tract tumours, including pancreatic, colorectal, and hepatic cancers (Satelli and Li, 2011). The use of vimentin as a marker of EMT has been corroborated in vitro using cancer cell lines (Thompson et al., 1992a, Zajchowski et al., 2001, Thiery, 2002b, Blick et al., 2010). Vimentin expression in breast cancer cells in culture has often been associated with an increase in invasive migratory potential (Hendrix et al., 1996, Hendrix et al., 1997, Willipinski-Stapelfeldt et al., 2005, Rodríguez-Pinilla et al., 2006, Blick et al., 2010), although its significance in vivo is controversial (Perou et al., 2000, Petersen et al., 2001, Gilles et al., 2005, Korsching et al., 2005, Reis-Filho, 2005).

In the course of earlier studies undertaken on the EDW-01 breast cancer xenograft model (De Sousa, Walker, Williams, Thompson), it was noted that the xenograft acquired parenchymal expression of vimentin, and became incrementally more vimentin-positive.
and E-cadherin-negative with successive passages. In expansion of these findings, results from this chapter demonstrated heterogeneous expression of vimentin in the EDW-01 xenograft was comparable to the pattern of vimentin expression typically seen in ~15% of invasive human breast cancer specimens (Gilles and Thompson, 1996, Willipinski-Stapelfeldt et al., 2005, Sarrió et al., 2008). Q-RT-PCR analysis of the EDW-01 xenograft demonstrated that vimentin mRNA expression progressively increased with successive passage. Taken together, the immunohistochemistry data combined with the Q-RT-PCR results indicate clear evidence of an increasing extent of EMT in this in vivo system.

A few animal models have accordingly revealed such EMT traits in vivo (Chen et al., 2008, Trimboli et al., 2008, Ponzo et al., 2009). Trimboli and colleagues (Trimboli et al., 2008) demonstrated that EMT occurs in breast cancer using a myc oncogene-driven mouse model. This data is also supported by other studies on human breast biopsies, which have revealed breast tumour heterogeneity and have shown that EMT-related phenotypes exist in human tumours (Guarino, 2007, Guarino et al., 2007, Klymkowsky and Savagner, 2009, Bonnomet et al., 2012). Although many studies have also shown an EMT progression with therapy resistance (Bhatia et al., 2017), it is important to note that the EDW-01 xenograft was not challenged by therapy; the EMT progression was spontaneous.

Whilst many studies have not detected any relationship between vimentin expression and survival and/or recurrence (Seshadri et al., 1996, Scawn and Shousha, 2002, Kusinska et al., 2009), others have found that expression of vimentin is an indicator of poor prognosis (Domagala et al., 1990, Holck et al., 1993, Domagala et al., 1994, Heatley et al., 2002, Niveditha and Bajaj, 2003, Willipinski-Stapelfeldt et al., 2005, Yamashita et al., 2013). EMT phenotypes have been detected in a variety of human primary carcinomas, and have been associated with poor survival (Guarino et al., 2007, Sarrió et al., 2008). Several studies have reported overexpression of vimentin in breast cancer (reviewed in (Kokkinos et al., 2007)), where vimentin expression is associated to high histological grade, lack of ER expression, EGFR positivity, and p53 expression (Raymond and Leong, 1989, Domagala et al., 1990, Koutselini et al., 1995, Santini et al., 1996, Tsuda et al., 2005,
Umemura et al., 2005). In particular, vimentin expression, detected in approximately 15% of the breast parenchymal tissues, has been identified through molecular-profiling studies as a marker of basal-like breast cancer cells that may represent the clinical ‘triple-negative’ (ER-, PR- and HER2-negative) tumour-type that is associated with a poor prognosis (Perou et al., 2000, Van't Veer et al., 2002, Sorlie et al., 2003, Neve et al., 2006, Sarrió et al., 2008, Sotiriou and Pusztai, 2009). A study by Yamashita and co-workers (2013) was the first to show that elevated expression of vimentin is associated with the aggressive phenotype, and a poor prognosis in triple-negative breast cancer patients (Yamashita et al., 2013). Whilst vimentin expression was increased in the EDW-01 xenograft, our results were conflicting with the above studies, in that the immunohistochemical classification of the EDW-01 xenograft correlates well with the intrinsic gene expression microarray categorisation of a luminal B (ER positive, HER2 negative, and PR low) (Inic et al., 2014) rather than the basal phenotype, evidenced by ER-positive, HER2-negative, and PR-Negative staining as well as negative staining for CK14, Ck5/6, CK17, SMA, and EGFR (Staining conclusion contributed by Dr Sunil Lakhani). These evident discrepancies may be due to differences in the clinic-pathological characteristics of the tumours analysed.

Although vimentin is commonly over-expressed in breast cancers, its presence is not always a clear representation in determining the prognosis of the patient. However, overall patient outcome is the cumulative result of a multi-step process, and only the initial stage of invasion resembles EMT. However, as vimentin is also expressed by other cell types in the mammary tissue, further analyses are required to identify more specific markers for EMT.

As such, N-cadherin, which is also implicated in EMT through Cadherin switching (Hazan et al., 2000, Nagi et al., 2005, ElMoneim and Zaghloul, 2011, Shapiro et al., 2011), was investigated. N-cadherin staining was attempted on the EDW-01 xenograft, but did not reproducibly provide any N-cadherin staining patterns. However, similar to vimentin Q-RT-PCR data, an increased level of mRNA expression was also observed for N-cadherin in late xenograft material when compared to early xenograft material.
Numerous studies from past, and current literature have demonstrated the expression (de-novo expression, re-expression, up-regulation, and down regulation) of N-Cadherin in a number of cancers (reviewed in (Derycke and Bracke, 2004, Rai and Ahmed, 2014)) such as oral cancer (Islam et al., 1996, Lawson et al., 2006, Pyo et al., 2007, Diamond et al., 2008, Nguyen et al., 2011a, Nguyen et al., 2011b, Zhao et al., 2012), gastrointestinal cancer (Yanagimoto et al., 2001, Rosivatz et al., 2002a, Nakajima et al., 2004, Rosivatz et al., 2004, Kamikihara et al., 2012), prostate cancer (Tran et al., 1999, Tomita et al., 2000, Jaggi et al., 2005, Gravdal et al., 2007, Jennbacken et al., 2010, Cui and Yamada, 2013), renal cancer (Tani et al., 1995, Mialhe et al., 2000, Lascombe et al., 2006, Behnes et al., 2012), lung cancer (Nakashima et al., 2003, Yamauchi et al., 2011, Zhang et al., 2013b), and breast cancer (Nieman et al., 1999b, Hazan et al., 2000, Burstein et al., 2004, Nagi et al., 2005, Christiansen and Rajasekaran, 2006, ElMoneim and Zaghloul, 2011, Rezaei et al., 2012). In human breast cancer, N-cadherin is upregulated in invasive ductal carcinomas, and is further increased in tumours with metastatic potential (Nagi et al., 2005). This notion is further supported by the work of Hazan and coworkers (2000), who demonstrated that overexpression of N-cadherin in breast carcinoma correlates with invasiveness as a result of N-cadherin-mediated interactions between cancer and stromal cells (Hazan et al., 2000). Additionally, Rezaei et al (2012), who studied the critical role of N-cadherin in breast cancer progression, showed that N-cadherin is involved in maintaining the malignant tumour cell phenotype (Rezaei et al., 2012). Furthermore, ElMoneim and Zaghloul (2011), showed that high levels of N-cadherin expression are observed in most high-grade invasive ductal carcinomas (ElMoneim and Zaghloul, 2011), where N-cadherin is increased further in metastasising subtypes, such as micropapillary breast cancers (Nagi et al., 2005). N-cadherin expression is significantly higher in poorly differentiated carcinomas than in moderately, or well-differentiated invasive ductal carcinomas (Ma et al., 2010). As such, overexpression of N-cadherin in breast carcinomas may play a central role in the development of invasive ductal carcinoma.

Taken together, the increased expression of N-cadherin mRNA demonstrated in the EDW-01 xenograft system is in agreement with the findings from previous research. The enhanced expression of N-cadherin during tumour progression suggests that N-cadherin
may be important when tumours change from a benign, non-aggressive neoplasm, to an invasive tumour, highlighting the possibility that N-cadherin contributes to the invasive phenotype (Rai and Ahmed, 2014), and emphasising the relevance of N-cadherin during EMT in breast cancer progression.

In aggressive tumours, EMT is characterized by increased N-cadherin, and reduced E-cadherin expression, contributing to a stroma-oriented cellular adhesion profile, increased tumour cell motility, and other invasive properties (Christiansen and Rajasekaran, 2006). This gain of N-cadherin expression and loss of E-cadherin expression is reminiscent of the cadherin switching that is seen during EMT. This cadherin switching from E- to N-cadherin in epithelial malignancies has often been attributed to a switch from an epithelial to a mesenchymal phenotype (Cavallaro et al., 2002, Thiery, 2002b, Christofori, 2003, Maeda et al., 2005, Thiery and Sleeman, 2006), and this switching is an important event in cancer progression (Wheelock et al., 2008). This observation correlates with the results obtained in the EDW-01 xenograft in this study, where E-cadherin levels were dramatically reduced with increasing EDW-01 xenograft passage material, both in immunohistochemical, and Q-RT-PCR analyses. These findings are consistent with increased N-cadherin (mRNA and protein) and vimentin (mRNA) expression, indicating that as the xenograft model progresses to a more mesenchymal state, expression of the epithelial marker E-cadherin is reduced. This supports the use of the EDW-01 xenograft model to study EMT progression in breast cancer, as the pattern of mRNA expression seen for vimentin, N-cadherin, and E-cadherin satisfies the most basic criteria for an epithelial to mesenchymal transition and progression.

One role of cadherin switching, which occurs during normal developmental processes, is to allow a select population of cells to separate from their neighbours (Wheelock et al., 2008)). It has been shown that cells expressing different cadherins segregate from one another in *in vitro* aggregation assays (Steinberg and Takeichi, 1994), and it is easy to infer that, *in vivo*, this ability to segregate cells allows cadherin switching to promote separation of the egressing cells from those left behind. Tumour cells often recapitulate this activity and the result is an aggressive tumour cell that gains the ability to leave the site of the tumour and metastasise. The cadherin switch is also essential for increased
motility, but is not always required for the morphological changes that accompany EMT (Maeda et al., 2005). Some reports have described that N-cadherin expression is more important for cancer metastasis than E-cadherin and other EMT inducers (Islam et al., 1996, Nakajima et al., 2004). In some malignant tumours, especially undifferentiated tumours and metastases, E-cadherin is mostly negative and N-cadherin is sometimes positive, comprising the cadherin switch (Tomita et al., 2000). Published findings have reported an inverse correlation in some human cancers such as breast and prostate (Hazan et al., 1997, Tomita et al., 2000), between the expression of E-cadherin and N-cadherin, in the cadherin switch (Wheelock et al., 2008). In a study by Nieman and co-workers (1999b), a switch from E-to N-cadherin in breast cancer cells correlated with invasion and motility (Nieman et al., 1999b). In a more recent study, in extrahepatic cholangiocarcinoma (EHCC), E-cadherin and the cadherin switch promoted cancer progression via TGF-β-induced EMT (Araki et al., 2011).

The possibility therefore exists that the increased N-cadherin and reduced E-cadherin (cadherin switch) in the EDW-01 xenograft is due to selection of distinct subpopulations, rather than a reversible switch. Given that the assessment of N-cadherin and E-cadherin were in tissue, reversion of cadherin switching is difficult to assess. However, it is possible that future experiments such as adapting cells from the tumour to culture and performing an EMT array at varying stages of passage specifically looking at cadherin changes may help further confirm whether this switch during EMT, is indeed reversible.

Loss or down-regulation of E-cadherin expression in carcinomas is often observed, and correlates with the degree of malignancy of the tumour (Birchmeier and Behrens, 1994). Loss of E-cadherin-mediated cell adhesion is one of the key mechanisms involved in the metastatic conversion of epithelial cells and EMT (Agiostratidou et al., 2007, Becker et al., 2007). E-cadherin is a key mediator of cell-cell adhesions in epithelial tissues, and loss of E-cadherin expression and/or function has been observed during tumour progression of most carcinomas (reviewed in (Takeichi, 1993, Birchmeier and Behrens, 1994, Berx and Van Roy, 2009)). This loss of E-cadherin increases tumour cell invasiveness in vitro and contributes to the transition of adenoma to carcinoma in animal models (Christofori and Semb, 1999, Thiery, 2002b). E-cadherin exerts a potent
invasion-suppressing role in both tumour cell lines, and in vivo tumour model systems (Frixen et al., 1991b, Vleminckx et al., 1991, Meiners et al., 1998, Perl et al., 1998a). Studies using animal models of breast and pancreatic cancer have demonstrated that loss of E-cadherin function is a casual factor in the promotion of invasion and metastasis, largely through the conversion of epithelial tumour cells into highly migratory and invasive cells (Canel et al., 2013). Loss of E-cadherin is considered to be a fundamental event in EMT, leading to increased tissue mobility and tumour invasion (Hazan et al., 2000, Baranwal and Alahari, 2009). In breast cancer, the partial or total loss of E-cadherin expression correlates with loss of differentiation characteristics, acquisition of invasiveness, increased tumour grade and stage, metastatic behaviour, and poor prognoses (Berx and Van Roy, 2001, Kang and Massagué, 2004). The connection between loss of E-cadherin expression in cancer cells and poor patient prognosis, including increased tumour grade, metastasis, and mortality, has been established by several studies (Krisanaprakornkit and Iamaroon, 2012). The association of E-cadherin down-regulation with tumour grade, in particular, has been shown in a few studies, although published results have been somewhat inconsistent (Asgeirsson et al., 2000, Rakha et al., 2005, Jeschke et al., 2007). Hugo and co-workers (2017), in their study, examined whether E-cadherin maintains epithelial proliferation in MDA-MB-468 breast cancer cells, facilitating metastatic colonization in severe combined immunodeficiency (SCID) mice. They found that E-cadherin expression promotes growth of primary breast tumours and conceivably the formation of metastases, supporting a role for MET in metastasis (Hugo et al., 2017).

E-cadherin expression showed a significant relationship with lymph node status in one study (Jeschke et al., 2007), while it was independent of lymph node status in another study (Rakha et al., 2005). Additionally, a study by ElMoneim and Zaghloul (2011) found associations between E-cadherin expression and ER and PR expression. ER-positive tumours express normal amounts of E-cadherin protein, and the loss of ER and E-cadherin genes has been linked to disease progression in invasive carcinomas of the breast (ElMoneim and Zaghloul, 2011). However, in another study, E-cadherin expression was not associated with positive ER or PR status (Mohammadizadeh et al.,
Oesterreich and coworkers (2003) showed downregulation of E-cadherin protein and mRNA in ER-positive breast cancer cell lines (Oesterreich et al., 2003), supporting the data seen in the ER-positive EDW-01 xenograft in this study, where E-cadherin expression is downregulated with increased passage.

Loss of E-cadherin expression during carcinoma progression can occur by transcriptional regulation or epigenetic mechanisms, including promoter hypermethylation. Methylation of the E-cadherin promoter has been shown to correlate with loss of E-cadherin expression in breast cancer cell lines and primary ductal and lobular breast cancers (Nass et al., 2000, Droufakou et al., 2001). However, the loss of E-cadherin expression is not simply attributable to hypermethylation. Overexpression of the transcriptional repressors (SNAIL, SLUG (Snail-2), Zeb1, Zeb2 (SIP1), Twist, etc), which target the E-Cadherin promoter, are alternative mechanisms for its inactivation in breast tumours (Berx and Van Roy, 2009). Downregulation of E-cadherin has a number of significant consequences that are of direct relevance to EMT (Lombaerts et al., 2006). Levels of E-cadherin become limiting, which results in the loss of E-cadherin-dependent intercellular epithelial junction complexes, and a reduction of E-cadherin-mediated sequestering of β-catenin in the cytoplasm. As a result, β-catenin localises to the nucleus, and feeds into the Wnt signalling pathway by activating transcriptional regulation through lymphoid-enhancer-binding factor/T-cell factor-4 (LEF/TCF4) (Thiery and Sleeman, 2006).

While there are many successful studies showing reduced E-cadherin expression in vitro, there is a lack of information on cadherin regulation in vivo. Despite this lack of information, there is now a more comprehensive knowledge of the role of E-cadherin repressors in development (Peinado et al., 2007). The loss of E-cadherin expression at the transcript level was first identified in several human cancer cell lines, and later in different human cancers, including prostate, breast, colorectal and thyroid cancers (Reviewed in (Van Aken et al., 2001, Strumane et al., 2004). A study by Fujita and co-workers (2003) showed that aberrant expression of Snail in ER-negative breast cancer cell lines results in the loss of E-cadherin expression (Fujita et al., 2003).
E-cadherin is a potent invasion/tumour suppressor of breast cancer. Consistent with this role in breast cancer progression, partial or complete loss of E-cadherin expression has been found to correlate with poor prognosis in breast cancer patients (Yoshida et al., 2001). The association between loss or down-regulation of E-cadherin and the progression of sporadic breast cancer has been extensively documented (Reviewed in (Cowin et al. (2005)). A study by Jeschke and coworkers (2007) analysed the expression of E-cadherin in mammary ductal carcinoma in situ, invasive breast carcinomas without metastasis, invasive carcinomas with their corresponding lymph node metastasis, invasive carcinomas with recurrence, including the recurrence tissue, and in invasive carcinoma with distant body metastasis, including the corresponding metastasis tissues. They showed that there are significant differences of expression of E-cadherin between primary breast cancer cells and their metastases. In detail, they demonstrated strong expression of E-cadherin in carcinoma in situ in all cases investigated, and a lower expression in invasive tumour cells without metastasis. Expression of E-cadherin is further down-regulated in invasive tumour cells with lymph node metastasis, and is significantly reduced in carcinoma with recurrence, and recurrence tissue. In addition, a reduced, but not significantly so, expression of E-cadherin was observed in carcinoma with distant body metastasis, and in the metastasis itself, when compared to carcinoma in situ (Jeschke et al., 2007).

