ROLE OF CELL ADHESION MICROENVIRONMENT AND THE SRC/STAT3 AXIS IN AUTOCRINE HGF SIGNALING DURING BREAST TUMOURIGENESIS

By

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A thesis submitted to the Department of Pathology and Molecular Medicine in conformity with the requirements for the degree of Master of Science

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This thesis is dedicated to my parents: Liljana and Genci. I would not be the person I am, or where I am today without your constant love, support and financial help. Your encouragement and belief in me have made me realize that I can achieve anything in life. You have been the best parents anyone could hope for and I appreciate everything you have done. Thank you from the bottom of my heart.
ABSTRACT

Over-expression of both hepatocyte growth factor (HGF) and its receptor Met frequently occurs in invasive human breast cancer, suggesting that the establishment of an HGF “autocrine loop” may be linked to breast tumour progression. We have recently shown a novel activating function of two signaling molecules, Src tyrosine kinase and the signal transducer and activator of transcription-3 factor (Stat3), on HGF expression in breast epithelial cells. Interestingly, Stat3 is also important in normal breast development, but this function does not require Src. In addition, β1-integrin adhesion occurs minimally in differentiated breast epithelium, but is upregulated during oncogenic progression and is required for transformation by Src. We therefore hypothesize that β1-integrin engagement is necessary for Src/Stat3-dependent activation of HGF transcription and breast tumourigensis. Using specific inhibitors of Src (Dasatinib) and Stat3 (CPA7) we demonstrated that autocrine HGF expression is linked to activation of Src/Stat3 in a malignant breast cell line. Phenotypic reversion (e.g., cell rounding and loss of filopodial extensions) and inhibition of pY705Stat3, HGF and pYMet expression as determined by immunofluorescence was achieved with both inhibitor treatments separately, and a synergistic effect was observed with combined treatment. Furthermore, β-catenin localization was nuclear in malignant cells, but shifted to cortical cytoplasmic following inhibitor treatment, similar to non-malignant mouse breast epithelial cells (EPH4). We are currently extrapolating these findings to a 3D Matrigel culture model in which EPH4 cells form acini-like spheroids with hollow lumen surrounded by a well-polarized outer layer of cells. Under these conditions, Stat3 levels are decreased followed by a reduction in
cyclin D1 expression, while Src activation remains at a low baseline level. Interestingly, expression of Stat5, which has a reciprocal relationship with Stat3 in breast development and involution, is increased concomitant with elevated β-casein expression. Moreover, Fibronectin and HGF in combination stimulate tubular outgrowths with lumen filling. These findings suggest that aberrant changes in extracellular matrix milieu may stimulate integrin cross talk resulting in a switch of HGF/Met signaling to a transformation phenotype. Information from this study may lead to novel cancer therapies through targeting the HGF/Met and integrin signaling cascades.
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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>2D</td>
<td>two-dimensional (monolayer)</td>
</tr>
<tr>
<td>3D</td>
<td>three-dimensional (matrigel)</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AP-2</td>
<td>adipocyte fatty acid-binding protein</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>Balb/c</td>
<td>bagg albino (inbred research mouse strain)</td>
</tr>
<tr>
<td>Bcl</td>
<td>b cell leukemia</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Chk</td>
<td>Csk homologous kinase</td>
</tr>
<tr>
<td>CKI</td>
<td>casein kinase I</td>
</tr>
<tr>
<td>CR2</td>
<td>conserved region 2</td>
</tr>
<tr>
<td>CSF1R</td>
<td>colony-stimulating factor 1 receptor</td>
</tr>
<tr>
<td>CSK</td>
<td>c-terminal Src kinase</td>
</tr>
<tr>
<td>ddH2O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamino-2-phenylindole</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>DKK1</td>
<td>dickkopf</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine-tetraacetic acid</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>ERM</td>
<td>ezrin radixin moesin</td>
</tr>
<tr>
<td>F</td>
<td>phenylalanine</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>FGFR</td>
<td>fibroblast growth receptor</td>
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<tr>
<td>FN</td>
<td>fibronectin</td>
</tr>
<tr>
<td>Fyn</td>
<td>Yes related Src family kinases member</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>Grb</td>
<td>growth factor receptor-bound protein</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>glycogen synthase kinase-3</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2 (also known as ErbB2)</td>
</tr>
<tr>
<td>HGF/SF</td>
<td>hepatocyte growth factor/ scatter factor</td>
</tr>
<tr>
<td>HGFA</td>
<td>hepatocyte growth factor activator</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL5/6</td>
<td>interleukin 5/6</td>
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</table>
JAK  janus kinase
kDa  kilodaltons
Ki-ras  kirsten ras
KO  knockout
LEF  lymphoid enhancer-binding factor
LIF  leukemia inhibitory factor
Lrbm  laminin-rich basement membrane
Lyn  Yes related Src family kinases member
MET  mesenchymal-to-epithelial transition
MMP  matrix metalloproteinase
Myc  myelocytomatosis
MW  molecular weight
NF-1  neurofibromin 1
Pan  all protein members of a group
PBS  phosphate buffered saline
PBS*  phosphate buffered saline with 0.1mM CaCl and 0.1mM MgCl
PDGF  platelet-derived growth factor
PDGFR  platelet-derived growth factor receptor
PFA  paraformaldehyde
PI3 kinase  phosphoinositide 3- kinase
PIAS  protein inhibitor of activated Stat3
PKA  cAMP-dependent protein kinase
PLC-γ  phospholipase C gamma
PPAR  peroxisome proliferator-activated receptor
PTPα  protein tyrosine phosphatase alpha
PTP1  protein tyrosine phosphatase 1
PTP1B  protein tyrosine phosphatase B1
pY  phosphorylated tyrosine
PVDF  polyvinylidene
RasGAP  Ras GTPase activating protein
RIPA  radioimmuno precipitation assay
RGD  arginine-glycine-aspartic acid motif
RNAi  RNA interference
RTK  receptor tyrosine kinase
RT-PCR  reverse transcriptase- polymerase chain reaction
SDS PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser/S  serine
SH  Src homology domain
Shc  Src homology 2 (SH2)-containing
SHP 1/2  SH2-domain containing protein phosphatase 1/2
shRNA  short hairpin RNA
siRNA  small interfering RNA
SOCS  suppressors of cytokine signaling
Src  Rous sarcoma non-receptor tyrosine kinase
SFK  Src family kinases
SFRP1  secreted frizzled-related protein
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Stat</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate-EDTA electrophoresis buffer</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with tween-20</td>
</tr>
<tr>
<td>TC45</td>
<td>trypanosoma cruzi 45</td>
</tr>
<tr>
<td>TEB</td>
<td>terminal end bud</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetrarhodamine isothiocyanate; a fluorescent label</td>
</tr>
<tr>
<td>TNM</td>
<td>tumour size, regional lymph node involvement, presence of metastasis (classification system for tumour stage)</td>
</tr>
<tr>
<td>β-TrCP</td>
<td>beta-transducin repeat-containing protein</td>
</tr>
<tr>
<td>Tyr/Y</td>
<td>tyrosine</td>
</tr>
<tr>
<td>USF</td>
<td>upstream stimulatory factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial cell growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial cell growth factor receptor</td>
</tr>
<tr>
<td>WCL</td>
<td>whole cell lysate</td>
</tr>
<tr>
<td>Wnt</td>
<td>“wingless-type”</td>
</tr>
<tr>
<td>Yes</td>
<td>Src family kinases member</td>
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CHAPTER 1- INTRODUCTION

1.1 The Breast

1.1.1 Breast Cancer

Breast cancer has become the most common cancer diagnosed among women and is expected to account for 26% of all new cancer cases this year [1]. Approximately 22,000 Canadian women are diagnosed with breast cancer each year [2]. Even though detection methods continue to increase in sensitivity, one in three women diagnosed with breast cancer will develop and ultimately die due to metastasis [2].

The TNM staging system currently in use for breast cancer is based on: the size of the primary tumour and its invasion into the surrounding stroma (T), the extent of regional lymph node involvement (N), and metastases to distant sites (M) [3]. This is then further divided into invasive (infiltrating) versus non-invasive (in situ) subgroups [3]. Non-invasive tumours are benign growths that have not invaded through the surrounding basement membrane; an example of which is ductal carcinoma in situ (DCIS) [4]. Invasive carcinomas, on the other hand, are the most dangerous seeing as these tumours have the ability to infiltrate into the surrounding stroma and metastasize to other regions of the breast and/or other tissues of the body. The most common types of invasive carcinomas are lobular and ductal carcinomas, which occur in 50-80% of all invasive breast cancers (reviewed in [4]). Less common types of breast cancer include: inflammatory breast cancer characterized by redness and swelling of the diseased breast, medullary carcinoma (characterized by a distinct boundary of immune cells localized between tumour tissue and normal tissue), mucinous carcinoma (formed by the mucus-
producing cancerous cells), and tubular carcinoma (named so due to the tubular appearance of cells under the microscope) [4].

Every woman is at risk for developing breast cancer. These risks are classified as either established or speculated. Some of the established risk factors include aging, genetic risk factors (e.g. BRCA1), early menarche, late menopause, ethnicity and exposure to radiation earlier in life [5]. Some of the speculated risk factors include hormone replacement therapy, oral contraceptives, abortion, alcohol consumption, and tobacco smoking [5].

Surgery, chemotherapy and radiation are the current conventional treatment options for malignant breast cancer [6]. However, most of these procedures lack tumour specificity and chemotherapy is particularly toxic to rapidly proliferating cells found in the bone marrow, hair follicles, and the gastrointestinal tract [6]. As such, biochemical targets are becoming increasingly popular in the development of cancer therapeutics. Therefore, the elucidation of new oncogenic signaling pathways and an exploration of the effects of pharmacological targeting may lead to novel treatment options for the large portion of the population affected by this pathology.

1.1.2 Mammary Gland Development

Both the human and rodent mammary gland morphogenesis begins in the embryo, pauses at birth, is modified at puberty, and resumes in response to ovarian hormones during pregnancy, lactation and involution [7,8]. The mammary gland is composed of two main compartments: the epithelium and the surrounding mesenchyme/stroma [9]. During embryogenesis, development of the ductal system begins at the nipple as a bulbous
epithelial rudiment which penetrates into the mammary mesenchyme and the surrounding fat pad [7]. At birth, a layer of laminin-rich extracellular matrix (ECM), the basement membrane, develops between the epithelium and the stroma [7]. During mammary gland development in both human and rodent, it is the signaling from ECM that maintains cellular differentiation and epithelial polarity. Although the ECM function remains the same in both, the composition of the stroma differs by being dense and fibroblastic in the human and adipose-rich in the rodent gland [8].

The female mammary gland development pauses at birth and remains inactive until puberty [8]. At puberty, ductal morphogenesis is driven by specialized structures termed the terminal end buds (TEBs) [8]. During this stage epithelial proliferation is directed by mesenchymal growth factors produced in response to ovarian hormones that act in harmony to orchestrate ductal development. For example, TGF-β, prevents side branching and induces apoptosis in the posterior region of the TEB, resulting in the formation of the lumen of the duct [7,8]. In contrast, another factor, HGF, which is also produced by the mesenchyme but acts on the receptors found on epithelial cells, induces site-specific branching [7]. The mammary ducts formed are composed of two layers; an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells [8].

During pregnancy, lobular epithelial proliferation occurs under tight hormonal regulation by estrogen and progesterone [9]. The lobular cells form secretory acinar structures - circular structures with hollow lumen surrounded by polarized epithelial cells - at the end of each branch [9]. During lactation, reproductive hormones (such as prolactin) transform lobular epithelial cells into alveolar cells that produce milk proteins, such as β-casein. This process is facilitated by the contraction of the surrounding
myoepithelial cells (reviewed in [9]). Following lactation, hormonal withdrawal leads to epithelial apoptosis and involution which restores the mammary epithelium to the stage it was prior to pregnancy [9]. It is believed that stem cells, which are thought to reside in the mammary gland, play an important role in the initial ductal development and later on in the expansion of the existing ductal system, not to mention in the mammary tissue remodeling and repair (reviewed in [10]). This remarkable plasticity of the mammary gland makes it susceptible to tumourigenesis and poses a significant clinical challenge in therapeutic design and clinical application.

1.1.3 The Epithelial-Mesenchymal Transition (EMT) is Modulated by the Extracellular Matrix (ECM)

Epithelial-Mesenchymal Transition (EMT) occurs when epithelial cells assume a mesenchymal phenotype in order to migrate into the surrounding environment. For instance, expression of E-cadherin, a protein involved in cell-cell contacts and formation of adherens junctions, is frequently downregulated during EMT. On the other hand, proteins that promote EMT such as N-cadherin, Snail1/2 and Twist are usually upregulated. These changes are usually accompanied by a translocation of β-catenin from the cytosol to the nucleus (reviewed in [11]). EMT is essential for branching morphogenesis at all stages of mammary gland development. However, when EMT becomes deregulated, it can lead to tumourigenesis [12].

In the quiescent mammary gland, the extracellular matrix (ECM) produced by both stromal fibroblasts and epithelial cells holds the cells in place and prevents tumourigenesis [9]. During EMT however, extensive breakdown and remodeling of the
ECM occur, which allows the cell to escape into the surrounding stroma. ECM proteins that play a key role in metastasis mainly include integrin-ligands and degrading-enzymes (e.g. MMPs) [12]. For instance, the overexpression of integrin α5β1 and its ligand Fibronectin (FN), have been shown to elicit EMT in mammary epithelial cells [14]. Mutations affecting stromal cells may also lead to tumourigenesis [15,16]. These cells secrete a variety of pro-inflammatory and growth factors such as TGF-β, TNF-α, HGF, IGF, and FGF [17]. During normal development these cytokines act in a tightly controlled paracrine manner on epithelial cells to regulate their proliferation and differentiation [17]. Neoplastic cells undergoing EMT, however, have lost ligand specificity and often start to secrete cytokines in an autocrine fashion [17]. In addition, these cells might secrete large amounts of ECM proteins that enhance their ability to survive in anchorage-independent conditions during migration to distal sites (reviewed in [17]).

Interestingly, the process of EMT seems to conflict with findings that secondary metastatic lesions share the epithelial characteristics of the primary tumour they were derived from. These findings suggest that a reverse process called mesenchymal-to-epithelial transition (MET) might exists which may be required for the latter stages of metastasis during which tumour cells must establish themselves in the new site [18]. In both EMT and MET, the surrounding stroma seems to play a key role in the regulation of processes controlling cell plasticity [18]. Defining the molecular mechanisms underlying this plasticity might lead to the development of therapeutics that would potentially target both the tumour and its surrounding stroma.
1.2. Modeling the Mammary Gland in Three-Dimensional (3D) Culture

The development of the mammary gland is an active process which requires signaling from both the cells and the surrounding stroma. EMT is specifically difficult to study \textit{in vivo}, as the process is slow, transient and hard to detect, since mesenchymal cells formed during EMT are impossible to differentiate from stromal cells after histological staining (reviewed in [19]). Growing the epithelial cells in a three-dimensional (3D) laminin-rich ECM, which mimics the \textit{in vivo} process (although in a more accelerated motion), could present one of the ways to alleviate this problem. In the presence of lactogenic hormones, normal epithelial cells grown in laminin, organize into spherical structures resembling the mammary acini [21,22]. These acini assume several characteristics of the \textit{in vivo} mammary gland including the formation of a central lumen surrounded by a well polarized layer of epithelial cells, as well as the expression of milk proteins such as $\beta$-casein [23-25]. In addition, the 3D laminin-rich ECM assays seem to substitute for myoepithelial cells and other signals that are needed for mammary epithelial cells to form these organized acini structures [26]. In contrast, breast tumour cells grown under the same conditions form nonpolarized and undifferentiated aggregates [20]. Various groups have also utilized 3D culture systems to model mammary branching morphogenesis by adding growth factors such as HGF and TGF-$\beta$ to the laminin-rich culture [27,28]. Moreover, cells undergoing EMT in 3D often form hyperplasia-like lesions that may progress to tumours or metastases upon subcutaneous and intravenous injections, respectively, into mice [29]. These \textit{in vitro} culture systems have evolved into essential models of mammary gland development and tumourigenesis that can be used effectively in testing therapeutic agents in a biologically relevant context.
1.3 Integrin Signaling

1.3.1 Biochemical Features and Integrin Activation

Integrins represent a large family of transmembrane receptors, which function as non-covalently linked heterodimers of α- and β- subunits and act to link cells to ECM proteins [30]. In mammals, 8 β-subunits (and their splicing variants) can form dimers with 18 α-subunits, resulting in 24 potential integrin heterodimers with each resultant heterodimer having binding specificity for particular ECM ligands [30]. Examples of integrin binding ligands include fibronectin, collagen, and laminin. The sequence arginine-glycine-aspartic acid (RGD) was originally thought to be the only integrin-binding motif for all integrin types. However, it was later discovered that integrins also contain specific binding sites for their particular ligands (reviewed in [31]). While some integrins commonly recognise several ligands, others such as the Fibronectin (FN) receptor α5β1 bind specifically to a single ECM protein ligand [32,33]. The cytoplasmic tail of β1 integrin is short and lacks an enzymatic domain. Therefore, β1 integrin forms complexes through its cytoplasmic domain with adapter proteins such as focal adhesion kinase (FAK) and Src, to form the focal adhesion complexes, which connect the cell to the surrounding ECM. In addition, actin-binding proteins, such as talin and vinculin, bind to the cytoplasmic domains of integrins and connect them to the cytoskeleton, thus creating a direct link between the surrounding matrix and the inside of the cell (reviewed in [31]).
1.3.2 Integrins in Normal Development

Integrin engagement regulates processes such as cell adhesion, migration, and differentiation by providing bidirectional signaling (i.e. outside-in and inside-out) through the plasma membrane [31]. While some integrins are restricted to certain cell types, others are more widely distributed [31]. Gene knockout experiments in mice have shown that β1-integrin plays a critical role in embryonic development (reviewed in [34]).

