Heregulin (Neuregulin)/HER3 Signalling Increases Invasive Behaviour of HER2-positive Breast Cancer Cells

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M.Sc. of Human Genetics

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School of Medicine
Abstract

Breast cancer is a heterogeneous disease with distinct histological and molecular subtypes differing in prognosis, response to therapy and metastatic behaviour. Like most cancers, poor outcome in breast cancer is related to tumour cell spread and colonisation of distant organs, which ultimately disrupts local and systemic physiological processes to the demise of the patient. Colonisation of the brain is arguably the most aggressive manifestation of metastatic disease. Brain metastasis is a growing public health problem associated with a high degree of morbidity and virtually 100% mortality. The current treatments for these patients are surgery, radiotherapy and chemotherapy, but their outcome remains poor. In this regard, the management of brain metastases is an unmet medical need for which establishment of more effective preventive and therapeutic treatment strategies is of paramount importance. In order to achieve that, we need to deepen our knowledge and insights about the molecular mechanisms and signal transduction pathways underlying initiation and progression of brain metastases.

Invasion of breast tumour cells across the basement membrane and endothelium to gain entry to the blood stream and lymphovascular system is a critical early requirement for metastasis. Disseminated cells must then undergo extravasation at distant tissue sites. In the case of brain metastases, cells must breach the highly specialised and impermeable blood-brain-barrier. Improving our understanding of the molecular mechanisms underlying these processes may reveal new drug targets to prevent development or suppress growth of these lethal tumours.

Of all the breast cancer subtypes, patients with HER2-positive disease (amplification and/or overexpression of the ERBB2 receptor tyrosine kinase) exhibit the highest frequency of brain metastases. Both HER2 and its obligate dimerisation partner, HER3 (ERBB3), are associated with development of brain metastases, though a mechanistic link has not yet been established. The main goal of this project was to elucidate the molecular mechanisms by which the HER2/HER3 heterodimer promotes metastatic progression and more specifically, development of brain metastases. In order to achieve this, activation of the HER2/HER3 signalling axis was assessed in a panel of HER2-positive breast cancer cell lines (MDA-MB-361, MCF7 and SKBr3) after treatment with the HER3 ligand heregulin (HRG). The effects of HRG on proliferation, migration, invasion and transendothelial migration (TEM) across a tight layer of primary human brain microvascular endothelial cells were also assessed as...
functional readouts of this axis in vitro. Finally, the molecular mechanisms underlying some of the HRG-induced changes (proliferation and TEM) were investigated.

The results of this study showed that HRG increased proliferation of luminal HER2-positive breast cancer cells via induction of cyclin D1 and down-regulation of p27 proteins. In addition, HRG-induced mesenchymal-like phenotype, migratory and invasive capabilities in these cells in vitro, and this was associated with induction of matrix metalloproteinase-9 (MMP-9) and cathepsin B. Moreover, HRG induced TEM activity of these cells in a HER3- and MMP-dependent manner, raising the possibility that HRG plays a role in proteolytic permeabilisation of the BBB in brain metastases from HER2-positive breast cancer. Further in vivo studies are required to determine if this mechanism is involved in the initial seeding of micrometastases in the brain and/or in maintaining a favourable microenvironment to sustain tumour growth. Given that HER3 is overexpressed and activated more frequently than HER2 in brain metastases from breast cancer, it will also be important to establish whether this axis promotes brain metastatic cell behaviour independent of HER2 status. The results of this study have potential implications in oncology, where targeting the HRG/HER3 signalling could be exploited in preventive and therapeutic strategies for management of brain metastases.
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Publications included in this thesis

“None”.
Contributions by others to the thesis

“No contributions by others.”

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breast cancer brain metastasis, blood brain barrier, HER2, HER3, HRG, MMP-9, transendothelial migration activity

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<th>Full name</th>
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<tbody>
<tr>
<td>ADAM-17</td>
<td>ADAM metallopeptidase domain 17</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
</tr>
<tr>
<td>BM</td>
<td>Brain metastases</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony-stimulating factor 1</td>
</tr>
<tr>
<td>CSF-1R</td>
<td>Colony-stimulating factor-1 receptor</td>
</tr>
<tr>
<td>CTC</td>
<td>Circulating tumour cell</td>
</tr>
<tr>
<td>CXCL12</td>
<td>Chemokine (C-X-C motif) ligand 12</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>HBEGF</td>
<td>Heparin-binding epidermal growth factor</td>
</tr>
<tr>
<td>HBMEC</td>
<td>Human brain microvascular endothelial cell</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HK2</td>
<td>Hexokinase 2</td>
</tr>
<tr>
<td>HPC</td>
<td>Hematopoietic cell</td>
</tr>
<tr>
<td>HRG-β1</td>
<td>Human recombinant heregulin-β1</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IGF1</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
</tr>
<tr>
<td>LOX</td>
<td>Lysyl oxidase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-epithelial transition</td>
</tr>
<tr>
<td>Mkk4</td>
<td>Mitogen activated protein kinase kinase 4</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MTT</td>
<td>Microculture tetrazolium test</td>
</tr>
<tr>
<td>MUC1</td>
<td>Mucin 1</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Neuronal cadherin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphoinositol triphosphate</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RARB</td>
<td>Retinoic acid receptor-β</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum-free media</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline tween</td>
</tr>
<tr>
<td>TEER</td>
<td>Transendothelial electrical resistance</td>
</tr>
<tr>
<td>TEM</td>
<td>Transendothelial migration</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>Tissue inhibitor of MMP-2</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>TME</td>
<td>Tumour microenvironment</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>Vascular endothelial growth factor receptor 1</td>
</tr>
<tr>
<td>WBRT</td>
<td>Whole brain radiation therapy</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula occludens</td>
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</table>
Chapter 1
General Introduction
1.1 Breast cancer

Breast cancer is the most commonly diagnosed cancer in women and the second leading cause of cancer-related death (Mego, Mani, & Cristofanilli, 2010). The vast majority of cancer-related death is due to tumour cell dissemination and colonisation of distant organs (metastasis), which disrupts local and systemic physiology (Jones, 2008). The major metastatic sites in breast cancer are bone, lung, liver and the brain (Smid et al., 2008). Despite advances in systemic therapies and earlier detection through mammographic screening, metastatic breast cancer still remains incurable (Mego et al., 2010).

Cancer metastasis is the most poorly understood hallmark of cancer (Chaffer & Weinberg, 2011). Metastatic dissemination has the following sequential steps: local invasion of surrounding tissue, entering lymph and blood systems (intravasation), survival in the bloodstream and translocation to distant organs, exit from the bloodstream (extravasation) and finally adaption to the foreign microenvironment of target tissues to facilitate cell proliferation and colonisation (Chaffer & Weinberg, 2011).

Metastatic cascade can be conceptually classified into two major parts (Fig. 1.1): (i) physical translocation of cancer cells from the primary tumour and (ii) colonisation (Chaffer & Weinberg, 2011). While our understanding of physical dissemination is in sight, our understanding of colonisation is still not well understood.
knowledge and insight about colonisation is still in its infancy (Chaffer & Weinberg, 2011). In terms of translational oncology, apprehension of molecular mechanisms of the physical translocation might be important to block early metastasis in cancer patients whereas understanding the mechanisms of colonisation is hoped to lead to establishment of more effective therapeutic strategies for patients who are suffering from already-established metastases (Chaffer & Weinberg, 2011).

The metastasis process is determined by both intrinsic properties of the tumour cells, as well as complicated interactions with the local microenvironment (Langley & Fidler, 2007). Accumulating clinical and experimental evidence indicate that metastases are independent of vascular anatomy, rate of blood flow and number of tumour cells delivered to each organ. Indeed, it is well established that tumour cells reach the microenvironment of multiple organs but colonisation occurs only in specific target organs (Langley & Fidler, 2007).

In the 19th Century, Stephen Paget analysed more than 900 autopsy records of women with breast cancer to determine whether the organ distribution of metastases from different human malignancies was random. He concluded that certain tumour cells (the seed) had an affinity for certain organs (the soil) (Paget, 1989). A current definition of the ‘seed and soil’ hypothesis has at least three principles. First, tumours are heterogeneous and consist of subpopulations of cells (clones) with different genetic profiles and biological properties. Second, metastasis is highly selective for the specific sub-clones that can fulfil all of the requirements for generation of metastases; and third, the outcome largely depends on the interactions between tumour cells and the microenvironment (Chaffer & Weinberg, 2011).

1.1.1 Breast cancer subtypes

Invasive ductal breast cancers can be divided into several categories with different prognoses and responses to chemotherapy on the basis of tumour morphology, grade (degree of differentiation) and expression status of certain receptors (oestrogen receptor (ER), progesterone receptor (PR) and HER2) (Harrell et al., 2012). Amongst these histopathologic breast cancer classes are HER2-positive (HER2+) (HER2 amplified and/or overexpressed) and triple-negative (ER, PR and HER2 negative) tumours, which have been shown to have increased risk of brain metastases (BM) compared to luminal (ER+) and rarer ‘breast cancer special types’ (Harrell et al., 2012). The molecular mechanisms for the high tendency of BM formation from these two subtypes of breast cancer are largely unknown.

In addition to histopathologic classification, microarray-based gene expression profiling is gaining momentum as a way to classify tumours into clinically useful groups, and is helping
to elucidate the molecular basis of breast cancer. Unsupervised cluster analysis of the gene expression profiles of large tumour cohorts has revealed phenotypically distinct subgroups that correlate with clinical outcomes, such as prediction of treatment response and metastatic behaviour. Different tumour cohorts and analysis techniques have produced slightly different subgroupings (Perou et al., 2000); however there are certain molecular subtypes that are robustly identified with different classification approaches:

<table>
<thead>
<tr>
<th>Molecular subtype</th>
<th>Features</th>
<th>Metastatic behaviour</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal-like</td>
<td>Expression profile resembles basal cells of normal breast. Significant overlap between this group and triple negative histological subtype</td>
<td>High risk of BM</td>
<td>(Harrell et al., 2012)</td>
</tr>
<tr>
<td>HER2+</td>
<td>HER2-positive</td>
<td>High risk of BM</td>
<td>(Harrell et al., 2012)</td>
</tr>
<tr>
<td>Claudin-low</td>
<td>Usually triple negative, low expression of cell-cell junction proteins (e.g. claudins 3, 4, 7), high expression of mesenchymal differentiation markers (e.g. Twist 1/2)</td>
<td>High risk of BM</td>
<td>(Harrell et al., 2012)</td>
</tr>
<tr>
<td>Luminal A</td>
<td>ER+, PR+, most favourable outcomes of all the molecular Subtypes</td>
<td>Lowest risk of BM. Highest rates of bone Metastases</td>
<td>(Harrell et al., 2012)</td>
</tr>
<tr>
<td>Luminal B</td>
<td>Also ER+, PR+ but is more highly proliferative and is Ki67+</td>
<td>Low risk of BM</td>
<td>(Harrell et al., 2012)</td>
</tr>
<tr>
<td>Normal-like</td>
<td>Contentious subtype that some argue represents tumour samples contaminated with normal breast tissue as the gene expression profile has similarities to adipose tissue</td>
<td>ND</td>
<td>(Harrell et al., 2012)</td>
</tr>
</tbody>
</table>

Table 1.1 Different molecular subtypes of breast cancer and their metastatic behaviour.

1.1.1.1 Breast cancer cell lines as in vitro models of diseases

The number of breast cancer cell lines is limited (Lacroix & Leclercq, 2004). Whether they are representative of the tumours from which they originated remains an open question. Whether their diversity reflects the tumour heterogeneity is another matter of debate (Lacroix & Leclercq, 2004). However, recent advances in technical approaches including microarrays and comparative genetic analysis have shown that breast cancer cell lines derived from human tumours largely show the genetic and transcriptional characteristics of the breast tumours and that they retain important clinical characteristics (e.g. ER, HER2 status) (Lacroix & Leclercq, 2004).

In this regard, breast cancer cell lines can be used to model the disease (Lacroix & Leclercq, 2004). On the basis of gene expression profiling, they can be stratified into 3 groups: luminal, basal and claudin-low (Hollestelle et al., 2010; Neve et al., 2006). The luminal group includes
ER+ and HER2+ cell lines. The basal group is further divided into basal-A and -B. It has been shown that basal-A cell lines represent certain features of basal-like primary tumours. Basal-B cell lines are less similar to basal-like tumours, and Prat et al showed that these cells most resemble the claudin-low subtype (Prat et al., 2010).

Through *in vivo* passaging (serial passaging in mice), certain breast cancer cell lines have been established that have the characteristic for site-specific metastasis. For example, in the case of BM from breast cancer, brain-seeking variants of 4T1.2 (Saxena & Christofori, 2013) MDA-MB-231 (Bos et al., 2009), CN34 (H. Zhao et al., 2012) and BT-474 (S. Zhang et al., 2013) cell lines have been established in different laboratories around the world. In the case of BM from HER2+ breast cancers, MDA-MB-361, a HER2-amplified luminal epithelial breast cancer cell line, is the only commercially available cell line.

1.1.2 ErbB/HER Receptor Tyrosine Kinases in breast cancer

1.1.2.1 ErbB family and their ligands

This family of receptor tyrosine kinases (RTKs) has 4 members: EGFR (HER1), HER2 (Neu or ERBB2), HER3 (ERBB3) and HER4 (ERBB4). HER receptors have 3 domains: extracellular ligand binding, transmembrane and intracellular kinase domains (Fig. 1.2) (Baselga & Swain, 2009). Following ligand binding, HER receptors form homo and heterodimers with other HER RTKs which causes activation of numerous downstream signalling pathways that control diverse processes including cell proliferation, survival, migration, invasion and differentiation (Citri & Yarden, 2006). So far, 11 ligands have been recognized: EGF, TGF-α, amphiregulin, betacellulin, HBEGF, epieregulin, epigen, neuregulin-1 (NRG-1)/Heregulin (HRG), NRG-2, NRG-3 and NRG-4 (Fig. 1.2)(Baselga & Swain, 2009).

Neuregulins are the largest subclass of EGFR family of ligands (Breuleux, 2007). NRGs are involved in regulation of cell proliferation, differentiation, survival and metastasis, and implicated in several disease including cancer, heart failure and schizophrenia (Breuleux,
NRGs are synthesised as membrane-bound pro-NRGs, which then are cleaved by matrix metalloproteinases (MMPs) such as ADAM metallopeptidase domain 17 (ADAM-17) and ADAM-10 to soluble and proteolytically active isoforms (Breuleux, 2007). This ‘ectodomain shedding’ process is controlled by intracellular kinases such as ERK1/2 and p38 signal transduction pathways (Fig. 1.3) (Breuleux, 2007).

So far, three mechanisms of action have been reported for NRGs (Montero et al., 2008)(Fig. 1.3). In paracrine mechanism, one cell provides the soluble ligand and another cell bears the receptor. In autocrine signalling, one cell provides both the ligand and receptor. On the other hand, juxtacrine signalling occurs between two cells that are in physical contact with each other (Montero et al., 2008).

Ligand-induced dimerization of HER monomers causes activation of their tyrosine kinase and auto-phosphorylation, attracting second messengers and activating the key intracellular signal transduction pathways (Baselga & Swain, 2009). HER receptors form homo or heterodimers. Since HER3 does not have any tyrosine kinase activity, it must form heterodimers with the other HER family members to initiate downstream signalling pathways. HER2 does not have any known ligand and functions via heterodimerization (Baselga & Swain, 2009). The HER3/HER2 dimer is thought to be the most oncogenic complex in this family of receptors (Fig. 1.4) (Baselga & Swain, 2009).
It is now well established that HER2 overexpression in breast cancer patients is associated with HER3 hyperactivity (Witton, Reeves, Going, Cooke, & Bartlett, 2003). HER2-induced transformation of mammary epithelial cells largely depends on activation of PI3K/Akt signalling pathway and HER3 has been shown to potently activate PI3K/Akt (Hellyer, Kim, & Koland, 2001). In agreement, it has been reported that loss of HER3 in HER2+ breast cancer cell lines results in attenuation of the PI3K/Akt signalling pathway and induction of apoptosis (Lee-Hoeflich et al., 2008). Altogether, these studies indicate for a pivotal role for HER3 in HER2-derived growth and survival in breast cancer cells (Garrett et al., 2011).

1.1.2.1 Heregulin/HER3/HER2 axis

HER3 maps to human chromosome 12q13.2, is 23.2 kb in size and consists of 28 exons and has 43-67% homology with the other ERBBs (Kraus, Issing, Miki, Popescu, & Aaronson, 1989). The HER3 gene is transcribed as a 6.2kb mRNA of 4080 nucleotides and 1342 codons specifying the full-length protein (Kraus et al., 1989). HER3 is a glycoprotein of 180 kDa (Stein & Staros, 2000).
One unique feature of HER3 is activation of PI3K/Akt signalling pathway (A. C. Hsieh & Moasser, 2007). Although all ERBBs activate Ras-Raf-MAPK pathway, only HER3 has the capacity to directly activate PI3K/Akt (A. C. Hsieh & Moasser, 2007). While EGFR and HER2 do not directly bind PI3K, HER3 has six binding sites for PI3K. Following phosphorylation by EGFR or HER2, these

HER3 phosphotyrosines bind p85 subunit of PI3K, resulting in activation of membrane phosphoinositides to phosphoinositol triphosphate (PIP3). PI3P in turn recruits and activates Akt (Fig. 1.5). Akt is a master regulator of a wide range of cellular programs including protein translation, survival and response to chemotherapy, gene transcription, cell cycle and metabolism (A. C. Hsieh & Moasser, 2007).

HER3 ligands are the members of the HRG family, a large group of isoforms encoded by four genes, with an EGF-like C-terminal portion and a variable N-terminal region (Breuleux, 2007). Alpha and β isoforms of HRG utilize different exons for the EGF-like domain. HRG1-β bind to HER3 with much greater affinity than HRG1-α (Breuleux, 2007). In consistency, HRG1-β exerted a much stronger stimulatory effect on DNA synthesis in NIH3T3 cells, compared with HRG1-α (H. S. Lu et al., 1995). In addition, Weiss et al showed that in T47D mammary and OVCAR3 ovary cancer cells HRG1-β induced greater activation of HER3, HER4, HER2 and ERK1/2 than did HRG1-α (Weiss, Wallasch, Campiglio, Issing, & Ullrich, 1997).

1.1.2.2 Roles of ErbBs in primary breast cancer
The members of ErbB family are aberrantly expressed and/or mutated in primary breast cancer (Abd et al., 2004; Witton et al., 2003). EGFR overexpression is associated with poor prognosis, higher invasive ability and negative ER status (Tsutsui, Ohno, Murakami, Hachitanda, & Oda, 2002). HER2 is overexpressed and/or amplified in approximately 25% of breast cancers and is associated with unfavourable prognosis (Slamon et al., 1987) and metastatic potential (Tan, Yao, & Yu, 1997a). HER3 expression in primary breast cancers is increased compared to normal breast tissue (Witton et al., 2003) but this is unrelated to copy number (Lemoine et al., 1992). Immunohistochemical studies show that HER3 protein is detectable in 50–70% of human breast cancers (Naidu, Yadav, Nair, & Kutty, 1998a).

Revillion et al quantified mRNA levels of all four ERBB receptors and their ligands in a series of 365 primary breast cancers (Revillion, Lhotellier, Hornez, Bonneterre, & Peyrat, 2008). The mRNA levels of HER3 were positively correlated with HER4 mRNA and negatively with EGFR (Revillion et al., 2008). There was also a positive association between HER3, ER and PR (Revillion et al., 2008). High HER3 expression correlates with poor prognosis (Bieche et al., 2003), high histological grade (Naidu, Yadav, Nair, & Kutty, 1998b), lymph node metastasis (Lemoine et al., 1992), tumour size and local recurrence (Travis et al., 1996).