The findings in the EDW-01 xenograft, and comparative studies of the expression patterns of E-cadherin, indicate the importance of E-cadherin in affecting its metastatic ability, and in EMT. Characteristically therefore, this unique EDW-01 xenograft can be used to initiate and investigate the progression of E-cadherin loss, Cadherin-switching, and EMT.

In the EDW-01 xenograft, we did not assess metastasis other than looking for macro metastasis. It is somewhat outside the scope of the thesis to address this and the experiments were not designed to directly test this. It is important to note, however, the data presented in the thesis strongly support that increasing passage in the EDW-01 xenograft correlated with elevated metastatic potential, coupled with alterations in EMT markers such as cadherin expression. When tumour cells switch from expressing E-
cadherin to N-cadherin, they present with increased aggressive behaviour. Future experiments would be aimed at further understanding the pathways both upstream and downstream of cadherin switching in order to expand on and identify the cadherin changes and which cadherins are expressed early and then later on as the cells become more aggressive.

Generally, there are 2 models of studying metastasis: spontaneous, and experimental (Price, 2014). The appropriate selection of a spontaneous or experimental model of metastasis depends primarily on the specific steps of metastasis being studied, the metastatic potential of the cancer cells, and the gene being investigated. Spontaneous models of metastasis involve the inoculation of tumour cells into an orthotopic site or ectopically (Price, 2014). These models allow the analysis of both early and late stages of metastasis, making it possible to study the function of any given gene. In experimental models of metastasis, cells are introduced directly into the blood stream of the host, making this model more ideally suited for testing therapies specifically targeting late stage metastasis (Khanna and Hunter, 2005b). For the EDW-01 model, future studies assessing the spontaneous model will define whether the late passage tumours are more metastatic than the early tumours. Perhaps isolating circulating tumour cells (CTCs) from the circulation of the SCID mice at each passage of the EDW-01 xenograft and analysing them for EMT genes would allow further investigation into this, as a future experiment.

Having verified and further characterised the in vivo EMT seen with increasing passage of the EDW-01 xenograft, this unique system was used to assess whether integrins, which were found to change in accordance with the EGF-induced EMT of PMC42 cells (Chapter 3), also mapped with EMT. However, due to technical complications, successful staining was not possible for ITGβ1, ITGα2 and ILK in the EDW-01 xenograft. More study is required to determine the specificity and sensitivity of the antibodies used in the study. Due to time constraints, the IHC approach was abandoned in favour of species-specific RT-PCR, as this proved quite effective, showing increased expression levels of ITGβ1 and ITGα2 mRNA, and a trend towards increased ILK mRNA expression in the later xenograft material in comparison to the early xenograft material. EMT regulation is
in fact often reported at the transcriptional level (Thiery et al., 2009). In particular, the role of the transcription regulation of EMT in the regulation and control of gene expression is very important. IHC, although an important tool to show protein expression, is still a semi-quantitative mode of analysis and not always the most reliable form of protein expression, as it has limitations (Taylor and Levenson, 2006, De Matos et al., 2010). Although IHC did not serve to be helpful in showing the presence of ITGα2 and ITGβ1 and ILK, the data in this thesis presented clear support that these markers are expressed or altered in EMT regulation. As such, for the nature of this study, this thesis implemented the use of both transcriptional and functional modes of analysis, where deemed appropriate.

Integrins, a large family of cell-matrix adhesion receptors, have been demonstrated to play important roles in many types of tumour cells (Hinton et al., 2008). Through the interaction with the basement membrane, integrins can mediate both cell-extracellular matrix (ECM) and cell-cell adhesion. As mediators of cell adhesive behaviour, integrins play a critical role in tumour progression and metastasis (Hynes, 2002, Guo and Giancotti, 2004, Langsenlehner et al., 2006, Ganguly et al., 2013, Naci et al., 2015, Raab-Westphal et al., 2017). In benign breast lesions (fibroadenoma or papilloma) and mammary adenocarcinomas, altered patterns of integrin expression were discerned when compared to normal breast tissue. Since altered expression of various integrins occurs during tumour growth and progression, the integrins, and their associated proteins, could be potential targets for cancer diagnosis and therapy. It is this altered expression of integrins that may further contribute to the invasive and metastatic potential of tumour cells (Mizejewski, 1999).

The α2β1 integrin, primarily a receptor for collagen and laminin, is highly expressed on normal breast epithelium (Zutter and Santoro, 1990). The integrin is also expressed on many epithelial cells, activated endothelial cells, and some inflammatory cells (Arase et al., 2001, Sasaki et al., 2003, Edelson et al., 2004, Zhang et al., 2006). Expression of α2β1 integrin is associated with motility, invasiveness, and cellular differentiation of a variety of tumours (Felding-Habermann et al., 2001, Hood and Cheresh, 2002).
To date, studies on α2β1 integrin have been controversial, demonstrating equivocal results on the role of the integrin. Previous studies have suggested that α2β1 integrin expression is altered in vivo during progression of breast, lung, and prostate cancers (Nissinen et al., 1998, Zutter et al., 1998, Lochter et al., 1999, Gogali et al., 2004, Van Slambrouck et al., 2009, Sottnik et al., 2013), as well as oral, squamous cell carcinoma (Evans et al., 2004). In particular, early studies provided the initial correlative suggestion that decreased expression of α2β1 integrin might play an important role in breast cancer progression (Zutter et al., 1990). Their detailed immunohistochemical study of α2β1 integrin expression on human breast cancer revealed that the α2β1 integrin was highly expressed on the epithelium of ducts and ductules of normal breast tissue, and was expressed at normal or close-to-normal levels in benign lesions such as fibroadenoma or papilloma. In contrast, markedly reduced, or undetectable levels of α2β1 integrin expression were typical of poorly differentiated adenocarcinomas. Well-differentiated adenocarcinomas exhibited intermediate levels of α2β1 integrin expression. Similar alterations of integrin expression in adenoma of the breast were subsequently described by (Pignatelli et al., 1991) and (Koukoulis et al., 1991). These studies suggest that decreased integrin expression contributes to the invasive and metastatic potential of tumour cells. Other studies using cell lines either in vitro or in vivo suggest that the α2β1 integrin may serve to enhance metastasis to different organs (Ho et al., 1997, Yoshimura et al., 2009). Alternatively, loss of α2β1 integrin expression may simply be a consequence of malignant progression. Hence, the results of the in vitro and in vivo studies of various designs may be subject to conflicting interpretations. On this basis, Ramirez and colleagues explored the role of α2β1 integrin in cancer initiation and progression using a clinically relevant, spontaneous mouse model of breast cancer progression and metastasis (mouse mammary tumour-virus-Neu-MMTV-Neu). They demonstrated that the α2β1 integrin is a metastasis suppressor of breast cancer (Ramirez et al., 2011), consistent with previous studies indicating high expression of α2β1 integrin in normal breast epithelium, and loss of α2β1 integrin in poorly differentiated breast cancer. Although decreased α2β1 integrin expression has been correlated with de-differentiation and tumour progression in the mammary gland (Xu et al., 1998, Zutter et
al., 1998), a causal link between α2β1 integrin and tumourigenesis has not been established.

Loss of α2β1 integrin in specific tissues may not result in a severe phenotype because the function of α2β1 may be fulfilled by other integrins with similar properties. A related possibility is that the loss of α2β1 integrin expression is compensated for by an increase in the expression, or function, of a related integrin (Mercurio, 2002).

Whereas previous findings have indicated a loss of α2β1 integrin in breast cancer progression, results from this study show increasing α2β1 integrin expression in the EDW-01 xenograft model of breast cancer. Whilst this data shows contrasting results to published studies, this data may suggest that gain of α2β1 integrin expression might play an important specific role in breast cancer EMT. Although increasing levels of α2β1 integrin expression were shown in the EDW-01 xenograft, the possibility remains that the level of expression may actually still be lower than what is seen in the normal mammary gland, however it was outside the scope of the work undertaken to determine this. Supportive of the data seen in the EDW-01 xenograft, some cancers are associated with high α2β1 integrin expression levels. Dedhar and Saulnier (1990) showed that the expression of α2β1 integrin increased in the chemically transformed human osteosarcoma cells, and that the upregulation of this integrin might be correlated with tumour progression and metastasis (Dedhar and Saulnier, 1990). Another study by Sottnick and colleagues showed that increased expression of α2β1 integrin stimulated in vitro invasion, and promoted the growth of prostate cancer cells within the bone. α2β1 integrin protein and mRNA expression was enhanced in bone metastases to the level observed in normal, non-malignant prostate tissue, and significantly higher than primary prostate cancer lesions or metastasis to other sites such as lymph nodes (Sottnik et al., 2013). Similarly, α2β1 integrin expression accelerated experimental metastasis or tumour dissemination of melanoma (Baronas-Lowell et al., 2004) and rhabdomyosarcoma (Chan et al., 1991, Hannigan et al., 1996b), gastric cancer (Ura et al., 1998, Matsuoka et al., 2000) and colon cancer (Bartolome et al., 2014). In contrast, D’Ardenne et al. (1991) demonstrated that the α2 integrin subunit, amongst others, is not downregulated in breast tumours (d’Ardenne et al., 1991).

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Therefore, in the tumourigenic process, the expression pattern of integrins is not uniform, but complex. Because of this heterogeneity amongst studies, further investigation is required to characterise the expression and function of α2β1 integrin in breast carcinoma, both in vitro as well as in vivo. Taken together with the in vitro results from the previous chapter (in vitro PMC42 model system), overexpression of α2β1 integrin shows evidence of an increasing extent of EMT, and may facilitate migration and metastatic spread, making α2β1 integrin a potential biomarker for cancer progression diagnosis and treatment monitoring.

Despite the conflicting observations seen both in this results chapter and the literature, imperative questions remain concerning the role of the α2β1 integrin in cancer biology, such as what is/are the molecular mechanism/s through which α2β1 integrin loss/gain facilitates increased intravasation? How does α2β1 integrin down/up-regulation during breast cancer progression occur? Answers to these questions will provide novel insights into tumour biology, and clarify prospects for clinical application of the α2β1 integrin as a biomarker or therapeutic target. Given their diverse roles in human disease, integrins have increasingly been viewed as therapeutic targets (Madrazo et al., 2017). Approaches include targeting the integrins or their ligands by using antibodies, peptides and organic inhibitors. Integrin blocking therapies are already under clinical development in cancer, with approaches that include targeting the integrins or their ligands with antibodies, peptides and molecular inhibitors, such as the anti-integrin antibodies, antagonists and small molecules against αvβ3, αvβ5, α5β1 and αvβ6 (Gutheil et al., 2000, Livant et al., 2000, Cianfrocca et al., 2006, Khalili et al., 2006, Ricart et al., 2008, Bell-McGuinn et al., 2011, O’day et al., 2011, Sheldrake and Patterson, 2014, Uhl et al., 2014, Yacobovich et al., 2016, Miller et al., 2017) as well as a specific α2β1 integrin antagonist, Rhodocetin, a snake venom protein that binds to α2β1 integrin, inhibiting its interaction with its endogenous ligand collagen (Eble et al., 2001, TUCKWELL, 2003, Bracht et al., 2011). Despite the promising preclinical data, clinical trials have been unsuccessful at showing any improvement over conventional therapies. Therefore, research is currently focused on developing new tumour targeted drug-delivery strategies approach to overcome any clinical limitations.
ILK, a multifunctional intracellular effector of cell-matrix interactions, regulates many cellular processes, including growth, proliferation, survival, differentiation and angiogenesis (McDonald et al., 2008). ILK is identified as an important mediator of EMT, and the increase of ILK activity is also suggested as a molecular marker for EMT (Lee et al., 2006). Recent reports suggest that overexpression of ILK in epithelial cells induces the EMT by repressing E-cadherin expression, activating nuclear β-catenin, and inducing a transformed, tumorigenic phenotype (McDonald et al., 2008). ILK overexpression has been shown to promote invasion and cell migration (Troussard et al., 2000, Wong et al., 2007). White and co-workers were the first to demonstrate a direct potential oncogenic role for ILK, which is upregulated in human tumours and tumour cell lines (White et al., 2001). The observed high ILK expression in the EDW-01 xenograft in this chapter, is consistent with the above published research, and the research on cancer cell lines of ovarian, prostate, breast, gastric, and thyroid origin, squamous cell carcinoma and glioblastoma (Persad et al., 2000, Ahmed et al., 2003, Ito et al., 2003, Edwards et al., 2005, Younes et al., 2005, Troussard et al., 2006, Younes et al., 2007). This data is also in agreement with Lee and co-workers, who suggested ILK as a molecular marker for EMT (Lee et al., 2006), where it is involved in embryonic development, oncogenesis, and metastasis of malignant cells (Oloumi et al., 2006). The role for ILK in breast cancer, however, is controversial. Chen and coworkers (2004) presented divergent data for the role of ILK in malignant growth and invasion (Chen et al., 2004). In their system, ILK expression was lost or downregulated, and suppressed the growth and invasive properties of tumour versus normal breast epithelial cells, suggesting that ILK may not be essential to the progression of breast cancer metastasis, but rather contributing to the suppression of breast carcinoma cell growth and invasion. This data is an interesting contrast to the well-published role for ILK in linking extracellular signalling to growth, proliferation, survival, differentiation, migration, invasion and angiogenesis (Hannigan et al., 2005, Legate et al., 2009).

Nevertheless, the overexpression of ILK is often a prominent feature of human malignancies, and its increased abundance in tumour tissues correlates with poor patient
outcome (McDonald et al., 2008). Thus, ILK is an attractive therapeutic target in breast cancer EMT.

This study revealed that through successive *in vivo* tumour formation, the EDW-01 xenograft underwent drastic changes, adopting a mesenchymal morphology and expression profile, as detected by loss of epithelial marker expression and the gain of mesenchymal marker expression. Moreover, the EMT-like phenotype is correlated to progression and metastasis, suggesting that EMT is an integral aspect of breast cancer progression. These results indicate that the host tumour microenvironment may be playing an important role in promoting tumour progression and metastasis, while increased tumorigenic potential is accompanied by EMT of the tumours. Further investigation is needed to define how interactions with the tumour microenvironment could result in such drastic morphologic and behavioural changes in cancer cells.
CHAPTER 5: DISTINGUISHING TRANSCRIPTIONAL DRIVERS AND PASSENGERS OF EMT: SIRNA APPROACHES OF CANDIDATE GENES ITGβ1, ITGα2 AND ILK.
5.1 INTRODUCTION

Integrins are the major receptors for extracellular matrix (ECM) proteins. The expression profile of integrins on cancer cells and the composition and organisation of the ECM of the tumour stroma are major factors in cancer development, metastasis, and in therapy resistance (Naci et al., 2015). The functions of integrins during EMT are diverse and dynamic, and being involved in signal transduction, they are able to initiate and enforce EMT and invasion (Yilmaz and Christofori, 2009).

The α2β1 integrin is a major collagen receptor that is widely expressed and known to promote cell migration and control tissue homeostasis. Numerous studies have implicated a role for α2β1 integrin in a range of biological functions (Zutter et al., 1998, Santoro, 1999, Mercurio, 2002, Leabu et al., 2005), and have suggested that it can be a key player in cancer metastasis either by promoting or inhibiting the dissemination process of cancer cells. Early studies have shown that expression of α2β1 integrin can modulate cell adhesion, spreading, post-extravasation migration, collagen gene transcription, matrix metalloproteinase production, and collagen matrix contraction (Chan et al., 1991, Schiro et al., 1991, Chan et al., 1992, Langholz et al., 1995, Hangan et al., 1996, Ivaska et al., 1999a, Ivaska et al., 1999b, Lochter et al., 1999). In particular, α2β1 integrin has been implicated in multiple aspects of tumour progression and metastasis. In many tumours, there are correlations between high expression of α2β1 integrin and tumour progression (Fishman et al., 1998, Lochter et al., 1999, Böhle et al., 2002, Grzesiak and Bouvet, 2006, Kirkland and Ying, 2008). In addition, α2β1 integrin has been implicated as a promoter of malignant phenotype in pancreatic cancer cells (Grzesiak and Bouvet, 2006) and prostate cancer (Hall et al., 2006).

Furthermore, α2β1 integrin has been associated with the promotion of EMT, which is an important driver of invasion and metastasis and tightly associated with cadherin alterations. It has been shown that α2β1 integrin enhances cell migration and proliferation, through both N-cadherin upregulation (Shintani et al., 2008) and by disrupting E-cadherin-mediated cell-cell contacts (Koenig et al., 2006). To effectively
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mediates the cellular interaction essential for migration and tumour metastasis, cadherins and integrins must function in a coordinated manner (Gil et al., 2011).