In the mammary gland, luminal epithelial cells express α2, α3, α6, β1 and β4 integrin subunits. Basal myoepithelial cells, on the other hand, express all the integrins found in the luminal cells and in addition α1, α5 and αv (reviewed in [33]). However, the primary integrin involvement in the mammary gland seems to be through the laminin-receptor α6β4, which is essential for hemidesmosomes (cell junctions) formation and maintenance of epithelial polarity (reviewed in [32,33]). Furthermore, the integrin α5β1 and its ligand Fibronectin — a central focus of Objective 4 in this thesis — are induced during the development of the vascular system but they are not expressed by quiescent endothelium [35]. As such, antibody and peptide inhibitors of α5β1 are currently in clinical trials for the inhibition of angiogenesis in cancer [35].

1.3.3 Integrins in Cancer

Alterations in the expression of integrins in breast cancer have been reported in several studies. These studies have shown that cancer cells increase the expression of integrins that favour the maintenance of the malignant phenotype while downregulating integrins that have the opposite effect (reviewed in [32]). Overexpression of β1 integrin in vitro has been shown to cause cell scattering and disruption of adherens junctions [36].
Moreover, studies using a transgenic mouse model for breast cancer, showed that β1 integrin expression was essential for mammary tumourigenesis [37]. Inhibition of the β1 integrin has also been shown to abrogate the formation of metastasis in gastric, colon and breast cancer models [39-41]. Furthermore, β1-integrin has been shown to cooperate with receptor tyrosine kinases (RTK’s) that play crucial roles in regulating cell proliferation, apoptosis and motility [42-48].

In addition to its role in angiogenesis (see section 1.3.2), α5β1 integrin has been shown to prevent apoptosis [38] and induce cell migration [49]. Studies have shown that inhibition of this integrin negatively regulates tumour cell migration, even when other integrin receptors are intact and bound to their corresponding ligands [49]. In fact, α5β1 antagonists activate PKA (cAMP-dependent protein kinase), which then inhibits cell migration by disrupting the formation of stress fibers [49]. In addition to cancer progression, β1 and α5β1 integrin signaling is thought to enhance resistance to cytotoxic drugs by enhancing cell survival in breast, lung and skin cancers [50-53]. Thus, several aspects of α5β1 integrin signaling point to this pathway as a multifaceted target for breast cancer therapy.

1.4 HGF/Met

1.4.1 Biochemical Features and Met Activation

The Met receptor tyrosine kinase (RTK) is a disulfide-linked heterodimer composed of an extracellular 50 kDa α-chain and a transmembrane 145 kDa β chain [54]. The extracellular domain binds the Met ligand hepatocyte growth factor, also known as scatter factor (HGF/SF: hereon referred to as ‘HGF’) [55, 56]. The transmembrane
domain contains the hydrophobic region of Met that spans the plasma membrane. The intracellular portion of the receptor consists of three domains: (a) the juxtamembrane domain which includes S985, a negative regulatory site, and Y1003, the phosphorylation of which targets the protein for degradation by the Cbl ubiquitin ligase [57]; (b) the kinase domain which gets transphosphorylated at residues Y1230, Y1234 and Y1235, upon ligand binding [58]; and finally, (c) the carboxy-terminal docking site which includes tyrosine residues Y1349 and Y1356 that bind numerous adaptor proteins upon receptor activation [60]. Downstream adaptor proteins containing a Src homology-2 (SH2) domain include proteins like PI3-kinase, RasGAP, PLC-γ, Src-related tyrosine kinases, and Grb-2 (Figure 1) [58-63]. Studies have shown that its C-terminal tail is adequate for Met signaling in vitro and in vivo (reviewed in [64]). Binding of HGF to Met causes the dimerization of two Met molecules and transphosphorylation of tyrosines 1234 and 1235 in the Met kinase domain (Figure 1) (reviewed in [59]). In addition, activation of Met can occur in the absence of ligand-binding through transactivation by other receptors, such as integrins, CD44, and EGFR (reviewed in [64]).

### 1.4.2 Biochemical Features and HGF Expression

HGF, the only known ligand for Met, is produced as an inactive 728 amino acid single chain precursor, pro-HGF [65]. Extracellular cleavage between residues 494 and 495 yields the fully active, two-chain form of HGF: a 69 kDa α-chain composed of an N-terminal hairpin domain and 4 kringle domains, and a 34 kDa β-chain containing a serine protease-like motif, held together by a disulfide bond [65,66]. Studies have shown that the N-terminal fragments of HGF (e.g., NK1, NK2, and NK4) exhibit both agonistic and antagonistic properties on HGF binding to Met, which demonstrates that the binding site
Figure 1. Structural domains for Met receptor kinase and corresponding ribbon diagram.

The Met receptor is a disulfide-linked heterodimer composed of an extracellular α-chain and a transmembrane β-chain. The extracellular region of the β-chain is composed of the sema domain (Sβ), a cysteine rich region (C), and four immunoglobulin domains (Ig). The transmembrane region contains the hydrophobic region of Met that spans the plasma membrane. The juxtamembrane region includes S985, a negative regulatory site, and Y1003, which targets the protein for degradation by the Cbl ubiquitin ligase. The kinase domain (K) shown in red includes Y1230, Y1234, and Y1235 which are transphosphorylated upon HGF binding. Phosphorylation of Y1313 serves as a docking site for P13K. Y1349 and Y1356 phosphorylation serve as docking sites for various signaling molecules and adaptor molecules. Phosphotyrosine 1365 is involved in cell morphogenesis. Ribbon diagram shown on the right and domain structure were adapted from [59].
for Met is found in this region of the HGF protein [67]. Although the β-chain shares structural homology with serine proteases, amino acid substitutions of serine and histidine residues in the catalytic region have suggested that this chain is proteolytically inactive [66]. In addition, deletion and mutational studies have shown that even though the HGF β-chain is not required for initial binding of full length HGF to Met, the β-chain is still needed for the complete activation of the receptor [68-70]. The HGF promoter is regulated by numerous transcription factors. Regulators such as Stat3, PPAR, USF, SP1, p53, act positively on the promoter by activating HGF expression, whereas other regulators such as TGFβ, NF-1 and AP-2 act negatively by repressing HGF promoter activity [71-77]. In addition, Src can act synergistically with Stat3 in the activation of HGF transcription [146,177,213]. This complex regulation of the HGF promoter explains in part the tissue specific expression pattern of HGF, and the possible aberrant increase in HGF expression in malignancies.

1.4.3 HGF/Met in Normal Development

HGF is a cytokine which induces morphogenesis, proliferation, motility and angiogenesis [59,80]. HGF/Met signaling plays an essential role in nervous system development, liver and kidney regeneration, and bone remodeling (for review see [54,82]). HGF is known to be a potent inducer of EMT in embryonic development as well as in tissue regeneration [54]. In normal mammary development, HGF in collaboration with other growth factors such as neuregulin stimulates tubulogenesis in a tightly controlled paracrine manner [78]. HGF is primarily expressed by mesenchymal/stromal cells, whereas its receptor, Met, is expressed selectively by epithelial cells, thereby
creating a paracrine regulatory system [79]. In normal breast tissue, the HGF-and-Met paracrine system has a low basal level of expression [80, 81]. During embryogenesis, Met and HGF provide essential signals for liver and placental development. As such, Met or HGF knockout embryos show an embryonic lethal phenotype and die \textit{in utero} [82]. Moreover, the knockout mice showed an ablation of sensory nerves in both the limbs and thorax, consistent with the importance of HGF signaling in nervous system development [82]. Point mutations in the multifunctional docking site of Met, on the other hand, result in a postnatal lethality with newborns dying due to inefficient breathing and suckling [82]. These findings suggest that the motility function of Met is crucial for the long-range migration of skeletal muscle progenitor cells involved in tongue, forelimb and diaphragm development [83]. The morphogenic signaling pathways activated by Met are under tight control during normal development [81]. However, when deregulation of HGF-Met signaling occurs, it might lead to malignancy.

\subsection*{1.4.4 HGF/Met in Cancer}

Over-expression and constitutive activation of Met occurs in many types of invasive human cancers including breast [84-86], lung [87,88] and ovarian [89] carcinomas. Thus, sustained high level activation of Met may be pivotal in the switch of epithelial cells from a morphogenic to a tumourigenic phenotype [64]. Clinical studies have reported the development of diverse human neoplasms as a result of abnormal HGF signaling [90]. In addition, over-expression of Met [64,85] and HGF [91] in breast tumours and of HGF in the sera [92] of breast cancer patients has been found to be an independent predictor of recurrence and decreased patient survival. In addition, Met
activation has been found to define a separate group of tumours from those that are positive for HER2 or hormone receptor (e.g., ER, PR) expression [93].

The mechanisms leading to sustained HGF/Met signaling in human breast cancer, however, are not clearly known. Although activating mutations in c-met have been associated with a small proportion of sporadic papillary kidney carcinomas and in some childhood hepatocellular carcinomas [94], somatic mutations in c-met have not been correlated with disease progression or poor prognosis in human breast cancer patients [95]. Thus, research has focused on non-genetic cell regulatory effects on Met signaling in cancer progression. One type of mechanism is through co-operativity of Met with other signaling molecules, such as activated forms of Erb-B2 [96] and Ki-ras [97], which have been shown to synergize with Met to enhance tumourigenicity. In addition, we have shown that the integrin-based adhesion to cell-matrix proteins such as Fibronectin promotes Met activation in breast carcinoma cells and that Src is required for this effect (Hui, A. et al, manuscript in preparation).

1.4.5 HGF-Met Autocrine loop

Another mechanism of sustained Met activation is through the formation of autocrine HGF loops [84,98]. The HGF autocrine loop occurs when epithelial cells, which already express the receptor Met, begin to express HGF, thereby shifting the paracrine system to an autocrine loop. The autocrine HGF-Met activation enhances cell motility via disruption of E-Cadherin dependent cell-cell contacts, and also upregulates collagenase activity leading to increased invasiveness and metastasis [99]. Consequently, HGF and Met expression has been experimentally shown to be particularly strong at the
migrating tumour front of invasive human breast carcinomas [100]. Nevertheless, whether the HGF autocrine loop plays a significant role in human breast cancer is not clearly known, since the majority of experimental studies have used cell lines that show no endogenous HGF expression. Indeed, a previous analysis showed only 25% Met/HGF responsiveness in a series of breast carcinoma cell lines [101], only one of which expresses HGF and Met - MDA-MB-231 [102,103]. Our group, however, has demonstrated autocrine HGF expression and constitutive activation of Met in a murine breast carcinoma cell line AC2M2 [220], a metastatic variant of SP1 cells [104,219] that was used in the present study. Taken together, these observations suggest that deregulation of HGF and the establishment of the HGF-Met autocrine loop might be linked to tumour growth and metastasis in breast cancer.

1.5 Src

1.5.1 Biochemical Features and Src Activation

Src is a 60 kDa protein that belongs to a large family of proto-oncogenic non-receptor tyrosine kinases, which also includes Lyn, Fyn, and Yes [106,114]. c-Src is the cellular homologue of v-Src, an activated form initially identified from the v-Src oncogene of Rous Sarcoma Virus (RSV) [105]. Src is composed of five highly conserved domains: the C-terminal tail, four Src homology (SH) domains, and a unique myristoylated N-terminal domain (Figure 2). Myristoylation at this site is required for targeting Src to the membrane [106]. Normally, Src is present in an inactive form in the perinuclear region of the cell. The C-terminal tail in humans contains the negative-regulatory tyrosine residue 530 (mouse Y529, chicken Y527). When phosphorylated by
Figure 2. Structure and regulation of Src.

A. Schematic representation of Src domain structure. Src family kinases share a common domain structure composed of (from left to right): SH4 domain (also called membrane targeting domain or M), unique domain (U), SH3 domain, SH2 domain, linker region, catalytic domain (SH1 domain) and regulatory domain. Src activity is regulated by tyrosine phosphorylation and/or dephosphorylation of tyrosine residues in the regulatory and kinase domains (adapted from [114]).

B. In the inactive conformation shown on the left, the SH2 domain interacts with phosphorylated Y530 in the regulatory domain and Y418 in the activation loop is dephosphorylated. Dephosphorylation of Y530 by protein tyrosine phosphatases leads to release of the intramolecular bonds and unfolds the protein (shown on the right). Autophosphorylation of Y418 in the kinase domain renders the kinase fully active (adapted from [121]). By convention, the amino-acid residue numbers shown are relative to human Src. Ribbon diagram depicts the crystal structure of Src in both its inactive (left) and active (right) conformations. (Ribbon diagram adapted from [109]).
kinases such as C-terminal Src Kinase (CSK), the pY530 residue forms intramolecular bonds with the SH2 and SH3 domains of Src, which keep the molecule in a closed, inactive conformation [110]. All the intramolecular bonds are relatively weak so a single activating event can open up the closed conformation and expose other parts of the molecule to potential activation. This is the case when Y530 is dephosphorylated by phosphatases such as PTPα, SHP1&2, PTP1B [111-113]. Once unfolded Src may be phosphorylated at its autophosphorylation site Y418 (Y416 in mouse) found in the kinase domain (SH1). This site provides a binding site for the downstream SH2 domain-containing proteins [114]. The SH2 domain of Src, on the other hand, binds specifically to tyrosine-phosphorylated peptides and determines the substrate specificity of the protein [107], whereas the SH3 domain binds to proline-rich regions [108]. Binding of the Src SH2 domain directly to β1 integrin, FAK or activated Met, has been shown to modulate Src activity (reviewed in [114]). Another level of regulation occurs through ubiquitination of Src by the Cbl ubiquitin ligase [116].