HER3 transcript is reported to be up-regulated in 35% of human breast cancer cell lines compared to a nontransformed mammary cell line (Kraus et al., 1989). In SKBr3 human epithelial breast cancer cells harbouring genomic HER2 amplification, knockdown of HER3 reduced p-Akt, cyclin D3 and induced cell cycle arrest (Holbro et al., 2003). HER3 is an obligate dimerisation partner for HER2 to maintain cell proliferation in breast cancer cell lines (Lee-Hoeflich et al., 2008). In HER2+ breast cancer cell lines, knockdown of HER3 was more potent in inhibition of cell proliferation than blocking EGFR (Lee-Hoeflich et al., 2008). In addition, in HER2-amplified breast cancer tissues, preferential phosphorylation of HER3, but not EGFR, was observed (Lee-Hoeflich et al., 2008).

There is substantial evidence that in HER2+ breast tumour cells, HRG increases cell motility and invasion by establishment of HER3/HER2 heterodimers (Adelsman, McCarthy, & Shimizu, 1999; Spencer, Graus-Porta, Leng, Hynes, & Klemke, 2000). For example, treatment of MCF7 and SKBr3 cells with human recombinant HRG was shown to enhance their invasive abilities into Matrigel membranes (Mazumdar, Adam, Boyd, & Kumar, 2001). In addition, Yuan et al reported that HRG increases invasive characteristics of MCF7 cells.
through induction of MMP-7 in these cells (Yuan et al., 2008). While there is ample evidence for the key roles of the HRG/HER3/HER2 axis in different aspects of tumourigenesis in HER2+ breast cancers including cell proliferation and motility, the underlying molecular mechanisms are largely unknown.

1.1.2.3 ErbBs as important therapeutic targets for primary breast cancer

ErbBs are the most extensively studied family of receptor tyrosine kinases in human malignancies (Citri & Yarden, 2006). Due to the cardinal roles of HER2 in controlling various hallmarks of cancer in HER2+ breast tumours, there are several FDA-approved drugs against this receptor. Trastuzumab (Herceptin) is a monoclonal humanized antibody that binds to the extracellular domain of HER2 and interferes with its dimerisation. Trastuzumab is the first line treatment against HER2+ breast cancers and in early-stage cases, it significantly reduces the risk of recurrence after surgery (Moja et al., 2012). In metastatic HER2+ breast cancers, trastuzumab was shown to improve overall survival from 20.3 to 25.1 months (Hudis, 2007). In addition, combination of trastuzumab with chemotherapy has been shown to improve both survival and response rate, in comparison to trastuzumab alone (Nahta & Esteva, 2003).

Lapatinib is a tyrosine kinase inhibitor that blocks both EGFR and HER2. Lapatinib is used for the treatment of patients with HER2+ metastatic breast cancer who progressed after initial treatment with trastuzumab or taxane-derived chemotherapies (Wood et al., 2004). Combination therapy of lapatinib with capecitabine in patients with HER2+ advanced breast cancer was shown to reduce the risk of disease progression compared to capecitabine alone (Geyer et al., 2006). Pertuzumab is another monoclonal antibody that inhibits dimerization of HER2 with other HER receptors, resulting in attenuation of tumour growth in HER2+ metastatic breast cancers (Gianni et al., 2012). Interestingly, the combination of pertuzumab plus trastuzumab plus docetaxel, as compared with placebo plus trastuzumab plus docetaxel, when used as first-line treatment for HER2+ metastatic breast cancer, significantly prolonged progression-free survival in the randomized, multinational, phase III CLEOPATRA trial (Keating, 2012).

Regarding the importance of HER3 as the preferred partner of HER2 in HER2+ solid tumours, recently HER3 has gained attraction as a potential therapeutic target in HER2+ cancers including breast cancer (Ocana et al., 2013) and there are a variety of HER3 blockers in pre-clinical and clinical trials. These include MM-121 (Schoeberl et al., 2010) and EV20
These agents are humanized monoclonal antibodies that bind to the extracellular domain of HER3 and prevents its binding to HRG.

1.2 Breast cancer brain metastases

In the last decade, due to significant advances in systemic therapies, overall survival of patients with metastatic breast cancer has improved (Berghoff et al., 2012). Despite this, an increased incidence of BM has become a challenging clinical problem in these patients (Berghoff et al., 2012). It has been estimated that 10-15% of metastatic breast cancer patients will eventually develop BM (Berghoff et al., 2012). Breast cancer is a heterogeneous disease comprising distinct subtypes, each differing in prognosis, clinical and metastatic behaviour (Berghoff et al., 2012). Amongst these subtypes, HER2+ and claudin-low breast cancers represent high risk for development of BM by unknown molecular mechanisms (Harrell et al., 2012).

Patients with BM often present with seizures, headache, cognitive impairment and some other neurological abnormalities that vary according to anatomical location of the metastatic lesions (Gavrilovic & Posner, 2005). Some of the most important prognostic factors that strongly affect the outcome of BM patients are age of patients, number of metastatic lesions, presence of extracranial metastases (e.g., liver or bone metastases), the time period between diagnosis of the primary tumour and development of brain relapse and histological subtypes (Melisko, Glantz, & Rugo, 2009).

1.2.1 Current treatments

The current treatments for BM patients are surgery, radiotherapy and chemotherapy but their outcome remains poor. It has been shown that combination of surgical resection with whole brain radiation therapy (WBRT) improves survival in patients with a single cerebral lesion (Andrews et al., 2004). In patients with up to four metastatic lesions, treatment with surgical resection results in similar survival compared with combination of surgical resection plus WBRT but at the cost of higher possibility for intracranial recurrence (Aoyama et al., 2006). However, overall survival in patients treated with WBRT alone is short (between 4 and 5 months) and unfortunately, multidisciplinary management does not prolong survival beyond 12 months (Andrews et al., 2004).

1.2.2 Rationale for exploring HER3/HER2 heterodimer as a therapeutic target for treatment of BM in HER2+ breast cancers
1.2.2.1 Molecular/preclinical evidence

Breast cancers that are driven by HER2 amplification and/or overexpression have an inherent propensity to colonise the brain microenvironment, though the reasons for this are not well understood (Palmieri et al., 2007). One possibility that has received much attention in the literature is that the brain acts as a sanctuary site for metastatic cells, with the specialised blood-brain barrier (BBB) blocking uptake of trastuzumab to efficacious levels. Another possible explanation is that HER2+ cells develop trastuzumab resistance in the brain. HER3 has been shown to be indispensable for HER2-induced neoplastic transformation, cell growth and proliferation and tumour cell spread in breast cancer (Lee-Hoeflich et al., 2008; Spencer et al., 2000; Vaught et al., 2012). Since HER3 is a preferred partner for HER2 and regarding the critical roles of HER2 in development of BM in HER2+ breast tumours, we hypothesize that HER3 plays a central role in establishment of BM in HER2+ breast cancers.

1.2.2.2 Clinical evidence

HER3 is significantly over-expressed in a subset of BM compared to matching primary breast tumours (Da et al., 2010). Similar findings have been reported in BM from lung cancer (Sun et al., 2009) but the molecular mechanisms of involvement of HER3 in establishment of BM are not understood yet. Over-expression of HER3 in BM samples suggests that acquisition of high HER3 expression is beneficial at some point during the development of metastatic outgrowths in the brain, perhaps at the colonisation stage in order to exploit the local abundance of HRG ligand in brain tissue.

Clinical data suggests that comprehensive blockade of HER3/HER2 heterodimers with trastuzumab and HER2 dimerisation inhibitor pertuzumab may be efficacious against BM in HER2+ breast cancer patients. In this setting, it has been shown that trastuzumab monotherapy delays central nervous system (CNS) relapse and prolongs survival after BM diagnosis (Dawood et al., 2008). In addition, the results of the ‘CLEOPATRA’ phase III clinical trial showed that addition of pertuzumab to adjuvant trastuzumab/chemotherapy delays the onset of BM. Overall survival also showed a trend in favour of combination therapy, despite the relatively small cohort size (Swain et al., 2014).

1.3 Biology of metastatic brain tumours

Secondary brain tumours are growing public health problems and it has been estimated that around 200000 patients in the United States are diagnosed with BM every year (Fox, Cheung,
Patel, Suki, & Rao, 2011). During the last decade, the incidence of BM has been increasing due to improvements in systemic therapies which causes longer survival in cancer patients as well as establishment of better diagnostic and imaging tools that result in earlier detection of metastatic spread to the brain (Melisko et al., 2009).

BM is an unmet medical emergency for which establishment of more effective preventive and therapeutic treatment strategies is of paramount importance and in order to achieve that, we need to increase and deepen our knowledge and insights about the molecular mechanisms and signal transduction pathways underlying initiation and progression of BM (Eichler et al., 2011).

1.3.1 Molecular mechanisms underlying development of BM

BM is a multi-step process and the metastatic cascade that leads to development of BM is characterized by the escape of tumour cells from the primary tumour, dissemination through the blood circulation, attachment to the blood vessel wall via activation of certain adhesion molecules on the surface of tumour cells (e.g. intercellular adhesion molecule-1 (ICAM-1)), extravasation along the BBB into brain parenchyma followed by invasion and interaction with the microenvironment that subsequently, lead to survival and proliferation (Eichler et al., 2011). At this stage, the cancer cells grow either by forming new blood vessels (angiogenesis) or growing along the pre-existing blood vessels (vascular co-option)(Fig. 1.6)(Eichler et al., 2011).

In an attempt to determine genes that favour development of BM from breast cancer, Bos et

**Fig. 1.6** Steps in formation of BM. After shedding from the primary tumour, a) the tumour cells arrest in the capillary bed. The next step (b) is extravasation across the BBB. Activation of certain types of integrins promotes adhesion of the tumour cells to the vasculature. c) Tumour cells (the seeds) establish metastasis-promoting interactions with the stromal cells (the soil) which causes growing of tumour cells along the pre-existing blood vessels (d) or recruitment of new blood vessels (angiogenesis) to obtain sufficient nutrients to support their proliferation and further invasion (Eichler et al., 2011).
al found that cyclooxygenase-2 (COX2), the heparin-binding epidermal growth factor (HBEGF) and the alpha-2,6-sialyltransferase ST6GALNAC5 were the regulators of transmigration of breast cancer cells across the BBB (Bos et al., 2009). In agreement, they also showed that EGFR blocking antibody cetuximab decreased formation of BM (Bos et al., 2009). Moreover, melanotransferin, a member of the sialyltransferase family, enhanced transendothelial migration (TEM) activity in melanoma cells and targeting melanotransferin reduced melanoma BM (Rolland, Demeule, Fenart, & Beliveau, 2009). Activation of Rho/ROCK signalling mediates cytoskeleton organization and subsequently, facilitates small cell lung cancer migration through human brain microvascular endothelial cells (HBMECs) (Li et al., 2006).

Vascular endothelial growth factor (VEGF) signalling has been shown to play a critical role in development of BM. Measurement of VEGF levels in conditioned media of the cells with high brain metastatic activity has shown that VEGF is produced and secreted by these cells in vitro (Yano et al., 2000). In addition, enhanced VEGF secretion has been shown in BM xenografts in mouse models and suppression of VEGF in PC14PE6 lung adenocarcinoma cells reduced the frequency and size of brain metastases, confirming the critical role of VEGF in formation of BM (Yano et al., 2000).

The PI3K/Akt pathway is hyperactivated in a variety of human malignancies. Davies et al have shown higher levels of phosphorylated Akt (active p-Akt), in BM compared to other distant metastases from melanoma (Davies et al., 2009). In addition, the BM had lower levels of phosphatase and tensin homolog (PTEN) compared to unmatched lung and liver metastases (Davies et al., 2009).

Hexokinase 2 (HK2) is involved in metabolic shift from oxidative phosphorylation to glycolysis in tumour cells, and is overexpressed in human malignancies. Palmieri et al showed that BM express more HK2 mRNA compared to matched primary breast cancers (Palmieri et al., 2009). In agreement with these findings, Chen et al showed that metabolic enzymes associated with glycolysis, the tricarboxylic acid cycle and oxidative phosphorylation are significantly enhanced in BM, which indicates that remodelling of metabolic pathways occurs in BM (E. I. Chen et al., 2007).

1.3.2 Metastasis suppressor genes
Metastasis suppressor genes suppress formation of macroscopic metastases (Yoshida, Sokoloff, Welch, & Rinker-Schaeffer, 2000). They are distinct from oncogenes and tumour-suppressor genes that promote cellular transformation and suppress tumour growth, respectively. For example, RECK encodes a membrane glycoprotein that inhibits tumour cell invasion and metastasis by down-regulating MMP activity (Oh et al., 2001). Moreover, RECK mRNA has been shown to be significantly reduced in cancer cells compared to normal tissues, tumours with high RECK expression are less invasive and these patients show better survival (Oh et al., 2001).

On the other hand, KAI-1, a membrane-bound metastasis suppressor protein, has been shown to suppress cell migration by down-regulating EGFR signalling via increased receptor endocytosis (Odintsova, Sugiura, & Berditchevski, 2000). There is substantial evidence that HER2 suppresses RECK and KAI-1 expression, suggesting this could be a molecular mechanism underlying HER2-driven metastatic behaviour (Hsu, Chang, & Hung, 2006; Wilson, Roberts, Leek, Harris, & Geradts, 2002).

A study by Stark et al showed that in BM from breast cancer, the mRNA levels of KAI-1 are reduced (Stark, Tongers, Maass, Mehdorn, & Held-Feindt, 2005). In addition, the expression of metastasis suppressor genes KISS1, BRMS1, and mitogen activated protein kinase kinase 4 (Mkk4) have been reported to be downregulated in BM from ductal invasive breast cancer compared to the primary tumours (Stark et al., 2005). In consistency, re-expression of KiSS-1 significantly hampered melanoma metastasis to the brain (J. H. Lee et al., 1996). Finally, Sarris et al have reported that downregulation of nm23 expression in melanoma increases development of BM (Sarris et al., 2004).

1.3.3 The blood-brain-barrier

The BBB comprises endothelial cells and other supportive cell types, including pericytes, glial cells and astrocytic endfeet (Fig. 1.7). BBB is critical for the maintenance of the homeostasis of CNS as well as regulation of the neural microenvironment (Abbott, Ronnback, & Hansson, 2006).
major cellular components of this barrier are HBMECs with specialized features including high transendothelial electrical resistance (TEER) and a highly selective permeability (Abbott et al., 2006). BBB structure and functions are maintained by tight junction (TJ) proteins between HBMECs (Abbott et al., 2006). These protein complexes include claudins, occludin, junctional adhesion molecules (JAMs) and the cytoplasmic zonula occludens-1 and -2 proteins (ZO-1 and ZO-2)(Huber, Egleton, & Davis, 2001). The expression of TJs in HBMECs is reduced in certain neuro-pathological conditions (Greenwood, 1991).

BBB permeability maybe beneficial not only for initial extravasation but also for maintaining a permissive microenvironment for established tumours. BBB has also a key role in resistance to chemotherapy in BM by reducing the CNS availability of anti-cancer drugs (Steeg, Camphausen, & Smith, 2011). However, there is evidence that the BBB is physically disrupted in BM, and BBB leakage seems to be time dependent and largely correlated with tumour size (Pardridge, 2003). For example, detectable levels of trastuzumab, which is not expected to cross the BBB, were found in CNS of breast cancer patients with BM who were receiving this treatment (Stemmler et al., 2007). Smaller drugs like paclitaxel, lapatinib and doxorubicin have shown increased delivery in BM compared to normal brain tissue, however, their concentrations may be too low to exert a therapeutic response (Lockman et al., 2010; Taskar et al., 2012). Systemic delivery of therapies to treat BM needs further research and development.

1.3.3.1 Mechanisms of blood-brain-barrier disruption

Traversing the BBB is a prerequisite for metastatic colonisation of tumour cells to the brain and formation of BM (Pardridge, 2003) however, it is not known how tumour cells gain the abilities to infiltrate the BBB and penetrate into the brain parenchyma to form BM. Moreover, the dynamic interactions between tumour cells and HBMECs within the brain microenvironment, which lead to tumour cell survival, co-option, colonisation and metastatic outgrowth in the brain are largely unknown.

VEGF has been shown to increase the BBB permeability by disrupting TJ assembly in CNS inflammatory disease (Argaw et al., 2012). In addition, VEGF has been reported to enhance neutrophil transendothelial migration via upregulation of interleukin-8 (IL-8) in HBMECs (T. H. Lee, Avraham, Lee, & Avraham, 2002). In BM from breast cancer, VEGF secreted from breast cancer cells increases penetration of these cells across the HBMECs monolayer (T. H. Lee, Avraham, Jiang, & Avraham, 2003).
There is evidence that MMPs are dysregulated in certain neurological disorders including stroke, meningitis and Alzheimer's disease (Seo et al., 2012). MMPs belong to a family of zinc-dependent endopeptidases that degrade almost all components of the extracellular matrix (ECM) in a substrate-specific manner, and therefore their expression and activity are tightly regulated in order to control the extracellular environment. The roles of MMPs in cancer progression are complex, including not only degrading ECM and promoting cancer cell spread, but also cleaving latent forms of signalling molecules and growth factors that support tumour survival and proliferation (Kessenbrock, Plaks, & Werb, 2010).

MMP-9 was shown to be up-regulated in animal models of cerebral ischemia and its inhibition prevents neuronal cell death and reduces infarct size (Gasche et al., 1999). In addition, MMP-9 knockout mice exhibited decreased brain cell death after cerebral ischemia (Asahi et al., 2000). One of the molecular mechanisms through which MMP-9 contributes to initiation and progression of these neurological conditions is damaging the neurovascular matrix and penetration of the BBB (Cunningham, Wetzel, & Rosenberg, 2005). It has been shown that in focal cerebral ischemia MMP-9 degrades basal lamina and TJs proteins, leading to BBB leakage, edema and hemorrhage. In this setting, inhibition of MMP-9 reduced matrix proteolysis and BBB disruption (Asahi et al., 2000).

A study by Feng et al has shown a cardinal role for leukemic-derived MMP-2 and MMP-9 in breakdown of BBB by disrupting the TJs proteins ZO-1, claudin-5 and occludin and subsequently, CNS relapse in patients with acute lymphoblastic leukaemia (Feng et al., 2011). In this regard, down-regulation of MMP-2 and MMP-9 diminished redistribution of the TJs proteins and reduced BBB breaching both in vitro and in vivo (Feng et al., 2011).

Interestingly, there is evidence of a correlation between HER2 amplification and circulating levels of MMP-9 and MMP-2 (La, Pucci-Minafra, Marrazzo, Taormina, & Minafra, 2004) and these MMPs have been suggested to contribute to BM from breast cancer in a rat syngeneic model (Mendes, Kim, Lungu, & Stoica, 2007; Mendes, Kim, & Stoica, 2005). These data raise the possibility that increasing MMP secretion may be a mechanism underlying metastasis in some HER2+ cases. Further evidence of a possible link between MMP activity and BM comes from a recent study by Liu et al, who demonstrated that siRNA-mediated knockdown of MMP-1 suppressed the invasive abilities of MDA-MB-231 cells in vitro, and diminished formation of BM in vivo (H. Liu et al., 2012).
In harmony, transfection of ENU1564 rat mammary adenocarcinoma cells with tissue inhibitor of MMP-2 (TIMP-2) diminished their metastatic spread to the brain (Mendes et al., 2007). Suppression of p-ERK1/2 by treatment with MEK inhibitor (PD98059) decreased the expression of MMP-2 in cancer cells grown in rat astrocyte-conditioned media, suggesting that ERK-induced MMP-2 in cancer cells is modulated by astrocyte factors (Mendes et al., 2007). In an attempt to clarify the differential expression of MMPs in brain and bone-seeking clones of metastatic MDA-MB-231 breast cancer cells, Stark et al have shown that mRNA levels of MMP-1 and 9 was significantly higher in brain-seeking 231 clones compared to bone and parental cells (Stark et al., 2007). Interestingly, Tester et al. reported that transfection of MDA-MB-231 cells with MMP-2 enhances their orthotopic primary growth and formation of BM in nude mice (Tester et al., 2004).