Among a number of possible candidates that might regulate/coordinate integrin/cadherin expression or functions, a very likely one being integrin-linked kinase (ILK). ILK is a serine/threonine protein kinase which interacts with the cytoplasmic domains of integrins β1 and β3 (Hannigan et al., 1996b, McDonald et al., 2008), and plays an important role in linking extracellular signalling to the regulation of survival, cell cycle progression, migration and invasion. Overexpression of ILK has been shown to promote invasion and cell migration (Troussard et al., 2000, Wong et al., 2007) and has been documented in many types of cancer, including prostate, colon, gastric and ovarian cancers, malignant melanomas, malignant pleural mesothelioma and non-small-cell lung carcinoma NSCLC (Papanikolaou et al., 2010, Zhao et al., 2013).

Moreover, increased ILK activity has been suggested as a molecular marker of EMT (Lee et al., 2006). It has been reported that overexpression of ILK induces EMT in mammary epithelial cells (Somasiri et al., 2001). In their studies, Somasiri and colleagues (2001) examined the role of ILK in the basement membrane-dependent differentiation and morphogenesis of the scp2 mouse mammary epithelial cell line. They found that ILK activity was increased by cellular interactions with either a reconstituted basement membrane gel or by purified laminin. Forced expression of a dominant negative form of ILK did not prevent differentiative b-casein induction, but it did subtly affect spheroidal morphogenesis. In contrast, forced overexpression of wild-type ILK inhibited differentiation, prevented morphogenesis, and initiated a mesenchymal transformation of the cells that was associated with a loss of cell-cell junctions (Somasiri et al., 2001).

Given the importance of ILK in cancer development and progression, it is anticipated that ILK inhibition and/or silencing may be an effective way to treat cancer (Han et al., 2015). In order to investigate the aims of this chapter, chemically synthesised short interfering RNA (siRNA) technology was employed. siRNA are short strands of RNA that mimic the strands of siRNA products of dicer cleavage. The siRNAs subsequently assemble with protein components into an RNA-induced silencing complex (RISC).
strands are then unwound to form activated RISCs. These activated RISCs then bind to complementary RNA molecules by base pairing interactions between the siRNA antisense strand and the target mRNA. The bound mRNA is cleaved and sequence-specific degradation of mRNA results in gene suppression (Figure 5.1). The attractiveness of RNA interference (RNAi) relies on its efficient and specific gene silencing. However, a number of barriers for clinical application still need to be overcome, such as instability & low bioavailability, off-target effects, immune response, and delivery problems (Deng et al., 2014).

Up until these studies were carried out, the PMC42 cells have previously not been amenable to transfection due to the poor uptake of chemically synthesised and in vitro transcribed siRNA or transgene-encoding plasmids or viruses. To the best of my knowledge, this study was the first, to successfully show that RNA interference proved to be an effective way to reduce gene expression in the PMC42 cell model used in the present study.

In chapter 3 of this thesis, experimental findings demonstrated an early upregulation of ITGβ1, ITGα2 and ILK in the PMC42 cells. Their overexpression occurred in a manner that coincided with the EMT induction in the PMC42 system. However, little is known about the functional role these transcripts play in the EMT process of PMC42 cells. Whether or not their function is centrally implicated or required in EMT induction remained to be elucidated. Therefore, the current chapter tests whether ITGβ1, ITGα2, and ILK are functional drivers of the EMT or passengers in the EMT process, contributing to the mesenchymal phenotype in the PMC42 model system.

The hypothesis of this chapter was that ITGβ1, ITGα2 and ILK expression are essential for EGF-induced EMT in the human PMC42 breast cancer cell system, being functional “drivers”. The aim was to identify whether they have an impact on adhesion, migration and late stage mesenchymal gene expression, and thus have a functional role in EMT progression.
Figure 5.1 RNA Interference (RNAi).

RNAi mechanism is expedited by small molecules of interfering RNA to express a gene of interest effectively. Double stranded RNA (dsRNA) is introduced into a cell and gets chopped up by the enzyme dicer to form small interfering RNA (siRNA). siRNA then binds to the RISC complex and is unwound. The antisense RNA complexed with RISC binds to its target mRNA which is the cleaved by the enzyme slicer rendering it inactive.

a) **Model of Dicer and RNA-induced silencing complex (RISC)** a. silencing initiate with enzyme Dicer and dsRNA is processed to convert the silencing trigger to ~22-nucleotide, small interfering RNAs (siRNAs), b. Dicer binding and cleaving dsRNA (*Cleavage into precisely sized fragments is determined by the fact that one of the active sites in each Dicer protein is defective. Different colours show two separate molecules of Dicer) (Taken from Younis et al., 2010).

b) **Diagrammatic representation of mechanism of RNAi.** Silencing triggers in the form of double-stranded RNA presented in the cell as synthetic RNAs, replicating viruses or transcribed from nuclear genes. These are recognized and processed into small interfering RNAs by Dicer. The duplex siRNAs are passed to RISC (RNA-induced silencing complex), and the complex becomes activated by unwinding of the duplex. Activated RISC complexes can regulate gene expression at many levels (Taken from Younis et al., 2010).
Chapter 5: Distinguishing Transcriptional Drivers and Passengers of EMT: siRNA Approaches of Candidate Genes ITGβ1, ITGα2 and ILK

a)

![Diagram of Dicer and RISC complex activation by ATP]

b)

![Diagram of siRNA pathways in cytoplasm and nucleus]

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5.2 RESULTS

5.2.1 Transfection of the PMC42 Human Breast Cancer Cells: siRNA-Mediated ITGβ1, ITGα2 and ILK Gene Silencing and Regulation of EMT Indicators.

To test whether ITGβ1, ITGα2, and ILK expression are essential for EMT, small interfering RNA (siRNA)-mediated knockdown of ITGβ1, ITGα2, and ILK was performed in PMC42-ET and PMC42-LA cells. The siRNA target sequences for ITGβ1, ITGα2, and ILK are presented in Table 5.1. A commercial non-targeting control sequence, termed control siRNA, was also used.

Table 5.1 Chemically Synthesised siRNA Constructs.

<table>
<thead>
<tr>
<th>siRNA constructs</th>
<th>siRNA target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITGβ1 siRNA</td>
<td>AAGCTTTTAATGATAATTCAT</td>
</tr>
<tr>
<td>ITGα2 siRNA</td>
<td>TCGCTAGTATTCCAACAGAAA</td>
</tr>
<tr>
<td>ILK siRNA</td>
<td>CCTGACGAAGCTCAACGAGAA</td>
</tr>
</tbody>
</table>

Briefly, PMC42-ET and PMC42-LA cells were transfected with 100nM siRNA, targeting ITGβ1, ITGα2, ILK, and control siRNA. After 8hrs, cells were then either left unstimulated, or stimulated with EGF for 72hrs to examine whether ITGβ1, ITGα2, and ILK expression was abrogated after transfection. Controls included cell alone (no transfection), transfection reagent alone (DharmaFECT 4), and the control siRNA. Protein and RNA were extracted 72hrs post EGF-stimulation, and analysed by Western immunoblotting and Q-RT-PCR, respectively.

ITGβ1, ITGα2, and ILK protein levels were detected by Western immunoblotting as a single band of approximately 98 kDa for ITGβ1 (Figure 5.2b), 150-180 kDa for ITGα2 (Figure 5.3b), and 50 kDa for ILK (Figure 5.4b). The bands were quantified and data presented in Table 5.2. Protein expression is presented as a percentage relative to control siRNA after normalisation to pan-actin.
Transfection of ITGβ1 siRNA suppressed the level of ITGβ1 protein by 64% in the unstimulated PMC42-LA cells and by 81% in the EGF-stimulated PMC42-LA cells, when compared with either control siRNA- or non-transfected cells. Moreover, in the unstimulated PMC42-ET cells, transfection of ITGβ1 siRNA suppressed ITGβ1 expression by 84% in comparison to the control siRNA- or non-transfected cells, and abrogated EGF stimulation by 74%, as determined by Western blotting of cell lysates after a period of 72hrs (Figure 5.2a). Inhibition of ITGα2 by siRNA did not affect ITGβ1 protein levels; However, ILK inhibition suppressed ITGβ1 protein levels in the unstimulated PMC42-LA cells by 49%, and by 26% in the EGF-stimulated PMC42-LA cells. In the unstimulated PMC42-ET cells, ILK inhibition suppressed ITGβ1 protein levels by 80%, whilst in the EGF-stimulated PMC42-ET cells, there was 68% abrogation of ITGβ1 protein levels. Control siRNA treatment of these cell lines did not modulate ITGβ1 protein levels. As for other Western results presented in this chapter, equal protein loading for each lane was confirmed by pan-actin probing.

ITGα2 siRNA significantly reduced the protein expression of ITGα2 by >90% in both the unstimulated and EGF-stimulated PMC42-ET and PMC42-LA cell lines, when compared to control siRNA (Figure 5.3a). Inhibition of ITGβ1 by siRNA also significantly reduced ITGα2 expression (>90%) in both PMC42-LA and -ET cell lines. ILK inhibition by siRNA modestly reduced ITGα2 expression by >40% in both the unstimulated and EGF-stimulated PMC42-LA cells, and by 60% in the unstimulated and EGF-stimulated PMC42-ET cells. Control siRNA treatment of these cell lines did not modulate ITGα2 protein levels.

Transfection of ILK siRNA resulted in the suppression of ILK protein levels by 91% in the unstimulated PMC42-LA cells and by 89% in the EGF-stimulated PMC42-LA cells, when compared with control siRNA- or non-transfected cells. In the unstimulated PMC42-ET cells, ILK siRNA suppressed ILK protein levels by 92% compared with control siRNA- or non-transfected cells, and abrogated the expression of ILK protein levels by 88% in the EGF stimulated PMC42-ET cells, as determined by Western blotting of cell lysates after a period of 72hrs (Figure 5.4a). Inhibition of ITGβ1 and ITGα2 by
siRNA did not affect ILK protein levels in the PMC42-LA and -ET cells. Control siRNA treatment of these cell lines did not modulate ILK protein levels.

To determine whether the cells having either low ITGβ1, ITGα2 and ILK are able to undergo EMT, changes in the “classical” indicators of EMT, vimentin, E-cadherin, and N-cadherin, were measured by Western immunoblotting concurrently with the targets of the siRNA on the PMC42 cells transfected with control siRNA, ITGβ1, ITGα2 and ILK siRNA. Immunoblotting of protein extracts of PMC42 cells transfected with control siRNA, ITGβ1, ITGα2 and ILK siRNA, revealed prominent bands of vimentin protein at 55kDa (Figure 5.5), N-cadherin protein at 130kDa (Figure 5.6), E-cadherin protein at 120kDa (Figure 5.7), and pan-actin protein at 42kDa (Figures 5.8, 5.10, 5.12). The loading of each lane was assessed by the expression of pan-actin. The results showed equal band densities at actin, supporting that the protein concentrations were equal across the samples. The bands were quantified and protein expression presented as a percentage relative to control siRNA after normalisation to pan-actin (Figures 5.5b, 5.6b 5.7b). No significant differences in vimentin protein (Figure 5.5) levels were observed following treatment with ITGβ1, ITGα2 or ILK siRNA when compared to treatment with control siRNA, in the PMC42 cells after a period of 72hrs. As with vimentin, N-cadherin protein (Figure 5.6) expression pattern remained unchanged across ITGβ1, ITGα2 and ILK siRNA transfected PMC42 cells when compared to the control siRNA. Transfection of ITGβ1, ITGα2 and ILK siRNA did not suppress the level of E-cadherin protein (Figure 5.7) in the PMC42 cells, when compared with the control siRNA.

These results clearly show that ITGβ1, ITGα2, and ILK siRNA, effectively reduced their respective targets after 72hrs with and without EGF stimulation in the PMC42 cell system. However, there was no evidence that these candidates are EMT drivers.
Table 5.2 Normalised Protein Expressions, Relative to Control siRNA, of siRNA Transfected PMC42-LA and PMC42-ET Cells With and Without EGF Treatment.

<table>
<thead>
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<th>ITGβ1</th>
<th>ITGα2</th>
<th>ILK</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMC42-LA</td>
<td><strong>-EGF</strong></td>
<td>+EGF</td>
<td><strong>-EGF</strong></td>
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<tr>
<td></td>
<td>Cell alone</td>
<td>0.92</td>
<td>1.02</td>
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<td>Transfection reagent alone</td>
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<tr>
<td></td>
<td>Control siRNA</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>ITGβ1</td>
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<td>0.18</td>
</tr>
<tr>
<td></td>
<td>ITGα2</td>
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<td>0.91</td>
</tr>
<tr>
<td></td>
<td>ILK</td>
<td>0.51</td>
<td>0.8</td>
</tr>
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</table>

**ITGβ1**

<table>
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<tr>
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<th>PMC42-ET</th>
</tr>
</thead>
<tbody>
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<td><strong>+EGF</strong></td>
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</tr>
<tr>
<td>Cell alone</td>
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<tr>
<td>Transfection reagent alone</td>
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<tr>
<td>Control siRNA</td>
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<td>1</td>
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<td>ITGβ1</td>
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<td>ILK</td>
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**ITGα2**

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<tr>
<td>Cell alone</td>
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<td>ILK</td>
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**ILK**

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<tbody>
<tr>
<td><strong>-EGF</strong></td>
<td><strong>+EGF</strong></td>
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</tr>
<tr>
<td>Cell alone</td>
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<td>0.94</td>
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<tr>
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</tr>
<tr>
<td>Control siRNA</td>
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<tr>
<td>ITGβ1</td>
<td>1.01</td>
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<tr>
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</tr>
<tr>
<td>ILK</td>
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Figure 5.2 Western Blot Analysis of ITGβ1 Protein Expression in Unstimulated and EGF-Stimulated PMC42-LA and PMC42-ET Cells following siRNA Treatment.

Untreated and EGF-treated PMC42-LA and PMC42-ET cells were transfected with control siRNA and siRNA directed against ITGβ1, ITGα2, and ILK at 100nM, as described in the materials and methods chapter of the thesis. Total cellular protein was harvested from cells 72hrs post transfection then later electrophoresed on a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with ITGβ1, then stripped and re-probed with a pan actin antibody. Depicted is one Western blot representative of three individual siRNA treatments and subsequent Western blots. The numbers below the blots represent the ITGβ1 protein expression after normalisation with pan actin, relative to cells treated with control siRNA.

a) ITGβ1 and pan-actin protein expressions of siRNA transfected PMC42-LA and PMC42-ET cells without and with EGF treatment.

b) Graphical representation of ITGβ1 protein expression relative to the housekeeper protein pan-actin, as a percentage of the control siRNA.
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(a)

PMC42-LA

- EGF

+ EGF

Cell alone

Transfection reagent alone

Control siRNA

1 siRNA

ITGβ1 siRNA

ITGα2 siRNA

ILK siRNA

Cell alone

Transfection reagent alone

Control siRNA

1 ... - EGF

+ EGF

PMC42-ET

(b)

Graph showing % Relative to control siRNA
Figure 5.3 Western Blot Analysis of ITGα2 Protein Expression in Unstimulated and EGF-Stimulated PMC42-LA and PMC42-ET Cells following siRNA Treatment.

Untreated and EGF-treated PMC42-LA and PMC42-ET cells were transfected with control siRNA and siRNA directed against ITGβ1, ITGα2, and ILK at 100nM, as described in the materials and methods chapter of the thesis. Total cellular protein was harvested from cells 72hrs post transfection then later electrophoresed on a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with ITGα2, then stripped and re-probed with a pan actin antibody. Depicted is one Western blot representative of three individual siRNA treatments and subsequent Western blots. The numbers below the blots represent the ITGα2 protein expression after normalisation with pan actin, relative to cells treated with control siRNA.

a) ITGα2 and pan-actin protein expressions of siRNA transfected PMC42-LA and PMC42-ET cells without and with EGF treatment.

b) Graphical representation of ITGα2 protein expression relative to the housekeeper protein pan-actin, as a percentage of the control siRNA.
Chapter 5: Distinguishing Transcriptional Drivers and Passengers of EMT: siRNA Approaches of Candidate Genes ITGβ1, ITGα2 and ILK

a)

b)
Figure 5.4 Western Blot Analysis of ILK Protein Expression in Unstimulated and EGF-Stimulated PMC42-LA and PMC42-ET Cells following siRNA Treatment.

Untreated and EGF-treated PMC42-LA and PMC42-ET cells were transfected with control siRNA and siRNA directed against ITGβ1, ITGα2, and ILK at 100nM, as described in the materials and methods chapter of the thesis. Total cellular protein was harvested from cells 72hrs post transfection then later electrophoresed on a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with ILK, then stripped and re-probed with a pan actin antibody. Depicted is one Western blot representative of three individual siRNA treatments and subsequent Western blots. The numbers below the blots represent the ILK protein expression after normalisation with pan actin, relative to cells treated with control siRNA.

a) ILK and pan-actin protein expressions of siRNA transfected PMC42-LA and PMC42-ET cells without and with EGF treatment.

b) Graphical representation of ILK protein expression relative to the housekeeper protein pan-actin, as a percentage of the control siRNA.
Chapter 5: Distinguishing Transcriptional Drivers and Passengers of EMT: siRNA Approaches of Candidate Genes ITGβ1, ITGα2 and ILK

a) 

b)
Figure 5.5 Western Blot Analysis of Vimentin Protein Expression in Unstimulated and EGF-Stimulated PMC42-LA and PMC42-ET Cells following siRNA Treatment.