The lack of the negative-regulatory site (Y530) is what renders v-Src constitutively active and unable to form intramolecular bonds with the SH2 and SH3 domains. In a similar way, many experimental studies, have used a constitutively activated form of chicken Src in which Y527 residue is mutated to the non-phosphorylatable phenylalanine (F) that inhibits the closed conformation of Src and makes the protein oncogenic (for review see [114,121]).
1.5.2 Src in Normal development

Src is expressed in most adult tissues with the greater concentration in platelets, neurons and osteoclasts [121]. It plays a vital role in numerous cellular processes such as transcription, cell cycle progression, adhesion, angiogenesis, migration, apoptosis and differentiation (for reviews see [114]). In vitro studies have shown that the inactive form of Src might act as an adaptor protein through its SH2 and SH3 domains in order to stabilize cell–cell adhesions prior to integrin engagement [115]. In vivo studies on the other hand, have shown that mice homozygous for an inactivating Src mutation survive through embryogenesis, raising the possibility for a functional overlap among various Src family members [117]. However, these mice did suffer from osteopetrosis – a bone remodeling disease in which excess bone accumulates – implicating Src directly in osteoclast regulation [118,119]. In addition, the germline deletion of Src has been shown to cause defects in the initial stages of mammary ductal outgrowth, as well as uterine and ovarian development [120]. Moreover, Src-deficient mammary epithelial cells were unable to respond to exogenous estrogen stimulation [120] and displayed severe cell spreading and migration deficiencies when plated on ECM proteins [120]. Taken together, these studies demonstrate that the developmental deficiencies seen as a result of Src ablation in vivo might be due to impaired transmission of ECM signals.

1.5.3 Src in Cancer

Studies indicate that Src activity is elevated in a number of human cancers including breast, colon, ovary, head, neck, and pancreas [122-128]. There are multiple ways to activate Src either through mutations in the protein itself or through mutations in
the proteins that regulate Src. In colon cancers, activating mutations that cause the loss of Y530 have been demonstrated in highly metastatic cancers, but are absent in non-metastatic tumours (reviewed in [129]). Proteins that regulate Src can also be deregulated in cancer cells. Phosphatases such as SHP-1, SHP-2, PTPα and PTP1B that activate Src by dephosphorylating Y527 are elevated in several breast carcinoma lines (reviewed in [131]). On the other hand, Src inactivators such as Csk and Chk, which phosphorylate Src at Y527, were found at reduced levels [131].

Constitutively active Src has been shown to transform cells in vitro by stimulating cell scattering, spreading, and migration as well as deregulation of cell-cell contacts (reviewed in [130]). Nevertheless, the activated Src is not sufficient for mammary tumourigenesis in transgenic mice indicating that Src must interact with other signaling pathways [132]. It has been demonstrated that activated Src co-operatively interacts with many RTKs (e.g., EGFR, ErbB2, PDGFR, FGFR, CSF1R, VEGFR, and Met) in the development of the invasive phenotype [133-140].

Moreover, it has been shown that signaling in response to ECM proteins was reduced in cells lacking Src, Yes and Fyn, but could be rescued following the overexpression of Src in these cells, which points to the importance of Src in integrin signaling [141]. Accordingly, Src activation by integrin signaling can induce the phosphorylation of β-catenin and p120 catenin, leading to the disassembly of adherens junctions and subsequent translocation of the above molecules to the cytoplasm [142]. Also, Src stimulates the ubiquitination of E-cadherin itself, leading to its endocytosis and further loss of cell-cell contacts [143]. Furthermore, Src kinase activity has been shown to be needed for HGF-induced anchorage-independent growth of carcinoma cells [144].
Taken together, these observations point towards the fact that deregulated Src activity leads to a more migratory phenotype.

1.6 Stat3

1.6.1 Biochemical Features and Stat3 Activation

The signal transducer and activator of transcription (Stat) family of proteins has been shown to transduce signals from a variety of RTK’s (e.g. Met) as well as non-RTK’s (e.g. Src) [145,146]. At the time of discovery, Stat3 was identified as acute phase response factor activated by the IL-6 family of cytokines [147,148]. Further studies have shown that Stat3 is also activated in response to other cytokines and growth factors including IL5, EGF, and HGF (reviewed in [149]). Stats are latent in the cytoplasm. A recent study has pointed to receptor-mediated endocytosis as a mechanism for Stat3 transport from the plasma membrane to the perinuclear region [150]. The binding of Stat3 SH2 domains to phosphotyrosine residues on activated receptors, leads to Stat3 activation by the receptor itself or by associated tyrosine kinases belonging to the Janus Kinase (JAK) [151]. When they get activated, cytoplasmic Stat3 monomers are phosphorylated on conserved tyrosine residues (Y705) at C-terminus (Figure 3), and dimerize through reciprocal SH2-phosphotyrosine interaction [151]. The activated dimers are then recruited to the nucleus where they can activate the transcription of growth factor genes (e.g., HGF, VEGF) [145,152] and cell growth regulatory genes including myc [153], cyclin D1 [154] and bcl-xL [155]. In addition to Tyr-705, the phosphorylation of Ser-727 and the acetylation of a Lys-685 within the transactivation domain (Figure 3A) by MAP kinases
Figure 3. Structure and regulation of Stat3

A. Schematic representation of Stat3 domain structure. The coiled-coil domain is followed by a DNA-binding domain, a linker domain, a SH2 domain and a transactivation domain containing the CR2 region. The approximate residue positions of each domain are shown together with the activating phosphorylation sites of Y705 and S727 (adapted from [161]).

B. Ribbon diagram of the Stat3 homodimer–DNA complex. As the three-dimensional structure of Stat3 has not yet been determined, the structure of its splice variant Stat3β (which lacks a transactivation domain) bound to DNA is illustrated following the same colour scheme as listed in the domain structure indicated (Ribbon diagram was adapted from [161]).
and p300/CBP, respectively, contribute to maximal Stat3 transcriptional activation [156,157]. Cells employ both cytoplasmic and nuclear regulation in order to maintain regulatory control of Stat3 activation. At the cytoplasmic level, protein tyrosine phosphatases (e.g., SHP-2, PTP1) suppress signals by de-phosphorylating residues on activated JAKs and/or cytokine receptor complexes [158]. Suppressors of cytokine signaling (SOCS) can also be recruited to active receptor complexes inducing Stat3 turnover through an ubiquitin-mediated process [159]. Nuclear regulators of Stat3 consist of nuclear tyrosine phosphotases (i.e. TC45) which also function in Stat3 export from the nucleus to the cytoplasm, as well as proteins that inhibit activated Stat3, such as PIAS, by sequestering Stat3 dimers [158]. Alternative splicing also serves as a form of Stat3 regulation. Naturally occurring Stat3 variant, Stat3β (Figure 3B), contains a truncation in the C-terminus which makes it act as a dominant-negative form of Stat3 [160,161]. Therefore, a careful balance between Stat3 activation and inhibition is necessary for proper control of this protein during normal development.

1.6.2 Stat3 in Normal Development

The Stat3 protein is involved in numerous cellular functions such as proliferation, movement, and apoptosis and plays an important role in tissues throughout the body [162]. In the immune system, Stat3 is involved in modulating innate and adaptive immune responses (reviewed in [163]). Hematopoietic cell-specific disruption of Stat3 led to osteoporosis and an increase in osteoclast – cells responsible for bone resorption (break down) – production in the mutant mice, which points to the importance of Stat3 in bone remodeling [164,165]. However, tissue-specific Stat3 KOs have indicated an essential role of Stat3 in mammary gland involution as well as tubulogenesis during
which Stat3 acts in concert with Stat5 in regulating cell survival and apoptosis at different times in mammary gland development [166]. The level of activated Stat3 declines as the level of activated Stat5 rises during pregnancy. During weaning, Stat5 phosphorylation levels drop rapidly while Stat3 phosphorylation increases corresponding to the established role of Stat3 in involution [167,168].

1.6.3 Stat3 in Cancer

While normal cells exhibit transient activation of Stat3, a persistent activity of this molecule has been demonstrated in a number of human cancers [169-172]. The fact that a constitutively active form of Stat3, Stat3C (with two cysteine residues in the C-terminus, allowing Stat3 dimerization in the absence of phosphorylation), is able to transform cultured cells, further points to an etiologic role for Stat3 in these tumours [173]. Moreover, disrupting hyperactive Stat3 signaling in tumours has been shown to induce apoptosis with little effect on normal tissues, suggesting that Stat3 may be an effective target for therapeutic intervention [174]. How the differentiation versus the transformation role of Stat3 is regulated is not clear, but most likely it involves additional signaling molecules such as Src, as well as stimuli from the cell adhesion microenvironment [160]. On the one hand, Diaz et al. [175] have shown in a prospective study of patients undergoing chemotherapy that increased expression of nuclear pYStat3 and pYSrc is associated with regions of invasive breast cancer compared to matched non-invasive tissues. Furthermore, pYStat3 levels were higher in partial than complete pathologic responders to treatment. On the other hand, Dolled-Filhart et al. [176] have shown in a retrospective study that the activation of Stat3 signaling is an independent
marker of better overall survival of node-negative breast cancer patients. Therefore, tumours that become highly aggressive may engage additional signaling molecules (e.g. Src) that act with Stat3 to stimulate an invasive phenotype. Furthermore, activated Src and Stat3 have been shown to act cooperatively in HGF signaling through the Met receptor to induce transformation of breast carcinomas [177]. Therefore, the activation of the Src-Stat3 axis may be important in overcoming the repression of HGF expression that exists in normal mammary epithelium, and thereby promoting the activation of the HGF autocrine loop and tumourigenesis.

1.7 Src and Stat3 as targets for anticancer drugs

The elevated activities of Src and Stat3 in a variety of human breast carcinomas and cell lines indicate that these proteins may play a significant role in the development of mammary tumours and thus, they are a likely target for therapeutic targeting. In carcinoma cell lines that express activated Src and constitutively active Stat3, pharmacological inhibitors of Src can effectively block Stat3 phosphorylation and activity. In contrast, cell-cell adhesions, characteristic of differentiated epithelial cells, induce increased Stat3 phosphorylation and activity, which is unaffected by Src inhibition [178]. Also notable is that inhibition using a dominant negative Src mutant or a pharmacological Src inhibitor, such as PP2, decreases the level of HGF mRNA and protein in tumour cells that express constitutively active Src: suggesting that HGF expression is regulated by Src activity [100].

Disruption of constitutively active Stat3 signaling in tumour cells effectively induces apoptosis with little detriment to normal tissues, suggesting that Stat3 may be a
viable target for the treatment of various human cancers [174]. Inhibition of the Stat3 pathway by the use of Stat3 specific inhibitors, such as AG490 and the Stat3 dominant negative variant Stat3β, significantly suppresses tumour growth and induces tumour cell death with minimal effects on the surrounding tissues [179,180]. Moreover, blocking Stat3 signaling has been shown to suppress Src transformation [160,180,181]. However, a specific investigation of the signaling pathways affected by both Src and Stat3 inhibition has not yet been undertaken.

1.8 β- catenin

1.8.1 Biochemical Features and β-catenin Activation

β-catenin is composed of several domains: (a) an N-terminal domain [182,183], (b) a conserved central armadillo repeat domain, and (c) a C-terminal domain [184,185]. β-catenin binds to the cytoplasmic domain of E-cadherin at cell junctions and is necessary for the linkage of E-cadherin to the actin cytoskeleton (reviewed in [192]). Studies have shown that the presence of β-catenin in the cellular junctions is controlled by tyrosine phosphorylation of Y654, a residue located in the last armadillo repeat of β-catenin [186,187]. In addition to its role in cellular junctions, β-catenin is a critical component of Wnt signaling which is activated during early embryogenesis, adult morphogenesis and cancer [193,194]. In the absence of a Wnt signal, phosphorylation by CKI and GSK-3β at several Serine and Threonine sites located in the N-terminal domain, targets cytosolic β-catenin for ubiquitin-mediated proteosomal degradation [188,189]. This phosphorylation is modulated by the multiprotein complex composed of GSK-3β, axin and APC also known as the “β-catenin destruction complex” [190,191]. Wnt signaling leads to
dephosphorylation of axin and APC, which are usually phosphorylated by GSK-3β. The dephosphorylated proteins bind less efficiently to β-catenin, resulting in the accumulation of β-catenin in the cytosol (Figure 4). Accumulated β-catenin translocates to the nucleus where it can act in conjunction with DNA-binding proteins of the Tcf/LEF family to activate transcription of the Wnt-response genes [185].

1.8.2 β-catenin in Normal Development

β-catenin plays essential roles both in the formation of adherens junction complexes and in the Wnt signaling. During early embryogenesis, interaction of β-catenin with the Tcf/LEF family factors converts them to transcriptional activators and stimulates the expression of several genes involved in neural crest development [195,196]. Studies have shown that Wnt/β-catenin pathway also plays an essential role in embryonic liver development as well as liver regeneration following partial hepatectomy [197]. Recent evidence points to a role of Wnt/β-catenin signaling in the development of mammary gland and possibly breast tumourigenesis [198]. KO mice deficient in the transcription factor LEF fail to develop mammary glands [199]. In addition, transgenic mice expressing the Wnt signal inhibitor Dickkopf (DKK1) display an early block in mammary bud formation [200]. These findings and others suggest that β-catenin signaling plays a crucial role in mammary gland development and preservation of mammary stem cells during embryogenesis and possibly later in life [201,202].
Inside the cells, β-catenin protein is bound to either the GSK-3β/Axin/APC complex or E-cadherin. In the absence of a Wnt signal, cytosolic β-catenin is targeted for ubiquitin-mediated proteosomal degradation by paired phosphorylation by CKI and GSK-3β. The presence of a Wnt signal, weakens the bonds between these regulatory proteins and β-catenin, which results in accumulation of β-catenin in the cytosol. This ultimately leads to translocation of β-catenin to the nucleus where it binds to members of the Tcf/LEF transcription factor family to activate transcription of Wnt-response genes. Another way to modulate β-catenin activity is through Src-mediated phosphorylation at Y654 which also targets β-catenin to the nucleus.
1.8.3 β-catenin in Cancer

The activation of the β-catenin pathway plays a key role in the initiation and progression of a large number of human cancers, particularly colon cancer [190]. More than 50% of human breast cancers have been found to express β-catenin signaling [201]. In addition, β-catenin target genes such as cyclin D1, c-myc, and Twist are upregulated in a majority of invasive breast carcinomas [203-205]. Increased level of β-catenin signaling has also been associated with poor prognosis in breast cancer [206,207]. However, mutations in β-catenin or other components of this pathway in breast cancer have not been identified [201]. One way of β-catenin activation might be through autocrine Wnt signaling, which has been found to be present in both breast and ovarian cell lines [208].

As mentioned above, an activated Src may also lead to the phosphorylation of β-catenin on Y654, which in turn serves as a signal for disruption of the adherens junctions [143,186]. In addition, β-catenin has been shown to directly associate with Met [210-212] through Y654 and Y670 residues of β-catenin [211]; HGF-induced activation of Met on the other hand, can induce dose-dependent nuclear localization of β-catenin [210]. Therefore, β-catenin can be employed as a marker of cell transformation with nuclear translocation being indicative of the malignant phenotype.

1.9 Rationale, Hypothesis and Objectives

Our lab has recently shown that HGF and Met expression is particularly strong at the migrating tumour front of invasive human breast carcinomas [100] and may be casually linked to metastasis. In contrast HGF expression (but not Met) is strongly suppressed in normal breast epithelial cells. HGF expression is therefore a potential target
for therapeutic intervention in the treatment of breast cancer [28]. We have recently demonstrated that sustained hyper-activation of Src and Stat3, which occurs in invasive breast cancer, can stimulate strong expression of HGF in several carcinoma cell lines [213]. In contrast, transient induction of Stat3 occurs in normal epithelium and promotes tubulogenesis. In addition, we have shown that integrin-based adhesion to cell-matrix proteins such as Fibronectin promotes Met activation in breast carcinoma cells and that Src is required for this effect (Hui, A. et al. manuscript in preparation). Together, our findings suggest at least two distinct pathways of Stat3 activation in breast carcinomas: one is Src-independent and occurs during normal mammary development; the other is Src-dependent and occurs in tumour or premalignant cells in which Src is activated through cell-matrix adhesion. Therefore, we hypothesize that increased HGF-Met signaling is a critical downstream function of Src-Stat3 activation in mammary tumourigenesis and that inhibitors of Src-Stat3 signaling as well as HGF expression are expected to elicit a reversion of the malignant phenotype to a more differentiated phenotype. This hypothesis is summarized in Figure 5.