1.4 Project objectives

Clinical and experimental studies have shown that in HER2+ breast tumour cells, HER3 is an obligate partner and HER2/HER3 heterodimers are the most oncogenic complex in EGFR family of receptors. There is substantial evidence that in HER2+ breast tumour cells, the HRG/HER3/HER2 axis controls various aspects of tumourigenesis including cell proliferation, angiogenesis, response to chemotherapy, cell motility and cancer metastasis. An important and unanswered question is the molecular mechanisms through which the HRG/HER3/HER2 axis promotes metastatic progression and subsequently, development of BM in HER2+ breast tumours.

This project aimed to characterise baseline levels of HRG and HER3 expression in a large panel of breast cancer cell lines. We also asked whether activation of the HRG/HER3 axis in certain breast cancer cell lines is paracrine or autocrine. Moreover, we aimed to determine the molecular mechanisms through which the HRG/HER3/HER2 axis promotes proliferative and invasive behaviours of HER2+ human luminal breast cancer cell lines.
Chapter 2

General materials and methods
2.1 Materials

2.1.1 Chemicals and reagents

All the materials used in the experiments in this thesis were of analytical grade and purchased from Sigma (Australia), Invitrogen (Australia), Merck-Millipore (Australia) and BD Bioscience (Australia) unless otherwise specified. Plasticware for tissue culture was purchased from Nunk (Denmark) and Corning Life Sciences (Australia).

40-kDa Fluorescein isothiocyanate (FITC)-dextran was from Invitrogen and dissolved in DMSO. The broad spectrum MMP inhibitor GM6001 was purchased from Calbiochem and dissolved in DMSO. Heceptin (trastuzumab)(dissolved in PBS) was a gift from Dr. Jane Holland (Max Delbruck Centre for Molecular Medicine, Berlin, Germany) and HER3 blocker EV20 was kindly provided by MediaPharma, Chieti, Italy and dissolved in PBS. For all the experiments, the final concentrations of DMSO did not exceed than 0.1% [v/v].

2.1.2 Antibodies

HER3 (clone 2F12; Millipore); HER2, p-HER2 (Tyr1248), p-HER3 (Tyr1289; clone 21D3), Akt, p-Akt (Ser473; clone D9E), ERK1/2, p-ERK1/2 (Thr202/Tyr204; clone 197G2), vimentin (clone R28), cathepsin B (clone G60), Snail1 (clone SN9H2), Slug (clone C19G7) and MMP-2 (Cell Signalling Technology, Inc.); cyclin D1 (clone H-295), ICAM-1 (clone H-108), p27 (clone C-19), RECK (clone H-300) and KAI-1 (clone C-16) (Santa Cruz Biotechnology); MMP-9, uPA and uPAR (clone PL231) (Sigma); E-cadherin (clone HEC-1-1); ZO-1 (clone ZO1-1A12), Occludin (clone OC-F10 ), Claudin-5 (clone 4C3C2) (Invitrogen); N-cadherin and alpha-tubulin (Abcam).

The controls for Westerns were as follows: p-HER3, HER3, p-HER2, HER2 (human ovarian cancer cell line SKOV3) (Sheng et al., 2010); Akt and p-Akt (human glioma cell line U87MG) (Guan et al., 2011); p-ERK1/2 and ERK1/2 (human hepatocellular carcinoma cell line HepG2) (Granado-Serrano, Martin, Bravo, Goya, & Ramos, 2006); vimentin, N-cadherin and snail1 (U87MG) (Qi et al., 2012); cathepsin B, uPA and uPAR (human glioma cell line U251) (Malla et al., 2011); Slug (U251) (H. W. Yang, Menon, Black, Carroll, & Johnson, 2010); MMP-2 and MMP-9 (U251) (Y. Zhao et al., 2010); p27 (SKOV3) (Meng, Xia, Fang, Rojanasakul, & Jiang, 2006); cyclin D1 (MDA-MB-231) (Pervin, Singh, & Chaudhuri, 2001); ICAM-1 (U251) (Ueda et al., 2009); KAI-1 (MCF7) (Joshi, Li, & Nabi, 2010); E-cadherin (MCF7) (Vermeulen, Bruyneel, van Roy, Mareel, & Bracke, 1995); ZO-1 (MCF7)
(Mauro, Bartucci, Morelli, Ando, & Surmacz, 2001); occludin (HepG2) (Schmitt, Horbach, Kubitz, Frilling, & Haussinger, 2004); claudin-5 (human colon cancer cell line HT-29) (Amasheh et al., 2005) and RECK (MDA-MB-231) (Gomes, Terra, Wailemann, Labriola, & Sogayar, 2012).

2.1.3 Peptide

Human recombinant heregulin-β1 (HRG-β1) was from Sigma and dissolved in ddH₂O. Human plasma fibronectin was purchased from Invitrogen and dissolved in ddH₂O. Growth factor reduced matrigel was from BD Bioscience.

2.2 Cell culture

2.2.1 Breast cancer cell lines

The breast cancer cell lines in this project were obtained from the American Type Culture Collection (ATCC) or collaborator labs around Australia. All of the cell lines have undergone STR authentication and have been checked frequently for mycoplasma contamination. The cell cultures were maintained at 37 °C in 5% CO₂ in a humidified incubator in the appropriate culture media determined by the ATCC. Subculturing of the cells was performed using 0.25% trypsin (Invitrogen).

2.2.2 Human Brain Microvascular Endothelial Cells (HBMECs)

HBMECs and HBMECs culture reagents were purchased from Cell Systems (Kirkland, WA). HBMECs were maintained according to the manufacturer’s protocol. Briefly, HBMECs were cultured in CSC Complete Media on fibronectin pre-coated surfaces. For all experiments, cells were grown to confluence and maintained for 4 days in 1% FBS-CSC media to allow for complete tight junctions (TJs) formation.

2.3 Experimental assays

2.3.1 RNA extraction

TRIzol (Invitrogen) was used to isolate total RNA from exponentially growing cells according to the manufacturer’s protocol. The cells were collected by trypsinisation and 0.2 mL chloroform per 1 mL of TRIzol was added to the cells for homogenization. The samples were vigorously shaken by hands for 15 s and incubated at room temperature for 3 min. The samples were then centrifuged at 12,000 × g for 15 min at 4° C. The mixture was separated
into three phases: a colourless upper aqueous phase (which has RNA), an interphase and a lower red phenol chloroform phase.

The aqueous phase was removed and transferred to a new tube. For RNA precipitation, 0.5 mL of 100% isopropanol was added to the tube; the samples were incubated at room temperature for 10 min and then centrifuged at 12,000 × g for 10 min at 4º C. After removing the supernatant, the RNA pellets were washed with 1 mL of 75% ethanol and centrifuged at 7500 × g 5 min at 4º C. Finally, the RNA pellets were air dried for 30 min at room temperature.

2.3.2 Quantitation of extracted RNA

RNA samples were quantified spectrophotometrically using a Nanodrop ND-1000 (Nanodrop Technologies). 2 µL of the extracted RNA samples were applied on the Nanodrop and the concentration of the RNA, its 260/230 and 260/280 ratios were measured.

2.3.3 Reverse transcription

SuperScript III Reverse Transcriptase (Invitrogen) was used for reverse transcription of 1µg RNA from each sample. Briefly, the following components were added to nuclease-free water to reach 13 µL.

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>oligo(dT)20</td>
<td>1 µL</td>
<td>50 µM</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1 µL</td>
<td>10 mM</td>
</tr>
<tr>
<td>RNA</td>
<td>Variable</td>
<td>1 µg</td>
</tr>
</tbody>
</table>

*Table 2.1. Components of reverse transcription reaction.*

The mix was heated to 65 ºC for 5 min and then incubated on ice for 1 min. 4 µL 5X First-Strand Buffer, 1 µL 0.1 M DTT, 1 µL SuperScript III RT and 1 µL RNaseOUT Recombinant RNase Inhibitor were added to each tube and the mixtures were incubated at 25 ºC for 5 min followed by 50 ºC for 1 h. The reaction was inactivated by heating at 70 ºC for 15 min.

2.3.4 Quantitative reverse transcription PCR

For quantitative RT-PCR, primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primerblast/index). qRT-PCR was performed on a StepOnePlus instrument (Applied Biosystems). The PCR assay was performed in a final
volume of 20 µL containing 1x SYBR Green master mix, 2 µL of cDNA samples and 10 pmol of each forward and reverse primers. PCR cycles (40 total) consisted of 15 s denaturation at 95 ºC and a combined annealing/extension step for 30 s at 60 ºC. Melt curve analysis was applied to validate that primers produced single PCR products. Hypoxanthine phosphoribosyltransferase1(HPTI) was amplified as a normalizer and fold change in expression of each target mRNA relative to HPRT1 was calculated based on the $2^{-\Delta \Delta Ct}$ relative expression formula.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Forward primer (5´-3´)</th>
<th>Reverse primer (5´-3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT1</td>
<td>NM_000194</td>
<td>TGGACAGGACTGAACGTCTTG</td>
<td>CCAGCAGGTCAAGAAAAGATTTA</td>
</tr>
<tr>
<td>MMP2</td>
<td>NM_004530</td>
<td>CCTCAAGTCTGGAGCGATGT</td>
<td>TACGGTCAAGGAGGATCATCA</td>
</tr>
<tr>
<td>MMP9</td>
<td>NM_004994</td>
<td>GGGACCGACACATCGTCATC</td>
<td>TCGTCACTGCAAAATGGGC</td>
</tr>
<tr>
<td>uPA</td>
<td>NM_002658</td>
<td>TCAAAAACCTGTATGAGGGGA</td>
<td>GGGCATGGTACGTTTGCCTG</td>
</tr>
<tr>
<td>uPAR</td>
<td>NM_002659</td>
<td>TGTAAGACACAGGGAGATTGC</td>
<td>AGCCAGTCCTGATAGCTCAGG</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>NM_001792</td>
<td>AGCAACCTTAACTGAGGAGT</td>
<td>GGCAAGTTGATTGGAGGATG</td>
</tr>
<tr>
<td>KAI-1</td>
<td>NM_002231</td>
<td>GCCGACAAGAGCAGTTTCATC</td>
<td>AGGAAGCAAAGTACAGCCTG</td>
</tr>
<tr>
<td>RECK</td>
<td>NM_021111</td>
<td>TGTAAGCTGGCTATTGCCTTG</td>
<td>GCATAACTGCAAACAAACCGAG</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>NM_000201</td>
<td>TTGGGCATAGAGACCCCCGT</td>
<td>GCACATTGCTCAGTTCATACACC</td>
</tr>
<tr>
<td>HER3</td>
<td>NM_001982</td>
<td>GGACAGTACGGAGATCACAG</td>
<td>GCACTAATTTCCTGGAGGATCG</td>
</tr>
<tr>
<td>Slug</td>
<td>NM_003068</td>
<td>TGTAAGAAATATGTGAGGCC</td>
<td>TGGACCTCAGATTTGACCTG</td>
</tr>
<tr>
<td>Snail1</td>
<td>NM_005985</td>
<td>ACTGCAAACAGGAATACCTCAGG</td>
<td>GCACTGGTACTTCTTGACATCG</td>
</tr>
<tr>
<td>Fibronectin 1</td>
<td>NM_212482</td>
<td>GGCTGTTGAGTACAAATGTCAAGT</td>
<td>CTGCGCAACTACCTGTAGT</td>
</tr>
<tr>
<td>Vimentin</td>
<td>NM_003380</td>
<td>TGGCGGTGAAGCTGCTAACA</td>
<td>CCAGGGAGGTGAATCCAGATTGA</td>
</tr>
<tr>
<td>ZEB1</td>
<td>NM_001128128</td>
<td>AGAAATCTGAGGGCGCTGAAG</td>
<td>ATGACCAGTTGCTTCTGTGTA</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>NM_147780</td>
<td>CTGGTGGATGAGCTGGCTCAAC</td>
<td>TGGTAAACATAACTTCTGGGG</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>NM_004360</td>
<td>AAGGGGCCCCATTTCTAAACCTC</td>
<td>TGGGTCTCCTATCCAGAGGCT</td>
</tr>
<tr>
<td>Twist 1</td>
<td>NM_000474</td>
<td>GTCCGAGTCTTACAGAGGAGG</td>
<td>GCTTGAGGGTCTGAATCTTGCT</td>
</tr>
</tbody>
</table>

Table 2.2: Primers used for real time RT-PCR.

2.3.5 Taqman gene expression assays
Levels of HER3 in 30 human BCa cell lines were determined using the Taqman expression assay. PCR was performed as described earlier, except reactions comprised TaqMan® Gene Expression Master Mix (ABI Biosystems), HER3 TaqMan® Gene Expression Assay (ABI Biosystems) and 4 µL of cDNA. Ribosomal protein L13a (RPL13A) was amplified as a normalizer and fold change in expression of each target mRNA relative to RPL13A was calculated using the $2^{-\Delta\Delta Ct}$ relative expression formula. For HRG and HER3 screening in the panel of breast cancer cell lines in this study, cDNA was already available from the research group’s cell line macromolecule bank.

### 2.3.6 Cell proliferation assay

A microculture tetrazolium test (MTT) was performed to determine cell proliferation after treatment of cells with recombinant HRG. Briefly, cells were plated onto 96-well plates at a density of $4 \times 10^4$ well for 24 h and then starved in 0.1% FBS for 24 h. The cells were then treated with 50 ng/mL of HRG for 24, 48 and 72 h. Untreated cells were used as the control group. 100 µL of MTT (0.5 mg/ml) (Sigma) was added to each well and the cells were further incubated at 37 ºC for 2 h. After dissolving the precipitated formazan with 100 µL of dimethyl sulfoxide (DMSO), the optical density was measured at 570 nm.

### 2.3.7 Western Blot

#### 2.3.7.1 Treatment of cells with HRG for Western blot

This was done to investigate the effects of HRG on activation of certain signalling pathways in human breast cancer cell lines. Prior to the day of treatment, cells were incubated in serum-free media overnight. Cells were then treated with 50 ng/mL of HRG for 30 min at 37 ºC. To analyse the effects of HRG on expression of desired proteins, the cells were starved in 0.1% FBS and then treated with 50 ng/mL of HRG for 48 h at 37º C.

#### 2.3.7.2 Preparation of extracts

Treated cells were washed two times with ice-cold 1xPBS. Total protein extracts were prepared in RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) containing protease and phosphatase inhibitors (Thermo Scientific) for 30 min at 4 ºC. Cell lysates were then transferred to pre-chilled microcentrifuge tubes and centrifuged at 14,000 rpm for 30 minutes at 4º C. Insoluble
material was pelleted by centrifugation for an additional 10 min. Lysates were stored in aliquots at -80 °C until required.

2.3.7.3 Determination of protein concentration

The concentration of the extracted proteins was measured using Biorad protein assay reagent (Biorad). Briefly, standards (0, 50, 100, 200 and 500 µg/mL) of Bovine Serum Albumin (BSA; sigma) and equal volumes of 1:10 dilutions of protein lysates were added into a 96-well plate in triplicate. The Biorad protein assay reagent was diluted 1:5 and then mixed with the standards and the samples. After 10 min incubation at room temperature, the optical density was measured at 595 nm. The protein concentration was quantitated by fitting the absorbance data to the standard curve obtained from the BSA samples.

2.3.7.4 Gel electrophoresis and Western transfer

30 µg of lysate was resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-PROTEAN Tetra System (Bio-Rad) and 4-15% Mini-PROTEAN TGX Precast gels (Bio-Rad). The electrophoresis apparatus was filled with 1xTris/Glycine/SDS buffer (Bio-Rad). Protein samples and PageRuler Prestained Protein Ladder (Thermo Scientific) were electrophoresed at 175 volts for 1 h. The resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membrane. The gels were washed in transfer buffer. The membrane was soaked in methanol and then washed in transfer buffer.

A transfer ‘sandwich’ was assembled, including: sponge, 1 piece of filter paper, gel, membrane, 1 piece of filter paper, and sponge, and placed within the Bio-Rad Mini Trans-Blot Cell. Protein transfer was performed at 100 volts for 1 h or 1.5 h (for HER3, p-HER3, HER2, p-HER2) at 4º C.

2.3.7.5 Immunoblotting

The membranes were blocked in 5% skim milk (for total proteins) and 5% BSA (for phospho-proteins) at room temperature for 1 h. To increase the quality of the bands for both the phospho and total forms, the samples were running separately on the same blot. Blots were then incubated with primary antibodies diluted in 1% skim milk (for total proteins) or 1% BSA (for phospho-proteins) overnight at 4ºC with agitation. Unbound antibodies were removed by the washing the blots three times with 1xTBST washing buffer and the blots were incubated with the relevant horseradish peroxidase (HRP)-conjugated secondary
antibodies diluted in the required blocking solution for 1 h at room temperature with agitation. After washing the blots three times with 1xTBST, they were exposed to enhanced chemiluminescence for 5 minutes using SuperSignal West Pico Chemiluminescent Substrate (Pierce). The signal was developed with a Konica Minolta SRX-101A tabletop processor. Alpha-tubulin was used as the loading control.

2.3.8 Gelatin Zymography

2.3.8.1 Cell treatment and sample preparation

MCF7 (3×10^5 cells), MDA361 (8×10^5 cells) and SKBr3 cells (2.5×10^5) were seeded into 6-well plates. After 24 h, the cultures were washed three times with 1xPBS and then starved in serum-free media for 24 h. The cells were then treated with 50 ng/mL HRG for 48 h. After this time, the media from the treated cells was collected and centrifuged at 10,000 rpm for 15 min to remove cellular debris. The media was then concentrated using Concentrator plus/Vacufuge® plus (Eppendorf). The samples were diluted 1:1 with 2X sample buffer and then incubated at 55 °C for 5 min.

2.3.8.2 Gel preparation and electrophoresis

10% zymogram gels were prepared according to the table below (Hawkes, Li, & Taniguchi, 2010):

<table>
<thead>
<tr>
<th>Type of gel</th>
<th>10% Zymogram</th>
<th>5% Stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/bis (30%)</td>
<td>3.33 mL</td>
<td>1.67 mL</td>
</tr>
<tr>
<td>0.5 M Tris-Hcl, pH 6.8</td>
<td>-</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>1.5 M Tris-Hcl, pH 8.8</td>
<td>2.5 mL</td>
<td>-</td>
</tr>
<tr>
<td>Gelatin (100X)</td>
<td>100 µL</td>
<td>-</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>100 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>3.92 mL</td>
<td>5.68 mL</td>
</tr>
<tr>
<td>APS (10%)</td>
<td>50 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µL</td>
<td>5 µL</td>
</tr>
<tr>
<td>Total volume</td>
<td>10 mL</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

Table 2.3. Ingredients for preparation of separating and stacking gels for zymography.
All the ingredients except APS and TEMED were mixed and degassed under vacuum for 15 min. Polymerization was initiated by adding APS and TEMED. The mix was carefully poured between the glass plates of a gel apparatus. The monolayer was overlaid with ddH₂O to exclude oxygen from the surface of the polymerizing gel.