Untreated and EGF-treated PMC42-LA and PMC42-ET cells were transfected with control siRNA and siRNA directed against ITGβ1, ITGα2, and ILK at 100nM, as described in the materials and methods chapter of the thesis. Total cellular protein was harvested from cells 72hrs post transfection then later electrophoresed on a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with vimentin, then stripped and re-probed with a pan actin antibody. Depicted is one Western blot representative of three individual siRNA treatments and subsequent Western blots. The numbers below the blots represent the vimentin protein expression after normalisation with pan actin, relative to cells treated with control siRNA.

a) Vimentin and pan-actin protein expressions of siRNA transfected PMC42-LA and PMC42-ET cells without and with EGF treatment.

b) Graphical representation of Vimentin protein expression relative to the housekeeper protein pan-actin, as a percentage of the control siRNA.
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a)

b)
Figure 5.6 Western blot analysis of N-cadherin protein expression in unstimulated and EGF-stimulated PMC42-LA and PMC42-ET cells following siRNA treatment.

Untreated and EGF-treated PMC42-LA and PMC42-ET cells were transfected with control siRNA and siRNA directed against ITGβ1, ITGα2, and ILK at 100nM, as described in the materials and methods chapter of the thesis. Total cellular protein was harvested from cells 72hrs post transfection then later electrophoresed on a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with N-cadherin, then stripped and re-probed with a pan actin antibody. Depicted is one Western blot representative of three individual siRNA treatments and subsequent Western blots. The numbers below the blots represent the N-cadherin protein expression after normalisation with pan actin, relative to cells treated with control siRNA.

a) N-cadherin and pan-actin protein expressions of siRNA transfected PMC42-LA and PMC42-ET cells without and with EGF treatment.

b) Graphical representation of N-cadherin protein expression relative to the housekeeper protein pan-actin, as a percentage of the control siRNA.
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a)

b)
**Figure 5.7 Western blot analysis of E-cadherin protein expression in unstimulated and EGF-stimulated PMC42-LA and PMC42-ET cells following siRNA treatment.**

Untreated and EGF-treated PMC42-LA and PMC42-ET cells were transfected with control siRNA and siRNA directed against ITGβ1, ITGα2, and ILK at 100nM, as described in the materials and methods chapter of the thesis. Total cellular protein was harvested from cells 72hrs post transfection then later electrophoresed on a 10% SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with E-cadherin, then stripped and re-probed with a pan actin antibody. Depicted is one Western blot representative of three individual siRNA treatments and subsequent Western blots. The numbers below the blots represent the E-cadherin protein expression after normalisation with pan actin, relative to cells treated with control siRNA.

a) E-cadherin and pan-actin protein expressions of siRNA transfected PMC42-LA and PMC42-ET cells without and with EGF treatment.

b) Graphical representation of E-cadherin protein expression relative to the housekeeper protein pan-actin, as a percentage of the control siRNA.
Chapter 5: Distinguishing Transcriptional Drivers and Passengers of EMT: siRNA Approaches of Candidate Genes ITGβ1, ITGα2 and ILK

a)

b)
5.2.2 Transfection of the PMC42 Human Breast Cancer Cells: siRNA-Mediated ITGβ1, ITGα2, and ILK Gene Silencing and Regulation of EMT Indicators—mRNA Expression.

In conjunction with measuring the effects of siRNA on ITGβ1, ITGα2, and ILK protein levels, cells treated with siRNA were also harvested for mRNA, and Q-RT-PCR performed. Controls included cell alone (No Transfection), transfection reagent only (DharmaFECT 4), and a negative siRNA control (control siRNA). The mRNA expression of ITGβ1, ITGα2 and ILK in the PMC42 transfected cells was assessed with mRNA harvested from the same experiment.

Results were analysed using repeated measures one-way ANOVA with the “Dunnett’s multiple comparison test”, which corrects for multiple comparisons. The analysis of the results is "matched", so in determining whether there is an effect of siRNA treatment, each siRNA treated sample was compared to the siRNA control treated sample for the same cell line (PMC42-LA and PMC42-ET, without or with EGF), and then those 4 "differences" assessed as a group to determine if there has been an effect. This approach assessed whether or not there is a consistent effect of siRNA treatment across the cell lines, without and with EGF. Values are presented as δCt, which are representative of mRNA expression levels relative to L32 ribosomal- protein mRNA expression levels. Negative δCt values represent a lower mRNA abundance than for the RPL32 mRNA to which it is normalised. Each bar represents the mean of the δCt values across the cell lines, without and with EGF treatment.

ITGβ1 mRNA expression pattern paralleled that of ITGβ1 protein expression when treated for 72hrs (Figure 5.8). In the PMC42 cells, the expression of ITGβ1 mRNA was inhibited by 82% (P<0.0001), represented as a 2.45 decrease in δCt values, when compared to the non-target control siRNA. ITGβ1 mRNA expression was not affected by knockdown of ITGα2 or ILK siRNA.

Figure 5.8 shows similarities between both ITGα2 mRNA and protein expression patterns following siRNA treatment. In the PMC42 cells, the expression of ITGα2 mRNA was inhibited by 86% (P<0.0001), expressed as a 2.9 decrease in δCt values, relative to
control siRNA. ITGα2 mRNA expression was not affected by knockdown of ITGβ1 or ILK siRNA.

ILK mRNA expression pattern paralleled that of ILK protein expression when treated for 72hrs (Figure 5.8). In the PMC42 cells, treatment with ILK siRNA demonstrated a 76% reduction in ILK mRNA expression levels (P<0.0001), represented as a 2.1 decrease in δCt values, relative to control siRNA. No change in ILK mRNA expression levels was observed with cells treated with siRNA targeting ITGβ1 or ITGα2.

To determine whether the cells having low ITGβ1, ITGα2, or ILK are able to undergo EMT, changes in RNA levels for the “classical” indicators of EMT, vimentin, E-cadherin, and N-cadherin, were measured concurrently with the targets of the siRNA on the PMC42 cells transfected with control siRNA, ITGβ1, ITGα2, and ILK siRNA.

No significant differences in vimentin mRNA (Figure 5.9) levels were observed following treatment with ITGβ1, ITGα2, or ILK siRNA when compared to treatment with control siRNA, in the PMC42 cells as determined by Q-RT-PCR after a period of 72hrs. As with vimentin, the N-cadherin mRNA (Figure 5.9) expression pattern remained unchanged across ITGβ1, ITGα2 and ILK siRNA transfected PMC42 cells compared to the control siRNA. Transfection of ITGβ1, ITGα2, and ILK siRNA also did not suppress the level of E-cadherin mRNA in the PMC42 cells (Figure 5.9), when compared with the control siRNA.

The post-transcriptional gene silencing proved to be highly efficient and reproducible in the siRNA experiments performed in the PMC42 cells. Control siRNA treatment of these cell lines did not modify ITGβ1, ITGα2, or ILK protein or mRNA. The results indicate that each siRNA treatment effectively and significantly suppressed the mRNA levels of their respective targets after 72hrs with and without EGF stimulation in the PMC42 cell system. However, there was no evidence that these candidates are EMT drivers.
Figure 5.8 Q-RT-PCR Analysis of ITGβ1, ITGα2, and ILK mRNA in the PMC42 Cell Lines following siRNA Treatment.

Untreated and EGF-treated PMC42-LA and PMC42-ET cells were transfected with control siRNA and siRNA directed against ITGβ1, ITGα2, and ILK at 100nM, as described in the materials and methods chapter of the thesis. RNA was harvested from cells 72hrs post transfection using the QIAGEN RNeasy kit. 100ng of RNA was reverse transcribed using gene specific primers, and probed with primers specific for ITGβ1, ITGα2, ILK, and RPL32. The average δCt values are shown with standard deviation, representing the relative amounts of ITGβ1, ITGα2, and ILK mRNA expression present in relation to the RPL32 housekeeping gene mRNA expression levels. Negative δCt values represent an apparent lower mRNA abundance than for the RPL32 mRNA to which it is normalised. Results are of one Q-RT-PCR representative of two individual siRNA treatments and subsequent QRT-PCR. Results were analysed with the One way ANOVA, with Dunnett’s multiple comparison test, which corrects for multiple comparisons. Each bar represents the mean of the δCt values across the cell lines, without and with EGF treatment. **** P<0.0001 statistically significant.

a) The expression of ITGβ1 mRNA was inhibited by 82% (P<0.0001), relative to control siRNA.

b) The expression of ITGα2 mRNA was inhibited by 86% (P<0.0001), relative to control siRNA.

c) The expression of ILK mRNA was inhibited by 76% (P<0.0001), relative to control siRNA.
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Figure 5.9 Q-RT-PCR Analysis of Vimentin, N-cadherin, and E-cadherin mRNA in the PMC42 Cell Lines following siRNA Treatment.

Untreated and EGF-treated PMC42-LA and PMC42-ET cells were transfected with control siRNA and siRNA directed against ITGβ1, ITGα2, and ILK at 100nM, as described in the materials and methods chapter of the thesis. RNA was harvested from cells 72hrs post transfection using the QIAGEN RNeasy kit. 100ng of RNA was reverse transcribed using gene specific primers, and probed with primers specific for vimentin, N-cadherin, E-cadherin, and RPL32.

The average δCt values are shown with standard deviation, representing the relative amounts of Vimentin, N-cadherin, and E-cadherin mRNA expression present in relation to the RPL32 housekeeping gene mRNA expression levels. Negative δCt values represent an apparent lower mRNA abundance than for the RPL32 mRNA to which it is normalised. Each bar represents the mean of the δCt values across the cell lines, without and with EGF treatment.

Results are of one Q-RT-PCR representative of two individual siRNA treatments and subsequent QRT-PCR. Results were analysed with the One way ANOVA, with Dunnett’s multiple comparison test, which corrects for multiple comparisons.
After ascertaining the effectiveness of the siRNA directed against ITGβ1, ITGα2, and ILK in the PMC42 cells, further experiments determining the effects of ITGβ1, ITGα2, and ILK knockdown were performed.

In order to conserve the siRNA reagents, only one of the isogenic PMC42 cell lines was used in subsequent siRNA studies. The PMC42-ET sub-line was chosen as, on average, slightly better siRNA efficacy was observed for it.

5.2.3 Transfection of the PMC42 Human Breast Cancer Cells: siRNA-Mediated ITGβ1, ITGα2, and ILK Gene Silencing-Morphology

In addition to the protein and mRNA data on the PMC42 transfected cells, morphological analysis of the PMC42-ET cells at 72hrs post transfection with the appropriate siRNA for ITGβ1, ITGα2, and ILK was also performed. Inhibition of ITGβ1, ITGα2, and ILK with siRNA had no effect on the morphology of PMC42-ET cells compared to control siRNA. Treatment with 10ng/ml EGF altered the morphology of PMC42-ET cells, which appeared more elongated and mesenchymal (Figure 5.10). No cell toxicity was observed with the siRNA treatment following transfection.

5.2.4 Silencing ITGβ1, ITGα2, and ILK Expression in PMC42-ET Cells Affects Cell-Matrix Adhesions:

The next stage of the analysis involved comparing the adhesive properties of control, ITGβ1, ITGα2, and ILK siRNA-transfected cells to the various extracellular matrix (ECM) substrates - collagen type I, collagen type IV, Laminin, and Fibronectin, in order to dissect the role and confirm the functionality of ITGβ1, ITGα2, and ILK in PMC42-ET cell adhesion. PMC42-ET cells were transfected with siRNA. Eight hours later, cells were either left un-stimulated, or stimulated with EGF for 72hrs, after which cells were lifted and allowed to attach to the different ECM substrate-coated plates for 1hr. Attached cells were trypsinised and adhesion was estimated by the average of cell counts from five random high-power fields under light microscopy. Results were analysed with the two way ANOVA with Dunnett’s multiple comparison test, which adjusts the P values for
Figure 5.10 Effect of ITGβ1, ITGα2 and ILK siRNA on Morphology of PMC42-ET Cells, without and with EGF Stimulation.

PMC42-ET cells were transfected with 100nM siRNA, targeted against ITGβ1, ITGα2, ILK, and control siRNA, as described in the materials and methods chapter of the thesis. After 8hrs, cells were then either left unstimulated, or stimulated with 10ng/ml EGF for 72hrs, to utilise an EMT model and examine whether EGF expression is abrogated after transfection. Controls included Cell alone and a negative control siRNA. Morphology of cells was recorded by phase contrast microscopy and images captured on MCID image software under 20x magnification. The scale bar is 100μm in size.

Inhibition of ITGβ1, ITGα2, as well as ILK with siRNA had no effect on the morphology of PMC42-ET cells compared to control siRNA. Treatment with EGF altered the morphology of PMC42-ET cells, appearing more elongated and mesenchymal. No cell toxicity was observed with the siRNA treatment following transfection.

Multiple comparisons. Results presented are the average (±SD) from 4 biological replicates. Figures 5.11 to 5.14 show the adhesion of the PMC42-ET cells transfected with ITGβ1, ITGα2, and ILK siRNA, to the different ECM-substrates.

Figure 5.11 shows the inhibitory effects of the transfected PMC42-ET cells on the cellular attachment to collagen I. ITGβ1 siRNA treatment significantly (P<0.0001) reduced the number of adherent non EGF-stimulated PMC42-ET cells to 21% (2.5-fold) when compared to the control siRNA treatment (52%). The ITGα2 siRNA treatment significantly (P<0.0001) reduced the number of adherent non EGF-stimulated PMC42-ET cells to 25% (2.1-fold), whereas the ILK siRNA treatment significantly (P<0.0001) inhibited the number of adherent non EGF-stimulated PMC42-ET cells to 5% (10-fold).
When stimulated with EGF, the attachment of the PMC42-ET cells treated with ITGβ1, ITGα2, and ILK siRNA was significantly (P<0.0001) abrogated. The adhesion of PMC42-ET cells was 31% (1.8-fold), 33% (1.7-fold), and 11% (5.1-fold) with siRNA for ITGβ1, ITGα2 and ILK, respectively, when compared to control siRNA treatment.

On the collagen IV substrate (Figure 5.12), ITGβ1 siRNA treatment significantly (P<0.0001) reduced the number of adherent non EGF-stimulated PMC42-ET cells to 7.1% (3.3-fold) when compared to the control siRNA treatment (52%). The ITGα2 siRNA treatment significantly (P<0.0001) reduced the number of adherent non EGF-stimulated PMC42-ET cells to 8.3% (2.8-fold), whereas the ILK siRNA treatment significantly (P<0.0001) inhibited the number of adherent non EGF-stimulated PMC42-ET cells to 3.7% (6.3-fold). When stimulated with EGF, the attachment of the PMC42-ET cells treated with ITGβ1, ITGα2, and ILK siRNA was significantly abrogated (P<0.0001, P=0.0004, P<0.0001, respectively). The adhesion of PMC42-ET cells was 11.8% (2.3-fold), 13.6% (2.0-fold), and 8.2% (3.2-fold) with ITGβ1, ITGα2, and ILK siRNA, respectively, when compared to control siRNA treatment.

On the Laminin substrate (Figure 5.13), ITGβ1 siRNA treatment significantly (P<0.0001) reduced the number of adherent non EGF-stimulated PMC42-ET cells to 6.3% (6.9-fold) when compared to the control siRNA treatment (52%). The ITGα2 siRNA treatment significantly (P<0.0001) reduced the number of adherent non EGF-stimulated PMC42-ET cells to 13% (3.3-fold), whereas the ILK siRNA treatment significantly (P<0.0001) inhibited the number of adherent non EGF-stimulated PMC42-ET cells to 3.1% (14-fold). When stimulated with EGF, the attachment of the PMC42-ET cells treated with ITGβ1, ITGα2, and ILK siRNA was significantly abrogated (P<0.0001). The adhesion of PMC42-ET cells was 11.8% (4.4-fold), 16.3% (3.2-fold), and 5.6% (9.2-fold) with ITGβ1, ITGα2, and ILK siRNA, respectively, when compared to control siRNA treatment.

On the Fibronectin substrate (Figure 5.14), the ITGβ1 siRNA treatment significantly (P=0.001) reduced the number of adherent non EGF-stimulated PMC42-ET cells to 7.5% (1.9-fold) when compared to the control siRNA treatment (52%). The ITGα2 siRNA
treatment significantly (P=0.0001) reduced the number of adherent non EGF-stimulated PMC42-ET cells to 6.4% (2.2-fold), whereas the ILK siRNA treatment significantly (P<0.0001) inhibited the number of adherent non EGF-stimulated PMC42-ET cells to 3.3% (4.3-fold). When stimulated with EGF, the attachment of the PMC42-ET cells treated with ITGβ1, ITGα2, and ILK siRNA was significantly abrogated (P<0.0001). The adhesion of PMC42-ET cells was 11% (1.7-fold), 9.3% (2.0-fold), and 6.3% (3.0-fold) with ITGβ1, ITGα2, and ILK siRNA, respectively, when compared to control siRNA treatment.