**Hypotheses:** *The induction of an HGF-autocrine-loop and transformation of breast epithelial cells is dependent on the Src-Stat3 signaling axis. Src activation through cell-matrix adhesion may shift the Stat3 phenotype toward tumourigenesis, via increased HGF expression and the activation of this autocrine loop.*
Figure 5. A proposed model of HGF-Met autocrine loop activation

Sustained hyper-activation of Src and Stat3 occurs in breast cancer by a variety of mechanisms, such as increased stimulation by upstream transducer molecules such as integrins. Sustained high levels of Src/Stat3 activation induce HGF transcription and HGF protein expression in epithelial cells that express Met, resulting in activation of an HGF-Met autocrine-loop and disruption of cell-cell contacts. These combined effects promote EMT, mammary tumourigenesis, and metastasis. Thus blocking Src/Stat3-dependent HGF transcriptional activity by specific inhibitors, would be expected to preferentially neutralize autocrine HGF-Met activation in breast carcinoma cell lines. Red bars indicate specific inhibitors to be used.
Objectives

1. Characterize mouse breast cell lines (EPH4 and AC2M2) in 2D & 3D culture.

2. Determine the dependency of transformation of malignant mouse breast cells (AC2M2) on:
   a) Src/Stat3, and
   b) HGF-autocrine-loop.

3. Assess the effect of activated Src and/or Stat3 on the morphology of non-malignant mouse breast epithelial cells (EPH4).

4. Assess the effect of cell-matrix adhesion on the morphology of EPH4 cells in 3D culture.

Our model will be very useful in assessing the role of Src/Stat3 activation and HGF expression in mammary development and in malignancy. The study will provide insight into how normal breast development deteriorates to produce pre-cancerous and eventually fully cancerous cells. It will also likely lead to novel strategies for targeting HGF expression in tumour cells with minimal effects on normal tissues as a potential new treatment of invasive breast cancer.
CHAPTER 2 – MATERIALS AND METHODS

2.1. List of Reagents.

The sources of reagents used in this study are described in Table 1.

Table 1. List of reagents used in this study

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute Ethanol</td>
<td>Botterell Solvent Store, Kingston, ON</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>Fisher scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Agarose</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Ammonium Persulfate (APS)</td>
<td>ICN Biomedicals Inc, Irvine, CA</td>
</tr>
<tr>
<td>Bio-Rad Dc Protein Assay Kit</td>
<td>BioRad, Mississauga, ON</td>
</tr>
<tr>
<td>Bio-Rad Kaleidoscope Protein Marker</td>
<td>BioRad, Mississauga, ON</td>
</tr>
<tr>
<td>Bis-Acrylamide</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Calcium Chloride (CaCl₂)</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>CPA7</td>
<td>Courtesy of Dr. Raptis</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>Toronto Research Chemicals Inc., North York, ON</td>
</tr>
<tr>
<td>Digitonin</td>
<td>Calbiochem, Cedarlane Laboratories Ltd., Hornby, ON</td>
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<tr>
<td>Dimethyl Sulfoxide (DMSO)</td>
<td>BioShop Canada Inc, Burlington, ON</td>
</tr>
<tr>
<td>Disodium Hydrogen Orthophosphate (Na₂HPO₄)</td>
<td>Fisher Scientific, Ottawa, ON</td>
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<tr>
<td>Dithiothreitol (DTT)</td>
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<tr>
<td>Dulbecco’s Modified Eagles Medium (DMEM)</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagles Medium/F12 (DMEM/F12)</td>
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<tr>
<td>Enhanced Chemi-Luminescence Kit</td>
<td>Perkin Elmer, Wellesley, MA</td>
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<tr>
<td>Ethylenediaminetetraacetic Acid (EDTA)</td>
<td>Sigma Aldrich, Oakville, ON</td>
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<tr>
<td>F-12 Nutrient Mixture</td>
<td>Gibco-Invitrogen, Grand Island, NY</td>
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<tr>
<td>Fetal Bovine Serum</td>
<td>Sigma Aldrich, Oakville, ON</td>
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<tr>
<td>Fibronectin</td>
<td>Invitrogen, Burlington, ON</td>
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<td>G418 Sulfate</td>
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<td>Hydrocortisone</td>
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</tr>
<tr>
<td>Insulin</td>
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</tr>
<tr>
<td>Isopropanol</td>
<td>Botterell Solvent Store, Kingston, ON</td>
</tr>
<tr>
<td>Leupeptin/Pepstatin</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
</tbody>
</table>
Lipofectamine 2000 Transfection Reagent | Invitrogen, Burlington, ON
Magnesium Chloride (MgCl₂) | Sigma Aldrich, Oakville, ON
Methanol | Ficher scientific, Ottawa, ON
Mowiol 4-88 Reagent | Calbiochem, Cedarlane Laboratories Ltd., Hornby, ON
N,N,N′,N′- Tetramethylethylenediamine (TEMED) | BioRad, Mississaugu, ON
NP-40 | Sigma Aldrich, Oakville, ON
Paraformaldehyde | Fisher Scientific, Ottawa, ON
Penicillin-Streptomycin (Pen/Strep) | Sigma Aldrich, Oakville, ON
Phenyl-methyl-sulfonyl-fluoride (PMSF) | Fisher Scientific, Ottawa, ON
Potassium Chloride (KCl) | Merck Frost Canada Ltd., Kirkland, QC
Potassium Dihydrogen Orthophosphate (KH₂PO₄) | ICN Biomedicals Inc, Irvine, CA
Prolactin | Sigma Aldrich, Oakville, ON
Recombinant HGF | Invitrogen, Burlington, ON
Sodium Chloride (NaCl) | Sigma Aldrich, Oakville, ON
Sodium Dodecyl Sulphate (SDS) | ICN Biomedicals Inc, Irvine, CA
Sodium Fluoride (NaF) | ICN Biomedicals Inc, Irvine, CA
Sodium Orthovanadate (Na₃VO₄) | Sigma Aldrich, Oakville, ON
Trasylol | KGH Pharmacy, Kingston, ON
Tris Base | Sigma Aldrich, Oakville, ON
Trypsin | Sigma Aldrich, Oakville, ON
Tween-20 | Sigma Aldrich, Oakville, ON

### 2.2 Cell Lines

The cell lines used in this study include EPH4 and AC2M2 - both derived from mouse mammary epithelial cells. EPH4 cells are a clonal epithelial derivative of IM-2 mouse mammary gland epithelial cells, which were originally isolated from mammary tissue of a mid-pregnant Balb/c mouse [214,215]. In the presence of lactogenic hormones such as prolactin and hydrocortisone, these cells will polarize and form differentiated mammospheres. In addition, EPH4 cells are able to produce milk proteins such as β-casein and thus are representative of an in vivo lactating mammary gland [216]. The AC2M2 cell line is a lung metastatic variant derived following intramammary injection of SP1 cells into syngeneic mice (SP1 was isolated from a mammary intraductal
adenocarcinoma that arose spontaneously in an 18-month-old CBA/J female retired breeder [217]) and subsequent culturing of tissue explants [218,219]. AC2M2 cells exhibit a scattering, invasive phenotype characteristic of malignant epithelial cells. Moreover, these cells have been shown to express HGF and phosphorylated Met, consistent with the establishment of an HGF autocrine loop [220].

2.3 Pharmacological Inhibitors

The Stat3 inhibitor, CPA7, is a cisplatin family member that has been shown to specifically disrupt Stat3 signaling activity in vitro at low micromolar concentrations [221]. CPA7 does not inhibit phosphorylation of Tyr705, but rather inhibits pY705Stat3 from interacting with DNA [222]. Dasatinib is a small-molecule, ATP-competitive inhibitor of Src family and Bcr-Abl tyrosine kinases with potency in the low nanomolar range [223]. Dasatinib is currently in phase II clinical trials for treatment of melanoma, chronic myelogenous leukemia and solid tumours (reviewed in [224]). The molecular structures of Dasatinib and CPA7 are presented in Figure 6. The specificity and potency of the aforementioned Src and Stat3 inhibitors makes them ideal for elucidation of the signaling pathways involved in the treatment of breast cancer. The pharmacological Stat3 inhibitor, CPA7 and the Src family kinase inhibitor, Dasatinib were employed at several concentrations ranging from 5-100 μM for CPA7 to 300-1000 nm for Dasatinib. Concentrations were chosen based on previous Western blots and available literature, then optimized using confocal imaging. Final concentrations of 100μM for CPA7 and 1000nm for Dasatinib were chosen, Dasatinib concentration was halved when used in combination treatment. CPA7 was reconstituted in DMSO/ddH₂O whereas Dasatinib was
further diluted in DMEM. Control treatment groups were incubated with 10μL of DMSO, diluted 1:1 in distilled water to account for the DMSO in CPA7.

(a) Dasatinib                                                        (b) CPA7

Figure 6. Molecular structures of inhibitors

(a) Dasatinib, the small molecule Src family kinase inhibitor, and (b) CPA7, the cisplatin-containing pY705Stat3 inhibitor were employed to disrupt the Src-Stat3 signaling axis.
2.4 Infection of EPH4 cells with Retroviral Particles

2.4.1 Transduction of EPH4 cells with retroviral particles expressing Stat3C

The packaging line phoenix-Stat3C (HEK293T-based line) expressing the gene of interest (Stat3C in this case (Figure 7) tagged to GFP was grown to confluence for a couple of days in DMEM with 10% FBS. The viral supernatant was collected and filtered using a nalgene 0.45 μm sterile filter and the viral supernatant containing polybrene (8 mg/ml final concentration) was added to the target cells. The target cell line (EPH4) was seeded at 20% confluence. For every 6-cm EPH4 plate, one 10-cm plate of the packaging line was plated in 5ml of DMEM 10% FBS. Twenty-four hours post transduction the media was replaced with regular EPH4 media. This transduction process was repeated two more times to increase the transduction efficiency. Cells were checked for GFP expression every day and forty-eight hours later the green cells (cells expressing the gene of interest) were cloned.

![Diagram of normal and constitutively active Stat3](image)

**Figure 7. Constitutively active Stat3 (Stat3C)**

The substitution of two cysteine residues 661 and 663 within the C-terminal loop of the SH2 domain of Stat3 produces a molecule that dimerizes spontaneously through inter-chain disulfide bridges in the absence of tyrosine phosphorylation at Y705. This dimer is then able to bind to DNA and activate gene transcription. Details of the construct are described in [154].
2.4.2 Transduction of EPH4 cells with retroviral particles expressing activated Src

PT67 fibroblasts (NIH3T3-based line expressing the 10A1 viral envelope, Becton Dickson, Mountain View, VA) were stably transfected with a constitutively active chicken Src mutant (Src-Y527F) containing a puromycin selection marker as previously described in our laboratory [146,225] to generate mass populations of retrovirus-producing cells (Figure 8). Viral particles in supernatants from PT67 cells were concentrated using a 0.45 \( \mu \)m filter. The target cell line (EPH4) was seeded at 20% confluence. For every 6-cm EPH4 plate, one 10-cm plate of the packaging line was plated in 5ml of DMEM 10%FBS. EPH4 cells were exposed to retrovirus expressing activated Src in the presence of 8 mg/ml polybrene. Twenty-four hours post transduction the media was replaced with regular EPH4 media. This transduction process was repeated three times to increase the transduction efficiency. Cells were then selected with 8 \( \mu \)g/ml puromycin initially which was then reduced to 4 \( \mu \)g/ml.

![Inactive and Active Src](image)

**Figure 8. Constitutively active chicken Src (SrcY527F)**

Normally Src activity is regulated by phosphorylation on Tyrosine Y527 (chicken nomenclature, as this protein is of avian origin to differentiate it from endogenous Src in the mouse epithelial cells). In this construct a point mutation converting Y527 to phenylalanine (F), results in a site that cannot be phosphorylated and thus cannot form intramolecular interactions with the Src SH2 domain. The protein is therefore always in an open conformation and constitutively active. Details of the construct are described in [146,225].
2.5 Infection of AC2M2 cells with HGF Lentiviral vectors

2.5.1 Constructs

Five different HGF short hairpin RNAs (shRNA) in pGIPZ lentiviral vectors were obtained from Open Biosystems, shown in Table 2.

Table 2. HGF shRNA Lentiviral vectors

<table>
<thead>
<tr>
<th>Vectors</th>
<th>Clone ID</th>
<th>Type</th>
<th>Species</th>
<th>Bacterial Selection Marker</th>
<th>Mammalian Selection Marker</th>
<th>Tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>V2LHS_179579</td>
<td>pGIPZ</td>
<td>Homo Sapiens</td>
<td>Zeocin (25μg/ml), Ampicillin (100μg/ml)</td>
<td>Puromycin (4-8μg/ml)</td>
<td>GFP</td>
</tr>
<tr>
<td>#2</td>
<td>V2LHS_179580</td>
<td>pGIPZ</td>
<td>Homo Sapiens</td>
<td>Zeocin (25μg/ml), Ampicillin (100μg/ml)</td>
<td>Puromycin (4-8μg/ml)</td>
<td>GFP</td>
</tr>
<tr>
<td>#3</td>
<td>V2LHS_179581</td>
<td>pGIPZ</td>
<td>Homo Sapiens</td>
<td>Zeocin (25μg/ml), Ampicillin (100μg/ml)</td>
<td>Puromycin (4-8μg/ml)</td>
<td>GFP</td>
</tr>
<tr>
<td>#4</td>
<td>V2LHS_179584</td>
<td>pGIPZ</td>
<td>Homo Sapiens</td>
<td>Zeocin (25μg/ml), Ampicillin (100μg/ml)</td>
<td>Puromycin (4-8μg/ml)</td>
<td>GFP</td>
</tr>
<tr>
<td>#5</td>
<td>V2LMM_20402</td>
<td>pGIPZ</td>
<td>Mus musculus</td>
<td>Zeocin (25μg/ml), Ampicillin (100μg/ml)</td>
<td>Puromycin (4-8μg/ml)</td>
<td>GFP</td>
</tr>
</tbody>
</table>
Vector Element | Utility
--- | ---
CMV Promoter | RNA Polymerase II promoter

cPPT | Central Polypurine tract helps translocation into the nucleus of non-dividing cells

WRE | Enhances the stability and translation of transcripts

turbo GFP (tGFP) | Marker to track shRNAmir expression

Puro<sup>+</sup> | Mammalian selectable marker

AMP<sup>+</sup> | Ampicillin bacterial selectable marker

5’LTR | 5’ long terminal repeat

pUC ori | High copy replication of maintenance in E.coli

SIN-LTR | 3’ Self inactivating long terminal repeat

RRE | Rev response element

ZEO<sup>+</sup> | Bacterial selectable marker

**Figure 9. pGIPZ lentiviral HGF shRNA vector**

Some of the advantages of the pGIPZ vector include: (a) the ability to perform transfections (transient and stable) or transductions using the replication incompetent lentivirus, (b) TurboGFP (tGFP) and shRNA belong to a bicistronic transcript allowing the visualization of shRNA expressing cells, (c) can be used in both *in vitro* and *in vivo* applications, and (d) contains a puromycin drug resistance marker for selecting stable cell lines. (Taken from www.biocat.de)

**2.5.2 Generation of virus**

Lentiviral particles containing the different HGF shRNA constructs were grown in HEK293T packaging cells by transfecting a three-plasmid packaging system [226] according to the manufacturer's instructions. The packaging line HEK293T was grown in
DMEM with 10% FBS. Cells were seeded in 10-cm dishes at cell densities of 70-80%. Next day cells were transfected using Lipofectamine/Plus 2000 Transfection Reagent (Invitrogen) according to manufacturer’s protocol. A total of 3 μg of DNA was mixed with Lipofectamine/Plus 2000 Transfection Reagent and incubated on cells. Eighteen hours post-transfection, transfection media was replaced with DMEM 30% FBS. Virus containing supernatants were collected 48 hr and 72 hr after transfection, centrifuged at 1250 rpm for 5 min to remove any cell debris, and filtered through a 0.45 μm syringe filter. Freshly collected supernatants were used to infect the target cells (Figure 10).