After gel polymerization (30 min), the liquid above the gel was decanted and the stacking gel was pipetted on top of the separating gel. The comb was immediately inserted into the stacking gel, taking care not to trap bubbles beneath the teeth. After polymerization of the stacking gel, the comb was removed and the wells were rinsed with 1xTris/Glycine/SDS electrophoresis buffer (Bio-Rad). The electrophoresis apparatus was assembled with the gel and filled with 1X electrophoresis buffer.

The samples were loaded into the bottom of each well using gel loading tips, taking care not to introduce bubbles or remove the tip from each well until the entire sample has been loaded. Due to the high sensitivity of the assay, it is very critical not to contaminate the adjacent well while loading the sample in one well. The electrophoresis was done at 175 ºC for 90 min at room temperature.

2.3.8.3 Development and staining of gels

After electrophoresis, gels were rinsed in 2.5% Triton X-100 (3×30 min) to remove SDS followed by incubation at 37 °C overnight in incubation buffer (0.15 M NaCl, 10 mM CaCl₂, 0.02 % NaN₃ in 50 mM Tris-HCl, pH 7.5). After incubation, the gels were stained in staining solution (0.5% Coomassie Brilliant Blue) and destained with 7% methanol and 5% acetic acid. Areas of enzymatic activity appeared as clear bands over dark background.

2.3.9 Adhesion assay

Adhesion assays were done as described by Ueno et al (Ueno et al., 2008). MDA361, MCF7 and SKBr3 cells were seeded into 6 well plates at mentioned densities. After 24 h, the cells were washed three times with 1xPBS and starved in 0.1% FBS overnight. The starved cells were treated with 50 ng/mL HRG for 48 h and then seeded in collagen I coated 60 mm dishes (Biocoat Cell Environments; Becton Dickinson). After incubation for 15 min, cells were washed twice with cold PBS, stained with 0.5% crystal violet, lysed with 30% acetic acid and then the optical densitometry was measured at 590 nm on a Paradigm™ Detection Platform (Beckman Coulter Inc.).
2.3.10 Cathepsin B activity assay

To monitor the effect of HRG on activity of secreted cathepsin B in growth medium, a fluorometric cathepsin B activity assay (Abcam) was applied following manufacturer’s instructions. MDA361, MCF7 and SKBr3 cells were seeded into 6-well plates at mentioned densities. After 24 h, the cells were washed three times with 1xPBS and then starved in serum-free media overnight. The starved cells were treated with 50 ng/mL HRG for 48 h and the media from the cultures was collected and centrifuged at 10,000 rpm for 15 min to remove cell debris. Conditioned media was then concentrated using Concentrator plus/Vacufuge® plus (Eppendorf).

Equal amount of protein from conditioned media of each sample was incubated with 50 µL of reaction buffer and 2 µL of a specific substrate in 96-well plates at 37 ºC for 2 h. Both the reaction buffer and the substrate were provided by the kit. The substrate contains amino-4-trifluoromethyl coumarin, which is released due to cathepsin B activity. After reading the samples using a Paradigm™ Detection Platform (Beckman Coulter Inc.; 360 nm excitation filter, 465 nm emission filter), the induction rate of cathepsin B activity was obtained by comparing the relative fluorescence units (RFU) of samples with the control group.

2.3.11 Cell migration assay

To investigate the effects of HRG on migratory features of MDA361, MCF7 and SKBr3 cells, cell migration was assayed in 24-well, 6.5-mm-internal-diameter Transwell plates (Costar Corp.). The cells were seeded into 6-well plates at 100,000 cells per well. Starvation was done using 0.1% FBS overnight and the starved cells were placed into 8.0-µm porous cell culture inserts (Costar Corp.). The lower chamber contained 0.1% FBS or HRG (50 ng/mL). Cells were allowed to migrate for 48 h. The cells on the upper surface of the filter were removed by wiping with a cotton swab and migrated cells were fixed with methanol, stained with crystal violet, lysed with 30% acetic acid and then the optical densitometry was measured at a wavelength of 590 nm.

2.3.12 Cell invasion assay

For cell invasion assay, cells were starved in 0.1% FBS and then placed into 8.0-µm porous cell culture matrigel-coated inserts (24-well plates, Costar) at 100,000 cells per well. The lower chamber contained 0.1% FBS or HRG (50 ng/mL). Cells were allowed to invade the matrigel layer for 48 h. Invasive cells were fixed with methanol, stained with crystal violet,
lysed with 30% acetic acid and then the optical densitometry was measured at a wavelength of 590 nm.

2.3.13 Blood-brain-barrier transendothelial migration (BBB TEM) assay

2.3.13.1 Validation of BBB formation: Dextran-FITC permeability assay in vitro

To validate whether the TJs between the HBMEC cells have been established, we performed a permeability assay by measuring the flux of fluorescein isothiocyanate (FITC)-Dextran through confluent HBMEC monolayers. $2 \times 10^4$ HBMEC cells were seeded on matrigel-coated 0.4 µm Transwell filters (Costar Corp.) in 200 µL of CSC complete medium. The lower compartment was filled with 600 µL of the same medium. Cells were grown for 4 days to allow for complete TJ formation. After this time, the media was changed to 1% FBS-CSC media plus CultureBoost (provided by the supplier) for 24 h. Dextran-FITC was added to the top chamber of the Transwell at a final concentration of 1 mg/mL and after 20 min, 50 µL from the media in the bottom chamber was removed and read in a Paradigm™ Detection Platform (Beckman Coulter Inc.; 485 nm excitation filter, 520 nm emission filter).

2.3.13.2 HRG-induced TEM activity assay

TEM assay was applied to find out whether HRG treatment increases the TEM activity of MDA361, MCF7 and SKBr3 across a tight monolayer of HBMECs. The HMBEC were dispersed using TrypLE™ Express (Invitrogen), counted and resuspended in the appropriate volume of CSC complete media. $2 \times 10^4$ HBMEC cells were seeded into 24-well Transwell inserts with pore sizes of 8 µm (Costar Corp.) pre-coated with matrigel for 4 days to obtain confluence and allow for TJ formation. After this time, the media from apical and basal chambers was changed to 1% FBS–CSC media plus CultureBoost for 24 h.

100,000 cells/100 µL of serum-starved epithelial breast tumour cells were seeded into the apical chamber in serum-starved conditions (0.1% serum) in DMEM/F12 media. The breast tumour cells were then treated with 50 ng/mL HRG, GM6001 (a broad spectrum MMP inhibitor), Herceptin (HER2 blocker), EV20 (HER3 inhibitor) and a combination of Herceptin and EV20 for 48 h. After this time, the apical side of the Transwell filter was gently wiped clean with a cotton swab to remove all non-migrating cells and the cells on basal side were fixed with methanol, stained with crystal violet, lysed with 30% acetic acid and then the optical densitometry was measured at a wavelength of 590 nm. The 6 different treatment groups for this assay are shown in Table 2.4.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experimental group</th>
<th>HRG (50 ng/mL)</th>
<th>GM6001 (20 µg/mL)</th>
<th>Herceptin (20 µg/mL)</th>
<th>EV20 (20 µg/mL)</th>
<th>Questions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>HRG-induced TEM activity?</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>HRG-inducd MMP9-mediated TEM activity?</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>HRG-induced TEM activity by activation of HER2?</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>HRG-induced TEM activity by activation of HER3?</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>HRG-induced TEM activity by activation of HER2/HER3?</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Control group</td>
</tr>
</tbody>
</table>

**Table 2.4.** Different experimental groups for HRG-mediated TEM activity assay.

2.4. Statistical analysis

Data are expressed as mean ± standard deviation (SD). All experiments were performed in triplicate. For statistical analysis, the Student’s t-test and one-way analysis of variance were applied. *p* values of less than 0.05 were considered significant. We applied both biological and technical replicates in this study. Technical replicates measure a quantity from one source to evaluate the reproducibility of the results. The differences are based only on technical issues in the measurement. In comparison, biological replicates measure a quantity from difference sources under the same conditions. For instance, if one is performing a cell-based assay, then different flasks containing the same cell type and the exact lineage and passage number grown under the same conditions could be considered biological replicates of one another.
Chapter 3

Optimisation of an *in vitro* model to investigate the effects of HER3 on proliferative and invasive behaviour of HER2+ breast cancer cells
3.1 Introduction

The lethal outcome of the majority of human malignancies is due to metastatic dissemination of tumour cells and outgrowth of secondary tumours at distant sites, causing disruption to local and systemic physiology. In breast cancer, initiation of metastasis involves local invasion of carcinoma cells across the basement membrane that defines their local position, intravasation through the vascular or lymphatic endothelium, and eventually, extravasation at the target tissue to allow tissue colonization (Quail & Joyce, 2013). A critical prerequisite for metastasis is therefore the acquired ability to cross physical barriers; largely the basement membrane and endothelium. Extracellular proteinases, including matrix metalloproteinases (MMPs), control many of the modifications in the tumour microenvironment (TME), including tumour cell invasion and metastasis (Kessenbrock et al., 2010), and interestingly, MMPs may derive from a variety of different cell types in the TME. For example, in lung metastases from breast cancer, tumour-derived MMP-2 disrupts endothelial cell-cell junctions (Huang et al., 2009) whereas in prostate cancer bone metastases, metastasis-promoting MMP-7 derives from osteoclasts (Lynch et al., 2005).

Evidence from both in vitro and in vivo studies suggests that MMPs are involved in development of brain metastases (BM) from breast cancer. In this setting, it has been shown that ectopic expression of MMP-2 in MDA-MB-231 breast cancer cells augments the growth of mammary fat-pad xenografts and formation of BM in nude mice (Tester et al., 2004), and siRNA-mediated knockdown of MMP-1 reduced formation of BM by these cells (H. Liu et al., 2012). In agreement, ectopic expression of tissue inhibitor of MMP-2 (TIMP-2) in a rat mammary adenocarcinoma cell line blocked the metastatic spread of these cells to the brain (Mendes et al., 2007). Comparison of MMP expression in brain- and bone-seeking derivatives of MDA-MB-231 breast cancer cells revealed that MMP-1 and MMP-9 transcripts are more highly expressed in brain-seeking compared to bone-seeking and parental cells (Stark et al., 2007). Altogether, these data highlight the potential importance of MMPs in invasion and metastasis to the brain in breast cancer. In a study aimed at correlating circulating MMPs in breast cancer patient sera with clinicopathological parameters (tumour grade and size, stage, lymph node involvement, hormone receptors and HER2 status), it was revealed that increased MMP-2 and MMP-9 activity correlated only with HER2 positivity (amplification and/or overexpression; HER2+), suggesting MMP activity may be associated with HER2 signalling in breast cancer (La et al., 2004).
Evidence indicates that HER3 plays a major role in HER2+ breast cancer as an indispensible ligand-binding substrate for HER2, unique amongst ErbBs in that it is an orphan tyrosine kinase with no known ligand. HER3 promotes metastasis in breast cancer (Pan et al., 2011), hepatocellular carcinoma (HCC) (S. Y. Hsieh et al., 2011) and colon cancer (Beji, Horst, Engel, Kirchner, & Ullrich, 2012). HER3 is induced in BM compared to matching breast cancers (Da et al., 2010) and also lung adenocarcinomas (Sun et al., 2009), suggesting it is beneficially induced during metastatic progression to the brain. Interestingly, the incidence of brain relapse in breast cancer is highest for HER2+ cases (Harrell et al., 2012), though the mechanisms underlying this association have not been extensively investigated.

In vitro, depletion of HER3 suppresses transformation of HER2-overexpressing mammary epithelium via induction of apoptosis (Vaught et al., 2012). In agreement, loss of HER3 blocks activation of Akt and ERK1/2 in pre-neoplastic HER2-overexpressing mammary epithelium and enhances the response to lapatinib, a HER2 tyrosine kinase inhibitor (Vaught et al., 2012). A recent study by Aceto et al. showed that simultaneous ectopic expression of HER3 and HER2 in non-invasive MCF10A breast cancer cells increased their migratory and invasive abilities in the presence of HRG (Aceto et al., 2012). The in vitro and in vivo evidence demonstrating that the oncogenic unit in HER2+ cancer cells is the HER2-3 heterodimer are substantiated by the clinical success of the HER2 dimerisation blocker pertuzumab (Baselga et al., 2012), which significantly improves overall and disease-free survival in HER2+ breast cancer patients.

Both autocrine and paracrine modes of HER3 signalling have been demonstrated in different cancer models. In autocrine activation, HER3+ tumour cells are activated by self-secreted HRG. In some ovarian high-grade serous adenocarcinoma cell lines, autocrine HER3 activation is responsible for tumour cell proliferation both in vitro and in vivo (Sheng et al., 2010). Disruption of this circuit with HER3-directed RNAi decreased cell growth, reduced disease progression and prolonged survival in a xenograft mouse model of ovarian cancer (Sheng et al., 2010).

Another example is HCC. Constitutive phosphorylation of HER3 and PI3K/Akt was observed in some HCC cell lines, which was abolished by treatment of these cells with an anti-HRG antibody or genetic knockdown of HRG expression. In addition, silencing of HER3 diminished migration and invasion of these cells in transwell assays in vitro (S. Y. Hsieh et al., 2011). Autocrine activation of HER3 plays a major role in tumour cell invasion in HCC.
cells and contributes to intrahepatic metastasis and early recurrence in HCC patients (S. Y. Hsieh et al., 2011).

In the case of paracrine HER3 activation, the main source of HRG is cellular components of the TME (Montero et al., 2008). For example, in pancreatic ductal adenocarcinoma (PDAC), carcinoma-associated fibroblast-derived HRG promotes tumourigenesis by activation of tumour cell HER3. Hence it has been suggested that the HRG/HER3 axis is a potential therapeutic target to interrupt stroma-mediated tumourigenesis in PDAC (Liles et al., 2011).

Compared to HCC and PDAC, the precise activation mode of HER3 in breast cancer is not well understood. Moreover, there is limited data demonstrating whether expression of HER3 and HRG is associated with specific breast cancer subtypes, which are associated with clinical outcomes including the frequency and sites of distant metastasis (e.g. HER2+ breast cancers frequently spread to the brain). A landmark study by Hijazi et al. showed that HRG-expressing breast cancer cell lines (e.g. MDA-MB-231) exhibit high invasive and metastatic indices, and treatment of MDA-MB-231 cells with an HRG-neutralizing antibody significantly inhibited proliferation and motility in vitro (Hijazi et al., 2000). In this setting, treatment of SKBr3 cells with HRG increases their invasiveness in vitro (F. J. Xu et al., 1997).

A recent paper by Kim et al. suggested that HRG increases the invasive behaviour of MCF7 and SKBr3 cells through induction of mesenchymal-like features (J. Kim et al., 2013). In breast tumour-initiating cells, autocrine activation of HER3 may promote self-renewal and survival (C. Y. Lee et al., 2014). Collectively, these data suggest that HRG acts as an autocrine or paracrine ligand to promote invasive behaviour in breast cancer cells. A better understanding of the activation pattern of HER3 signalling in breast cancer is required to further support the development of therapeutic strategies that block this axis in breast cancer.

Since HER2 induces invasion and metastasis through induction of certain types of MMPs, HER3 is required for HER2-mediated oncogenic activity, and HER3 has been independently associated with invasion and metastasis in breast cancer, we hypothesised that HER3 induces MMPs to promote cancer invasion and metastasis in HER2+ breast tumour cells. The aims of experiments described in this chapter were:

1-To investigate the association between HRG and HER3 expression and different subtypes of breast cancer cell lines.
2-To clarify the activation pattern of canonical HER3 signalling pathway in \textit{HER2}+ human epithelial luminal cells lines.

3-To explore the effect of HRG on induction of \textit{MMP-2} and \textit{MMP-9} in \textit{HER2}+ breast tumour cell lines.
3.2 Results

3.2.1 Screening HRG isoforms and HER3 in breast cancer cell lines

In order to ascertain baseline levels of HRG and HER3 in breast cancer cell lines and select appropriate lines to examine this axis with in vitro and in vivo models, qRT-PCR analysis of HRG and HER3 expression in a large panel of breast cancer cell lines was performed. Because both alpha and beta isoforms of HRG activate HER3, the screening was performed for both the isoforms. The panel included three molecular subtypes previously defined by expression array profiling and unsupervised cluster analysis: luminal, basal A and claudin-low (basal B) (Holleystelle et al., 2010; Neve et al., 2006; Prat et al., 2010).

![Graph showing relative mRNA levels of HER3, NRG1a and NRG1b in Claudin-low, Luminal-like, Basal A and primary BM cultures. Data are shown as mean ± SD of three technical replicates.](image)

**Fig. 3.1** Relative mRNA levels of HER3, NRG1a and NRG1b in Claudin-low, Luminal-like, Basal A and primary BM cultures. Data are shown as mean ± SD of three technical replicates.

Luminal cell lines are characterised by expression of epithelial markers and cobblestone-like morphology with cohesive cell-cell attachments, whereas claudin-low cell lines express markers of epithelial-mesenchymal transition (EMT), are discohesive, spindle-like and exhibit more invasive and metastatic behaviour in animal xenografts. The basal-A cell line subtype is ‘intermediate’ with respect to morphology, phenotype and invasive behaviour. A small panel of unique, primary human BM cell cultures (passage <10) was also assayed.
This screening experiment revealed significantly higher levels of HER3 transcript in luminal compared to claudin-low cell lines (consistent with the classification of HER3 is an epithelial marker (Balko et al., 2012)), with mixed expression in basal A and primary BM cultures. Interestingly, HRG (NRG1) isoform analysis revealed a striking inverse correlation with HER3 expression, with highest levels in claudin-low cell lines where HER3 mRNA was virtually undetectable, and very low levels in 7/8 luminal lines with high HER3 (Fig. 3.1). This correlation was also observed in the primary BM cultures, which resembled claudin-low subtype in terms of their HRG/HER3 expression profile.

Western analysis confirmed a lack of HER3 expression in 3 representative claudin-low cell lines (Fig. 3.2: Hs578T, MDA-MB-231 and SUM159) and strong HER3 protein expression in 3 representative luminal cell lines (Fig. 3.3: MDA-MB-361, MCF7 and SKBr3). This analysis led to the selection of HER3+ luminal cell lines for HRG activation experiments. In addition, we hypothesised that HER3+ luminal lines would be susceptible to paracrine HRG, whereas cell surface receptor-independent signalling by autocrine HRG may operate in claudin-low lines, which may have lost their HER3 expression as part of the EMT process.

Given our interest in BM from HER2+ breast cancer, we decided to investigate the effects of paracrine HRG on three HER2+ luminal epithelial cell lines including MDA-MB-361 (MDA361) (a brain metastatic breast cancer cell line with HER2 amplification), MCF7 (HER2+) and SKBr3 (HER2 amplification).

3.2.2 Canonical HER3 signalling in luminal breast cancer cells

The HER3 positivity of luminal cell lines suggested they could be receptive to paracrine HRG ligand. In situ, this could involve education of the TME by tumour cells to release HRG, or proliferation of tumour cells in tissues naturally rich in HRG (e.g. brain tissue). To
determine whether the HRG/HER3 axis can be activated by exogenous HRG in three luminal breast cancer cell line models (MDA361, MCF7 and SKBr3), Western analysis was performed on whole cell protein lysates from each cell line to assess the phosphorylation status of HER3. Cells were cultured in normal conditions, serum-free media (SFM) and SFM supplemented with HRG. The cells were starved in serum-free media for 24 h and then treated with 50 ng/mL of HRG for 30 min.