Results above show that the binding of the ITGβ1, ITGα2, and ILK-siRNA transfected PMC42-ET cells to collagen I, collagen IV, laminin, and fibronectin substrates was significantly (P<0.05) abrogated by each of the individual respective targets both under EGF-stimulated and unstimulated conditions, but there was no indication of an interaction between them. The reported P value for the effect of EGF varied across the ECM substrates. Although these results are from a single experiment, they matched what was expected for a successful functional knockdown of these proteins. The adhesion levels were higher in the EGF-stimulated transfected cells in comparison to the unstimulated transfected cells, across all tested ECM-substrates (Figures 5.11 to 5.14). However, ITGβ1, ITGα2, and ILK siRNA significantly (P<0.05) suppressed the cell attachment ability of the PMC42-ET breast cancer cell line to all substrates under both unstimulated and EGF-stimulated conditions, indicating that both ITGβ1 and ITGα2 subunits, and ILK, are important components for the adhesion of PMC42-ET cells to these substrates. The knockdown of these genes most significantly affected the adhesion to the ECM-substrates collagen I and Laminin.
Figure 5.11 Functionality of ITGβ1, ITGα2 and ILK in PMC42-ET Cell Adhesion (+/- EGF) on the ECM-Substrate, Collagen Type I.

Inhibition of PMC42-ET breast cancer cell adhesion, on Collagen Type I, by integrin subunit-specific and ILK siRNA. Cell adhesion assays were conducted as described in the materials and methods chapter of the thesis. Results are expressed as % of added cells adhered and represent the mean ± SD from 4 biological replicates. Results were analysed with the Two way ANOVA, with Dunnett’s multiple comparison test, which corrects for multiple comparisons, with P values adjusted for multiple comparisons. P<0.05 statistically significant compared to control siRNA. Results indicate that all 3 siRNAs impacted upon adhesion to Collagen I (P<0.0001) both without and with EGF treatment, but there was no indication of an interaction between them.
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Figure 5.12 Functionality of ITGβ1, ITGα2 and ILK in PMC42-ET Cell Adhesion (-/+ EGF) on the ECM-Substrate, Collagen-IV.

Inhibition of PMC42-ET breast cancer cell adhesion, on Collagen Type IV, by integrin subunit-specific and ILK siRNA. Cell adhesion assays were conducted as described in the materials and methods chapter of the thesis. Results are expressed as % of added cells adhered and represent the mean ± SD from 4 biological replicates. Results were analysed with the Two way ANOVA, with Dunnett’s multiple comparison test, which corrects for multiple comparisons, with P values adjusted for multiple comparisons. P<0.05 statistically significant compared to control siRNA.

Results indicate that all 3 siRNAs impacted upon adhesion to Collagen IV (P<0.05) both without and with EGF treatment, but there was no indication of an interaction between them.
Figure 5.13 Functionality of ITGβ1, ITGα2 and ILK in PMC42-ET Cell Adhesion (+/- EGF) on the ECM-Substrate, Laminin.

Inhibition of PMC42-ET breast cancer cell adhesion, on Laminin, by integrin subunit-specific and ILK siRNA. Cell adhesion assays were conducted as described in the materials and methods chapter of the thesis. Results are expressed as % of added cells adhered and represent the mean ± SD from 4 biological replicates. Results were analysed with the Two way ANOVA, with Dunnett’s multiple comparison test, which corrects for multiple comparisons, with P values adjusted for multiple comparisons. P<0.05 statistically significant compared to control siRNA. Results indicate that all 3 siRNAs impacted upon adhesion to Laminin (P<0.0001) both without and with EGF treatment, but there was no indication of an interaction between them.
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Figure 5.14 Functionality of ITGβ1, ITGα2 and ILK in PMC42-ET Cell Adhesion (+/- EGF) on the ECM-Substrate, Fibronectin.

Inhibition of PMC42-ET breast cancer cell adhesion, on Fibronectin, by integrin subunit-specific and ILK siRNA. Cell adhesion assays were conducted as described in the materials and methods chapter of the thesis. Results are expressed as % of added cells adhered and represent the mean ± SD from 4 biological replicates. Results were analysed with the Two way ANOVA, with Dunnett’s multiple comparison test, which corrects for multiple comparisons, with P values adjusted for multiple comparisons. P<0.05 statistically significant compared to control siRNA. Results indicate that all 3 siRNAs impacted upon adhesion to Fibronectin (P<0.05) both without and with EGF treatment, but there was no indication of an interaction between them.
5.2.5 **Silencing ITGβ1, ITGα2, and ILK Expression in PMC42-ET Cells Inhibits Cell Migration:**

Coordinated regulation of cell adhesion and adhesion complex remodelling are crucial for cell movement. Active migration of tumour cells is a prerequisite for tumour-cell invasion and metastasis, and accelerated migration is a key feature of EMT (Thiery et al., 2009). As such, the effect of ITGβ1, ITGα2, and ILK silencing on PMC42-ET cell migration was evaluated. PMC42-ET cells were transfected with control siRNA, ITGβ1, ITGα2, and ILK siRNA, and either left unstimulated or stimulated with 10ng/ml EGF for 48hrs, after which confluent monolayers were disrupted with a pipette tip for an in vitro wound-healing assay. After 24hrs, the distance of the wound that was closed was determined. A two-way ANOVA followed by Holm-Sidak's multiple comparisons test was conducted that examined the effect of siRNA-transfected PMC42-ET cells (without and with EGF treatment) on their wound healing migration ability. There was a statistically significant interaction between the effects of control and siRNA treatments on migration (P=0.0203), but no interaction between the effects on EGF treatment. Main column effect analyses however showed a more powerful significant interaction between the effects of both control and EGF treatment, and control and siRNA treatment on the PMC42-ET cells migratory ability. The ability of the PMC42-ET cells to migrate into the wound upon silencing of ITGβ1, ITGα2, and ILK, is shown in Figure 5.15. Data are expressed as percentage of control.

ITGβ1 knockdown by siRNA significantly impeded the ability of PMC42-ET cell migration (P=0.026) (Figure 5.15). The unstimulated ITGβ1-targeted cells resulted in a 47% reduction in cell migration when compared to that of the control siRNA, whilst there was a 60% reduction in cell migration in the EGF-stimulated ITGβ1-targeted cells in comparison to the control treatment.

ITGα2 knockdown by siRNA significantly impeded the ability of PMC42-ET cell migration (P= 0.0130). Figure 5.15 shows that ITGα2 inhibition resulted in a 32% reduction in cell migration in the non-EGF treated PMC42 cells when compared to
control. Upon EGF stimulation, ITGα2-targeted cells showed a 57% reduction in PMC42-ET cell migration in comparison to control.

Similarly, ILK knockdown by siRNA significantly impeded the ability of PMC42-ET cell migration (P= 0.0260). In the unstimulated ILK-targeted cells, cell migration was inhibited by 55% when compared to the control (Figure 5.15). Upon EGF stimulation, ILK-targeted cells resulted in a 63% reduction in PMC42-ET cell migration in comparison to control.

These findings suggest that ITGβ1, ITGα2, and ILK may enhance the migration potential of human breast cancer cells.

To strengthen these findings from the wound-healing model, cell migration using the Boyden chamber method (Transwell filters) was studied (Figure 5.16). PMC42-ET cells were transfected with control, ITGβ1, ITGα2, and ILK siRNA and either left unstimulated, or stimulated with EGF for 48hrs, after which cells were lifted and allowed to migrate through collagen I-coated filters overnight.

Results were analysed with the two way ANOVA, with Dunnett’s multiple comparison test, which corrects for multiple comparisons and reports the 95% confidence intervals of the difference between sample and control siRNA. Results represent the number of migrated cells ±SD from 3 biological experiments.

Results indicate that all 3 siRNAs impacted upon migration to collagen I (P<0.0001) both without and with EGF treatment, with a statistically significant (P< 0.0001) interaction between them. This means that statistically, the scale of the effect of the siRNA treatments is different dependent upon whether or not the cells were treated with EGF. Nonetheless, knockdown impacted irrespective of induced mesenchymalisation, indicating that these candidate genes are important for mesenchymal properties but not drivers of the process.

The migration levels were higher in the EGF-stimulated transfected cells in comparison to the unstimulated transfected cells (P<0.0001) and there was a significant inhibition of
migration of the ITGβ1, ITGα2, and ILK transfected cells through collagen I-coated Transwell filters under both unstimulated and EGF-stimulated conditions (Figure 5.16). Under non EGF-stimulated conditions, knockdown by siRNA significantly impeded PMC42-ET cell migration by 78% for ITGβ1 (P=0.0003), and completely inhibited migration for ITGα2 (P<0.0001) and ILK (P<0.0001). Under EGF-stimulated conditions, PMC42-ET cell migration was significantly inhibited in ITGβ1 (P<0.0001), ITGα2 (P<0.0001), and ILK siRNA transfectants (P<0.0001). Knockdown of ITGβ1, ITGα2, and ILK, by siRNA impeded PMC42-ET cell migration by 62%, 75% and 91%, respectively.

5.2.6 ILK Inhibition Decreases Cell Invasion and Down-Regulates ITGβ1 in the PMC42-ET Cell Line; Use of Pharmacological Inhibitor QLT-0267:

This section outlines preliminary work carried out to further investigate ILK knockdown-mediated suppression of migration, and determine whether the enzymatic activity of ILK is required for the PMC42-ET cells invasive/migratory ability and is crucial to the regulation of integrin function. For this purpose, the effect of inhibition of ILK activity on integrin function in the PMC42-ET cells was studied by treatment of the PMC42-ET cells with the small-molecule inhibitor of ILK, QLT-0267, which has previously been reported to inhibit ILK activity in a highly specific dose-dependent manner (Koul et al., 2005a). Since these studies on the PMC42 cells were carried out, there has been a large body of evidence that the kinase like domain of ILK lacks catalytic activity (Wickström et al., 2010), which has resulted in an ongoing debate about whether ILK indeed functions as a protein serine/threonine kinase (bona fide protein kinase) or as a pseudokinase (Hannigan et al., 2005, Boudeau et al., 2006, Wickström et al., 2010, Hannigan et al., 2011). Hannigan and co-workers however, still hold firm that ILK is an active kinase (Hannigan et al., 2011).

The PMC42-ET cell line was treated in vitro with 6.25µM of QLT-0267 for 24hrs, harvested, and replated onto collagen I-coated inserts. Cells were allowed to incubate for an additional 48hr, and cells that invaded to the opposite side of the insert were stained
Figure 5.15 Wound Migration Properties of PMC42-ET Cells (+/- EGF) following siRNA Knockdown of ITGβ1, ITGα2 and ILK; without, and with Stimulation by EGF.

Inhibition of PMC42-ET wound closure, on Collagen Type I, by integrin subunit-specific and ILK siRNA. Wound healing assays were conducted as described in the materials and methods chapter of the thesis. A 2-way ANOVA followed by Holm-Sidak's multiple comparisons test was conducted that examined the effect of siRNA transfected PMC42-ET cells (without and with EGF treatment) on their wound healing migration ability. Data are expressed as percentage of control and represent the mean ± SD from 3 biological replicates. P<0.05 statistically significant compared to control siRNA.

ITGβ1 knockdown by siRNA significantly impeded the ability of PMC42-ET cell migration (P=0.026).

ITGα2 knockdown by siRNA significantly impeded the ability of PMC42-ET cell migration (P= 0.0130).

ILK knockdown by siRNA significantly impeded the ability of PMC42-ET cell migration (P= 0.0260).
with crystal violet. The membranes with invaded cells were dissolved in 10% acetic acid and read colourimetrically at 560nm for quantification of invasion.

Results were analysed using an unpaired two-tailed T test with Welch's correction (P<0.05) based on results of f-test between groups (i.e. control and tests are unlikely to have equal variances). Experiments were performed in triplicate from 3 independent experiments. Cells treated with the ILK inhibitor had a significantly (P=0.0001) reduced ability to invade through collagen I-coated Boyden chambers, when compared with the control cells (Figure 5.17). Invasion, as measured in the Boyden chamber assay, was 5% that of untreated cells.

Based upon these results, and the results in sections 5.2, where ILK siRNA knocked-down ITGβ1 expression, the next step was to determine whether this effect was structural (due only to the loss of physical interactions with ILK) or may involve ILK kinase activity. Therefore, the PMC42-ET cell line was either left untreated or treated with QLT-0267 for 24 and 72hrs, after which ITGβ1 protein expression was analysed by Western immunoblotting. Results clearly showed that the ILK inhibitory compound markedly reduced the ITGβ1 protein levels (Figure 5.18). This has not been previously reported, however this is suggestive of the fact that ILK co-immunoprecipitates with β1-integrin in mammalian cells, and directly phosphorylates its cytoplasmic domain in vitro (Juliano and Haskill, 1993, Hannigan et al., 1996b, Dedhar et al., 1999, Mulrooney et al., 2000, Hannigan et al., 2005, McDonald et al., 2008).
Figure 5.16 Boyden Chamber Migration Properties of PMC42-ET Cells following siRNA Knockdown of ITGβ1, ITGα2 and ILK; without, and with Stimulation by EGF.

Untreated and EGF-treated PMC42-ET cells were transfected with control siRNA and siRNA directed against ITGβ1, ITGα2, and ILK at 100nM, as described in the materials and methods chapter of the thesis. Cells were then treated with 6.26uM QLT0267 ILK inhibitor for 24hrs, harvested, and replated onto Collagen I-coated inserts. Cells were allowed to incubate for a further 24hrs, and cells that migrated to the opposite side of the insert were stained with crystal violet. The membranes with invaded cells were dissolved in 10% acetic acid and read colorimetrically at 560nM. The determination of adhesion was achieved by the average of cell counts from 5 random high-power fields under light microscopy. Results represent the number of migrated cells ±SD from 3 biological experiments. Results were analysed with the Two way ANOVA, with Dunnett’s multiple comparison test, which corrects for multiple comparisons. P<0.05 statistically significant compared to untreated control siRNA.
Figure 5.17 Inhibition of ILK in PMC42-ET Cell Invasion (+/- QLT0267); Boyden Chamber Migration.

Cultured cells in mid-log growth phase were either untreated or treated with 6.25uM QLT0267 ILK inhibitor for 24hrs, after which cells were lifted and replated onto collagen-I coated inserts, as described in the materials and methods chapter of the thesis. Cells were allowed to incubate for an additional 24hr, and cells that invaded to the opposite side of the insert were stained and quantified. Error bars represent standard deviation for triplicate measurements.

Results were analysed using an Unpaired Two-tailed t test with Welch's correction. P<0.05 statistically significant.

Cells treated with QLT0267 inhibitor had a statistically significant (P=0.0001) reduced ability to invade through collagen I-coated Boyden chambers, when compared with the control cells.
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Figure 5.18 ILK Inhibitor QLT0267 Down-Regulates ITGβ1 Expression in the PMC42-ET Cell Line.

Cultured PMC42-ET cells in mid-log growth phase were either untreated or treated with 6.25uM QLT0267 inhibitor for 24 and 72hrs, after which ITGβ1 protein expression was analysed by Western Immunoblotting, as described in the materials and methods chapter of the thesis. Results clearly showed that the ILK inhibitory compound markedly reduced the ITGβ1 protein expression.
5.3 DISCUSSION

RNA interference (RNAi) technology was utilised in this study to determine whether the ITGβ1, ITGα2, and/or ILK, contribute to the EMT induction or to the migratory phenotype associated with the EMT in the PMC42 human breast cancer cell line. Although PMC42 cells have previously not been amenable to transfection, this study successfully showed that RNA interference is an effective way to reduce the gene expression in the PMC42 cell model.

siRNA can facilitate the suppression of gene expression with high efficiency in living cells (Hannon, 2002). RNAi rapidly became one of the most powerful and widely used tools for the study of gene function in the early 2000s (Deng et al., 2014). One advantage of using RNAi in therapy is that once the RNA interference silencing complex is formed inside a cell, it is stable for up to a few weeks in some cell types (Dykxhoorn and Lieberman, 2006a, Dykxhoorn and Lieberman, 2006b). That being said, however, several limiting factors can affect the actions of siRNA. This can include the initial design of the siRNA, as the activity of the siRNA can depend on the mRNA structure on which it is based (Sioud and Leirdal, 2004). The specificity of siRNA is another factor that can limit the effectiveness of this technology. Off-target activity can complicate the interpretation of phenotypic effects in gene-silencing experiments and can potentially lead to unwanted toxicities (Jackson and Linsley, 2010). Off-target effects are related to the siRNA itself and most often arise from partial complementarily of the sense or antisense strands to an unintended target. These effects are concentration dependent and therefore the best way to minimize these off-target effects is by reducing siRNA concentrations. Although siRNA are relatively stable in cell culture conditions, the ability to resist endonuclease degradation is vital for in vivo experiments. As our studies did not involve in vivo testing, it is yet unknown if the siRNA used in this thesis have the thermodynamic stability or endonuclease resistance necessary for in vivo functionality. Chemical modifications of siRNA are being explored to enhance these potential obstacles (Czauderna et al., 2003, Hoshika et al., 2005, Dande et al., 2006, Hall et al., 2006, Jackson and Linsley, 2010).
More recently, Genome editing tools such as the clustered regularly interspaced short palindromic repeat (CRISPR)-associated system (Cas), have provided new revolution in a variety of applications, including genome editing, gene function investigation, and regulation of gene expression in model systems including animal zygotes and human cells (Chang et al., 2013, Cho et al., 2013, Cong et al., 2013, Friedland et al., 2013, Hwang et al., 2013, Jinek et al., 2013, Li et al., 2013a, Li et al., 2013c, Mali et al., 2013, Wu et al., 2013, Hsu et al., 2014, Ikmi et al., 2014, Irion et al., 2014, Long et al., 2014, Ma et al., 2014, Niu et al., 2014, Shalem et al., 2015, Smith et al., 2015, Wu et al., 2015b, Dominguez et al., 2016, Jiang and Doudna, 2017, Dong et al., 2018).