2.5.3 Infection of Target Cells

AC2M2 cells were grown in DMEM with 10% FBS to 50% confluency. The filtered virus supernatant was added with polybrene (8 mg/ml final concentration), and the cells were placed at 37 °C overnight. Fresh medium was added to the cells on the following day (Figure 10). This transduction process was repeated two more times to increase the transduction efficiency. Un-infected cells that went through a mock process without vector were used as control. Green GFP expression normally reached its peak 48 hr post-transduction (∼70%) and was generally higher after the first round of puromycin selection (8 μg/ml final concentration). All the pictures shown (except control) are of cells after their first round of puromycin selection.

2.6 3D Cell Differentiation Assay

The wells of a 6-well and a 24-well plate (with one glass coverslip in each well) were coated with 150 μL and 50 μL of 100% Matrigel at 4°C (on ice) respectively, following which Matrigel was allowed to solidify at 37°C for one hour. Matrigel is a
Figure 10. Production of lentivirus and transduction of target cells

The production of functional lentiviral particles was performed by cotransfection of a packaging cell line with three different vectors as described in [226]. The viral proteins Gag (core protein) and Pol (reverse transcriptase) are not properly assembled in murine cells. Therefore, a human cell line is used instead, such as the embryonic kidney cell line, HEK293 [227]. The cell line used in this study is HEK293T. The 293T cell line is a variant of 293, stably expressing the SV40 large T antigen. This line allows for the replication of plasmids containing the SV40 origin of replication such as the transfer vector used [228]. Viral particles are secreted into the culture medium from which they are collected. Production of viral particles is maximal 24 hr to 72 hr following transfection. The supernatant is filtered through a 0.45 μm filter and then used to infect the target cells (AC2M2 in our case). Diagram adapted from www.openbiosystems.com.
basement membrane solution, rich in laminin and other extracellular matrix proteins. EPH4 or AC2M2 cells were then seeded on the Matrigel-coated, sterile coverslips at $5 \times 10^5$ cells per well (6-well plate) or $1 \times 10^5$ cells per well (24-well plate) in 3D medium. The medium was replaced every other day. Cells were seeded as a single-cell suspension in a culture medium containing growth factors, hormones, and 0% or 5% Matrigel depending on the cell line (see Appendix). Experiments were terminated at day 10, with replacement of medium plus factors every other day. In one experiment, HGF and FN (at the concentrations indicated) were added daily, beginning at day 2. HGF only and FN only groups stained for DAPI were contaminated and no data are shown for these groups. This overlay method (Figure 11) has several beneficial features: (a) the mammospheres that form are large enough to detect lumen formation and central apoptosis, (b) the cells can be stained in situ to visualize the subcellular localization of different proteins by confocal microscopy, and finally (c) proteins can be easily extracted using mild buffers such as RIPA for Western blot analysis (reviewed in [22]).

2.7 Phase contrast microscopy

Images of live cell morphology were taken by phase contrast microscopy (Leica Leitz DM IL) at the magnifications indicated using an attached digital camera (Nicon coolpix 995).
Figure 11. Schematic of overlay method for growing cells on Matrigel

The well of a plate is initially coated with 100% Matrigel and allowed to solidify, forming a gelled bed of basement membrane measuring approximately 2-5 mm in thickness. EPH4 or AC2M2 cells are seeded onto this bed as a single-cell suspension in an Assay Medium containing growth factors, hormones, and 0% (EPH4) or 5% (AC2M2) Matrigel. The Assay Medium is replaced every other day. Cells proliferate and form clusters after 4–5 days in 3D culture (Adapted from [22]).
2.8. Western Blotting

2.8.1. Preparation of Whole Cell Lysates (WCL)

To avoid potential dephosphorylation of tyrosine residues, cells were rinsed twice with PBS* at room temperature, and immediately lysed in either RIPA buffer or 500 μl 2X SDS sample buffer (See Appendix). Bradford assays were performed using a Bio-Rad Dc protein assay kit to determine total protein levels, and WCLs were normalized according to their corresponding concentrations read using a Bio-Tek ELx800UV automated microplate reader.

2.8.2. SDS-PAGE

Right before running the gels, WCLs were supplemented with 3.0% β-mercaptoethanol and boiled for 5 minutes at 100 °C and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with remaining lysates stored at -20°C. Samples were then resolved on reducing mini SDS-PAGE gels (8%) alongside BioRad Kaleidescope (pre-stained protein) Marker. The mini gels were then transferred, using a Bio-Rad semi-dry transfer apparatus, onto PVDF immobilon membranes (Millipore) pre-incubated in 100% methanol for 10 min and submersed in semi-dry transfer buffer for 10 min prior to transfer (Details in Appendix).

2.8.3. Immunoblotting

Following SDS-PAGE and transfer, membranes were blocked with 5% BSA or milk diluted in TBST for 1 hour before being probed with corresponding primary antibodies at dilutions as indicated in Table 3. Membranes were then rinsed three times
(10 min) with TBST before incubation with the corresponding secondary antibodies diluted to 1:2500 in TBST at room temperature for one hour (See Table 4). Membranes were then washed three times (10 min) with TBST, and the immune complexes were visualized using enhanced chemiluminescence reagent (Perkin Elmer, Wellesley, MA) followed by exposure to autoradiographic film (Fuji Medical x-ray film, Christie Group LTD., Mississauga ON).

Table 3. Primary Antibodies used for Western Blot analysis

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Clonality</th>
<th>Source (Catalogue #)</th>
<th>Concentration</th>
<th>Blocking</th>
<th>Species of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>p120 RasGAP</td>
<td>Poly</td>
<td>Courtesy of Dr. Greer</td>
<td>1:2000</td>
<td>5% milk overnight</td>
<td>Rabbit</td>
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<tr>
<td>Pan Met (B2)</td>
<td>Mono</td>
<td>Santa Cruz Biotechnology Inc., Santa Cruz, CA (sc-8057)</td>
<td>1:500</td>
<td>5% milk overnight</td>
<td>Mouse</td>
</tr>
<tr>
<td>pY 1234/1235 Met</td>
<td>Poly</td>
<td>Cell Signaling, Beverly, MA (3126)</td>
<td>1:1000</td>
<td>5% BSA overnight</td>
<td>Rabbit</td>
</tr>
<tr>
<td>HGF</td>
<td>Poly</td>
<td>RD Systems Inc., Minneapolis, MN (AF2207)</td>
<td>1:1000</td>
<td>5% milk overnight</td>
<td>Goat</td>
</tr>
<tr>
<td>Pan Src (B12)</td>
<td>Mono</td>
<td>Santa Cruz Biotechnology Inc., Santa Cruz, CA (sc-8056)</td>
<td>1:1000</td>
<td>5% milk overnight</td>
<td>Mouse</td>
</tr>
<tr>
<td>pY418 Src</td>
<td>Poly</td>
<td>Biosource International, Burlington, ON (44-660G)</td>
<td>1:1000</td>
<td>5% BSA overnight</td>
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</tr>
<tr>
<td>Pan Stat3</td>
<td>Poly</td>
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<td>1:1000</td>
<td>5% milk overnight</td>
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<tr>
<td>pY705 Stat3</td>
<td>Poly</td>
<td>Cell Signaling, Beverly, MA (9131)</td>
<td>1:1000</td>
<td>5% BSA overnight</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Pan Stat5A</td>
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<td>Upstate Cell Signaling</td>
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<td>5% milk overnight</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Secondary Antibody</td>
<td>Source (Catalogue #)</td>
<td>Concentration</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>--------------------</td>
<td>----------------------</td>
<td>---------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donkey anti-goat IgG HRP-linked</td>
<td>Santa Cruz Biotechnology Inc., Santa Cruz, CA (sc-2020)</td>
<td>1:2500</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Donkey anti-rabbit IgG HRP-linked</td>
<td>GE Healthcare, Ltd., Buckinghamshire, UK (NA934V)</td>
<td>1:2500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep anti-mouse IgG HRP-linked</td>
<td>GE Healthcare, Ltd., Buckinghamshire, UK (NA931V)</td>
<td>1:2500</td>
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<td></td>
</tr>
</tbody>
</table>

### Table 4. Secondary Antibodies used for Western Blot analysis

2.9 Immunofluorescence

Cells were seeded on sterile coverslips at 2.5x10^4 cells per well on a 24-well plate, overnight, in the appropriate growth medium. Alternatively, cells were seeded on Matrigel-coated coverslips at 1x10^5 cells per well on a 24-well plate for 1 day, in the appropriate growth medium. Coverslips in all cases were gently rinsed three times with PBS* before fixation in 3% paraformaldehyde for 20 min. Coverslips were then washed three times in PBS* before permeabilization in 25μg/ml digitonin in PBS* for 10 min. Following three more 2 min washes in PBS*, cells were blocked with 0.3% bovine serum albumin (BSA) in PBS* for 20 min. Coverslips were incubated in primary antibodies at
indicated concentrations (Table 5) for 1 hr in a humidified chamber. Cells were then washed three times for five min in wash reagent (see Appendix) before incubation in the appropriate fluorescent-conjugated secondary antibodies (Table 6) and/or DAPI/Phalloidin (Table 7). After a final set of three 5 minute washes in wash reagent, coverslips were mounted on glass slides using 5μl of Mowiol 4-88 Reagent, and sealed with nail polish. Stained cells were visualized using a Leica TCS SP2 multi photon confocal microscope system, equipped with a 1.4NA 100X objective lens. Colour overlay and image analysis were performed using Leica Confocal Software (LCS ver. 2.61). Nuclear versus cytoplasmic localization was assessed using DAPI counter-staining to confirm the nuclear boundary. The proportion of nuclear stained cells for each of β-catenin and pYStat3 were assessed with a total of at least 10 cells in 3-5 images per group unless otherwise stated. A preliminary statistical analysis was done using a nonparametric Fisher Exact test (two sided) as described in http://www.quantitativeskills.com/sisa/statistics/fishrhlp.htm. pYSrc, HGF and pYMet were assessed qualitatively as membranous, cytoplasmic or cortical cytoplasmic (Table 8).

### Table 5. Primary Antibodies used for Confocal Microscopy

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<thead>
<tr>
<th>Primary Antibody</th>
<th>Source (Catalogue #)</th>
<th>Dilution</th>
<th>Species of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>pY418 Src</td>
<td>Biosource International, Burlington, ON (44-660G)</td>
<td>1: 100</td>
<td>Rabbit</td>
</tr>
<tr>
<td>pY705 Stat3</td>
<td>Cell Signaling, Beverly, MA (9131)</td>
<td>1: 100</td>
<td>Rabbit</td>
</tr>
<tr>
<td>HGF</td>
<td>RD Systems Inc., Minneapolis, MN (AF2207)</td>
<td>1: 100</td>
<td>Goat</td>
</tr>
<tr>
<td>pY1234/1235 Met</td>
<td>Cell Signaling, Beverly, MA (3126)</td>
<td>1: 100</td>
<td>Rabbit</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Cell Signaling, Beverly, MA (9582S)</td>
<td>1: 100</td>
<td>Rabbit</td>
</tr>
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</table>
Table 6. Fluorescent-Conjugated Antibodies used for Confocal Microscopy

<table>
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<tr>
<th>Secondary Antibody</th>
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<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor 568 goat anti-rabbit IgG</td>
<td>Invitrogen, Burlington, ON (A-11036)</td>
<td>1: 200</td>
</tr>
<tr>
<td>Alexa Fluor 546 donkey anti-goat IgG</td>
<td>Invitrogen, Burlington, ON (A-11056)</td>
<td>1: 200</td>
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<td>Alexa Fluor 488 goat anti-rabbit IgG</td>
<td>Invitrogen, Burlington, ON (A-11008)</td>
<td>1: 200</td>
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<tr>
<td>Alexa Fluor 488 conjugated EC10 anti-chicken Src</td>
<td>Upstate Cell Signaling Solutions, Lake Placid, NY (05-185)</td>
<td>1: 200</td>
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Table 7. Direct Stains used for Confocal Microscopy

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<th>Direct Stain</th>
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<td>DAPI (DNA)</td>
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<td>Red Phalloidin (actin)</td>
<td>Invitrogen, Burlington, ON (A12380)</td>
<td>1:600</td>
</tr>
<tr>
<td>Blue Phalloidin (actin)</td>
<td>Invitrogen, Burlington, ON (A22281)</td>
<td>1:40</td>
</tr>
</tbody>
</table>

2.10 Evaluation of cell morphology

Cell morphology was assessed by looking at both phase contrast and confocal images. The filopodial extensions were visually assessed as being strongly present, weakly present or not present. Using confocal microscopy, cell spreading was assessed by looking at comparative fields under the same magnification. X-y images per group were assessed. In addition, when determining the effect of the inhibitors (CPA7, Dasatinib, HGF shRNA) on the cells, cell spreading was also assessed initially by the ability of cells to round up (strong refractive index) or not under phase contrast illumination.
2.11 Co-localization calculations

Preliminary overlap analyses were done using Image Pro Plus version 6.1 (www.mediacy.com/pdfs/colocfluorprobes.pdf.) software as described in the online manual. This imaging software analyzes the presence of red versus green or green versus blue fluorescence in each pixel over the whole image. The Pearson correlation coefficient was used to express the proportion of red/green and green/blue overlap with respect to the total images.

2.12 Densitometric analysis

Densitometric analysis for Western blotting was performed using CorelDraw Photo-Paint 12 and represents the product of band intensity (mean) and area (pixels). All values were normalized to the background intensity of the gel, and the data presented was also normalized against the densitometric values of the loading control (p120RasGAP) to account for differences in overall protein concentration. The phosphorylated proteins were also normalized against the densitometric values of their corresponding pan proteins.

2.13 Statement on reproducibility of results

All results presented herein were reproduced to an ‘n’ of 2, unless otherwise indicated in figure captions.
CHAPTER 3 – RESULTS

3.1 Cell lines characterization in 2D culture

Since cell confluence is known to affect Src/Stat3 signaling status [178,229], both EPH4 and AC2M2 cell lines were grown under conditions recommended for each line to the indicated densities. Cells were harvested in 500µl of 2X SDS sample buffer and equal protein amounts for both cell lines were subjected to 8% SDS-PAGE under reducing conditions, followed by Western blotting with the indicated antibodies under identical conditions. The results, as shown in Figure 12, revealed several differences between the EPH4 and AC2M2 cells. While most of the proteins analyzed were detectable in both EPH4 and AC2M2 cells, pY\textsubscript{1234/1235}Met expression was much higher in AC2M2 cells, which also produce higher amounts of HGF, the ligand for Met. While normal breast stromal cells often produce growth factors and normal epithelial cells express cognate receptors, neoplastic cells such as AC2M2 may have acquired the expression of both HGF and its receptor, thereby creating an autocrine loop that may contribute to their transformed nature and tumourigenic capacity. Relative expression levels of Src/pY\textsubscript{418}Src and Stat3/pY\textsubscript{705}Stat3 proteins were also higher in the AC2M2 line, and this demonstrates the use of the AC2M2 line as an appropriate autocrine model for the study of cooperative Src-Stat3 signaling on HGF transcription. Total Src and Stat3 expression in the EPH4 cells, on the other hand, seemed to be dependent on cell-cell contact since it increases as confluence increases. The expression of the phosphorylated forms of Src and Stat3 in this cell line followed the same pattern as their pan proteins. To control for the cell-cell adhesion induced Src/Stat3 activation observed in the EPH4 cells, we decided to keep the cells at a confluence of 80% or lower (at which point Src and Stat3 activation is still
Figure 12. EPH4/AC2M2 Characterization in 2D

EPH4 cells or AC2M2 cells in 2D grown to the indicated densities were harvested in 500µl of 2X SDS sample buffer. Protein concentrations were normalized and equal protein amounts (20µg) were subjected to 8% SDS-PAGE under reducing conditions, and Western blotting was performed, probing with the indicated antibodies. p120RasGAP was used as an independent loading control. Results are representative of at least two experiments.
minimal) in subsequent experiments. Failure to detect expression of the milk protein β-casein in the malignant AC2M2 cells was expected; however failure to detect such a protein in the EPH4 cells might indicate that these cells are unable to fully differentiate in a 2D environment. Additionally, Stat5 protein expression showed very little change in either cell line under the various conditions tested.