As shown in Fig. 3, MDA361, MCF7 and SKBr3 cells responded to a paracrine HRG signal through phosphorylation of HER3 and its major dimerisation partner HER2, but not the other HRG receptor HER4. In addition, there was no detectable p-HER3 in regular, suggesting that cells in these conditions do not activate HER3 via autocrine mechanisms.

![Paracrine HRG/HER3 signalling in MDA361, MCF7 and SKBr3 cells. Cells were cultured in normal condition (FBS), serum free media (SFM) or SFM supplemented with 50 ng/mL HRG for 30 min. Lysates were prepared and WB was done for HER3 and p-HER3. α-tubulin was used as the loading control. Lysate from BT-474 cells was used as the positive control (+).](image)

### 3.2.3 HRG induces MMP-9 transcript in MDA361, MCF7 and SKBr3 cells

Since one of our longer-term goals was to evaluate whether the HRG/HER3/HER2 signalling mediates BBB impairment and MMPs are known to be involved in invasive behaviour and extravasation in other contexts, we first asked if this axis induces MMP-2 and MMP-9 in six different HER2 expressing breast cancer cell lines: MDA-MB-453, T47D, MCF7, MDA361, BT474 and SKBr3.
MDA453 (5 × 10⁵), T47D (4 × 10⁵), MCF7 (3 × 10⁵), MDA361 (8 × 10⁵), BT474 (5 × 10⁵) and SKBr3 (2 × 10⁵) cells were seeded into 6-well plates, then serum-starved before treatment for another 24 h. Total RNA was extracted and used for analysis of MMP-2 and MMP-9 expression by qRT-PCR. As shown in Fig. 3.4, HRG treatment induced MMP-9 mRNA in MDA361, MCF7 and SKBr3 cells. In addition, HRG enhanced mRNA levels of MMP-2 in MDA361 cells. There was no significant change observed in MDA-MB-453, T-47D or BT-474 cells (data not shown). These data raise the possibility that in vivo, induction of MMP-9 may occur in some breast cancer cells situated in HRG-rich microenvironments.

### 3.2.4 Determination of optimal in vitro conditions for HRG-mediated induction of MMP-9 in luminal, HER3+ breast cancer cells

Previous studies have used different concentrations of HRG, a variety of serum-starvation conditions and assay end-points for analysis of the effects of HRG on cancer cell lines (e.g. (Beji et al., 2012; Dagnell et al., 2013; J. Kim et al., 2013; P. W. Tsai, Shiah, Lin, Wu, & Kuo, 2003)). It was therefore necessary to optimise each of these variables for use with the three luminal HER3+ cell lines of interest for this study, assessing MMP-9 mRNA expression as a read-out of HRG activity.

First, serum-deprived (0.1% serum) MDA361, MCF7 and SKBr3 cells were treated with 25, 50 or 100 ng/mL HRG for 48 h. RNA was extracted and qRT-PCR for MMP-9 was performed. As depicted by Fig. 3.5, the optimal HRG concentration for maximal MMP-9 expression was 50 ng/mL in all three lines, though the 25 ng/mL dose also induced MMP-9. Interestingly, MMP-9 levels at the 100 ng/mL dose were lower than at 25 ng/mL, suggesting negative feedback may occur with excessive exposure to the ligand. Altogether, these data show that 50 ng/mL HRG is an appropriate dose for assessment of MMP-9-mediated
functions in these three cell lines.

We next determined the best serum content for HRG-induction of MMP-9 in the cell line models. Previous studies have included starvation in serum-free media for 24 h (Liang et al., 2013), starvation in 0.1% FBS for 24 h (Carracedo et al., 2008) and deprivation in 1% FBS for 24 h (Dagnell et al., 2013). MDA361, MCF7 and SKBr3 cells were therefore pre-treated in serum-free media or 0.1% FBS-containing media for 24 h, treated with 50 ng/mL HRG for 48 h, then assayed for MMP-9 mRNA expression by qRT-PCR. As shown in Fig. 3.6, the 0.1% serum pre-treatment was more conducive to HRG-mediated MMP-9 induction in 2 out of 3 lines (MDA361 and MCF7) compared to complete serum starvation.

expression by qRT-PCR. As shown in Fig. 3.6, the 0.1% serum pre-treatment was more conducive to HRG-mediated MMP-9 induction in 2 out of 3 lines (MDA361 and MCF7) compared to complete serum starvation.

![Fig. 3.5](image1.png)  
**Fig. 3.5** Dose-dependent effects of HRG on mRNA levels of MMP-9 in MDA361, MCF7 and SKBr3. The cells were seeded into 6-well plates and after 24 h starved in 0.1% FBS for another 24 h. After this time, the cultures were treated with escalating concentrations of HRG for 48 h. Untreated cells were used as the control group. The relative mRNA expression of MMP-9 was measured using real-time RT-PCR in HRG-treated cells after normalizing the cycle thresholds of each triplicate against their corresponding *HPRT1*. Values are given as mean ± SD of three technical replicates. The statistical significance of difference between treatments and their controls was determined using unpaired, 2-tailed student's t-test; * p<0.05 and ** p<0.001 were determined compared with the control.

![Fig. 3.6](image2.png)  
**Fig. 3.6** Effects of serum starvation (0% serum) versus deprivation (0.1% serum) on induction of MMP-9 mRNA by HRG. Cells were starved as indicated (blue/red bars) for 24 h. Cultures were treated with 50 ng/mL HRG for 48 h. Untreated cells were used as the control group. Relative MMP-9 expression was analysed in triplicate by real-time qRT-PCR after normalizing to *HPRT1*. Values are given as mean ± SD of three technical replicates, normalised to the corresponding untreated control for each treatment. The statistical significance of difference between treatments and their controls was determined using unpaired, 2-tailed student's t-test; * p<0.05 was determined compared with the control.
The final parameter in the HRG-activation assay to be optimised was treatment time. Previous reports have employed 24 h (Mazumdar et al., 2001), 48 h (Xiong et al., 2001) and 72 h (J. Kim et al., 2013) for different purposes. For investigation of HRG-mediated induction and activation of protease enzymes, published reports suggested the 48 h time-point may be optimal. For example, 24 h HRG treatment was shown to induce levels of urokinase plasminogen activator (uPA) mRNA, but 48 h was required to activate enzymatic activity (Mazumdar et al., 2001). In addition, 48 h HRG treatment is required for MMP-7 activation in MCF7 cells (Yuan et al., 2008). Therefore, MDA361, MCF7 and SKBr3 cells were starved in 0.1% FBS for 24 h then treated with HRG (50 ng/mL) for 24, 48 and 72 h prior to assaying MMP-9 mRNA levels by qRT-PCR. HRG-mediated induction of MMP-9 was time-dependent in all the three lines, with maximal expression at 48 h post-treatment (Fig. 3.7), consistent with the aforementioned published reports. Data for the 72 h timepoint are not shown, as there was significant acidification of the growth medium and extensive cell detachment.

![Fig. 3.7](image-url) Time-dependent effects of HRG on induction of MMP-9 mRNA in MDA361, MCF7 and SKBr3. The cells were seeded into 6-well plates and after 24 h starved in 0.1% FBS for another 24 h. After this time, the cultures were treated with 50 ng/mL of HRG for 24 and 48 h. Untreated cells were used as the control group. The relative mRNA expression of MMP-9 was measured using real-time RT-PCR in HRG-treated cells after normalizing the cycle thresholds of each triplicate against their corresponding HPRT1. Values are given as mean ± SD of three technical replicates. The statistical significance of difference between treatments and their controls was determined using unpaired, 2-tailed student's t-test; * p<0.05, ** p<0.01 and *** p< 0.001 were determined compared with the control.
3.3 Discussion

The HRG/HER3 axis plays critical roles in breast tumourigenesis, including regulation of tumour cell invasion and metastasis (Pan et al., 2011), induction of angiogenesis (Amin, Campbell, & Moasser, 2010), resistance to anti-cancer drugs (Hutcheson et al., 2007) and tumour cell proliferation (Lee-Hoeflich et al., 2008). However, the relationships between expression of critical pathway members and their activation patterns in different breast cancer molecular subtypes are poorly understood. One aim of the experiments in this chapter was to investigate HRG and HER3 expression in a large panel of breast cancer cell lines, the transcriptional profiles of which resemble human breast cancers. The results of this qRT-PCR screening experiment indicated that luminal breast cancer cell lines express higher levels of HER3 compared to claudin-low cells (Fig. 3.1). Consistent with this, HER3 protein was undetectable in all three claudin-low cell lines tested in our lab (Fig. 3.2; Hs578T, MDA-MB-231 and SUM159). These issues are consistent with the recent observation that HER3 is expressed by the luminal epithelial cells (Balko et al., 2012).

These data also revealed a striking inverse association between HRG and HER3 expression in luminal and claudin-low breast cancer cell lines (Fig. 3.1). Luminal cell lines are characterized by a $HRG^{low}/HER3^{high}$ phenotype, while claudin-low lines are $HRG^{high}/HER3^{low}$. Basal-A lines showed mixed phenotypes, consistent with basal-like breast cancers, which are heterogeneous, both in terms of molecular profiles and clinical outcomes. Low expression of HER3 in claudin-low cells might be a consequence of EMT, as HER3 has been shown to be an important epithelial marker and discriminator between epithelial and mesenchymal phenotypes in breast epithelium (Balko et al., 2012). These data also show for the first time that there is an association between mRNA levels of HER3 and different molecular subtypes in breast cancer cell lines.

The $HRG^{low}/HER3^{high}$ phenotype in MDA361, MCF7 and SKBr3 luminal cells implied that these cells could be sensitive to a paracrine HRG signal. To test this hypothesis, HER3 activation was assayed in response to supplementation with recombinant human HRG1-ß, using Western analysis of phosphorylated HER3 isoform (antibody specific for HER3-phospho-tyrosine$^{Tyrl289}$) (Fig. 3.3). The results showed that in all the three cell lines, HER3 activation occurred only after treatment with exogenous HRG (Fig. 3.3). In addition, we observed concurrent activation of HER2. In SKBr3 cells, HER2 was phosphorylated even in low serum conditions, indicating a degree of ligand-independence in this cell line (Campbell,
Amin, & Moasser, 2010). Since HRG also activates HER4, we performed Western analysis for p-HER4 and HER4 in these cells. As shown in 3.3, we did not observe activation of HER4 after HRG treatment in none of the cell lines. This suggests that HRG-mediated functions are mainly through activation of HER3 and HER2.

The exogenous HRG-mediated activation of HER3 and HER2 observed here suggests that the TME could be an important source of HRG in some HER2+ breast tumours, for which evidence shows that HER3/HER2 is the functional oncogenic unit driving tumourigenesis (Vaught et al., 2012). Indeed, in pancreatic ductal adenocarcinoma, the main source of ligand driving HER3-dependent activation of growth and proliferation is cancer-associated fibroblasts in the TME (Liles et al., 2011). Similarly, establishment of the HER3/HER2 heterodimer, tumour formation and progression is largely dependent on stromal-derived HRG in breast cancer (Vaught et al., 2012).

Given the evidence implicating the HER3/HER2 dimer in driving tumour progression and metastasis, we investigated the hypothesis that HRG treatment activates expression of proteases separately implicated in these processes. The effect of in vitro activation of HER2/3+ cell lines with exogenous HRG on MMP-2 was variable, however we observed consistent and robust induction of MMP-9 (Fig. 3.4). This data was of considerable interest considering work from our group and others implicating HER3 induction in development of BM from breast and lung cancers (Da et al., 2010; Sun et al., 2009), which are common in HER2+ cases, and also other published reports implicating MMPs in BM (Feng et al., 2011). We further investigated this link in vitro, with experimental data presented in chapters 4 and 5. Because this HRG-activation assay was fundamental to many subsequent experiments, the conditions were extensively optimised. These experiments showed that starvation of the cells in 0.1% FBS followed by treatment with 50 ng/mL of HRG for 48 h is most favourable for induction of MMP-9 in the three cell line models (Fig. 3.5, 3.6, and 3.7). These conditions were applied for all the experiments performed in this project unless otherwise specified.
Chapter 4

Investigation of the role of the HRG/HER3 signalling pathway in regulating proliferative and invasive behaviour of HER2+ breast cancer cells
4.1 Introduction

Clinical and experimental evidence show that HER3 expression is increased in primary breast tumours compared to normal breast tissue (Bieche et al., 2003; Witton et al., 2003). High expression of HER3 in breast cancers correlates with poor prognosis, tumour grade, lymph node metastasis and local recurrence (Bieche et al., 2003; Lemoine et al., 1992; Naidu et al., 1998a; Travis et al., 1996).

Neuregulins (Heregulins (HRG)) comprise the largest subclass of growth factors in the epidermal growth factor (EGF) family (Montero et al., 2008). This family plays critical roles in regulating tumour cell invasion and metastasis, differentiation, cell proliferation and response to chemotherapy in a wide range of human malignancies (Wen et al., 1994). For example, mammary compartment-specific overexpression of ectopic HRG in the mouse causes mammary tumourigenesis and metastatic spread (Atlas et al., 2003). Consistent with this, blockade of HRG using antisense oligonucleotide in MDA-MB-231 breast tumour cells reduced tumour formation, tumour size, and suppressed development of lung metastases in vivo (M. S. Tsai, Shamon-Taylor, Mehmi, Tang, & Lupu, 2003).

Recent evidence implies that HER3 is critical for HER2-driven breast cancer (Vaught et al., 2012). Depletion of HER3 restraints transformation of HER2-overexpressing mammary epithelium in MMTV-HER2 transgenic mouse model by blocking cell proliferation and induction of programmed cell death in mammary epithelium (Vaught et al., 2012). In agreement, loss of HER3 inhibited phosphorylation of Akt and ERK1/2 in pre-neoplastic HER2-overexpressing mammary epithelium and increased the response to lapatinib, a HER2 tyrosine kinase inhibitor. Re-expression of HER3 rescued these events. Collectively, these findings suggest that HER3 is indispensable for HER2-induced pre-neoplastic changes to the mammary epithelium as well as tumour formation (Vaught et al., 2012). HER3 is the preferred heterodimerisation partner for HER2 to maintain tumour cell proliferation in breast cancer cells (Lee-Hoeflich et al., 2008). In line with this, knockdown of HER3 in HER2+ breast cancer cells inhibited cell proliferation (Lee-Hoeflich et al., 2008), and abolishing HER3/HER2 heterodimers in HER2+ breast tumour cell lines (including SKBr3, MDA361 and BT474) resulted in G1 cell cycle arrest (Holbro et al., 2003).

In HER2+ breast tumour cells, HRG fosters cell motility and invasion by establishment of HER3/HER2 heterodimers (Adelsman et al., 1999; Spencer et al., 2000). For example, treatment of SKBr3 cells with HRG increased their invasion into Matrigel (F. J. Xu et al.,
1997). Furthermore, Mazumdar et al. showed that treatment of MCF7 cells with HRG enhances mRNA, protein and enzymatic levels of uPA in these cells, significantly increasing their invasive abilities in vitro (Mazumdar et al., 2001).

As mentioned above, an important step in cancer metastasis is invasion into the surrounding stroma (Chaffer & Weinberg, 2011). Using an in vivo invasion assay, Hernandez et al. showed that gradients of EGF or colony stimulating factor-1 (CSF-1) induce invasion through an EGF/CSF-1 paracrine loop between cancer cells and macrophages (Hernandez et al., 2009). Interestingly, they also showed that invasion mediated by HRG also relies on this EGF/CSF-1 paracrine invasive loop. MTLn3 breast cancer cells expressing ectopic HER3 exhibited increased invasion in response to HRG. In addition, the in vivo invasive response of both MTLn3-HER3 and transgenic MMTV-Neu tumours to HRG was suppressed by blocking EGF receptor, CSF-1 receptor, or macrophage function, suggesting that HRG-induced invasiveness is dependent on the EGF/CSF-1 paracrine loop (Hernandez et al., 2009). These findings also highlight the critical roles of the tumour microenvironment (TME) in regulation of HRG-induced invasion and metastasis.

One of the most important signal transduction pathways activated by HRG/HER3 is PI3K/Akt (A. C. Hsieh & Moasser, 2007). HER3 contains six YXXM motifs that bind p85 subunit of PI3K. While previous studies demonstrated that HER3 in mammary tumour cells significantly enhances chemotaxis to HRG and overall metastatic potential, the molecular mechanism underlying HER3-mediated invasion and metastasis is not fully understood. In an attempt to determine the mechanism of HER3-driven invasion, Smirnova et al. showed that site-directed mutagenesis of tyrosine residues in the six YXXM motifs on the HER3 C-terminus blocked the HRG chemotactic response in vitro or in vivo (Smirnova et al., 2012). In addition, multiphoton micoroscopy showed drastic reductions in both primary tumour cell motility and intravasation (Smirnova et al., 2012). Treatment with the PI3K inhibitor PIK-75 suppressed motility and invasion both in vitro and in vivo. In sum, these findings suggest that HER3-mediated PI3K signalling is critical for HRG-induced motility, invasion, intravasation and metastasis (Smirnova et al., 2012).

A recent study by Aceto et al. showed that simultaneous ectopic expression of HER3 and HER2 in a non-invasive breast cancer cell line, MCF10A, significantly enhanced migratory and invasive abilities of these cells in the presence of HRG, suggesting that HRG is requisite for HER3/HER2-induced migration and invasion in HER2+ breast cancer cells (Aceto et al.,
Using microarray profiling, the authors found a specific “HER3/HER2 signature” in these cells including more than 80 transcripts, with interleukin-8 (*IL-8*) being the most highly induced gene (Aceto et al., 2012). Treatment of the MCF10A-HER3/HER2 cells with IL-8 neutralizing antibodies diminished proliferation and invasion. These observations demonstrate that induction of an IL-8 autocrine signalling loop by the HRG/HER3/HER2 axis is one molecular mechanism for promotion of invasion in HER2+ breast tumour cells (Aceto et al., 2012).

While several lines of evidence indicate for key roles of the HRG/HER3 signalling pathway in different aspects of tumourigenesis in HER2+ breast tumours including cell proliferation and motility, the underlying molecular mechanisms are not well characterized. The experiments in this chapter were designed to determine the mechanisms through which HRG fosters cell growth and proliferation as well as tumour cell motility in MDA361, MCF7 and SKBr3 cell lines.
4.2 Results

4.2.1 HRG activates PI3K/Akt and ERK 1/2 signalling in MDA361, MCF7 and SKBr3 cells

We have previously shown that HRG treatment causes phosphorylation of HER3 and its heterodimerisation partner, HER2, but not HER4 (Fig. 3.3). PI3K/Akt and ERK1/2 are HER2 downstream signalling networks that control tumour cell proliferation and invasion (Eccles, 2001). In order to examine if HRG-activated HER3 and HER2 receptors activate PI3K/Akt and ERK1/2 in MDA361, MCF7 and SKBr3 cells, we studied the effects of HRG on activation of these two signaling pathways. As shown in Fig. 4.1, treatment of HER2+ MDA361, MCF7 and SKBr3 cells with HRG activated Akt and ERK1/2 in all the cell lines. This suggests that HRG might contribute to activation of targets of these pathways in HER2/3+ breast cancer cells that are situated in a microenvironment containing adequate levels of HRG. The FBS group was included to compare the effects of HRG growth factor to the rich and complex growth factor mixture found in serum.