CRISPR/Cas9 is a gene-editing technology which involves two essential components: a guide RNA to match a desired target gene, and Cas9 (CRISPR-associated protein 9)—an endonuclease which causes a double-stranded DNA break, allowing modifications to the genome (Redman et al., 2016). Despite great progress in understanding the utilization of CRISPR/Cas9 in a variety of model organisms, much remains to be learned regarding the efficiency and specificity of CRISPR/Cas9-mediated gene editing in human cells. This new technology holds tremendous promise for both basic research and clinical applications, due to its ease, expedience, and efficiency.

The studies described in this thesis predated the availability of CrispR-Cas technology.

Integrins are key regulators of a variety of tumour cell functions, including adhesion, migration, invasion, proliferation and survival (Grzesiak et al., 2011). Therefore, deregulation of their expression and altered functions can play critical roles in cancer progression by enhancing cancer cell invasion, metastasis, and survival. This study set out to assess the functional role(s) that ITGβ1, ITGα2, and ILK may play in the progression of breast cancer, and determine whether they are actively or passively involved in the induction of the EMT program in PMC42 cells. This was investigated with respect to different processes involved in breast cancer progression, including attachment, migration, and invasion. Herein, the RNA interference strategy was applied to interfere with the ITGβ1, ITGα2 and ILK mRNA, thus reducing ITGβ1, ITGα2 and ILK levels in the PMC42 cells.
Whilst ITGβ1, ITGα2, and ILK depletion had no effect on the morphology of the PMC42 cells, treatment with EGF did alter their morphology to become elongated and mesenchymal, a key feature of EMT, in which cells lose epithelial characteristics and gain mesenchymal properties (Kokkinos et al., 2007). Furthermore, inhibition of ITGβ1, ITGα2, and ILK by targeted knockdown using siRNA resulted in several changes in cell behaviour, thus offering new insights into the functions of ITGβ1, ITGα2 and ILK in the PMC42 cells.

Initial results clearly showed a high efficiency in abrogating the corresponding ITGβ1, ITGα2 and ILK mRNA, as well as its translation into the ITGβ1 and ITGα2 subunits and ILK in the PMC42 cells, as ascertained by its detection on the cell membrane. Moreover, the specificity of the siRNA knockdown of ITGβ1, ITGα2 and ILK genes was ascertained by the fact that no effect was seen with a control siRNA. Interestingly, immunoblotting analysis demonstrated that the specific siRNA (i.e ITGβ1/ITGα2/ILK), besides silencing the expression of their target protein, also changed the expression level of another protein. When ITGβ1 was knocked down, the expression of ILK was also decreased, although this decrease was not as high as the decrease of the ITGβ1 observed when its specific siRNA was used. Likewise, the expression of both ITGβ1 and ILK, were also decreased when ITGα2 was knocked down. Whilst the decrease in ILK was not as high as the decrease of the ITGα2 observed when its specific siRNA was used, the decrease in the expression of ITGβ1 was very similar to the decrease in the expression of ITGα2. These data may be due to integrin cross-talk, a mechanism in which one integrin regulates the activation state of a different integrin in the same cell, as has been described previously for ITGβ1, which affects the stability of ITGβ3 mRNA (Retta et al., 2001). Since ITGα2 forms a heterodimer with ITGβ1, and is only known to associate with ITGβ1, changes in its expression level may lead to changes in ITGβ1 expression. This protein reduction that was seen in the absence of corresponding mRNA reduction, may indicate that the affected siRNA is mediating its effects at the translational level like a microRNA (Doench et al., 2003) or through protein stabilisation.
RNAi technology has previously been used to decrease ILK protein levels in several cell lines including human embryonic kidney cells (Troussard et al., 2003), and human lung fibroblasts (Nho et al., 2005b). Interestingly, Duxbery et al, who used identical siRNA to the ILK siRNA utilised in this chapter in their studies of the nucleoside analogue gemcitabine in pancreatic adenocarcinomas (Duxbury et al., 2005), demonstrated a significant inhibition of ILK protein expression, as well as the inhibition of phosphorylation of downstream anti-apoptosis pathway proteins, including Akt and GSK-3. The studies presented in this chapter indicate that ILK protein and mRNA were decreased with siRNA consistent with the above mentioned studies. An obvious question to ask is whether downstream proteins were also affected in the PMC42 cells. However, time constraints prevented the exploration of the phosphorylation and activation proteins such as Akt. It is likely, though, to assume that in the experiments described here, the downstream effects were also in place (Duxbury et al., 2005).

Inhibition of ITGβ1, ITGα2, and ILK by RNAi, whilst all demonstrated a respective dramatic reduction in ITGβ1, ITGα2, and ILK protein and mRNA levels, showed no modulation of vimentin, E-cadherin, nor N-cadherin protein and mRNA expression, implying that there was no impact upon EMT, as a decreased expression of the epithelial marker E-cadherin is usually concurrent with the increase in the mesenchymal markers vimentin and N-cadherin, and this shift is associated with EMT, a process implicated to the incidence of metastasis (Blick et al., 2008, Yang and Weinberg, 2008, Thiery et al., 2009). There is a possibility that upregulation of EMT markers has already occurred by the time siRNAs affect translation, as according to chapter 3, these changes can occur early (<24hr).

Thus, while the results indicate that these proteins do not play a functional role in the transition itself in the PMC42 breast cancer cells, they may directly contribute to the EMT, enabling the breast cancer cells to interact with the interstitial matrices, as it is essential for the integrins and cadherins to function in a coordinated manner to effectively mediate the cellular interaction necessary for migration and tumour metastasis. An increasing number of reports demonstrate functional and physical interaction between
cadherins and integrins, suggesting a connection between both integrin and cadherin signalling pathways (Gil et al., 2016). ILK is a likely candidate to coordinate integrin/cadherin expression or functions, thus is an important regulator of integrin-mediated signalling in breast cancer metastasis. In particular, increased ILK activity is suggested as a molecular marker for EMT (Lee et al., 2006). Pathological overexpression of ILK results in down regulation of E-cadherin and nuclear accumulation of b-catenin and NF-kB activating the expression of other mesenchymal genes such as N-cadherin (Novak et al., 1998a, Oloumi et al., 2004, Medici and Nawshad, 2010).

ILK is overexpressed in many types of cancers and it is a critical regulator of breast cancer cell survival through direct phosphorylation and activation of Akt. However, it is widely dispensable for the survival of normal breast epithelial cells, as shown by inhibition of ILK activity with a pharmacological small-molecule ILK inhibitor (QLT0267) (Troussard et al., 2006). In a study by Serrano and colleagues (2013), inhibition of ILK prevented TGFβ-1-induced EMT in NMe mouse, and MCF10A human mammary epithelial cells. They also identified Snail and Slug as key EMT effectors of this pathway. Furthermore, the authors showed a TGFβ-1-dependent increase in the phosphorylation of Akt Ser473 and Rictor Thr1135 that requires ILK activity. Interestingly, TGFβ-1 also induced Rictor expression and the formation of a complex between ILK and Rictor, which was also dependent on ILK activity (Serrano et al., 2013). The authors further characterized ILK-dependent phosphorylation of Rictor on Thr1135 in several human breast cell lines, suggesting that this phosphorylation is differentially regulated in normal and cancer cells. Pharmacological inhibition and genetic knockdown of ILK resulted in the downregulation of Rictor Thr1135 phosphorylation in breast cancer cells. Furthermore, the interaction between ILK and Rictor is malignancy-dependent, as this complex is present in cancer cells but not in normal breast cell lines. In addition, ILK/Rictor complex formation is induced after EMT and can be prevented by inhibition of ILK (Serrano et al., 2013).

ECM proteins, which are a major component of the tumour microenvironment, can undergo molecular alterations during tumourigenesis that may potentially have a
substantial impact on the metastatic process (Bernstein and Liotta, 1994). The process of tumour metastasis involves multiple steps that include the segregation of tumour cells from a primary tumour, their migration through connective tissue rich in collagen type I, intravasation into blood and/or lymphatic circulatory systems, adhesion to the substratum of ECM at a distant site, and subsequent extravasation and tissue invasion (Al-Mehdi et al., 2000, Steeg, 2006, Tharmalingam et al., 2011). Three of the critical steps of metastasis which are adhesion and migration of the primary tumour on ECM at the distant site, as well as invasion, involve the integrin cell surface molecules (Buckley et al., 1996, Voura et al., 2001, Leroy-Dudal et al., 2005, Leroy-Dudal et al., 2005).

There is no general consensus on the function of integrins in the metastatic process. In fact, previously published reports describing the role(s) of integrins in metastasis are controversial. The role of integrins is believed to depend on the type of cancer and can be associated with increased, or decreased malignancy (Mierke et al., 2011). In particular, reports on the roles of α2β1 integrin are controversial, and show both positive and negative correlations with metastasis formation or tumour malignancy. In breast cancer, the α2β1 integrin is mostly thought to function as a suppressor of metastasis, and loss of α2β1 integrin expression has been associated with tumour progression in both human (Arihiro et al., 1993) and mouse (Ramirez et al., 2011) breast cancer. Similar findings were demonstrated in human rhabdomysarcoma cells, where enforced expression of α2β1 integrin slowed post-extravasation movement (Hangan et al., 1996). Conversely, overexpression of the ITGα2 subunit in the same human rhabdomysarcoma cells has previously been shown to enhance metastasis to the lung (Chan et al., 1991). Expression of the α2β1 integrin has also been shown to correlate with the malignant phenotype in renal cell carcinomas (Anastassiou et al., 1995).

Primary cultures of human ovarian carcinoma and human pancreatic adenocarcinoma cell lines were demonstrated to both bind to and migrate toward collagen I, suggesting that collagen could promote the invasive phenotype, although it was not directly shown to affect metastasis (Fishman et al., 1998, Grzesiak et al., 2007). In addition, α2β1 integrin has been implicated as a promoter of the malignant phenotype in pancreatic cancer cells
Chapter 5: Distinguishing Transcriptional Drivers and Passengers of EMT: siRNA Approaches of Candidate Genes ITGβ1, ITGα2 and ILK

(Grzesiak and Bouvet, 2006) and metastasis to bone in prostate cancer (Hall et al., 2006). Furthermore, α2β1 integrin has been shown to play a key role in cell invasion in 3D ECMs (Mierke et al., 2011).

Integrin expression has been shown to be important for cell attachment and migration on ECM proteins (Etoh et al., 1992). Selective adhesion of tumour cells to organ-specific ECM components; in particular collagen I, may influence organ-specific metastasis. Indeed, work undertaken in prostate cancer showed that bone metastatic cells preferentially adhere to collagen I, which is the most abundant protein within the bone (Hall et al., 2006). The collagen I attachment mediated by the α2β1 integrin induces specific intracellular signalling and initiates motility programs such as Rho C, that might mediate the cell homing to the bone (Hall et al., 2008).

In the findings presented in this thesis, ITGβ1, ITGα2, and ILK knockdown in the PMC42 cells by RNA interference impaired both of these processes involved in breast cancer progression. Undoubtedly, cell adhesion is a prerequisite for extravasation and a reduced adhesion rate should result in a reduced transmigration rate (Heyder et al., 2005). During cell migration, cells undergo repeated cycles of adhesion and de-adhesion events, and “locking” integrins into a high affinity state can inhibit migration (Coppolino and Dedhar, 2000). Collagens I and IV, laminin, and fibronectin are the major components in the ECM that cells bind through the extracellular domain of integrins (Maemura and Dickson, 1994, Gahmberg et al., 2009). The findings in this study clearly showed that the binding of the PMC42-ET cells to the ECM substrates collagen I, collagen IV, laminin, and fibronectin, was significantly abrogated by individually knocking down ITGβ1 and ITGα2 subunits, and ILK in the cells, irrespective of the presence or absence of EGF. These results indicate that the ITGβ1 and ITGα2 subunits, and ILK, are required for the adhesion of PMC42-ET cells to these substrates, and are consistent with previously published research examining ITGβ1 and ITGα2 subunits in pancreatic cancer cell lines (Grzesiak and Bouvet, 2006, Grzesiak et al., 2007, Grzesiak et al., 2011), and ILK in gastric cancer (Zhao et al., 2011), and ovarian cancer cell lines (Choi et al., 2012).
The demonstrated inhibition of adhesion suggest that ITGβ1, ITGα2, and ILK play a central role in transducing signals when cancer cells engage with the ECM regulating cell attachment. This is an important biological activity that contributes to breast cancer metastasis. Upon engagement with the ECM, numerous signalling proteins including Src, Fak, MAPK, and P13K (Miranti and Brugge, 2002, Harms et al., 2004, Slack-Davis and Parsons, 2004) are recruited to the adhesion sites in which cell-matrix contact becomes established (Brakebusch and Fässler, 2003). Selective adhesion of tumour cells to organ-specific ECM components; in particular collagen I, may influence organ-specific metastasis, and hence may also mediate the EMT, as EMT may be a prerequisite for metastasis. Indeed, recent work undertaken in prostate cancer showed that bone metastatic cells preferentially adhere to collagen I, which is the most abundant protein within the bone (Hall et al., 2006). Collagen I attachment mediated by the ITG α2β1, induces specific intracellular signalling and initiates motility programs such as RhoC guanosine triphosphatase (GTPase), which might mediate the cell homing to the bone (Hall et al., 2008). It is known that integrin engagement can stimulate ILK resulting in cytoskeletal reorganisation and signal transduction through the activation of RhoA GTPase (Keely et al., 1998, Evers et al., 2000, Price and Collard, 2001). However, unlike RhoA GTPase, RhoC has not been previously shown as a downstream effector of integrin signalling. The Rho GTPases belong to the Ras superfamily of small GTP-binding proteins, which act as molecular switches involved in all aspects of cellular motility and invasion (Kjøller and Hall, 1999, Price and Collard, 2001, Takai et al., 2001). Whilst the mechanism through which RhoC GTPase becomes activated in metastatic cancer cells is unclear, Hall and colleagues, (2008) have demonstrated that RhoC is activated on α2β1 integrin engagement, and that it regulates the collagen-I mediated invasion of prostate cancer cells (Hall et al., 2008). Due to their involvement in motility, Rho GTPases have been implicated in tumour progression and metastasis. The Rho GTPase protein, RhoC, in particular, was shown to participate in the progression and metastasis of several cancers including breast, pancreas, and melanoma (Suwa et al., 1998, Clark et al., 2000, van Golen et al., 2002).
As active migration of tumour cells is a prerequisite for tumour-cell invasion and metastasis, and since accelerated migration is a key feature of EMT, the next behavioural process investigated in this study was the effect of silencing ITGβ1, ITGα2, and ILK on the PMC42 cells migratory ability. Testing of siRNA to ITGβ1, ITGα2, and ILK in wound healing assays was performed to identify the rate at which these transfected cells are able to heal the wound. Although the siRNA targeted against ITGβ1, ITGα2, and ILK in the wound healing assay exhibited closure of the wound, compared to control siRNA, these values failed to reach significance. Several factors may explain this such as the nature of the assay being semi-quantitative, or variability in the assay repeats. Due to this variability, a more reliable and measureable form of assessing the effect of knocking down the ITGβ1, ITGα2, and ILK on cell migration in a modified Boyden-chamber Transwell assay was studied. These results clearly demonstrate that supressing of ITGβ1, ITGα2, and ILK in the PMC42 cells resulted in a significant decrease in the ability of these cells to migrate, a finding that is in accordance with the above data showing decreased adhesive ability of the PMC42 cells transduced with ITGβ1, ITGα2, or ILK siRNA.

These results indicate that ITGβ1, ITGα2, and ILK each enhance adhesion and migration potential of PMC42 breast cancer cells, and thus are major contributors to the metastatic potential of breast cancer. While knockdown caused alterations in the adhesion/migration, the effects were much the same irrespective of EMT induction. This means that these candidate genes are not only part of the mesenchymal characteristics of the converted cell, but are also likely passengers in the EMT process.