3.2 Dependency of AC2M2 cell transformation on Src-Stat3 signaling

As mentioned in the introduction, both activated Src and Stat3 have been shown to transduce signals downstream of the HGF/Met pathway and seem to play essential roles in the initiation and transformation of breast tumourigenesis. Therefore, the goal of my second objective was to investigate the effects of Src and Stat3 inhibition in a breast carcinoma model, aiming to elicit a reversion of the malignant metastatic phenotype to a more normal, differentiated phenotype. The inhibitors employed individually or in unison, were hypothesized to elicit a reversion of the malignant AC2M2 phenotype. Concurrent use of pY418Src and pY705Stat3 inhibitors was expected to have a synergistic effect. The malignant cell line AC2M2 was evaluated in comparison to the EPH4 cells, which represent the normal differentiated phenotype. Concentrations (Dasatinib – 1000/500 nM; CPA7 – 100 μM) that were non-toxic were used in the present study, as determined by cell recovery (approximately 80-90%) as assessed by cell adhesion to the plate and lack of nuclear fragmentation indicative of nuclear apoptosis (assessed by DAPI staining). The results shown are sufficient for a preliminary statistical analysis (especially pYStat3 and β–catenin), but will require additional numbers of cells assayed for further validation.
3.2.1 Effect of CPA7 and/or Dasatinib on Morphological Changes

Morphology was assessed using immunofluorescence and confocal microscopy (Figure 13). Staining with TRITC-phalloidin allowed evaluation of the morphological changes in polymerized actin filaments, cell shape and filopodial extensions between the control and inhibitor treated groups. The EPH4 cells expressed well organized cellular structure and a cuboidal cell shape with very few filopodial extensions. In contrast, the malignant AC2M2 cells, showed irregular cellular structure with marked cell spreading, focal adhesions and filopodial extensions. The CPA7 treated and Dasatinib treated AC2M2 cells showed reduced filopodial extensions and less cell spreading. However, the morphological changes observed in the combined treatment groups were much more dramatic. The cells in the combined treatment groups showed a morphology closely approaching the cuboidal shape of the normal EPH4 cells, and also exhibited a loss of filopodia and focal adhesions. The morphology of the EPH4 cells, as evaluated by actin filament staining, seemed to be relatively unchanged with any of the inhibitor treatments.

3.2.2 Effect of CPA7 and/or Dasatinib on pY418Src localization and expression

Immunoreactivity for pY418Src, the auto-phosphorylation site in the kinase domain of Src, was used as an indication of active Src. Confocal images of pY418Src localization are presented in Figure 13. A low level of cytoplasmic pY418Src was present in the control (DMSO only) and CPA7 treated normal epithelial cells (EPH4). Dasatinib treatment alone or combined with CPA7 resulted in very low detectable cytoplasmic pY418Src. In the control malignant cells (AC2M2), pY418Src was localized in the cytoplasm and most strongly detected near the cell membrane. The CPA7 treatment did not alter the
Figure 13. Alterations in pY418 Src localization and cell morphology with inhibitor treatments

Representative confocal images of pY418Src localization (green) and actin filaments (red) in both cell lines detected using immunofluorescence techniques are shown. Cells were plated at 50% confluence in complete 2D medium (see Appendix). Next day medium was replaced with media containing DMSO/ddH2O (control), CPA7 (100μM), Dasatinib (1000nM), or CPA7 (100μM) and Dasatinib (500nM). Following an 18 hour incubation, cells were fixed in 3% PFA, permeabilized with 25μg/ml digitonin, and non-specific binding was blocked with 3% BSA. Cells were then immunostained for pY418Src (green) and phalloidin (red). Coverslips were mounted onto glass slides using Mowiol mounting medium. Photomicrographs were taken using a Leica TCS SP2 multi-photon confocal microscope. Combined drug treatment markedly inhibited pY418Src. In addition, AC2M2 cells show large morphological differences, approaching a cuboidal shape as seen in EPH4 cells. Scale bars as indicated (17-24μm). Results are representative of two experiments.
<table>
<thead>
<tr>
<th></th>
<th>EPH4</th>
<th>AC2M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (DMSO)</td>
<td><img src="image1" alt="Control (DMSO) EPH4" /></td>
<td><img src="image2" alt="Control (DMSO) AC2M2" /></td>
</tr>
<tr>
<td>CPA7 (100μM)</td>
<td><img src="image3" alt="CPA7 (100μM) EPH4" /></td>
<td><img src="image4" alt="CPA7 (100μM) AC2M2" /></td>
</tr>
<tr>
<td>Dasatinib (1000nM)</td>
<td><img src="image5" alt="Dasatinib (1000nM) EPH4" /></td>
<td><img src="image6" alt="Dasatinib (1000nM) AC2M2" /></td>
</tr>
<tr>
<td>CPA7(100μM) CPA7(500μM) Dasatinib</td>
<td><img src="image7" alt="CPA7(100μM) CPA7(500μM) Dasatinib EPH4" /></td>
<td><img src="image8" alt="CPA7(100μM) CPA7(500μM) Dasatinib AC2M2" /></td>
</tr>
</tbody>
</table>
localization patterns seen in the controls for the malignant cells. The Dasatinib and combined treatment groups showed a decrease in detectable cytoplasmic pY$_{418}$Src in both cell lines.

3.2.3 Effect of CPA7 and/or Dasatinib on pY$_{705}$Stat3 localization and expression

Immunoreactivity for pY$_{705}$Stat3, which homodimerizes and translocates to the nucleus, was used as an indication of active Stat3. Confocal images of pY$_{705}$Stat3 localization are presented in Figure 14. The proportion of cells exhibiting nuclear pY$_{705}$Stat3 was based upon three to five images captured per slide and is shown in Table 8. The EPH4 line expressed low levels of pY$_{705}$Stat3 localized mainly in the cortical cytoplasmic region for the control, CPA7 treated and Dasatinib treated cells; no nuclear localization was evident. The AC2M2 line showed nuclear expression of pY$_{705}$Stat3 in 7/14 (50%) of cells examined, as well as expression in the cortical cytoplasmic area in all cells. The separate CPA7 and Dasatinib treatments were able to abolish the detectable nuclear pY$_{705}$Stat3 (0/10 and 0/24, respectively) signal, while pY$_{705}$Stat3 remained in the cortical cytoplasm. The observed inhibitions were statistically significant (p=0.018, p =0.00027 respectively) using a Fisher Exact double sided test. The combined treatment group of AC2M2 cells showed a significant decrease to baseline levels in both cytoplasmic and nuclear (0/7, p=0.047) pY$_{705}$Stat3 levels, indicating a likely synergistic effect of the inhibitor treatments (Table 8). However, additional images are needed for further statistical analysis.
Figure 14. Alterations in pY\textsubscript{705}Stat3 localization with inhibitor treatments

Representative confocal images of pY\textsubscript{705}Stat3 localization (green) and nuclei (blue) in both cell lines detected using immunofluorescence techniques are shown. Cells were plated at 50% confluence in complete 2D medium (see Appendix). Next day medium was replaced with media containing DMSO/ddH\textsubscript{2}O (control), CPA7 (100µM), Dasatinib (1000nM), or CPA7 (100µM) and Dasatinib (500nM). Following an 18 hour incubation, cells were fixed in 3% PFA, permeabilized with 25µg/ml digitonin, and non-specific binding was blocked with 3% BSA. Cells were then immunostained for pY\textsubscript{705}Stat3 (green) and DAPI (blue). Coverslips were mounted onto glass slides using Mowiol mounting medium. Photomicrographs were taken using a Leica TCS SP2 multi-photon confocal microscope. Combined drug treatment caused a decrease in nuclear pY\textsubscript{705}Stat3 in the malignant cell line. Scale bars as indicated (16-30µm). Preliminary overlap analyses were done using Image Pro Plus version 6.1 software as described in the online manual www.mediacy.com/pdfs/colocfluorprobes.pdf. Results are representative of one experiment.
<table>
<thead>
<tr>
<th></th>
<th>EPH4</th>
<th>AC2M2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pY705Stat3</strong></td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td><strong>DAPI</strong></td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td><strong>Control (DMSO)</strong></td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>CPA7 (100 μM)</td>
<td>![Image]</td>
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<tr>
<td><strong>Dasatinib (500 nM)</strong></td>
<td>![Image]</td>
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<tr>
<td>CPA7 (100 μM)</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td><strong>Dasatinib (1000 nM)</strong></td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>
3.2.4 Effect of CPA7 and/or Dasatinib on HGF/pY1234/1235Met localization and expression

Co-localization of HGF and pYMet was used as an indicator of an active HGF autocrine loop. Confocal images of HGF and pY1234/1235Met staining localization are presented in Figure 15. Low levels of perinuclear HGF and cytoplasmic pY1234/1235Met were detected in EPH4 cells, but HGF and pY1234/1235Met were not colocalized. The malignant cell line, AC2M2, showed strong membranous staining with co-localization of HGF and pY1234/1235Met at focal adhesions, consistent with the establishment of an HGF autocrine loop. In both cells lines, the separate and combined inhibitor treatment groups showed a dramatic decrease in detectable pY1234/1235Met and HGF.

3.2.5 Effect of CPA7 and/or Dasatinib on β-catenin localization and expression

β-catenin was employed as a marker of transformation: with normal cells exhibiting localization in the cortical cytoplasmic region and at intercellular junctions, whereas malignant transformation was expected to induce nuclear translocation. Confocal images of β-catenin localization are presented in Figure 16. The proportion of cells exhibiting nuclear β-catenin based upon three to five images captured per slide was assessed and is shown in Table 8. As expected, the EPH4 cells expressed cortical cytoplasmic β-catenin in control and all treatment groups. Dasatinib treatment resulted in a tighter membrane association of β-catenin than control or CPA7-treated cells. The control (DMSO-treated) AC2M2 cells, on the other hand, showed strong nuclear β-catenin in 31/38 cells (82%) (Table 8).
Alterations in HGF localization (red) and pY_{1234/1235}Met (green) in both cell lines detected using immunofluorescence techniques are shown. Nuclei are shown in blue (DAPI). Cells were plated at 50% confluence in complete 2D medium (see Appendix). Next day medium was replaced with media containing DMSO/ddH_{2}O (control), CPA7 (100μM), Dasatinib (1000nM), or CPA7 (100μM) and Dasatinib (500nM). Following an 18 hour incubation, cells were fixed in 3% PFA, permeabilized with 25μg/ml digitonin, and non-specific binding was blocked with 3% BSA. Cells were then immunostained for HGF (red) and pY_{1234/1235}Met (green). Coverslips were mounted onto glass slides using Mowiol mounting medium. Photomicrographs were taken using a Leica TCS SP2 multi-photon confocal microscope. Combined drug treatment markedly inhibited both HGF and pY_{1234/1235}Met. Scale bars as indicated (15-21μm). Preliminary overlap analyses were done using Image Pro Plus version 6.1 software as described in the online manual www.mediacy.com/pdfs/colocfluorprobes.pdf. Results are representative of two experiments.
<table>
<thead>
<tr>
<th>HGF pY_{1234/1235} Met</th>
<th>Control (DMSO)</th>
<th>EPH4</th>
<th>AC2M2</th>
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</thead>
<tbody>
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<td><strong>Overlap = 37%</strong></td>
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<td><img src="image2" alt="Control (DMSO) AC2M2" /></td>
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<tr>
<td>CPA7 (100μM)</td>
<td><img src="image3" alt="CPA7 (100μM) EPH4" /></td>
<td><img src="image4" alt="CPA7 (100μM) AC2M2" /></td>
<td></td>
</tr>
<tr>
<td>Dasatinib (1000nM)</td>
<td><img src="image5" alt="Dasatinib (1000nM) EPH4" /></td>
<td><img src="image6" alt="Dasatinib (1000nM) AC2M2" /></td>
<td></td>
</tr>
<tr>
<td>CPA7 (500μM) / Dasatinib (500nM)</td>
<td><img src="image7" alt="CPA7 (500μM) / Dasatinib (500nM) EPH4" /></td>
<td><img src="image8" alt="CPA7 (500μM) / Dasatinib (500nM) AC2M2" /></td>
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</table>
Figure 16. Alterations in β-catenin localization with inhibitor treatments

Alterations in β-catenin localization (green) and nuclei (blue) in both cell lines detected using immunofluorescence techniques are shown. Cells were plated at 50% confluence in complete 2D medium (see Appendix). Next day medium was replaced with media containing DMSO/ddH2O (control), CPA7 (100μM), Dasatinib (1000nM), or CPA7 (100μM) and Dasatinib (500nM). Following an 18 hour incubation, cells were fixed in 3% PFA, permeabilized with 25μg/ml digitonin, and non-specific binding was blocked with 3% BSA. Cells were then immunostained for β-catenin (green). Coverslips were mounted onto glass slides using Mowiol mounting medium. Photomicrographs were taken using a Leica TCS SP2 multi-photon confocal microscope. Combined drug treatment caused a shift of β-catenin from the nucleus to the membrane in the malignant cell line. Scale bars as indicated (12-21μm).

Preliminary overlap analyses were done using Image Pro Plus version 6.1 software as described in the online manual www.mediacy.com/pdfs/colocfluorprobes.pdf. Results are representative of one experiment.
<table>
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<tr>
<th><strong>β-catenin</strong></th>
<th><strong>DAPI</strong></th>
<th><strong>EPH4</strong></th>
<th><strong>AC2M2</strong></th>
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<td><img src="image2" alt="Control" /></td>
<td>Overlap = 23%</td>
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<tr>
<td><strong>Dasatinib (1000nM)</strong></td>
<td><img src="image5" alt="Dasatinib" /></td>
<td><img src="image6" alt="Dasatinib" /></td>
<td></td>
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<tr>
<td><strong>CPA7 (100μM) - Dasatinib (500nM)</strong></td>
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### Qualitative assessment:

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<th>pY_{705}Stat3</th>
<th>HGF pY_{1234/1235}Met</th>
<th>β-catenin</th>
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<tbody>
<tr>
<td>Control (DMSO)</td>
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<td>Cortical cytoplasmic</td>
<td>No colocalization</td>
<td>Cortical cytoplasmic</td>
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<td></td>
<td>AC2M2</td>
<td>Cytoplasmic</td>
<td>Nuclear and membranous</td>
<td>Colocalize at focal adhesions</td>
<td>Nuclear and membranous</td>
</tr>
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<td>CPA7</td>
<td>EPH4</td>
<td>Cortical cytoplasmic</td>
<td>Cortical cytoplasmic</td>
<td>Little detectable</td>
<td>Cortical cytoplasmic</td>
</tr>
<tr>
<td></td>
<td>AC2M2</td>
<td>Cytoplasmic</td>
<td>Cortical cytoplasmic</td>
<td>Little detectable No colocalization</td>
<td>Cortical cytoplasmic</td>
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<tr>
<td>Dasatinib</td>
<td>EPH4</td>
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<td>Weak cortical cytoplasmic</td>
<td>Little detectable</td>
<td>Cortical cytoplasmic</td>
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<tr>
<td></td>
<td>AC2M2</td>
<td>Very little detectable</td>
<td>Weak cortical cytoplasmic</td>
<td>Little detectable No colocalization</td>
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<tr>
<td>CPA7 Dasatinib</td>
<td>EPH4</td>
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<td>Very little detectable</td>
<td>Cortical cytoplasmic</td>
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<tr>
<td></td>
<td>AC2M2</td>
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<td>Cortical cytoplasmic</td>
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### Quantitative assessment:

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<th>#cells expressing nuclear β-catenin</th>
<th>p-value two-tailed</th>
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<td></td>
<td>Positive</td>
</tr>
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<tr>
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<td>0</td>
<td>7</td>
<td>0.047</td>
<td>0</td>
</tr>
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</table>

Table 8. Summary of changes observed in expression and localization of our proteins of interest using immunofluorescence techniques.