4.2.2 Effects of HRG on proliferative features of MDA361, MCF7 and SKBr3 cells

PI3K/Akt and ERK1/2 signalling pathways have been shown to induce tumour cell proliferation in a variety of human malignancies including breast cancer (Serra et al., 2011). In this respect, we next sought whether treatment of MDA361, MCF7 and SKBr3 cells with HRG enhances their proliferation. As shown in Fig. 4.2, treatment of MDA361 and MCF7 cells with HRG significantly increased their proliferation. In comparison, HRG had no effect on proliferation of SKBr3 cells. It is thought that deregulation of cell cycle checkpoint proteins is a mechanism through which HER2 increases proliferation of breast tumour cells (Lenferink, Busse, Flanagan, Yakes, & Arteaga, 2001). We therefore investigated whether HRG treatment results in induction of cyclin D1 and inhibition of p27. As shown in Fig. 4.2 and consistent with the results of the proliferation assay, HRG induced cyclin D1 in MDA361
and MCF7 but not SKBr3 cells.

4.2.3 Effects of HRG on invasive properties of MDA361, MCF7 and SKBr3 cells

4.2.3.1 HRG induces MMP-9 in HER2+ breast cancer cells

PI3K/Akt and ERK1/2 pathways have been reported to increase invasive behaviour of breast tumour cells via induction of certain types of invasive markers including urokinase plasminogen receptor (uPAR) and its ligand (uPA), matrix metalloproteinase-2 (MMP-2) and MMP-9. Because we observed activation of these pathways after HRG treatment, we explored whether HRG induces invasion markers in MDA361, MCF7 and SKBr3 cells. As shown in Fig. 4.3A, HRG treatment significantly induced uPA, uPAR, MMP-2 and MMP-9 mRNA in MDA361 cells. There was no effect in SKBr3 cells, although MMP-9 was induced 2.3-fold. In comparison, HRG induced MMP-9 and uPA 3.4 and 2.7-fold respectively in MCF7 cells. The responses to HRG observed were cell line-specific, however HRG significantly induced MMP-9 transcript in all the cell lines.

Western analysis was used to determine whether these changes were accompanied by changes in protein levels of MMP-9, cathepsin B, uPA and uPAR. As shown in Fig. 4.3B, additional cell-line specific changes were observed. HRG had no effect on uPAR, however
HRG treatment induced uPA in MDA361 and MCF7 cells, and cathepsin B in MCF7 cells. Consistent with the qRT-PCR results, HRG treatment induced MMP-9 protein levels in all three lines. Gelatin zymography was then performed to determine whether this was associated with a concomitant increase in enzymatic activity. MMP-2 activity was also analysed since its transcript levels were induced by HRG in MDA361 cells. As shown in Fig. 4.3C, HRG induced MMP-9 activity in all three cell lines, and MMP-2 was induced in the two lines harbouring genomic HER2 amplification: MDA361 and SKBr3. Interestingly,

![Graph showing fold change in gene expression](image)

**Fig. 4.3 (A)**: Effects of HRG on mRNA levels of uPAR, uPA, MMP-2 and MMP-9 after treatment with HRG. Untreated cells were used as the control group. The relative mRNA expression of each gene was measured using qRT-PCR after normalizing the cycle thresholds of each triplicate against their corresponding hypoxanthine phosphoribosyl-transferase1 (HPRT1). Values are given as mean ± SD of three biological replicates. The statistical significance of difference between treatments and their controls was determined using unpaired, 2-tailed student's t-test; ** p< 0.01 and *** p< 0.001 were determined compared with the control.

**(B): Western analysis of MMP-9, uPA, cathepsin B and uPAR expression after HRG treatment.** Serum starved cells were treated with HRG for 48 h, then protein lysates were subjected to Western blotting and probed with the antibodies indicated. α-tubulin was used as the loading control. HRG: HRG-treated; SFM: serum-free media.

**(C): Effects of HRG on MMP-9 and MMP-2 enzymatic activity.** Serum starved cells were treated with HRG then equal amounts of total secreted protein were applied for zymography. Areas of enzymatic activity appeared as clear bands over the dark background.

MMP-2 activity was induced in SKBr3 cells despite no obvious increase at the mRNA level, suggesting HRG may act post-translationally to increase MMP-2 activity in some cases.
4.2.3.2 Effects of HRG on cathepsin B enzymatic activity
Cathepsin B activity assay was done to investigate whether HRG treatment enhances secretion and enzymatic levels of cathepsin B in the media of MDA361, MCF7 and SKBr3 cells. As depicted by Fig. 4.4, HRG significantly elevated enzymatic activity of cathepsin B in the media of all the three cell lines.

4.2.3.3 Effects on HRG on adhesive abilities of MDA361, MCF7 and SKBr3 cells
Tumour cell adhesion to components of the TME has been shown to enhance survival and metastatic abilities of circulating tumour cells (Zetter, 1993). It has been reported that intercellular adhesion molecule-1 (ICAM-1) activates intracellular signalling pathways in human cancer cells leading to enhanced motility and metastasis (Roland, Harken, Sarr, & Barnett, 2007). ICAM-1 was shown to be upregulated in metastatic breast cancer and its over-expression is associated with high expression of uPA (Schroder et al., 2011). To determine whether HRG induces ICAM-1, we performed qRT-PCR and Western analysis. HRG treatment induced ICAM-1 mRNA and protein levels in MDA361 and SKBr3 cells (Fig. 4.5A, B). To investigate whether HRG-mediated induction of ICAM-1 in MDA361 and SKBr3 cells is associated with increase in adhesive abilities of these cells, we next assayed the adhesive potentials of these cells to collagen I after HRG treatment. As depicted by Fig. 4.5C, HRG treatment significantly enhanced the adhesive abilities of MDA361 and SKBr3 cells to collagen I.
4.2.3.4 HRG induces mesenchymal-like phenotype in MDA361, MCF7 and SKBr3 cells

An important requirement for metastatic progression in breast cancer is acquisition of the ability to disseminate. This requires migration and invasion through the basement membrane of an in situ lesion, and ultimately the ability to cross endothelial barriers. During these processes, carcinoma cells are thought to revert to behaviours that facilitate correct spatial patterning of progenitor cells in the embryo (often referred to as ‘epithelial to mesenchymal transition’; EMT). Since others have reported that treatment of certain luminal breast cancer cells with HRG induces mesenchymal-like changes (e.g. spindle-like morphology and increased migratory behaviour in vitro (J. Kim et al., 2013), we hypothesised that the HRG/HER3 signalling may promote cancer progression in part by promoting EMT.

To explore this, qRT-PCR and Western analysis of mesenchymal markers were performed on the panel of HER2/3+ luminal breast cancer cells treated with HRG. As shown in Fig. 4.6A, HRG strongly induced N-cadherin mRNA in SKBr3 cells after 48 h, but not in MDA361 or MCF7 cells. In addition, HRG reduced mRNA expression of the epithelial marker, HER3, in all the three luminal breast cancer cell lines. In comparison, we did not observe any effects of
HRG on mRNA expression of Slug, Snail, fibronectin 1, vimentin, ZEB1, E-cadherin and Twist 1 (data not shown).

We next evaluated the effects of HRG on protein levels of epithelial (HER3, E-cadherin and Cytokeratin-8 (CK-8) and mesenchymal (Snail1, vimentin, Slug and Twist1) markers. As shown in Fig. 4.6B, HRG consistently reduced the epithelial markers E-cadherin and HER3, but not CK-8. Conversely, HRG treatment caused significant induction of Snail1 protein in all the three cell lines but failed to induce Slug, vimentin and Twist1. We did not observe induction of N-cadherin protein in SKBr3 cells after HRG treatment despite strong induction of N-cadherin mRNA. Altogether, these results show that HRG treatment reduced the expression of epithelial markers HER3 and E-cadherin and induced expression of Snail1 in luminal cells. This might indicate that HRG induces a partial mesenchymal phenotype in luminal cells, where tumour cells express both epithelial and mesenchymal markers and they do not acquire all the characteristics associated with mesenchymal transition (Leroy & Mostov, 2007).
4.2.3.5 HRG enhances migration and invasion of MD361, MCF7 and SKBr3 cells

In order to investigate whether HRG-mediated induction of protease activity (Fig. 4.3, 4.4) and mesenchymal-like phenotype (Fig. 4.6) in HER2/3+ luminal breast cancer cells lines is associated with changes in migratory and/or invasive cell behaviours in vitro, migration and invasion assays were done using Transwell inserts. As shown by Fig. 4.7, HRG significantly increased migration of all the three cell lines. In addition, HRG exposure also resulted in a significant enhancement in invasive abilities of these cells through Matrigel layer (Fig. 4.7). These data show that HRG promotes both migratory and invasive behaviour of luminal breast cancer cell lines, which otherwise migrate very poorly in vitro.

**Fig. 4.7 (A)** Relative migration rate in HRG-treated cells. Serum starved cells were placed into 8-µm porous culture inserts. The lower chamber contained serum free media or HRG. Cells were allowed to migrate for 48 h. Migrated cells were fixed, stained, lysed and then the optical densitometry was measured at a wavelength of 590 nm. Values are given as mean ± SD of three biological replicates. The statistical significance of difference between treatments and their controls was determined using unpaired, 2-tailed student’s t-tests: **p<0.01, *** p<0.001.

**Fig. 4.7 (B)** Relative invasion rate in HRG-treated cells. Serum starved cells were placed into 8-µm porous culture Matrigel-coated inserts. The lower chamber contained serum free media or HRG. Cells were allowed to invade through Matrigel layer for 48 h. Values are given as mean ± SD of three biological replicates. The statistical significance of difference between treatments and their controls was determined using unpaired, 2-tailed student’s t-test; * p<0.05 and ** p< 0.01 were determined compared with the control.
4.3 Discussion

The main goal of this chapter was to explore the molecular mechanisms through which HRG increases proliferation and invasion of the HER2+ human luminal breast cancer cells. MDA361, MCF7 and SKBr3 human luminal breast cancer cell lines with high expression of HER3 and paracrine HRG/HER3 signalling pattern were treated with exogenous HRG. This aimed to recapitulate the effects of HRG from the TME on proliferative and invasive behaviours of HER2+ breast cancer cells. HER2 is over-expressed, either through gene amplification or transcriptional deregulation, in 25-30% of breast cancer patients and confers poor clinical outcome (Slamon et al., 1987). HER2+ breast cancers have a high propensity to metastasize to the brain (Gabos et al., 2006; Niwinska, Murawska, & Pogoda, 2010). In addition, HER2 positivity is associated with poor cellular differentiation, high proliferative potential and spread to regional lymph nodes (Slamon et al., 1987), features strongly associated with a high risk of metastatic recurrence and death.

We first evaluated whether HRG treatment activates Akt and ERK1/2 in MDA361, MCF7 and SKBr3. As shown in Fig. 4.1, HRG phosphorylated Akt and ERK1/2 in these cells. Regarding HRG-mediated activation of Akt and ERK1/2 in MDA361, MCF7 and SKBr3 cells, we next asked if HRG promotes proliferation of these cells. As shown by an MTT assay, HRG increased proliferation of MCF7 and MDA361 cells in a time-dependent manner (Fig. 4.2). In contrast, no effect of was observed for SKBr3 cells.

Since HER2-mediated deregulation of cell cycle checkpoints is critically required for cell proliferation in HER2+ epithelial cells (Timms, White, O'Hare, & Waterfield, 2002), we next evaluated the effects of HRG on cyclin D1 protein levels in these cells. Fig. 4.2 demonstrates that HRG treatment induced cyclin D1 in MDA361 and MCF7 cells. On the other hand, HER2 is significantly activated in SKBr3 cells, even in serum-starved conditions (Fig. 3.2), providing a possible explanation for strong expression of cyclin D1 protein in serum-starved conditions in these cells. Enhancement of proliferation of MCF7 and MDA361 cells by HRG was associated with reduction of p27 protein levels in these cells (Fig. 4.2). Taken together, these data provide the mechanistic insights for HRG-induced cell proliferation in HER2+ luminal epithelial cells.

HER2 is a major driver of invasion and metastasis in HER2+ breast cancers by various mechanisms (Asrani et al., 2013; Finkle et al., 2004). It was demonstrated that transfection of mouse embryo fibroblast 3T3 cells with HER2 induces a more invasive phenotype in vitro.
and metastatic in vivo (D. Yu, Hamada, Zhang, Nicolson, & Hung, 1992). In addition, both clinical and experimental evidence indicate that HER2-induced up-regulation of MMP-2 and MMP-9 is strongly associated with aggressive behaviour in breast cancer (I. Y. Kim, Yong, Kang, & Moon, 2009; La et al., 2004; Pellikainen et al., 2004). In this regard, Kim et al reported that ectopic expression of HER2 in MCF10A, a non-invasive, immortalized human breast epithelial cell line, promotes more invasive phenotype in these cells through induction of MMP-9 via activation MAPK and Akt pathways (I. Y. Kim et al., 2009). HER2 over-expression in these cells was associated with down-regulation of E-cadherin and up-regulation of vimentin (I. Y. Kim et al., 2009).

In harmony, using zymography, La Rocca et al. showed elevated MMP-2 and MMP-9 enzymatic activities in the sera of HER2+ breast cancer patients compared to control sera (La et al., 2004). Our data showed that HRG treatment induced mRNA, protein and enzymatic levels of MMP-9 in all the three cell lines. Moreover, these data indicate for the first time, that HRG treatment induces MMP-2 and MMP-9 in the luminal, breast cancer brain metastatic cell line, MDA361 (Fig. 4.3).

Elevated expression and enzymatic levels of cathepsin B play critical roles in breast cancer invasion and metastasis (Castiglioni et al., 1994; Saad et al., 1998). Using the mouse mammary tumour virus (MMTV- PymT) model, Vasiljeva et al. showed that depletion of cathepsin B reduces tumour cell invasion and proliferation, leading to diminished metastatic burden in the lungs (Vasiljeva et al., 2008). In harmony, Victor et al suggested that in breast cancer cells, cathepsin B plays an important role in degradation of extracellular matrix (ECM) through activation of pro-uPA (Victor, Anbalagan, Mohamed, Sloane, & Cavallo-Medved, 2011). Others showed that RNA interference-mediated knockdown of cathepsin B in 4T1.2 breast cancer cells significantly abrogates collagen I degradation in vitro as well as development of bone metastases in vivo (Withana et al., 2012). Furthermore, Giusti et al suggested a cathepsin B-dependent activation of MMP-2 and MMP-9 in a pH-dependent manner (Giusti et al., 2008). More specifically, a recent study by Rafn et al demonstrated that motility and invasion of HER2+ breast cancer cells largely depends on cathepsin B (Rafn et al., 2012). Ablation of cathepsin B has been shown to significantly block invasiveness in HER2+ SKBr3 and MDA453 cells (Rafn et al., 2012).

Regarding the previous studies, we next evaluated whether HRG treatment increases mRNA, protein and enzymatic levels of cathepsin B in luminal cells. While we did not observe
increase in cathepsin B mRNA in any of the luminal cell lines following HRG treatment (data not shown), HRG increased cathepsin B protein levels in MCF7 cells (Fig. 4.3). In addition, as shown by our data, HRG strongly enhanced secretion and enzymatic levels of cathepsin B in all the three cell lines (Fig. 4.4). Steffan et al showed that suppression of PI3K/Akt cascade using LY294002, a specific inhibitor for this network, diminishes lysosomal exocytosis and secretion of cathepsin B into the media of prostate cancer cells (Steffan, Snider, Skalli, Welbourne, & Cardelli, 2009). In harmony, our data suggests that activation of PI3K/Akt by HRG might be one mechanism thereby HRG increased secretion of cathepsin B into the media of HER2+ luminal breast cancer cell lines.

Adhesion of tumour cells to endothelium is a key step in migration and invasion. ICAM-1 is a cell surface glycoprotein mainly expressed on vascular endothelial cells, and plays a key role in cell adhesion to vascular endothelium (Hubbard & Rothlein, 2000). Expression of ICAM-1 is elevated during progression of human breast cancers to a more invasive phenotype (Regidor, Callies, Regidor, & Schindler, 1998). In line with this, it was shown that inhibition of ICAM-1 via RNAi or neutralising antibody significantly reduces the ability of breast cancer cells to invade in vitro (Rosette et al., 2005). We investigated whether HRG treatment increases adhesion of luminal cells through enhancement of ICAM-1 expression. As shown in Fig. 4.5, HRG treatment increased ICAM-1 expression and adhesive characteristics of SKBr3 and MDA361 cells. These data suggest that HRG increases the invasive properties of HER2+ breast cancer cells by enhancing the adhesive abilities of these cells.

Accumulating evidence implies for a key role for EMT in cancer progression and metastasis (Rhim et al., 2012; M. Yu et al., 2013). During this process, tumour cells increase their migratory and invasive abilities through down-regulation of epithelial markers (E-cadherin, HER3, CK8) coupled with induction of mesenchymal markers (Snail, Slug, Twist, N-cadherin) (De Craene & Berx, 2013). We were interested to find out whether HRG treatment induces mesenchymal-like phenotype in the luminal cells. As shown by our results, HRG reduced mRNA and protein levels of the epithelial maker HER3 in all the three luminal cell lines (Fig. 4.6). In addition, HRG treatment down-regulated E-cadherin in MCF7 and MDA361 cells, possibly through induction of Snail1 (Fig. 4.6). We did not observe any effect of HRG on protein levels of Slug, vimentin, N-cadherin, Twist1 and CK8 in these cells (Fig. 4.6). A recent study by Kim et al. (published after we performed our experiments) also showed that HRG induces mesenchymal-like phenotype in MCF7 and SKBr3 cells through
down-regulation of E-cadherin. Besides, this study showed induction of Snail, vimentin and fibronectin in response to HRG treatment (J. Kim et al., 2013). Induction of vimentin by HRG in this study was very weak and disappeared after 24 h (J. Kim et al., 2013). Besides, while in SKBr3 cells we observed strong induction of Snail after HRG treatment for 48 h, Kim et al. did not see any difference between HRG-treated and the control group (J. Kim et al., 2013). The discrepancies between the two studies might be due to differences in the antibodies purchased from different companies.

In summary, the results of this chapter are hoped to help us increase our knowledge and insights about the effects of HRG from the TME on proliferation and metastasis of HER2+ breast tumour cells. Currently, we hypothesize that microenvironmental HRG at the primary site might increase the invasive abilities of these tumour cells by activation of certain proteolytic enzymes and induction of mesenchymal characteristics in these cells. These metastasis-promoting effects of HRG are thought to result in acquisition of a more invasive phenotype, invasion of tumour cells into the surrounding stroma (local invasion), and subsequently, intravasation to enter the blood circulation. It would be of paramount importance to explore if the second source of HRG at the metastatic site will favour survival and proliferation, extravasation and invasion of tumour cells into the brain parenchyma by disruption of the blood-brain-barrier and thereby, facilitating brain colonisation.
Chapter 5

Role of HRG/HER3/HER2 axis in blood-brain-barrier transendothelial migration of HER2+ breast tumour cells
5.1 Introduction

The second step in the metastatic cascade is tumour cell colonisation at the target sites (Chaffer & Weinberg, 2011). The molecular mechanisms of colonisation of cancer cells at their secondary sites are largely unknown. In the 19th century, Stephen Paget proposed his “seed and soil” theory of metastasis. According to this theory, certain tumour cells have an affinity to metastasize to certain organs (Paget, 1989). In other words, tumour cells with specific genetic and/or epigenetic profiles have the tendency to metastasize to certain organs with permissive microenvironment in order to establish metastatic lesions (Fidler, 2003). In 1928, James Ewing challenged Paget’s theory of tumour cell dissemination and suggested that tumour cell spread occurs by anatomic and mechanical factors that are the results of the circulatory system. Whether the metastasis of HER2+ and claudin-low breast tumour cells to the brain occurs due to distinct genetic and/or epigenetic attributes of breast tumour cells or it is solely due to the circulatory system still remains elusive.