Similar observations are also described in pancreatic cancer cells (Grzesiak et al., 2011), whereby knockdown of ITGβ1 and ITGα2 significantly inhibited cell adhesion, migration and proliferation on types I and IV collagen, fibronectin and laminin. The findings from the studies in this thesis demonstrate the importance of ILK in cell migration, consistent with the role of ILK in the cell migration of other mammalian cells like HeLa, Chinese hamster ovary, and rat embryonic fibroblasts (Zhang et al., 2002, Fukuda et al., 2003), and pancreatic cancer cell lines (Grzesiak and Bouvet, 2006, Grzesiak et al., 2007).
As RNAi technologies rely on repressed expression of a target and not specific functional inhibition, RNAi used alone does not allow delineation of the role of ILK function, from kinase function, in a cellular system. A number of studies have used potent, small molecule ILK kinase inhibitors to specifically target ILK kinase biological function in both experimental systems and therapeutically (Edwards et al., 2005, Koul et al., 2005a, Younes et al., 2007). These compounds decrease cancer cell proliferation, migration, invasion and angiogenesis, and induce apoptosis in vitro (Koul et al., 2005a, Younes et al., 2005, Rosanò et al., 2006, Younes et al., 2007, Edwards et al., 2008). Of the diverse functions of ILK, accruing evidence has suggested that some may require kinase activity, whereas others rely on protein-protein interactions and are, therefore, independent of kinase activity. This has resulted in an ongoing debate over the past few years about whether ILK is a bona fida protein kinase or a pseudokinase (Hannigan et al., 2005, Boudeau et al., 2006, Wickström et al., 2010, Hannigan et al., 2011). Although ILK was initially identified as a serine/threonine kinase (Hannigan et al., 1996b), Hanks and co-workers stated that it lacks a number of important motifs that are preserved in most kinases (Hanks et al., 1988). In addition, genetic studies in flies, worms and mice have demonstrated that the putative kinase activity is not required for development and homeostasis (Zervas et al., 2001, Mackinnon et al., 2002, Lange et al., 2009). Despite this evidence, the controversy remains, with many papers still being published claiming that ILK is a bona fida kinase (Hannigan et al., 2011).

Given the importance of ILK in cancer development and progression, ILK inhibition and/or silencing may be an effective way of treating cancer. Importantly, ILK inhibitors are well tolerated and effective in delaying tumour growth and decreasing Akt phosphorylation, cell survival and angiogenesis in prostate, melanoma, thyroid and glioblastoma xenograft models (Tan et al., 2004, Younes et al., 2005, Wong et al., 2007, Edwards et al., 2008). Previous efforts have been made to inhibit tumour growth in vivo by inhibiting ILK activity. For instance, the inhibition of ILK kinase activity by pharmacologic ILK inhibitor QLT-0267 suppresses tumour growth of anaplastic thyroid cancer by 50% to 60% (Younes et al., 2005). Another ILK inhibitor, QLT-0254, inhibits the tumour growth of pancreatic cancer by 40% (Yau et al., 2005). Many studies have
shown that inhibition of ILK in vivo, using antisense oligonucleotides or siRNA targeting ILK, as well as small molecule inhibitors such as QLT0267, reduces the growth of tumours in animal models of prostate cancer (Tan et al., 2004, Lee et al., 2011), pancreatic cancer (Yau et al., 2005), glioblastoma (Edwards et al., 2005, Edwards et al., 2008), gastric cancer (Zhao et al., 2011), melanoma (Wong et al., 2007), lung cancer (Liu et al., 2006), thyroid cancer (Younes et al., 2005), and breast cancer (Kalra et al., 2009).

Whist the mechanism through which ILK inhibition leads to reduced tumour growth in vivo is still undetermined, a number of studies have used ILK silencing (Wong et al., 2007, Zhao et al., 2011, Li et al., 2013d), or ILK inhibitors (Koul et al., 2005a, Younes et al., 2005, Oloumi et al., 2006, Troussard et al., 2006, Tabe et al., 2007, Younes et al., 2007, Dobreva et al., 2008, Edwards et al., 2008, Eke et al., 2009, Kalra et al., 2009, Li et al., 2009, Muranyi et al., 2009, Kalra et al., 2010, Muranyi et al., 2010, Faralli et al., 2011, Fielding et al., 2011, Kalra et al., 2011, Wang and Zhou, 2011, Becker-Santos et al., 2012, Steinbrunn et al., 2012, Li et al., 2013d, Lim et al., 2013, Jones et al., 2014, Sikkema et al., 2014) to show that decreased ILK activity leads to growth suppression.

Used together, RNAi and small molecule inhibitors provide a powerful tool to validate the biological functions of ILK. Hence, in addition to using siRNA to inhibit the expression of ILK, this study explored the effects of ILK inhibition on invasion using a pharmacological ILK kinase inhibitor, QLT-0267, which has previously been reported to inhibit ILK activity in a highly selective dose-dependent manner (Koul et al., 2005a). The selectivity of this small molecule for ILK relative to that of other kinases has been determined by analysing its effects on 150 recombinant kinases, among which, QLT0267 was found to be the most highly specific for ILK (Troussard et al., 2006, Younes et al., 2007). Furthermore, the in vitro kinase activity of purified ILK is sensitive to QLT0267 (Maydan et al., 2010). QLT0267 is an accepted inhibitor of ILK kinase activity in many systems (Koul et al., 2005a, Younes et al., 2005, Oloumi et al., 2006, Troussard et al., 2006, Dos Santos et al., 2007, Tabe et al., 2007, Younes et al., 2007, Dobreva et al., 2008, Edwards et al., 2008, Eke et al., 2009, Kalra et al., 2009, Li et al., 2009, Muranyi et al., 2009, Kalra et al., 2010, Muranyi et al., 2010, Faralli et al., 2011, Fielding et al.,
Since earlier results in this chapter clearly showed that ILK siRNA reduces the ITGβ1 protein levels, it was unclear whether this reduction was due to a structural role in ILK or an enzymatic functional role or a combination of the two. In this study, the inhibition of ILK in vitro was also achieved by the inhibition of ILK kinase activity using the small molecule inhibitor, QLT0267. ILK inhibition decreased the in vitro invasiveness of the PMC42-ET breast cancer cell line, concomitant with a decrease in ITGβ1 expression. These results emphasise the importance of the ITGβ1 in breast cancer cells and indicates a novel role for ILK in maintaining integrin levels in the PMC42 cells, and confirms that the role of ILK is not purely a structural role, but rather an enzymatic role that’s important. The precise mechanism involved in the role for ILK in maintaining integrin levels is not yet clear and remains to be elucidated, however, one possible mechanism would be inside-out/outside-in signalling, where ILK phosphorylates the ITGβ1 that its bound to. ILK has been reported to co-immunoprecipitate with ITGβ1, and is capable of phosphorylating its cytoplasmic domain in vitro (Hannigan et al., 1996b, Dedhar et al., 1999, Mulrooney et al., 2000). Nevertheless, further studies need to be carried out to confirm this theory.

The importance of integrins in several cell types that affect tumour progression has made them an appealing target for cancer therapy (Moore et al., 2014, Sun et al., 2014, Madrazo et al., 2017). Integrins have been a focus of therapeutic development for nearly 30 years, but despite some outstanding therapeutic successes, their complex biology has often confounded drug development (Raab-Westphal et al., 2017). Accordingly, several synthetic peptides and humanised antibodies have been developed to interfere with integrin–ligand interactions (Hamidi et al., 2016, Kobayashi et al., 2017). Currently, several potential drugs targeting integrins for cancer therapy and diagnosis are in different stages of clinical development (Pandolfi et al., 2017). Regardless of the encouraging in vitro and preclinical results, late phase clinical trials have, thus far, been disappointing (Desgrosellier and Cheresh, 2010). More knowledge of the integrin
structure, activation and signalling pathway will provide alternative strategies for cancer therapy and diagnosis.

In summary, results presented in this chapter substantiate the importance of the ITGβ1, ITGα2 and ILK axis in malignant processes. They identified for the first time that RNAi-mediated down-regulation of ITGβ1, ITGα2 and ILK expression was observed to varying degrees in the PMC42 model system, and translated into suppression of adhesion, migration, and invasion, as supported in the above mentioned studies by expression of ITGβ1, ITGα2, and ILK-deficient cell lines and by function blocking antibodies. Therefore, it was successfully demonstrated that knock down, not only in protein levels, but also in terms of known functions of integrins and ILK in attachment and migration was achieved. Despite these changes, no impact upon EMT was observed, indicating that these proteins do not play a functional role in the transition itself. Instead, the results indicate that they are potentially important and part of the induced mesenchymal characteristics/program, therefore being likely passengers in the EMT process, enabling the PMC42 breast cancer cells to interact with the interstitial matrices, and highlighting its possibly crucial role in cross-talk between integrins and cadherins in relation to the mesenchymal phenotype. Furthermore, these data suggest that the utilisation of siRNA to reduce the expression of the ITGβ1, ITGα2 and ILK may be a useful approach to prevent carcinoma cell progression. In particular, the ILK results allow for a cautious speculation on the important role of ILK in the crosstalk between integrins accompanying EMT in breast cancer.
CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS
6.1 GENERAL SUMMARY

Breast cancer is the most frequently diagnosed cancer in women worldwide. It holds a position as one of the leading causes of cancer deaths in women, in which the majority of deaths (90%) are caused by invasive and metastatic breast cancer. Therefore understanding the precise mechanisms involved in the progress of secondary metastases from the primary tumour is of utmost importance as a prognostic indicator in estimating metastatic risk, deciding the treatment plan, and monitoring of treatment response.

To date, the epithelial mesenchymal transition (EMT) phenomenon has been a favoured explanation of tumour cell progression, invasion and distant metastases for epithelial cancers including breast cancer (Savagner, 2010, Brabletz et al., 2018). During the process of EMT, epithelial cancer cells acquire molecular alternations that facilitate the loss of epithelial features and the gain of migratory and invasive traits (mesenchymal phenotype) either partially or completely (Jolly et al., 2016, Thompson and Nagaraj, 2018). While transitioning between the epithelial (E), and mesenchymal (M), phenotypes, cells can adopt a hybrid epithelial/mesenchymal (E/M) or a partial EMT phenotype. Cells attaining this hybrid phenotype through EMT exhibit a mix of epithelial (e.g. loss of cell-cell adhesion) and mesenchymal (e.g. migration) traits that enable them to move collectively to invade the basement membrane and enter the blood vessels as circulating tumour cells (CTCs) (Kalluri and Weinberg, 2009, Wu et al., 2016). CTCs are hypothesised to exit the vasculature at distant organs and undergo mesenchymal epithelial transition (MET- the conversion of mesenchymal cells to epithelial derivatives), to proliferate in order to form secondary tumours during metastasis (Nieto, 2013, Brabletz et al., 2018, Thompson and Nagaraj, 2018). The importance of MET in cancer metastasis has been gradually recognized, and studies on the mechanism of MET are increasing (Hugo et al., 2007, Gunasinghe et al., 2012, Yoshida et al., 2014).

Although it is not yet fully understood how the EMT occurs, and the implications of EMT for metastasis, it has recently been proposed in in vitro data (mainly through co-expression of epithelial and mesenchymal markers within the same cells), that EMT occurs through distinct intermediate hybrid states, through the generation of
subpopulations that represent different intermediate states across the epithelial - mesenchymal spectrum (Huang et al., 2013, Zhang et al., 2014b, Hong et al., 2015, Jolly et al., 2016, Bierie et al., 2017). However, there was no direct in vivo evidence for this idea, until very recently in a study by Pastushenko and colleagues (2018), who screened several of mouse tumour models undergoing spontaneous EMT, revealing the existence of multiple tumour subpopulations associated with distinct EMT stages: from epithelial to completely mesenchymal states, passing through intermediate hybrid states, with different invasive, metastatic and differentiation characteristics (Pastushenko et al., 2018).

E-cadherin, an important transmembrane protein that is localized to the adherens junctions and basolateral plasma membrane, represents the best-characterized EMT molecular marker expressed in epithelial cells (Lombaerts et al., 2006). The functional loss or downregulation of E-cadherin from epithelial cells is considered an integral step in EMT and its reverse, the MET. Expression of vimentin and N-cadherin, two proteins considered to be markers of a mesenchymal phenotype and crucial for cellular migration, are increased during this time as well, and often not apparent in distant metastases. However, the mechanisms underlying the transient expression of these EMT markers are not well established. Expression of vimentin in cancer correlates well with accelerated tumour growth, invasion, and poor prognosis (Domagala et al., 1990, Holck et al., 1993, Domagala et al., 1994, Heatley et al., 2002, Niveditha and Bajaj, 2003, Willipinski-Stapelfeldt et al., 2005, Yamashita et al., 2013); however, the precise role of vimentin in cancer progression remains obscure. Also, vimentin is expressed in many stromal cell types, and also in the normal basal / myoepithelial cells of the mammary gland, therefore, a need to develop better markers and detection methods as a prerequisite to causally link EMT with distinct steps of metastasis is crucial.

Three of the critical steps of metastasis - adhesion and migration of the primary tumour on ECM at the distant site, as well as invasion, involve the integrin cell surface molecules (Buckley et al., 1996, Voura et al., 2001, Leroy-Dudal et al., 2005, Leroy-Dudal et al., 2005, Desgrosellier and Cheresh, 2010). Integrins are the major receptors for ECM proteins, and the expression profile of integrins on cancer cells and the composition and
organisation of ECM of the tumour stroma are major factors in cancer development, metastasis and therapy resistance (Desgrosellier and Cheresh, 2010, Naci et al., 2015). Growing evidence suggests that α2β1 integrin, a major collagen receptor promoting cell migration, can be a key pathway in cancer pathogenesis (Naci et al., 2015). α2β1 integrin is expressed on different cell types such as epithelial, fibroblast and endothelial cells, as well as on immune cells, and it is found on many types of cancer cells (McCall-Culbreath and Zutter, 2008, Madamanchi et al., 2014). However, integrin expression can also vary considerably between normal and tumour tissue, usually either expressed at low or undetectable levels in most adult epithelia but highly upregulated in some tumours. Expression levels of some integrins, such as α2β1, decrease in tumour cells, potentially increasing tumour cell dissemination (Kren et al., 2007). In fact, it has been suggested that α2β1 integrin could function as a tumour suppressor by reversing some of the malignant properties of breast cancer cells (Zutter et al., 1995).

Cell culture models enable the complex molecular mechanisms that underlie EMT to be characterised, and they have revealed cooperation and crosstalk between signalling pathways and transcriptional, translational and post-translational regulation (Lamouille et al., 2014, Cursons et al., 2015).

Although commonly used, cell culture models are associated with the concern that cells have altered behaviour due to mutations acquired during the establishment of the cells line, the lack of a three dimensional context, loss of apical basal polarity and lack of in vivo micro-environment signals. In contrast, patient-derived xenografts (PDXs) are renewable tumour models engrafted in mice, generated from fresh human tumours without prior in vitro exposure. These models have been shown to be biologically stable and accurately reflect the patient tumour with regards to histopathology, gene expression, genetic mutations, and therapeutic response (Scott et al., 2013, Gao et al., 2015, Bruna et al., 2016, Sulaiman and Wang, 2017). PDX models allow an invaluable assessment of tumour evolution and adaptive response to therapy.

This thesis is a report on the work conducted during the course of my PhD project, whereby three primary aims were investigated using 2 model systems, an in vitro PMC42
cell line model and an *in vivo* EDW-01 PDX breast cancer model: (i) expression time-course analysis of the PMC42 EMT model system in order to determine the potential hierarchy of factors that regulate the EMT phenotype, (ii) characterisation of the EDW-01 xenograft model system with a focus on expression patterns of integrins and ILK in order to determine whether they correlate with the more progressive nature and mesenchymal properties of late passage tumours, and (iii) functional studies on the integrins and ILK in the PMC42 EMT model system by employing siRNA abrogation approaches to reduce expression of the candidate effectors and determine them as being either functional “drivers” or “passengers” of EMT.

Based on results presented as part of this thesis, there is sufficient evidence to support the hypothesis that ITGβ1, ITGa2 and ILK contribute to the mesenchymal phenotype and are functionally required for the transition from an epithelial to a mesenchymal state.

Firstly, **Chapter 3** explored the morphological and molecular aspects of the EGF-induced PMC42 cell line model *in vitro*. This cell line represents a human breast cancer progression model in which both the epithelial and mesenchymal cell intermediates, which emulate different stages of an EMT, have been derived from the same cell lineage. This cell line displays a predisposition to undergo EMT-like changes with growth factor treatment (EGF). In particular in this study, the PMC42-ET and PMC42-LA cells were extensively studied in respect to cell density, under both sparse and dense conditions, to determine whether the presence of fewer cells can in fact affect the extent of EGF-induced EMT. This was so that the molecular changes regulating the EMT phenotype may be determined in this process in human breast cancer.

Results clearly showed that the EGF-induced morphological changes (the development of a spindle-shaped, fibroblastoid morphology) in the PMC42 cells were more evident under sparse culture conditions, as was also previously seen in MCF10A cells (Sarrio et al., 2012). These results were confirmed by the changes in the expression of key molecular EMT markers; upregulated levels of vimentin and N-cadherin, and reduced expression levels of E-cadherin. Further to that, the ITGa2, ITGβ1 and ILK examined in this study were seen to be induced from 3hrs, and 1.5hrs, respectively, indicating that
these genes are early response genes in EMT, and may indeed be required for the early manifestation or EMT, all of which are modulated by EGFR signalling. Recent work from within our laboratory, Cursons et al., (2015) used RNA sequencing to identify gene expression changes, in the PMC42 cells, that occur as cells transition to a more-mesenchymal phenotype, and identified the cell signalling pathways regulated across these experimental systems (Cursons et al., 2015). Transition to a mesenchymal phenotype was induced across all three cell lines using EGF stimulation, and in MDA-MB-468 cells, by hypoxia. Comparing the transcriptional response to EGF and hypoxia, differences in the cellular signalling pathways that mediate, and are influenced by, EMT were identified, and significant differences were observed for a number of important cellular signalling components previously implicated in EMT (Cursons et al., 2015).