Qualitative and quantitative assessments of staining patterns with the indicated antibodies are shown. For nuclear staining of pYStat3 and β-catenin, a preliminary assessment of statistical significance was done using a nonparametric Fisher Exact test (two sided) as described in [http://www.quantitativeskills.com/sisa/statistics/fishrhlp.htm](http://www.quantitativeskills.com/sisa/statistics/fishrhlp.htm).
These cells also exhibited β-catenin expression in the cortical cytoplasmic region. Both the separate inhibitor treatments effectively eliminated (0/5, 1/19, respectively) detectable nuclear β-catenin in the malignant cell line, showing expression only in the cortical cytoplasmic region of treated cells. The observed inhibition was statistically significant (p=0.000822, p <0.0001, respectively, using a Fisher Exact double sided test). Likewise a similar blocking of nuclear β-catenin staining was observed in the double treatment group, although additional images would be required for statistical analysis (Table 8).

3.2.6 Effect of CPA7 and/or Dasatinib on pY418Src, pY705Stat3, HGF/pY1234/1235Met, and β-catenin expression as measured by Western blotting

The main features of immunofluorescence and confocal microscopy are qualitative, namely high sensitivity and the ability to localize fluorescent molecules inside cells. Unfortunately, this approach lacks the ability to quantify protein expression. We therefore used Western blotting and densitometric analysis to assess relative protein levels in the various treatment groups, as shown in Figures 17A and 17B. All treatments seemed to have no detectable effect on the expression of pan Stat3 and pan Src in either cell line. The pY705Stat3 signal in EPH4 cells was too weak for any conclusions to be drawn. However, both CPA7 and Dasatinib treatment alone, caused partial inhibition of pY705Stat3 in AC2M2 cells. In addition, when used in combination, the drugs had a strong inhibitory effect on pY705Stat3 inhibition, indicating that they can act synergistically (Figure 17B). As expected, CPA7 did not inhibit pY418Src in the AC2M2 cells. However, CPA7 seemed to enhance pY418Src in EPH4 cells possibly due to a compensation feedback effect. The redundancy and compensation in vivo among the
members of the Src family is a widely established fact [117]. In addition, the antibody used against pY418Src, also cross reacts with Fyn and Yes. Dasatinib alone had very little effect on pY418Src (or any other molecule in AC2M2 cells tested). One of the reasons for this marginal effect of Dasatinib might be that Dasatinib is blocking de novo Src phosphorylation; however, it has no detectable effect on already activated Src. The pY705Stat3 signal in EPH4 was too weak for any conclusions to be drawn.

CPA7 treatment (both alone and in combination with Dasatinib) reduced both pan Met and pY1234/1235Met expression in AC2M2 cells, suggesting that Stat3 inhibition might inhibit expression, of Met. This is also supported by densitometric analysis shown on the three panels on the left (Figure 17B). The Met and pY1234/1235Met signal in EPH4 cells was too weak for any conclusions to be drawn.

Surprisingly, Western blotting did not show a significant change in HGF expression after the treatments. The reason might be that soon after its expression, HGF, is usually quickly secreted as an inactive precursor form, and is then specifically activated by HGF activator (HGFA) to the active form. This suggests that examining the Conditioned Media of the cell lines, as opposed to the cell lysates, might be a more direct way to assess the effect of various inhibitors on HGF production.

The treatments did not affect the cellular pool of total β-catenin in either EPH4 or AC2M2 cells (Figure 17B, last panel), demonstrating that the elimination of nuclear β-catenin observed by confocal imaging in the AC2M2 cells was due to relocalization of β-catenin to the membrane rather than its degradation. Finally, CPA7 treatments (both alone and in combination with Dasatinib) inhibited cyclin D1 expression in both cells lines but more dramatically in the AC2M2
Figure 17A. Effect of CPA7 and/or Dasatinib on EPH4 and AC2M2

Cells were plated at 50% confluence in complete 2D medium (see Appendix). Next day medium was replaced with media containing DMSO/ddH2O (control), CPA7 (100μM), Dasatinib (1000nM), or CPA7 (100μM) and Dasatinib (500nM). Following an 18 hour incubation, cells were harvested in 500μl of 2X SDS sample buffer. Protein amounts were normalized and equal protein amounts (20μg) were subjected to 8% SDS-PAGE under reducing conditions, and Western blotting was performed, probing with the indicated antibodies. p120RasGAP was used as an independent loading control. Results are representative of at least two experiments.
Figure 17B. Densitometric analysis for AC2M2 protein expression

Densitometric analysis was carried out using CorelDraw Photo-Paint V12, and all signal intensities were normalized according to corresponding total protein loading control (p120RasGAP) for individual lysates. Phosphorylation intensities for pYMet and pYStat3 were also normalized to corresponding pan protein lysates.
3.3 Effect of activated Stat3 on the morphology of EPH4 cells

To further elucidate the transforming properties of the Src-Stat3 axis, we transduced non-malignant EPH4 cells with constitutively active Stat3 in an effort to induce a malignant morphology resembling the AC2M2 phenotype. A Stat3C form has been previously designed to imitate the behaviour of activated Stat3. In this molecule, substitution of two cysteine residues for A661 and N663 within the C-terminal loop of the SH2 domain of Stat3 induces spontaneous dimerization of the molecule [154]. The cysteine residues might possibly allow for inter-chain disulfide bridges to form between Stat3 monomers and render them capable of dimerizing in the absence of phosphorylation at Y705 residue [154]. The Stat3 dimers can then translocate to the nucleus where they can bind to DNA and activate gene transcription [173]. As expected, ectopic expression of Stat3C caused cellular transformation in the EPH4 cells as demonstrated by a spindlular morphology and a greater number of filopodial extensions compared to untransduced cells which showed a cuboidal morphology with no extensions (compare Figures 18 and 21). In addition, Stat3C (GFP-tagged) appeared to be localized in the nucleus in EPH4 cells transduced with GFP-Stat3C, as illustrated by the white arrows (Figure 18).

3.4 Effect of activated Src on the morphology of EPH4 cells

To further elucidate the transforming properties of the Src-Stat3 axis, we transduced the non-malignant EPH4 cells with constitutively active Src (Figure 19) in an
Figure 18. Effect of constitutively active Stat3 on EPH4 morphology

The packaging line phoenix-Stat3C (HEK293T-based line) expressing the gene of interest (Stat3C in this case) tagged to GFP was grown to confluence for a couple of days in DMEM with 10% FBS. The viral supernatant was collected and filtered using a nalgene 0.45μm sterile filter and the viral supernatant containing polybrene (8 mg/ml final concentration) was added to the target cells. The target cell line (EPH4) was seeded at 20% confluence. For every 6-cm EPH4 plate, one 10-cm plate of the packaging line was plated in 5ml of DMEM 10%FBS. Twenty-four hours post transduction the media was replaced with regular EPH4 media. This transduction process was repeated two more times to increase the transduction efficiency. Forty-eight hours later, cells were checked for GFP expression and the green cells (cells expressing the gene of interest) were cloned. A representative phase contrast photograph of the cloned EPH4 cells was captured using a Leica Leitz DM IL at the magnifications indicated (200X) using an attached digital camera (Nicon coolpix 995). STAT3C appeared to be primarily localized in the nucleus of EPH4 cells, as illustrated by the white arrows (right). Results are representative of at least two experiments.
PT67 fibroblasts (NIH3T3-based line expressing the 10A1 viral envelope, Becton Dickson, Mountain View, VA) were stably transfected with a constitutively active chicken Src mutant (Src-Y527F) containing a puromycin selection marker as previously described in our laboratory [146,225] to generate mass populations of retrovirus-producing cells. Viral particles in supernatants from PT67 cells were concentrated using a 0.45-μm filter. The target cell line (EPH4) was seeded at 20% confluence. For every 6-cm EPH4 plate, one 10-cm plate of the packaging line was plated in 5ml of DMEM 10%FBS. EPH4 cells were exposed to retrovirus expressing activated Src in the presence of 8 mg/ml polybrene. Twenty-four hours post transduction the media was replaced with regular EPH4 media. This transduction process was repeated three times to increase the transduction efficiency. Cells were then selected with 8 μg/ml puromycin initially which was then reduced to 4μg/ml. Cloned EPH4 cells were fixed in 3% PFA, permeabilized with 25μg/ml digitonin, and non-specific binding was blocked with 3% BSA. Exogenously expressed Src was visualized using the chicken-specific Src EC10 antibody. Morphology was assessed using phalloidin staining. Coverslips were mounted onto glass slides using Mowiol mounting medium. Photomicrographs were taken using a Leica TCS SP2 multi-photon confocal microscope. Negative control (no primary antibody) is shown on the left. Untransfected cells showed a cuboidal shape with no filopodia extensions similar to Figure 13 (data not shown). Scale bars= 30μm. Results are representative of two experiments.
effort to induce a malignant morphology resembling the AC2M2 phenotype. For this, we expressed a chicken Src protein, which has an activating tyrosine to phenylalanine mutation at position 527 in the Src coding sequences (SrcY527F) [146,225].

Exogenously expressed Src was visualized using the chicken-specific Src EC10 antibody. Morphology of actin filaments and filopodial extensions was assessed using phalloidin staining (Figure 19). As we expected, ectopic expression of the activated Src mutant caused cellular transformation in the EPH4 cells as demonstrated by marked cell spreading and a greater number of filopodial extensions and focal adhesions. Untransfected cells showed a cuboidal shape with no filopodia extensions similar to Figure 13 (data not shown).

3.5 Dependency of AC2M2 cell transformation on HGF expression

Our demonstration of Src/Stat3-dependent up-regulation of HGF expression in breast carcinoma cells suggests that targeting autocrine HGF loops directly might suppress breast cancer progression with minimal effect on normal tissues. To test this notion, RNA interference was used to knock down HGF expression in carcinoma cells. For higher efficiency of expression, we used lentiviral particles (carrying puromycin resistance and GFP tagged) expressing the relevant shRNA for HGF. Results from a preliminary experiment are shown in Figure 20. Control cells (no vector) showed strong cytoplasmic HGF and no GFP. They also presented strong cortical cytoplasmic and membranous staining of pY1234/1235Met particularly at the focal adhesions. Transduction with HGF shRNA vectors 3 and 4 induced a decrease in HGF and pY1234/1235Met expression followed by a morphological reversion to a more normal phenotype as
Alterations in HGF (red), pY1234/1235Met (red) and GFP (green) expression in AC2M2 cells were detected using immunofluorescence techniques. AC2M2 cells were grown in DMEM with 10% FBS to 50% confluency. The filtered virus supernatant was added with polybrene (8 mg/ml final concentration), and the cells were placed at 37 °C overnight. Fresh medium was added to the cells on the following day. This transduction process was repeated two more times to increase the transduction efficiency. Green GFP expression normally reached its peak (≈70% of cells) after the first round of puromycin selection (8 μg/ml final concentration). Un-infected cells that went through a mock process without vector were used as control. All the other pictures shown are of cells after their first round of puromycin selection. Vectors 3, 4 and 5 are shRNA lentivirus constructs (See Table 2 on p 38 for detailed vector description). Scale bars are as indicated (15 μm-30 μm). Results are representative of at least one experiment. Approximately 10-15 cells per group were imaged. Further experiments are required to optimize and validate these findings.
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indicated by increased cell rounding and cell-cell contacts and reduced filopodial extensions. Vector 5, on the other hand, was not as effective in blocking either HGF or pY\textsubscript{1234/1235}Met expression. No pictures are shown for vectors 1 and 2 because they proved to be detrimental for the cells (see Figure 7 and Table 2 for vector description). Further experiments are needed to optimize and validate these preliminary findings.

3.6 Morphological features of cell lines in 2D vs 3D culture

EPH4 and AC2M2 cells were cultured under conditions promoting growth in two-dimensions (2D) as monolayers and in three-dimensions (3D) on Matrigel matrix (Figure 21). In monolayer, the EPH4 cells assumed a cuboidal shape, showing minimal cell spreading and no focal adhesions or extensions. The transformed cell line on the other hand, AC2M2, showed marked cellular disorganization, greater cell spreading as well as a larger number of focal adhesions and filopodial extensions than EPH4 cells.

When grown on Matrigel and in the presence of lactogenic hormone, EPH4 cells polarized and formed differentiated mammospheres. These structures resembled little sacs of cells called acini (alveoli) in which milk is made. The mammospheres that developed were large and luminal apoptosis was detected as a darkening in the central region of the mammospheres. In the mammary gland this phase will be connected to ducts into which milk is secreted. By contrast, the highly metastatic human breast carcinoma cell line AC2M2 grown under the same conditions, did not form polarized mammospheres in 3D. These cells divided aggressively and in an uncontrolled fashion. They were also more migratory and invasive, and grew into disorganized clumps.
3.7 Biochemical features of cell lines in 3D

In contrast to 2D (Figure 12), EPH4 in 3D (Figure 22) did not express activated Src and Stat3, while pY_{1234/1235}Met and HGF expression was minimal. Also decreasing Stat3 and increasing Stat5 levels correlated with transient expression of cyclin D1 and increasing expression of β-casein, a milk protein (Figure 22). Since EPH4 cells were able to produce milk proteins in 3D, they are representative of an \textit{in vivo} lactating mammary gland. AC2M2 cells on the other hand showed constant levels of protein expression throughout. It is important to note that consistent with their malignant phenotype, AC2M2 cells expressed higher levels of pY_{1234/1235}Met, HGF, pY_{705}Stat3 and pY_{418}Src, compared to EPH4 cells. AC2M2 cells did not express β-casein. These findings suggest that differentiation of EPH4 cells is better maintained in 3D. Furthermore, different protein expression profiles were exhibited by non-malignant versus malignant breast cells.
Figure 21. Morphological features of cell lines in 2D vs 3D

**Top:** Cells were cultured in 10cm plates and maintained in complete 2D medium (see Appendix). The EPH4 cells expressed well organized cellular structure and a cuboidal cell shape with very few filopodial extensions. The malignant cell line, AC2M2, showed marked cellular disorganization in comparison to the EPH4 cells. The AC2M2 line displayed greater cell spreading as well as a larger number of focal adhesions and filopodial extensions than the EPH4 cells.

**Bottom:** Cells were cultured in Matrigel-coated 6-well plates and maintained in complete 3D medium (see Appendix). In the presence of lactogenic hormone, EPH4 cells polarized and formed differentiated mammospheres. By contrast, the highly metastatic human breast carcinoma cell line AC2M2 divided aggressively and in an uncontrolled fashion. They were also more migratory and grew into disorganized aggregates.

A representative phase contrast photograph of cells under each condition was captured using a Leica Leitz DM IL at the magnifications indicated (200X) using an attached digital camera (Nicon coolpix 995). Results are representative of at least four experiments.
Figure 22A. Cell lines characterization in 3D

EPH4 cells or AC2M2 cells grown in 3D were harvested in 500µl of RIPA buffer at the indicated days. Protein amounts were normalized and equal protein amounts (20µg) were subjected to 8% SDS-PAGE under reducing conditions, and Western blotting was performed, probing with the indicated antibodies. Homogenized mammary gland from a lactating mouse (MGT) was used as an *in vivo* comparison. p120RasGAP was used as an independent loading control. In contrast to 2D, EPH4 cells grown in 3D do not express activated Src and Stat3, and pY_{1234/1235}Met and HGF expression are minimal. Also decreasing Stat3 and increasing Stat5 levels correlate with transient cyclin D1 expression and increasing β-casein expression. AC2M2 cells on the other hand show constant levels of protein expression throughout. It is important to note that they express high levels of pY_{1234/1235}Met, HGF, pY_{705}Stat3 and pY_{418}Src. They do not express β-casein. Results are representative of at least two experiments.