While the molecular mechanisms of the first step of the metastatic cascade, physical translocation from the primary tumour, are emerging, our knowledge and insights about colonisation of tumour cells into the parenchyma tissue at the target sites are still in infancy (Chaffer & Weinberg, 2011). After binding to the microvasculature of the secondary sites, tumour cells penetrate into the parenchyma tissue (Al-Mehdi et al., 2000). Binding of tumour cells to endothelial cells (ECs) that line the blood vessels followed by transendothelial migration (TEM) is the first step in extravasation process (Kienast et al., 2010). Attachment of tumour cells to the ECs has two major steps: rolling of cancer cells on the endothelium followed by firmer adhesions between certain receptors and growth factors on both the tumour cells and the ECs (Ley, Laudanna, Cybulsky, & Nourshargh, 2007). For example, CD44 on breast tumour cells was shown to play an important role in attachment of tumour cells to the endothelium and their succeeding TEM (Draffin, McFarlane, Hill, Johnston, & Waugh, 2004). In breast cancer bone metastasis, microenvironmental hepatocyte growth factor (HGF) up-regulates CD44 expression on breast tumour cells, which in turn augments binding of tumour cells to the ECs and furthers their TEM abilities (Mine et al., 2003).

In breast cancer lung metastases, vascular cell adhesion molecule-1 (VCAM-1) on breast tumour cells interact with integrin α4β1 on macrophages, a process that activates PI3K/Akt pathway on cancer cells and provokes cell survival in these cells (Q. Chen, Zhang, & Massague, 2011). Interestingly, interaction of VCAM-1 on breast tumour cells with α4β1 on
the surface of osteoclasts plays a key role in breast cancer bone metastases (X. Lu et al., 2011). In BM from breast cancer, human brain microvascular endothelial cells (HBMECs) are the first host cells that breast tumour cells encounter after arresting within the brain microvasculature (Eichler et al., 2011). There is evidence that adhesion of brain-homing breast tumour cells to the HBMECs, early during brain colonisation, provides survival signals for the cancer cells (Carbonell, Ansorge, Sibson, & Muschel, 2009). Certain cellular components of tumour microenvironment (TME) at colonisation site, including tumour-educated microglia, are most likely to favour these adhesive interactions between the cancer cells and the HBMECs (Lorger & Felding-Habermann, 2010). Whether interfering with adhesion of brain-seeking tumour cells to the HBMECs can block brain colonisation of the cancer cells needs more in-depth investigations.

The microvasculature of destination site is an important impediment for metastatic colonisation due to highly impermeable microvessels at target sites (Carmeliet & Jain, 2011). In breast cancer lung metastasis, the cancer cells release certain types of matrix metalloproteinases (MMPs) including MMP-1, MMP-2, MMP-3 and MMP-10 to disarrange the endothelial junctions in the lungs (Huang et al., 2009). Infiltrating the blood-brain-barrier (BBB) is an imperative step in metastatic colonisation of the tumour cells to the brain (Pardridge, 2003). Tumour cells employ different molecular mechanisms to traverse the BBB and penetrate into the brain parenchyma (Avraham et al., 2014). For example, high expression of Angiopoietin-2 by triple negative breast cancer cells was shown to impair tight junction (TJ) structures, increase BBB permeability and eventually, foster seeding of the breast tumour cells into the brain tissue (Avraham et al., 2014). In addition, compelling evidence indicates for a key role of vascular endothelial growth factor (VEGF) in increasing HBMEC permeability through modulation of the TJ assembly (Wang, Dentler, & Borchardt, 2001) (Monaghan-Benson & Burridge, 2009).

Clinical and experimental data underline the importance of MMPs in BBB leakage in different neurological disorders (Seo et al., 2012). In animal models of cerebral ischemia, MMP-9 is markedly increased and causes BBB injury, edema and haemorrhage (Cunningham et al., 2005). Degradation of the TJ proteins is one molecular mechanism by which MMP-9 causes BBB leakage (Asahi et al., 2000). In mouse models of ischemic stroke, hypoxia leads to enhancement of BBB permeability associated with diminished expression of occludin and redistribution of ZO-1 protein (Bauer, Burgers, Rabie, & Marti, 2010). Hypoxic condition was shown to induce MMP-9 enzymatic activity in the vasculature, suggesting for a key role
of MMP-9 in disarranging the TJs and BBB leakage. In line with this, treatment with an MMP blocker significantly reduced BBB permeability and attenuated disarray of the TJs (Bauer et al., 2010).

In patients with acute lymphoblastic leukaemia, infiltration of leukemic cells into the central nervous system (CNS) and subsequent CNS relapse is a major hurdle for current treatment strategies for these patients (Pui & Howard, 2008). High expression of MMP-2 and MMP-9 by these cells is associated with a more invasive behaviour (Klein, Vellenga, Fraaije, Kamps, & de Bont, 2004). Interestingly, Feng et al manifested that leukemic-derived MMP-2 and MMP-9 exert a pivotal role in breaching the BBB via disturbing the TJ proteins ZO-1, claudin-5 and occludin in HBMECs both in vitro and in vivo (Feng et al., 2011). Accordingly, knockdown of MMP-2 and MMP-9 significantly reduced disorganization of the TJs proteins, leading to attenuation of the BBB opening and reduction of CNP relapse in mice models (Feng et al., 2011).

Metastasis suppressor genes suppress formation of macroscopic metastases (Yoshida et al., 2000). For example, RECK encodes a membrane glycoprotein that inhibits tumour cell invasion and metastasis by down-regulating MMP activity (Oh et al., 2001). Moreover, RECK mRNA was shown to be significantly reduced in cancer cells compared to normal tissues, tumours with high RECK expression are less invasive and these patients show better survival (Oh et al., 2001).

KAI-1, a membrane-bound metastasis suppressor, has been shown to suppress cell migration by down-regulating epidermal growth factor receptor (EGFR) signalling (Odintsova et al., 2000). There is substantial evidence that HER2 suppresses RECK and KAI-1 expression, suggesting this could be a molecular mechanism underlying HER2-driven metastatic behaviour (Hsu et al., 2006) (Wilson et al., 2002).

A study by Stark et al showed that in brain metastases (BM) from breast cancer, the mRNA levels of KAI-1 are reduced (Stark et al., 2005). In addition, the expression of metastasis suppressor genes KISS1, BRMS1, and mitogen activated protein kinase kinase 4 (M KK4) has been reported to be downregulated in BM from ductal invasive breast cancer compared to the primary tumours (Stark et al., 2005). In consistency, re-expression of KISS-1 significantly hampered melanoma metastasis to the brain (J. H. Lee et al., 1996). Finally, Sarris et al has reported that downregulation of nm23 expression in melanoma is associated with high predisposition to develop BM (Sarris et al., 2004). Altogether, these seminal studies suggest
that metastasis suppressor proteins inhibit development of BM though the underlying molecular mechanisms are largely unknown.

In sum, brain colonisation of tumour cells includes two steps: attachment of tumour cells to HBMECs followed by their TEM through disorganization of the TJ assembly via different mechanisms and subsequently, infiltrating the BBB. Due to the critical roles of MMP-9 in breaching the BBB and brain colonisation in ALL, the objective of experiments presented in this chapter was to explore whether HRG/HER3/HER2-induced MMP-9 in MDA361, MFC7 and SKBr3 cells induces TEM activity across a tight monolayer of the HBMECs in these cells.

5.2 Results

5.2.1 Effects of HRG on metastasis suppressor genes KAI1 and RECK

One of the molecular mechanisms of HER2-induced cell invasion and metastasis is down-regulation of metastasis suppressor genes, including RECK and KAI-1 (Wilson et al., 2002). A study by Hsu et al showed that HER2 transcriptionally down-regulates RECK and the ERK1/2 signalling cascade is involved in inhibition of RECK by HER2 (Hsu et al., 2006). Further, re-expression of RECK in HER2-overexpressing cells inhibited MMP-9 secretion and subsequently, tumour cell invasion (Hsu et al., 2006).

We observed activation of ERK1/2 in MDA361, MCF7 and SKBr3 cells after HRG treatment (Fig. 4.1). In this setting, we next hypothesised that HRG might reduce expression of RECK and KAI-1 in these cells, and that ultimately this may contribute to a more invasive
phenotype. As shown in Fig. 5.1A, HRG treatment attenuated KAI-1 expression in all three cell lines, and reduced RECK in MDA361 and SKBr3 cells. HRG also reduced protein levels of KAI-1 in MCF7 and SKBr3 cells but not MDA361 (Fig. 5.1B). Despite much effort, the purchased RECK antibody (Santa Cruz Biotechnology) did not work, not even for the positive control, so we couldn’t validate these results for RECK at protein levels.

5.2.2 HRG increased TEM activity of MDA361, MCF7 and SKBr3 cells
To investigate whether HRG treatment induces TEM activity in MDA361, MCF7 and SKBr3 cells via HER3/HER2-mediated induction of MMP-9, an in vitro TEM assay was performed. To validate whether the TJs between the HBMEC cells have been established and thereby, the HBMECs monolayer is impermeable, we performed a permeability assay by measuring the flux of fluorescein isothiocyanate (FITC)-Dextran through the confluent HBMEC monolayer. In addition, the expression of the tight junction proteins ZO-1, occludin and claudin-5 in the HBMECs monolayer were analysed using Western blot (Schubert-Unkmeir et al., 2010). As shown in Fig. 5.2, the HBMECs monolayer was impermeable and we did not observe FITC-Dextran in the lower chamber. Moreover, the Western blot analysis showed expression of all of the TJs proteins in the HBMECs monolayer.

In the next step, to determine whether HRG increases TEM activity in MDA361, MCF7 and SKBr3 cells, the cells were treated with HRG for 48 h. To evaluate whether HRG-induced TEM activity is through induction of MMP-9, the cultures were treated with GM6001, a MMP inhibitor. To ask if HRG-induced TEM activity occurs via activation of HER2 and HER3, the cells were treated with Herceptin (a HER2 blocker) and EV20 (a HER3 blocker), respectively. The cultures were treated with a combination of Herceptin and EV20 to assess whether HRG-induced TEM activity happens via activation of HER3/HER2. Untreated cells were used as control group. As shown in Fig. 5.2C, HRG treatment increased TEM activity in all the cell lines. HRG-mediated TEM activity was reduced when the cells were treated with GM6001, a MMP inhibitor. This suggests that HRG-induced MMP-9 favours TEM activity in these cells. In addition, HRG-derived TEM activity in these cells was inhibited after treatment with Herceptin as well as EV20, indicating that HRG-induced TEM activity in these cells is through activation of HER3/HER2. A combination of Herceptin and EV20 significantly inhibited HRG-mediated TEM activity in these cells (Fig. 5.2).
Fig. 5.2 Effects of HRG on TEM activity of MDA361, MCF7 and SKBr3 cells. Primary human brain microvascular endothelial cells (HBMECs) were seeded into Transwells pre-coated with matrigel and cultured to obtain confluence and allow formation of tight junction complexes. (A): HBMEC barrier formation was evaluated by measuring passive effusion of a small fluorescent dye in the lower Transwell chamber. (B): HBMEC expression of TJ proteins was confirmed by Western analysis. (C): serum-deprived breast cancer cells were seeded into the upper chamber of the Transwells containing pre-formed HBMEC monolayer. The cells were then treated with HRG, Herceptin, EV20 and GM6001 as indicated. After 48 h, non-migratory cells were removed from the apical side of the Transwells and the cells on the basal side were fixed, stained and lysed. Data shown are means ± SD from three biological replicates. The statistical significance of difference between treatments and the control was determined using unpaired, 2-tailed student’s t-test: * p<0.05, ** p<0.05 and *** p<0.001 were determined compared with the control.
5.3 Discussion

The molecular mechanisms of brain colonisation by breast tumour cells remain elusive (Eichler et al., 2011). The fact that HER2+ and claudin-low breast tumour subtypes compared to the other molecular subtypes have a high tendency to establish BM might suggest that BM formation in breast cancer occurs on the basis of the “seed” and “soil” theory (Ramakrishna & Rostomily, 2013). This is in agreement with the findings that brain has a unique microenvironment with paracrine growth factors that can attract brain-seeking tumour cells (Nicolson et al., 1996; C. Zhang, Zhang, Tsan, & Fidler, 2009).

Due to pivotal roles of HER2 in invasion and metastasis of HER2+ breast tumour cells (Moasser, 2007) and the necessity of HRG in establishment of the HER2/HER3 heterodimers and thereby, HER2-mediated metastatic spread in breast cancer cells, we hypothesize that metastasis of HER2+ breast tumour cells to the brain occurs in response to a gradient of HRG in the brain microenvironment. HRG is produced by a variety of cell types in the brain microenvironment including neurons (Bernstein et al., 2006), Schwann cells (Rosenbaum et al., 1997), astrocytes (Pollock, Franceschini, Graham, Marchionni, & Barnett, 1999) and HBMECs (Lok et al., 2009). Whether interplays between arriving HER2+ breast tumour cells and these HRG-expressing cells in the brain microenvironment increases expression and release of HRG by these cells is another open question.

Brain-homing or brain-seeking cancer cells are specific variants of certain cell lines established through in vivo passaging. In vivo, these cells represent exclusive tendency for metastasis to the brain compared to the parental ones. “Conversations” between brain-homing breast tumour cells and the cellular components of the brain microenvironment facilitate colonisation of the tumour cells. For example, using the MDA231-Br xenograft model of breast cancer brain metastasis, Fitzgerald et al showed that reactive glial cells are recruited by the breast tumour cells and favour tumour cell colonisation (Fitzgerald et al., 2008). MDA231-Br metastatic cells created an extensive reactive glial response in and around the metastatic lesions, supporting the hypothesis that tumour-educated glial cells alter the brain microenvironment in order to facilitate tumour cell proliferation, invasion and colonisation. In line with this, they also showed that BM from breast cancer patients contains masses of glial cells (Fitzgerald et al., 2008).

Microglia, the resident macrophages of the central nervous system, play central roles in neurodegenerative and neuroinflammatory responses (Pukrop et al., 2010). There is
compelling evidence that microglia contribute to cerebral malignancies (Pukrop et al., 2010). For example, the degree of infiltrating microglia in astrocytic tumours is positively associated with tumour grade (Roggendorf, Strupp, & Paulus, 1996). Microglia are thought to secrete a variety of cytokines and growth factors that favour tumour cell survival, invasion and evasion from the immune system (Wesolowska et al., 2008). In an attempt to determine the effects of microglia on glioma cell invasion in cultured brain slices, Markovic et al showed that when glioma cells were injected into slices devoid of microglia, the invasiveness of the glioma tumours was significantly decreased as compared with controls. In comparison, inoculation of exogenous microglia with glioma cells into cultured brain slices significantly enhanced the infiltrative behaviour of the tumour cells depending on the microglia/glioma cell ratio. In addition, the authors also showed that glioma-derived soluble factors stimulate MMP-2 enzymatic activity in microglia. These findings indicate that glioma cells stimulate microglial cells to increase breakdown of extracellular matrix by induction of MMP-2 and thereby promote tumour invasiveness (Markovic, Glass, Synowitz, Rooijen, & Kettenmann, 2005). In consistency, it was reported that microglia promote brain colonisation of MCF-7 cells in a Wnt-dependent manner (Pukrop et al., 2010).

In response to injuries and inflammation in the nervous system, reactive glia produce a variety of factors to support neuronal survival and these factors can be exploited by the tumour cells in order to favour their proliferation and invasion (Fitzgerald et al., 2008). These factors include glial cell line-derived neurotrophic factor, sphingosine-1-phosphate, CXCL12, TGF-α and TGF-β, interleukins, IGF1, autotaxin, HGF, EGF, HB-EGF, heparinase and certain types of MMPs (Graeber, Scheithauer, & Kreutzberg, 2002).

There is evidence that HRG (NRG-1) is a mitogen as well as a survival factor produced by different cell types in the brain microenvironment. For example, HRG plays a critical role in Schwann cell maturation, survival, and motility (Freidin, Asche, Bargiello, Bennett, & Abrams, 2009). In addition, HRG is thought to positively regulate neuronal survival, migration and differentiation (Buonanno & Fischbach, 2001). In animal models of stroke, HRG was found to play strong neuroprotective roles (Z. Xu, Croslan, Harris, Ford, & Ford, 2006). In addition, Lok et al showed that HRG possess significant proangiogenic and cytoprotective properties in the HBMECs (Lok et al., 2009). Both mRNA and protein levels of HRG are present in these cells and HER2 and HER3 receptors are phosphorylated. The autocrine signalling of HRG/HER3 in the HBMEC cells causes activation of ERK1/2 and PI3k/Akt signal transduction pathways which in turn favour proliferation and survival of
these cells, respectively. In line with this, it was shown that HRG protects the HBMECs against oxidative stress through activation of Akt. Collectively, these data show that HRG has a cytoprotective role in the HBMECs (Lok et al., 2009). In agreement, it was reported that axon-derived HRG promotes oligodendrocyte survival in the developing rat optic nerve and this protective effect is inhibited in the presence of a neutralizing antibody against HRG (Fernandez et al., 2000). Altogether, these seminal studies indicate for strong cytoprotective roles of HRG in central nervous system and raise the possibility that HER2+ breast tumour cells take advantage of these survival and migratory-promoting effects of HRG for their proliferation and invasion.

Tumour cells employ a wide range of mechanisms in order to breach the BBB and colonise brain tissue. Small-cell lung cancer (SCLC) patients with high serum levels of placentral growth factor (PLGF) are prone to form BM (Li et al., 2013). Using an in vitro BBB TEM assay, it was shown that tumour-derived PLGF causes disassembly of the TJ proteins and favours TEM activity of the cancer cells. In line with this, it was shown that knockdown of PLGF in tumour cells inhibits BM formation in vivo (Li et al., 2013).

It is now well-established that up-regulated MMPs play a cardinal role in certain neurological disorders through breaching the BBB (Cunningham et al., 2005) (Asahi et al., 2000). In reperfusion injury, astrocyte-derived MMP-2 and MMP-9 were shown to disrupt the BBB via degradation of the TJ proteins (Y. Yang, Estrada, Thompson, Liu, & Rosenberg, 2007). In a rat model of focal cerebral ischemia, MMP-9 was reported to rupture the BBB by degradation of occludin (W. Liu, Hendren, Qin, Shen, & Liu, 2009). In vascular cognitive impairment, MMP-2 and MMP-9 produced by astrocytes breach the BBB (Candelario-Jalil et al., 2011). BBB breakdown and cerebral oedema are the results of postischemic inflammation that cause mortality after ischemic stroke. Interestingly, Ludewig et al documented elevated neutrophil influx in infarct areas of the brain in a mouse model of ischemic stroke (Ludewig et al., 2013). These infiltrating neutrophils were shown to release MMP-9 and inhibition of neutrophil-derived MMP-9 alleviated the BBB opening and also reduced the stroke sizes, suggesting that neutrophil-derived MMP-9 traverses the BBB and causes postischemic inflammation after the stroke (Ludewig et al., 2013).

Due to induction of MMP-9 by HRG in the cell lines in this project, we next asked if HRG exposure increases the TEM abilities of these cells by induction of MMP-9 and consequently, breaching the BBB. As shown by our results for the first time, HRG increased TEM activity
in these HER2+ breast cancer cells via induction of MMP-9, suggesting that microenvironmental HRG-mediated activation of HER3/HER2 and thereby, induction of MMP-9 by HER2+ breast tumour cells is one molecular mechanism through which these cells traverse the BBB and subsequently, colonise the brain parenchyma.