They showed that the stimulation of PMC42-LA cells with EGF, promoted EMT as indicated by increased vimentin-positive cells. Unstimulated PMC42-ET cells express vimentin, thus increases in the number of vimentin-positive cells with EGF stimulation are relatively small, consistent with their previous reports on EMT within this cellular system (Hugo et al., 2011). Although EGF stimulation increased the mRNA transcript abundance of EMT markers, the inability of EGFR inhibitors to reduce the small percentage of vimentin positive PMC42-ET cells suggests that the unstimulated mesenchymal phenotype of these cells is maintained through EGFR-independent signalling mechanisms. In addition, their RNA-sequencing data revealed that some of the strongest transcriptional changes with EGF-induced EMT in the PMC42 system were observed for integrin subunits and corresponding ECM components.

This investigation has shown the potential use of the early response molecular markers (ITGβ1, ITGα2, and ILK) not only as predictive factors in breast cancer, but also as targets for clinical therapy, where identification of these markers could be a useful step to developing early detection methods.

**Chapter 4** examined the EDW-01 PDX model and expanded on previous work in our research facility to address whether the original EDW-01 model could be successfully re-propagated, obtaining its original features, hence making it a suitable model for EMT
studies. Evidence of glandular formation, adenocarcinoma, was detected in the early xenograft tumour sample, whilst the latest xenograft tumour showed local invasion into surrounding tissues, indicating that the xenograft acquires increased invasive potential with each passage. The EDW-01 xenograft was studied immuno-histochemically for the expression of the mesenchymal marker vimentin and epithelial markers E-cadherin and cytokeratins, together with markers currently used for the identification of specific subgroups of breast tumours with biological relevance (ER, PR, and HER2). Immunoreactivity to ER was identified in less than 10% of positive cells in the original patient material in the EDW-01 xenograft. This positivity was gradually increased with each passage, showing 40% ER positivity at late passage. Consistent with the original patient material, PR and HER2 expression was weak to negative throughout the EDW-01 xenograft, regardless of passage. This data allows the classification of the EDW-01 PDX systems molecular subgroup as ER+PR-HER2-, so-called Luminal-B, which tend to have higher proliferative rates and poorer prognosis (Prat et al., 2015). The immunohistochemical classification of the EDW-01 xenograft (ER+PR-HER2-) represents one of the four subgroups within the Luminal B subtype according to HER2 expression and ER and PR status (Cancello et al., 2012) and has a lower overall survival and progression-free survival than the ER+PR+HER2- subgroup. Patients with this luminal B subtype have a more clinically aggressive type of ER+ tumours, and would be at higher risk of relapse and death, and hence would benefit from both chemotherapy and hormonal therapy (Hequet et al., 2017). This finding in itself, though not unique, is rather exciting, as ER+ PDX lines have been difficult to establish among breast cancer PDX models, with a low average rate of engraftment reported (2–15%) (Marangoni et al., 2009, Cottu et al., 2012, Charafe-Jauffret et al., 2013, Landis et al., 2013). With a low intake rate, established ER+ PDX lines might not be representative of the overall ER+ tumour population. Therefore, characterisation of established ER+ PDXs is important for the proper usage of them as preclinical models to examine treatment outcome.

One of the first systematic attempts to determine the molecular features of ER+ tumours (including both ER+HER2- and ER+HER2+ classes) that impacts PDX engraftment, was a study by Kanaya and coworkers (2017), whereby a total of 97 tumour specimens from ER+ patients were implanted into NSG mice to generate PDX models. Eighty one were
ER+HER2− tumours and 16 were ER+HER2+ tumours. They compared the protein expression profiles by Reverse Phase Protein Array (RPPA) in tumours that resulted in PDXs compared to those that did not. Heat map analyses of their RPPA data showed that ER+HER2− tumours were divided into 2 groups by luminal A/B signature and this expression signature was also associated with the rate of PDX intake (Kanaya et al., 2017).

Overall, nine ER+ PDX models were successfully established and characterised from 97 tumour specimens: six ER+HER2− and three ER+HER2+. ER+HER2− tumours were lost more frequently during early passage than ER+HER2+ tumours, and subtype analysis demonstrated that the intake rate of ER+HER2+ tumours was higher than that of ER+HER2− tumours; 19% versus 7%, respectively. This may be explained by the more aggressive clinical characteristics of ER+HER2+ breast cancer compared with ER+HER2− breast cancer and supports their hypothesis that ER+HER2− and ER+HER2+ tumours are biologically different (Kanaya et al., 2017).

Therefore the EDW-01 results are supported by those of Kanaya et al., (2017), confirming that the luminal B tumours have significantly different protein expression profiles from luminal A tumours and are a more aggressive type of ER+ tumour clinically.

The gene expression of the classic EMT markers vimentin, N-cadherin and E-cadherin, as well as ITGβ1, ITGα2 and ILK in the in vivo EDW-01 PDX model was further explored, and in doing so, showed, for the first time, that the changes in the classic EMT markers was accompanied by up-regulated ITGβ1 and ITGα2, with a trend towards up-regulated ILK expression in this unique EDW-01 PDX model system.

Building on the findings from chapters 3 and 4, the data in chapter 5, on the EGF-induced PMC42 cells transduced with siRNA gives a further insight into the functional role of ITGβ1, ITGα2 and ILK and the impact on adhesion, migration and late stage mesenchymal gene expression. Results presented in this chapter have identified that using RNAi, down-regulating ITGβ1, ITGα2 and ILK axis in the PMC42 model system was observed to varying degrees and translates into suppression of adhesion and migration.
Therefore, it was successfully demonstrated that knock down, not only in protein levels, but also in terms of known functions of integrins and ILK in attachment and migration was achieved. The data in this study clearly showed that the binding of the PMC42-ET cells to the ECM substrates collagen I, collagen IV, laminin, and fibronectin, was significantly abrogated by knocking down both ITGβ1 and ITGα2 subunits, as well as ILK in the cells, indicating that both the ITGβ1 and ITGα2 subunits, as well as ILK, are required for the adhesion of PMC42-ET cells to these substrates. These observations are in agreement with a recent study by Kozlova and colleagues (2018), who showed that blocking the expression of integrin α2β1, which was accomplished by transduction of α2-specific shRNA, resulted in significant inhibition of proliferation and clonal activity in human MCF-7 breast carcinoma and SK-Mel-147 melanoma cells (Kozlova et al., 2018). These observations are also in agreement with previously published studies on pancreatic cancer cell lines (Grzesiak and Bouvet, 2006, Grzesiak et al., 2007, Grzesiak et al., 2011), where function-blocking monoclonal antibodies directed against the ITGβ1 and ITGα2 subunits inhibited interactions with types I and IV collagen, fibronectin and laminin ECM proteins. The ILK results are consistent with previously published studies on various cancer cell lines such as gastric cancer cell lines (Zhao et al., 2011) and ovarian cancer cell lines (Choi et al., 2012). A similar observation was made by Sottnik et al. (2013), who showed that overexpression of integrin α2β1 stimulated adhesion and migration, while its downregulation inhibited adhesion and migration of prostate cancer cells on collagen (Sottnik et al., 2013).

Results from this thesis also clearly demonstrate that silencing ITGβ1, ITGα2 and ILK in the PMC42 cells, using RNAi, resulted in a significant decrease in the ability of these cells to migrate, a finding that is in accordance with the above data showing decreased adhesive ability of the PMC42 cells treated with ITGβ1, ITGα2 and ILK siRNA. These results indicate that ITGβ1, ITGα2 and ILK may enhance adhesion and migration potential of PMC42 breast cancer cells, and thus are major contributors to the metastatic potential of breast cancer.

Previously published reports describing the role of integrins in metastasis are controversial. The results of in vitro and in vivo studies of various designs may be subject
to conflicting interpretation. Early studies initially suggested that loss of α<sub>2</sub>β<sub>1</sub> integrin expression may play an important role in cancer progression (Zutter et al., 1990). Alternatively, loss of α<sub>2</sub>β<sub>1</sub> integrin expression may simply be a consequence of malignant progression. Other studies, however, using cell lines either in vivo or in vitro, suggest that the α<sub>2</sub>β<sub>1</sub> integrin may serve to enhance metastasis to different organs (Chan et al., 1991, Ho et al., 1997, Yoshimura et al., 2009). In a mouse model of breast cancer, Ramirez and co-workers (2011) showed that α2β1 integrin suppressed metastasis. Deletion of the α2 integrin in MMTV-Neu mice carrying the Neu/erbB2 oncogene driven by the mouse mammary tumour virus promoter, characterized by a high breast tumour incidence, led to a drastic increase in spontaneous lung metastasis estimated by the number and size of metastases. On the other hand, they demonstrated that cell lines derived from primary breast tumours with high α2 gene expression did not differ in proliferative activity from the lines derived from tumours devoid of α2 gene expression (Ramirez et al., 2011). This was validated in Koslova et al., (2018) demonstrating that loss of α2β1 expression led to a decrease in active MMP-2. However, they showed that α2β1 downregulation inhibited proliferation of the cells with low metastatic potential while it reduced both proliferation and invasion of cells with high metastatic activity.

Integrin α2β1 expression in renal cell carcinomas correlated with the malignant phenotype (Anastassiou et al., 1995), where loss of integrin α2β1 expression in human breast cancer was associated with tumour progression (Arihiro et al., 1993). In addition to the above cited studies on the role of the α2β1 integrin in adhesion and migration, work by Dyce and colleagues (2002) demonstrate that mouse xenografts of a human squamous cell carcinoma line with high α2β1 expression exhibited more pronounced invasion into surrounding tissues compared to the cell lines with low α2β1 expression (Dyce et al., 2002). Furthermore, a key role of α2β1 integrin in metastasis was demonstrated in studies on bone metastasis developed in mice grafted with B16 mouse melanoma cells (Yoshimura et al., 2009). Further research is needed to clarify these contradictions.

Results from this part of the thesis also show that knockdown of ILK significantly inhibits breast cancer cell adhesion on types I and IV collagen, fibronectin and laminin,
indicating that ILK is required for the adhesion of PMC42-ET cells to these substrates. These observations are consistent with previously published studies on various cancer cell lines such as gastric cancer cell lines (Zhao et al., 2011) and ovarian cancer cell lines (Choi et al., 2012). Given that integrins lack intrinsic enzymatic activity (Schiller et al., 2011), it is fundamental that their signalling depends on recruiting adaptor and signalling proteins, such as ILK, which is directly recruited to the cytoplasmic domain of β1 integrin (Hannigan et al., 1996b). ILK is overexpressed in many types of cancer, and the oncogenic effects of ILK have been attributed to the catalytic activity of the kinase domain resulting in the activation of protein kinase B (PKB, also known as Akt) and glycogen synthase kinase-3beta (GSK3b), which in turn regulates the stability of protooncogenic b-catenin (Hannigan et al., 2005).

In the findings presented in this thesis, ILK inhibition, a pharmacological inhibitor that binds to the ATP-binding site of ILK (QLT-0267) decreased the in vitro invasiveness of the PMC42-ET breast cancer cell line, concomitant with a decrease in ITGβ1 expression. Treatment of the PMC42 cells with QLT-0267 was also as effective as siRNA-mediated depletion of ILK. Based on the assumption that ILK acts as a kinase, a potential explanation for the inhibitory effect of QLT-0267 could be an impairment of the stability of ILK (Widmaier et al., 2012).

These findings emphasise the importance of the ITGβ1 in breast cancer cells, and of ILK in maintaining integrin levels in the PMC42 cells, and supports an ongoing controversy as to whether that the role of ILK is not purely a structural role, but rather an enzymatic role that’s important. Of the diverse functions of ILK, there is accumulating and compelling evidence suggesting that some functions may require kinase activity whereas others depend on protein–protein interactions and are, therefore, independent of kinase activity. However, over the past several years there has been an ongoing debate about whether ILK indeed functions as a protein serine/threonine kinase (bona fide protein kinase) or not (pseudokinase) (Hannigan et al., 2005, Boudeau et al., 2006, Wickström et al., 2010, Hannigan et al., 2011). The findings in this thesis are in agreement with the study by Hannigan and coworkers (2005), where it was reported that the depletion or
inhibition of ILK with the small molecule inhibitor QLT-0267 inhibits anchorage-independent growth, cell cycle progression and invasion (Hannigan et al., 2005).

The precise mechanism involved in the role for ILK in maintaining integrin levels is complex and controversial. It remains to be elucidated, however, one possible mechanism would be outside-in signalling, where ILK phosphorylates the ITGβ1 that it is bound to. This mechanism is consistent with studies showing that ILK co-immunoprecipitates with β1-integrin in mammalian cells, and directly phosphorylates its cytoplasmic domain in vitro (Juliano and Haskill, 1993, Hannigan et al., 1996b, Dedhar et al., 1999, Mulrooney et al., 2000, Hannigan et al., 2005, McDonald et al., 2008).

As important as these results may be, more crucial is the finding that despite these changes, no evidence of any consistent shift in key EMT genes such as vimentin, N-cadherin and E-cadherin was seen in favour of the hypothesis, indicating that ITGβ1, ITGα2 and ILK do not play a functional role in the EMT transition itself. However, results do demonstrate that ITGβ1, ITGα2 and ILK directly contribute to the phenotype resulting after EMT, enabling the PMC42 breast cancer cells to interact with the interstitial matrices, and highlighting its possibly crucial role in cross-talk between integrins and cadherins in EMT.

6.2 FUTURE DIRECTIONS

There are many potential avenues of further investigation of ITGβ1, ITGα2 and ILK resulting from the work presented in this thesis in order to provide a stronger understanding of these markers in relation to EMT:

- Conduct miRNA expression profiling studies on the EGF-induced PMC42 cells (under sparse conditions), to identify candidate miRNAs with possible roles in the ITGβ1, ITGα2, ILK axis.

- Perform large-scale analysis of transcripts on the EGF-induced PMC42 cells (under sparse conditions) by RNA-sequencing or microarray to
uncover new candidates for EMT as well as highlight the involvement of ITGβ1, ITGα2 and ILK.

- Take the integrin work further to manipulate ITGβ1 and/or, ITGα2 with shRNA in an in vivo model system such as MDA-MB-468 which can induce an EMT, as well as MDA-MB-231 or a syngeneic metastasising model (e.g. 4T1), to show whether integrin ablation affects EMT and/or is important for metastasis.

- Another direction would be looking at “Inside-Out” signalling of ILK to ITGβ1. The data in this thesis has suggested an important role for ILK stabilising cell surface ITGβ1. ILK plays both a signalling role as a kinase in facilitating AKT activation but also has a structural role through the binding of actin-associated proteins (e.g. Parvins, PINCH, ILK-AP).

- Another possibility would be to investigate the expression profile of ITGβ1, ITGα2 and ILK, as well as others associated with EMT, in CTCs, which are important during metastasis development (Lowes and Allan, 2017, Škovierová et al., 2018). CTCs and CTC clusters (aggregates consisting of various types of cell, such as tumour, stroma and immune cells, as well as platelets) represent an attractive alternative to tissue biopsy of metastatic lesions, due to the fact that they may be non-invasively obtained. These results can then be compared to the RNA-sequencing studies in CTCs that are available (Yu et al., 2013).

- In the EDW-01 xenograft, a more rigorous analysis of the gene expression patterns, including transcriptome analysis by microarray or mRNA-sequencing, which have the capacity to distinguish mouse and human transcripts (Conway et al., 2012, Purdom et al., 2013), as well as the IHC data of the clinical specimens could be implemented, which may provide a
comprehensive analysis to determine a correlation between the involvement of these markers to EMT.

6.3 CONCLUDING REMARKS

The majority of the studies to date linking breast cancer progression with EMT are derived from studies in cancer cell lines and/or animal models. Therefore, their relevance in human breast cancer is still under debate and exploration. Validating biomarkers related with EMT in patient models is highly crucial for identifying patients at risk of developing drug resistance and metastasis.

This thesis has validated and confirmed both the in vitro PMC42 system and the in vivo EDW-01 system as viable model systems to assess the role of EMT in breast cancer. The results described in this thesis highlight the possibilities that ITGβ1, ITGα2 and ILK could serve as biological markers of the EMT process in breast cancer progression and metastasis and delineate more effective and valuable therapeutic strategies for future metastasis prevention in breast cancer.
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Appendix X: STR profiling and confirmation of human cell line identity.

DNA was extracted from cell lines and profiled using the StemElite ID System (Promega, Madison, WI, USA) which uses short tandem repeat (STR) analysis of specific loci in the human genome. This commercial kit permits authentication of human cell lines, 'genetic drift' of lines, and detection of contaminating mouse DNA. Nine STR loci are interrogated as well as Amelogenin for gender identification. Sizing of the loci by capillary electrophoresis and allele-calling analysis was performed by the QIMR Scientific Services, Herston, Qld. All lines were confirmed as female and none had detectable mouse contamination (1% detection limit specified by manufacturer).

PMC42 lines are clearly related. Two other human breast cell lines used in the laboratory (MCF-7 & MDA-MB-231) are represented along with reference to ATCC specifications (www.atcc.org). The PMC42 cell lines are not represented in the ATCC collection.

n.c., 'no call' made by software; n.d.s, no data specified
Author/s:
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