RECK, a negative regulator of MMP activity, is downregulated in many types of solid tumours including breast cancer (Span et al., 2003). Using immunohistochemical staining for RECK in 119 invasive breast cancer samples, Zhang et al showed that lower expression of RECK is associated with lymph node metastasis (Y. Zhang et al., 2012) and that RECK expression level correlates with survival (Y. Zhang et al., 2012). There is ample evidence that over-expression of RECK diminishes enzymatic levels of MMP-2 and MMP-9 and thereby, reduces invasive properties in vitro (Oh et al., 2001; Takagi, Simizu, & Osada, 2009) as well as metastatic ability in vivo (Chang, Hung, & Chang, 2008). Additionally, Hsu et al documented that HER2 represses RECK expression through ERK1/2 activation to favour cell invasion (Hsu et al., 2006). HER2-mediated suppression of RECK was associated with induction of MMP-9 (Hsu et al., 2006). Furthermore, re-expression of RECK was shown to abrogate secretion and enzymatic activity of MMP-9 and significantly attenuated HER2-induced invasiveness (Hsu et al., 2006). We examined whether HRG reduces RECK mRNA in luminal cells. As shown by our result HRG reduced RECK mRNA in SKBr3 and MDA361 cells (Fig. 5.1).

The KAI-1 metastasis suppressor has been reported to be downregulated during breast cancer progression (X. Yang et al., 2000). Ectopic expression of KAI-1 in aggressive human breast cancer cell lines was reported to diminish invasiveness in vitro and lung colonisation in vivo (X. Yang et al., 2001). Furthermore, enforced expression of KAI-1 in H1299, human lung carcinoma cells reduced the invasive capacity of these cells through suppression of MMP-9 activity (Jee et al., 2006). In HER2+ breast cancers, HER2-mediated downregulation of KAI-1 was suggested as a molecular mechanism underlying invasion and metastasis (Wilson et al., 2002). We explored if HRG treatment reduces mRNA and protein levels of KAI-1 in luminal breast cancer cells. We showed that HRG reduced mRNA levels of KAI-1 in all the three luminal cell lines (Fig. 4.11). In SKBr3 and MCF7 cells, reduction of KAI-1 mRNA by HRG was associated with down-modulation of its protein levels (Fig. 4.12). Altogether, our in vitro findings show, for the first time, that down-modulation of metastasis suppressor genes by HRG is associated with cell motility in HER2+ breast tumour cells.
Chapter 6

General Discussion
Breast cancer is the most common malignancy in women and the second major cause of cancer-related death after lung cancer in the United States (Cheng & Hung, 2007). Metastasis is the main reason for morbidity and mortality in breast cancer patients and the major metastatic sites in breast cancer are bone, lung and liver and the brain. Despite improvements in systemic therapies, currently there is no treatment strategy with proven efficacy against metastatic breast cancer (Aoyama, 2011).

Breast cancer is a heterogeneous disease with distinct molecular subtypes on the basis of metastatic behaviour and clinical outcomes. These subtypes include basal-like, HER2+, claudin-low, luminal A, luminal B and normal-like. Luminal A and normal-like subtypes have a better prognosis whereas basal-like, claudin-low and HER2-overexpressing breast cancers are aggressive and represent the least favourable prognosis. In addition, these subtypes show high tendency for development of brain metastases (BM) (Harrell et al., 2012).

It is estimated that between 15% and 20% of metastatic breast cancer patients develop BM (Lassman & DeAngelis, 2003). The median latency between diagnosis of primary breast cancer and the diagnosis of BM is 2 to 3 years and in the majority of the cases, BM occurs in the patients who represent extracranial metastases (Weil, Palmieri, Bronder, Stark, & Steeg, 2005). In breast cancer patients with BM, the metastatic lesions occur in the brain parenchyma or along the leptomeninges (Tsukada, Fouad, Pickren, & Lane, 1983). In majority of cases, parenchymal lesions follow a vascular distribution, suggesting hematogenous spread (Tsukada et al., 1983). Several risk factors for BM from breast cancer have been identified, including young age, tumour grade 3, tumour size ≥2 cm, oestrogen receptor (ER) negative and HER2 amplification (Cheng & Hung, 2007; Fulford et al., 2007).

Current therapeutic approaches for breast cancer patients with BM include surgery, whole brain radiation therapy (WBRT), stereotactic radiosurgery and chemotherapy. The median survival for untreated patients with multiple metastatic lesions is 5 weeks (Markesbery, Brooks, Gupta, & Young, 1978). Multimodal therapeutic approaches combining surgery, WBRT, and stereotactic radiosurgery can extend overall survival (Sperduto et al., 2010), however BM is ultimately incurable. BM represents a growing public health problem, and development of efficacious preventive and curative procedures are of critical importance, largely depending on a better apprehension of molecular mechanisms and signal transduction pathways that promote disease initiation and progression (Eichler et al., 2011).
HER2 is overexpressed in approximately 25-30% breast cancers and is associated with an unfavourable prognosis (Slamon et al., 1987), metastatic potential (Tan, Yao, & Yu, 1997b) and high risk for developing BM (Hicks et al., 2006). There is substantial evidence that in HER2+ breast cancers, HER2 is a major driver of tumour biology by controlling different aspects of tumourigenesis including cell proliferation, survival, response to chemotherapy, angiogenesis and tumour cell dissemination (Moasser, 2007).

Experimental and clinical data indicate for a pivotal role of HER3 in HER2-derived tumourigenesis in breast cancer (Garrett et al., 2011; Vaught et al., 2012). For example, in HER2+ breast tumour cells, depletion of HER3 inhibited cell proliferation and induced programmed cell death in these cells (Lee-Hoeflich et al., 2008). Moreover, treatment of HER2+ breast cancer cells with HRG enhanced their invasion through Matrigel (F. J. Xu et al., 1997). Ectopic expression of both HER3 and HER2 in non-invasive MCF10A breast cancer cells augmented their migration and invasion in vitro in the presence of HRG (Aceto et al., 2012).

Despite the fact that the HRG/HER3 pathway plays central roles in different aspects of HER2+ breast cancers and while the activation pattern of this axis is understood in some human malignancies, so far there is little data on the activation pattern of this network in breast cancer. Additionally, whether the expression of HRG and HER3 are associated with specific molecular subtypes of breast cancer is still elusive. In this regard, we first aimed to clarify the association between HRG/HER3 expression at the mRNA level and different molecular subtypes of breast cancer cell lines.

As shown by the results of chapter 3, luminal breast cancer cell lines express higher levels of HER3 compared to claudin-low cells (Fig. 3.1). In line with this, HER3 was undetectable in a representative panel of claudin-low cell lines (Fig. 3.2). This is consistent with the new finding that HER3 maintains the balance between luminal and basal breast epithelium and HER3 loss in mammary epithelial cells shifts gene expression patterns toward a mammary basal cell signature (Balko et al., 2012). In addition, HRG isoforms were higher in claudin-low cell lines compared to luminal lines (Fig. 3.1). Overall, we observed an inverse correlation between expression of HRG and HER3 in our panel of breast cell lines. Low expression of HER3 in claudin-low cell lines suggests that these cells may have lost their HER3 as part of the epithelial-mesenchymal transition (EMT) process. On the other hand, high expression of HRG by these cells while they do not express the receptor might suggest
that HRG is secreted into the surrounding stroma in order to modify the tumour microenvironment (TME) (Kung et al., 1994).

The HRG<sub>low</sub>/HER3<sub>high</sub> phenotype in the luminal breast cancer cell lines implies for a paracrine mode of activation of HRG/HER3 signalling in these cells. To test this hypothesis, we performed Western analysis for p-HER3 in MDA361, MCF7 and SKBr3 cell lines. HER3 was activated once the cells were treated with exogenous HRG (Fig. 3.3), demonstrating the cells were receptive to a paracrine signal. These data underline the potential importance of microenvironmental HRG in activation of HER3, and raise the possibility that therapeutically disabling the TME-derived HRG signal could augment the efficacy of HER2 blockers in HER2+ disease, reducing the risk of BM in these patients (Vaught et al., 2012). Indeed, in pancreatic ductal adenocarcinoma (PDAC), the microenvironmental HRG fosters tumourigenesis by activation of HER3 on tumour cells. Blockade of HER3 with MM-121, a humanized monoclonal antibody that antagonises binding of HRG to HER3, was shown to attenuate tumour growth both <em>in vitro</em> and <em>in vivo</em> (Liles et al., 2011).

While evidence suggests that in HER2+ breast tumours, the HRG/HER3/HER2 axis plays central roles in different aspects of tumourigenesis of these neoplasms, the underlying molecular mechanisms are not well characterized. In chapter 4 of this thesis, we aimed to find out the role of the HRG/HER3 signalling pathway in regulation of proliferative and invasive properties of MDA361, MCF7 and SKBr3 cell lines. In this regard, we first evaluated whether HRG treatment increases proliferation of MDA361, MCF7 and SKBr3 cell lines. As shown in Fig. 4.2A, HRG enhanced proliferation of MDA361 and MCF7 but not SKBr3 cells. Timms et al. documented that in the luminal breast cancer cells, HER2 promotes cell proliferation by modulation of cell cycle checkpoints (Timms et al., 2002). In an attempt to determine the molecular mechanisms by which HRG favours cell proliferation, we next evaluated the effects of HRG on protein levels of p27 and cyclin D1. As depicted by Fig. 4.2B, enhancement of proliferation of MDA361 and MCF7 cells by HRG was associated with induction of cyclin D1 and reduction of p27 protein. In comparison, HRG had no effect on these proteins in SKBr3 cells (Fig. 4.2B). On aggregate, our results provide the mechanistic insights for HRG-mediated cell proliferation in MDA361 and MCF7 cell lines.

HER2 is a major driver of cancer invasion and metastasis in HER2+ breast tumour cells by different mechanisms including attenuation of metastasis suppressor proteins (Finkle et al., 2004). For example, it has been documented that in HER2+ breast tumours, HER2-mediated
down-regulation of metastasis suppressor protein KAI-1 is one molecular mechanism for HER2-induced motility (Wilson et al., 2002). In this setting, we next explored if HRG treatment decreases mRNA and protein levels of KAI-1 in MDA361, MCF7 and SKBr3 cells. As shown by our results, HRG exposure reduced KAI-1 mRNA in all the three cell lines (Fig. 5.1A). In MCF7 and SKBr3 cells, reduction of KAI-1 transcript was associated with down-regulation of its protein level (Fig. 5.1B). Moreover, HRG treatment reduced mRNA levels of RECK, another metastasis suppressor protein, in MDA361 and SKBr3 cells (Fig. 5.1A). Altogether, these results show that down-modulation of metastasis suppressor genes might be one molecular mechanism for HRG-induced motility in HER2+ breast tumour cells.

Induction of mesenchymal-like phenotype (often referred to as ‘epithelial to mesenchymal transition’; EMT) in tumour cells is an important initial step in cancer invasion and metastasis. During this process, tumour cells lose their epithelial markers and acquire mesenchymal-like phenotype (M. Yu et al., 2013). This transdifferential plasticity confers an invasive and migratory phenotype to tumour cells (Mani et al., 2008). Mesenchymal differentiation is coordinated by induction of certain transcription factors such as Snail, Slug and Twist1 that suppress expression of E-cadherin and thereby, favour dissociation of single epithelial cells (Thiery, Acloque, Huang, & Nieto, 2009).

There is strong evidence that TME plays a central role in induction of EMT in cancer cells (Thiery et al., 2009). For example, Labelle et al showed that tumour-educated platelets secrete transforming growth factor-β (TGF-β) which induces EMT in cancer cells (Labelle, Begum, & Hynes, 2011). We then explored if HRG treatment induces EMT in MDA361, MCF7 and SKBr3 cells. As shown by our findings, HRG exposure decreased mRNA and protein levels of the epithelial marker HER3 in all the three cell lines (Fig. 4.6A, B). Moreover, HRG reduced E-cadherin in MDA361 and MCF7 cells possibly via induction of Snail1 in these cells (Fig. 4.6B). HRG had no effect on Slug, vimentin, Twist1, CK8 and N-cadherin in these cells. Regarding the effects of HRG on down-regulation of the metastasis suppressor genes and induction of EMT in MDA361, MCF7 and SKBr3 cells, in the next step, we evaluated whether HRG increases migratory and invasive phenotypes of these cells. Our results show that HRG treatment enhanced migration of all the three cell lines (Fig. 4.7A). In addition, a significant increase in invasive capabilities of these cells was observed after HRG treatment (Fig. 4.7B).
Matrix metalloproteinases (MMPs) are involved in all stages of the metastatic cascade (Kessenbrock et al., 2010). The metastatic dissemination of tumour cells starts with local invasion followed by intravasation. An essential step in intravasation is crossing the physical barriers including the endothelial basement membrane (Joyce & Pollard, 2009). A recent study by Juncker-Jensen et al showed that carcinoma-produced MMP-1 regulates endothelial permeability, transendothelial migration (TEM) and subsequently, vascular intravasation of tumour cells by activation of protease activated receptor-1 (PAR-1) on endothelial cells (Juncker-Jensen et al., 2013). Colonisation of the target tissues and extravasation by disruption of the physical barriers at the secondary sites is the end stage of metastasis. In lung metastases from breast cancer, tumour-derived MMP-2 disorganizes the cell junctions in the lungs and promotes lung colonisation of the breast cancer cells (Huang et al., 2009).

MMPs play central roles in development of BM in breast cancer. A recent study by Liu et al showed that siRNA-mediated depletion of MMP-1 in MDA-MB-231 cells reduced the invasive capabilities of these cells in vitro and decreased formation of BM in vivo (H. Liu et al., 2012). Additionally, it was reported that mRNA levels of MMP-1 and MMP-9 are higher in the brain-seeking variant of MDA-MB-231 breast cancer cell line, MDA-231BR, compared to the bone-homing clones and the controls (Stark et al., 2007). In line with this, it was documented that ectopic expression of MMP-2 in MDA-MB-231 cells enhanced their orthotopic primary growth and formation of BM in nude mice (Tester et al., 2004). Mendes et al showed that ectopic expression of tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) in a rat mammary adenocarcinoma cell line significantly attenuated the abilities of these cells to develop BM in mice models (Mendes et al., 2007).

Seminal studies indicate for a positive correlation between HER2 amplification and/or overexpression and expression levels of certain types of MMPs. Results from clinical studies have shown that expression of MMP-2 and MMP-9 in breast cancer patients are strongly associated with HER2 amplification and/or overexpression (La et al., 2004; Pellikainen et al., 2004). In this setting, Tan et al showed that overexpression of HER2 enhanced the invasive abilities of human breast cancer cells via induction of MMP-2 and MMP-9 in these cells (Tan et al., 1997a). In addition, Kim et al showed that overexpression of HER2 in MCF10A cells increased MMP-9 expression, invasive and migratory capacities of these cells through activation of p38 MAPK and PI3K/Akt signal transduction pathways (I. Y. Kim et al., 2009). Collectively, these studies suggest that HER2 amplification and/or overexpression enhances production of MMP-2 and MMP-9 in HER2+ breast tumour cells and this is one molecular...
mechanism for HER2-mediated metastatic progression and eventually, development of BM in these cells (Eccles, 2001).

Regarding the important roles of MMP-2 and MMP-9 in promotion of cancer invasion and metastasis in HER2+ breast tumour cells as well as development of BM, we next evaluated whether HRG treatment induces these matrix-degrading proteases in MDA361, MCF-7 and SKBr3. As shown by the results of this study, HRG treatment induced mRNA, protein and enzymatic levels of MMP-9 in all the three cell lines (Fig. 4.3A-C).

Owing to the important roles of MMP-9 in traversing the blood-brain-barrier (BBB) in different neurological conditions including development of BM in acute lymphoblastic lymphoma (Feng et al., 2011), in chapter 5 of this thesis, we asked if HRG-induced MMP-9 increases the TEM activities of MDA361, MCF7 and SKBr3 cells via activation of HER3 and HER2. As shown by our results for the first time, HRG increased the TEM activities of all the three cell lines in an MMP-9-dependent manner (Fig. 5.2). These results suggest that HRG-mediated activation of HER3/HER2 and thereby, induction of MMP-9 by HER2+ breast tumour cells might enable these cells to breach the BBB and consequently, colonise the brain tissue. In line with this, a recent study by Sevenich et al has shown an essential role for cathepsin S in breaching the BBB, suggesting a key role for the proteolytic permeabilisation of the BBB and thereby, development of BM. Higher expression of cathepsin S was shown in the brain-seeking variant of MDA-MB-231 cells compared to lung and bone-homing counterparts and its inhibition reduced development of BM in a mouse model (Sevenich et al., 2014).

The major difference between brain and extracranial metastases is the ability of the brain-homing tumour cells to infiltrate the impermeable BBB and eventually, establish metastatic lesions in the brain tissue (Nguyen, Bos, & Massague, 2009). In this regard, investigation of molecular differences between brain and extracranial metastases in patient-matched samples might lead to identification of signal transduction pathways involved in breaching the BBB. In keeping with this, molecular profiling of patient-matched brain and extracranial melanoma metastases showed that p-AKT is higher in BM compared to the lung and liver metastases from the same patients (G. Chen et al., 2014). It is hoped that similar approaches help us identify molecular mechanisms responsible for breaching the BBB in BM from breast cancer in order to introduce novel therapeutic targets to block development of BM.
MicroRNAs (miRNAs) are small noncoding RNAs that base-pair with the 3' untranslated regions of protein-encoding mRNAs, resulting in mRNA destabilization and/or translational inhibition (Psaila & Lyden, 2009). miRNAs are major RNA components of exosomes, small (30–100 nm) membrane-encapsulated vesicles that are released into the extracellular environment by different cell types, including cancer cells (Skog et al., 2008). Emerging evidence implies for a central role of miRNAs in promoting breast cancer metastasis and distant colonisation via destroying the vascular endothelial barriers (Zhou et al., 2014). Co-culture of primary human microvascular endothelial and MDA-MB-231 cells showed that exosome-mediated transfer of cancer-secreted miR-105 disorganizes the tight junctions (TJs) between the human microvascular cells via targeting the ZO-1 protein. In addition, injection of these miR-105-containing exosomes into tail veins of SCID mice led to development of BM and lung metastases (Zhou et al., 2014). It would be informative to investigate whether the HRG/HER3/HER2 axis favours infiltration of the BBB and ultimately development of BM in HER2+ breast cancers through regulation of miRNAs that target different protein components of the TJs in the human brain microvascular endothelial cells (HBMECs).

The results of this study showed that the paracrine HRG/HER3 signalling pathway increases proliferative and invasive features of HER2+ breast tumour cells. HRG enhanced proliferation of the luminal HER2+ breast cancer cells via induction of cyclin D1 and down-modulation of p27. Additionally, HRG-induced EMT, migratory and invasive abilities of these cells were associated with induction of MMP-9 and cathepsin B. Moreover, HRG augmented the TEM activity of these cells through HER3/HER2-induced MMP-9, suggesting that HRG could play a role in breaching the BBB and development of BM in HER2+ breast cancer cells.

Further in vivo studies are indeed required to confirm that HRG/HER3/HER2-induced MMP-9 in HER2+ breast tumours is a mechanism for traversing the BBB and initial seeding of micrometastases in the brain tissue. In these HER2+ breast cancer cells, HER3 and MMP-9 could be knockdown and their effects on development of BM could be determined in mouse models. Besides, expression of HER3 and MMP-9 in brain metastases could be compared to extracranial metastases in patient-matched samples. The results of this study have implications in translational oncology, where targeting HRG/HER3/HER2 axis could be exploited in order to establish more effective preventive and therapeutic strategies for management of BM in HER2+ breast tumours.
Chapter 7

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