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Figure 22B. Densitometric analysis for EPH4 protein expression

Densitometric analysis was carried out using CorelDraw Photo-Paint V12, and all signal intensities were normalized according to corresponding total protein loading control (p120RasGAP) for individual lysates. Since the data for β-casein were out of range compared to the other four, a second graph showing β-casein using a different scale is shown.
3.8 Events in 3D acinar morphogenesis

During the early stages of morphogenesis in 3D, EPH4 cells proliferated and clustered. A few days later, the cells organized into two groups; (a) an outer layer of polarized cells and (b) an inner cluster of disorganized cells which underwent cell death (corresponding to apoptosis as shown previously in [22]). This later led to the formation of a hollow lumen as characterized by the darkening of the center of the mammospheres as seen by phase contrast microscopy (Figure 23). We took this one step further and decided to add paracrine HGF (10 ng/ml) to the media, which resulted in the formation of tubular structures with no lumen filling. Approximately 20 aggregates were assessed per group. More than 60% of the HGF-induced aggregates were tubular. These results confirm previous reports and support the in vivo importance of HGF in mammary tubulogenesis [28,59].
Figure 23. Events in 3D acinar morphogenesis

EPH4 cells were grown in Matrigel-coated 6-well plates and maintained in complete 3D medium (see Appendix). During the early stages of morphogenesis in 3D, cells proliferated and clustered. A few days later, the cells organized into two groups; (a) an outer layer of polarized cells and (b) an inner cluster of disorganized cells which underwent cell death (corresponding to apoptosis as shown previously in [22]). This later led to the formation of a hollow lumen as characterized by the darkening of the center of the mammospheres as seen by phase contrast microscopy. We took this one step further and decided to add paracrine HGF (10 ng/ml) to the media which resulted in the formation of tubular structures with no lumen filling. More than 60% of the HGF-induced aggregates were tubular. Approximately 20 aggregates were assessed per group. None of the control aggregates showed tubulogenesis. A representative phase contrast photograph of cells at each stage was captured using a Leica Leitz DM IL at the magnifications indicated (300X) using an attached digital camera (Nikon coolpix 995). Results are representative of at least three experiments and 10-15 pictures taken per experiment. Upper panel was adapted from [21].
3.9 Fibronectin and HGF in combination stimulate tubular outgrowths with lumen filling in epithelial mammospheres

Stromal interactions play a key role in metastasis. ECM proteins involved in this process mainly include integrin-ligands [17]. For instance, the overexpression of integrin α5β1 and its receptor Fibronectin (FN) have been shown to elicit EMT in mammary epithelial cells [14]. To examine the involvement of integrin α5β1 in HGF-enhanced cell migration and transformation, FN, the major ligand of α5β1, was applied in addition to HGF to EPH4 cells grown on Matrigel. Our model EPH4 cells were cultured in Matrigel-coated 6-well plates and maintained in complete 3D medium (see Appendix). On day 2, the medium was replaced with serum free media. Experiments were terminated after 10 days, with replacement of medium plus factors every other day. HGF and FN (at the concentrations indicated) were added daily, beginning at day 2. Cells were immunostained for DAPI for the nucleus (blue) and results are shown in Figure 24. Fibronectin and HGF in combination stimulated tubular outgrowths with lumen filling in EPH4 epithelial mammospheres and this effect seemed to be dose-dependent. This effect is in contrast to the effect of HGF alone on tubulogenesis (Figure 23). This finding suggests that Fibronectin might modulate the response of epithelial cells to HGF by promoting enhanced epithelial cell migration and proliferation. Unfortunately the FN alone group was contaminated. Thus, future experiments are needed to examine the effect of FN alone to confirm whether there is a synergistic interaction between HGF and Fibronectin.
Figure 24. Fibronectin (FN) and HGF in combination stimulate tubular outgrowths with lumen filling in epithelial mammospheres

EPH4 cells were cultured in Matrigel-coated 6-well plates and maintained in complete 3D medium (see Appendix). On day 2, the medium was replaced with serum free media. Experiments were terminated after 10 days, with replacement of medium plus factors every other day. HGF and FN (at the concentrations indicated) were added daily, beginning at day 2. Cells were fixed in 3% PFA, permeabilized with 25 μg/ml digitonin, and non-specific binding was blocked with 3% BSA. Cells were then immunostained for DAPI for the nucleus (blue). Coverslips were mounted onto glass slides using Mowiol mounting medium. Photomicrographs were taken of the central xy-axis plane using a Leica TCS SP2 multi-photon confocal microscope. FN and HGF in combination stimulate tubular outgrowths with lumen filling in epithelial mammospheres and this effect seems to be dose-dependent. About half of the control aggregates (untreated) which were smaller than the average (i.e. not mature yet) also showed lumen filling but no tubular branching was observed. These phenotypes are characteristic of progression from an epithelial to a mesenchymal phenotype. Approximately 15 aggregates were assessed in each group. Scale bar = 50 μm. Results are representative of two experiments.
CHAPTER 4 – DISCUSSION

This thesis has demonstrated that the induction of an HGF-autocrine-loop and transformation of breast epithelial cells is dependent on the Src-Stat3 signaling axis and that cell adhesion to Fibronectin switches the epithelial phenotype toward transformation.

The most significant results of this study are:

1) Src/Stat3 inhibition induced reversal of the transformed phenotype in the carcinoma cell line, AC2M2, as indicated by: Less cell spreading, fewer focal adhesions and filopodia, decreased HGF expression and Met activation, and shift of β-catenin from the nucleus to the plasma membrane.

2) Activated Stat3 induced transformation in the EPH4 cells as demonstrated by nuclear translocation of activated Stat3, marked cellular disorganization and a greater number of filopodial extensions.

3) Activated Src signaling caused cellular transformation in the EPH4 cells as demonstrated by marked cell spreading and a greater number of filopodial extensions and focal adhesions.

4) HGF shRNA induced an apparent decrease in HGF and pYMet expression in AC2M2 cells as assessed by IIF, followed by a morphological reversion to a more normal phenotype as indicated by increased cell rounding and cell-cell contacts, and reduced filopodial extensions.

5) The differentiated phenotype of non-neoplastic (EPH4) epithelial cells and the transformed phenotype of (AC2M2) cells are better maintained in 3D than in 2D culture.

6) HGF stimulates tubulogenesis of EPH4 epithelial mammospheres in 3D culture.
7) Fibronectin and HGF in combination stimulate tubulogenesis and lumen filling of EPH4 mammospheres in 3D culture.

Together, these results implicate the Src-Stat3 signaling axis and cell-matrix adhesion as important mediators in HGF-Met autocrine signaling. Future studies will assess whether integrin adhesion is linked to transformation of epithelial cells through activation of Src/Stat3 and autocrine HGF/Met signaling.

4.1 Src/Stat3 inhibition induced a reversal of the transformed phenotype in the carcinoma cell line, AC2M2.

*Morphological Changes.*

Breast epithelial cells generally present a cuboidal shape and tend not to layer in 2D culture; the cells instead form cell-cell junctions when close to one another [232]. This is the non-neoplastic phenotype that was observed in the EPH4 cells cultured under 2D conditions in the present study (Figures 13, 21 upper panel), suggesting that these cells are an accurate representation of normal breast epithelium. Metastatic cells, on the other hand, spread out in culture, and have a tendency to overlap onto one another regardless of confluence [233], as was seen in the AC2M2 line studied. The cellular disorganization observed in the AC2M2 cell line (Figures 13, 21 upper panel) is indicative of a loss of differentiation and of a more transformed phenotype. Cellular motility is an indication of metastatic potential in cancer cells. As cells become more motile, they develop an increasing number of focal adhesions and cellular extensions, and also exhibit greater cell spreading [234]. The formation of cell extensions and alterations
in cellular adhesion complexes causing scattering has also been shown to occur when breast epithelial cells are exposed to excess HGF [235]. Breast epithelial cells grown \textit{in vitro} respond to HGF/Met signaling by increasing their motility, undergoing EMT and showing colony dispersal [59]. The increasing cellular disorganization and motility seen in the malignant cell line, AC2M2, further suggests that these cells are expressing the HGF autocrine loop.

The morphology of the EPH4 cells that were treated with inhibitors was found, as expected, to be relatively unchanged from the controls. Normal cell growth is not dependent upon Src-Stat3 signaling [236], thus the inhibitors did not alter the cuboidal EPH4 phenotype. However, when EPH4 cells reach confluence, they become dependent on active Stat3 as one of the pathways which inhibits apoptosis [155]. The irregular shape with the increased filopodial extensions observed for the AC2M2 cells indicate that this line has undergone malignant transformation. Upon treatment with each inhibitor alone, approximately 40-50% of the malignant cells assumed a more cuboidal cell shape and the expression of filopodial extensions decreased. The combination treatment was most effective at eliciting the phenotypic reversion in approximately 90% of the malignant cells, indicating that the concurrent use of the inhibitors most efficiently blocked the Src-Stat3 signaling axis as observed by actin filament staining in Figure 13. The double treated cells exhibited a cuboidal morphology closely comparable to the untreated EPH4 cells. There was also a marked loss of filopodial extensions and focal adhesions. These observations indicate that the inhibitor treatments induced a morphological reversion of the malignant phenotype to that similar to the non-malignant EPH4 cell line.
Src/\ pY_{418}Src\ localization\ and\ expression

A study by Irby and Yeatman [129] suggests that Src over-expression and activation generally correlates with cancer progression. In the present study, this gradation in activated Src expression levels was not readily apparent using immunofluorescence, although this technique tends to be more qualitative than quantitative. The untreated cell lines showed comparable levels of pY\_{418}Src detection, whether malignant or non-malignant. However, Western blot analysis suggested that relative expression levels of panSrc/pY\_{418}Src proteins were higher in the AC2M2 line than in the EPH4 line (Figure 17A). Further densitometric analysis would be required to analyse the actual change in protein expression.

Dasatinib has been shown to inhibit cell growth and filopodial extensions in squamous cell carcinomas and non-small cell lung cancers [237], suggesting that its effects induce phenotypic changes in malignant cells. In addition to morphological changes, Src inhibition has been experimentally shown to decrease phosphorylation of Stat3, which is one of Src’s downstream targets [238]. However, according to our Western blot analysis, Dasatinib treatment was not able to induce a visible decrease in the amount of pY\_{705}Stat3 or even of pY\_{418}Src detected (Figure 17). One of the reasons for this marginal effect of Dasatinib might be that Dasatinib is blocking \textit{de novo} Src phosphorylation, while having minimal effect on already activated Src. Therefore if there is a large pool of active Src, as is the case in AC2M2 cells, pY\_{418}Src would take longer to dissipate, and an 18 hour incubation with Dasatinib may not be sufficient to detect inhibition. Nonetheless, the results observed with Dasatinib treatment lend support to Src’s role in maintenance of the malignant phenotype, since Dasatinib was highly
effective in inducing a reversion of the malignant phenotype, as reflected in β-catenin localization changing from nuclear to cytoplasmic expression (Figure 16).

As expected, CPA7 did not inhibit pY418Src in the AC2M2 cells. However, CPA7 seemed to enhance pY418Src in EPH4 cells (Figure 17A) possibly due to a compensation feedback effect through increased activation of other Src family members (e.g. Fyn or Yes). \textit{In vivo} and \textit{in vitro} functional redundancy among the members of the Src family is a widely established fact [117]. Furthermore, since the Y418 epitope is highly conserved among the different SFKs [110], the antibody used did not allow us to fully distinguish which family members were specifically activated.

\textit{Stat3/ pY705Stat3 localization and expression.}\n
While normal cells exhibit transient activation of Stat3, a persistent activity of this molecule has been demonstrated in a number of human cancers [169-172]. The pY705Stat3 signal in EPH4 cells was very weak (Figure 17A). In contrast, nuclear pY705Stat3 was detected in the AC2M2 cell line (determined by co-localization with DAPI stain) consistent with the contention that pY705Stat3 was binding DNA and altering transcription. As CPA7 does not inhibit the activation and phosphorylation of Stat3 at Tyr705, but rather prevents its interaction with DNA, the relative amounts of pY705Stat3 in the control and CPA7 treated cells were not altered (Figures 14). Therefore, localization is key to the assessment of the efficacy of CPA7 inhibition, which makes the qualitative imaging techniques ideal for measurement. Since total Stat3 protein was constant (as determined by Western blotting, Figures 17A&B), localization of pY705Stat3 in the nucleus versus the cytoplasm could be assessed. CPA7 treatment effectively caused
a decrease in nuclear pY\textsubscript{705}Stat3 (Figure 14), consistent with the drug being effective at blocking pY\textsubscript{705}Stat3 from interacting with DNA. Dasatinib was also able to abolish detectable nuclear pY\textsubscript{705}Stat3 in the malignant AC2M2 cells (Figure 14). A synergistic effect was observed when the Src and Stat3 inhibitors, Dasatinib and CPA7, were administered concurrently. The combination treatment reduced pY\textsubscript{705}Stat3 to barely detectable levels in the malignant cells as determined by Western blotting (Figures 17A&B). The synergy observed with the combined treatment in this experiment suggests that blocking the Src-Stat3 signaling axis in multiple locations produces a stronger inhibition.

\textit{HGF/Met expression and localization}

The HGF autocrine loop occurs when epithelial cells, which already express the receptor Met, begin to express HGF, thereby shifting the paracrine system to an autocrine loop. Our present results provide further support of an HGF autocrine loop in the mammary carcinoma cell line, AC2M2 (Figure 15). Therefore, co-expression of both HGF and its receptor Met may provide a selective advantage for the progression of mammary carcinoma cells toward a more aggressive phenotype, making them independent of signaling from the surrounding stroma. CPA7 treatment (both alone and in combination with Dasatinib) reduced both pan Met and pY\textsubscript{1234/1235}Met expression in AC2M2 cells (Figure 17A), suggesting that Stat3 inhibition might inhibit expression, of Met. It has been previously demonstrated that the expression of the \textit{c-met} gene is inducible by HGF [230,231]. On the other hand, the \textit{HGF} promoter contains a Stat3 binding site at nt-95 [213]. These findings taken together confirm our hypothesis that an
inhibition of pY705Stat3 binding to DNA would lead to a decrease in Met production and consequently lower levels of pY1234/1235Met (Figure 17B).

Consistent with their normal phenotype, endogenous levels of Met and pY1234/1235Met signal in EPH4 cells were very low, making the effects of the inhibitors on these proteins difficult to assess. The inclusion of 5% FBS in the media may partially account for the activation of Met in the EPH4 cells (Figure 15), as serum is known to contain HGF; although its capacity to activate Met is questionable as it gets quickly inactivated through binding to extracellular matrix proteins [100].

Surprisingly, Western blotting of whole cell lysates did not show a significant change in HGF expression in both cell lines after the treatments. One reason might be that soon after its expression the majority of HGF is quickly secreted as an inactive precursor form, and is then specifically cleaved by HGF activator (HGFA) into the active 2-chain αβ HGF dimer [65]. This suggests that examining Conditioned Media (CM) of the cell lines, as opposed to the cell lysates, might be a more direct way to assess the effect of various inhibitors on HGF production. Since the FBS in the media can affect CM protein readings, the cells will need to be maintained in serum-free media for longer than 24 hr before CM collection. Over this time, the secreted proteins would accumulate in CM and putative HGF production could then be identified through Western blot analysis or ELISA.

**β-catenin localization and expression**

β-catenin is localized at the cell membrane in normally differentiated breast tissue, appearing most strongly at intercellular surfaces [239]. Upon transformation to a less
differentiated, malignant phenotype, β-catenin undergoes nuclear translocation [240], upon which it can act as a transcriptional regulator [241]. Consistent with their normal phenotype, the EPH4 cells expressed cortical cytoplasmic β-catenin in control and all treatment groups (Figure 16). β-catenin localization in the cytoplasm of all EPH4 groups confirmed that the cells were exhibiting a non-transformed phenotype. Dasatinib treatment resulted in a tighter membrane association of β-catenin than control or CPA7-treated cells. The control AC2M2 cells, on the other hand, showed strong nuclear β-catenin expression in approximately 82% of cells in the control group (Figure 16, Table 8). Both the separate and combined inhibitor treatments effectively eliminated detectable nuclear β-catenin in the malignant cell line, showing expression mostly in the cortical cytoplasmic region of treated cells. The treatments did not affect the cellular pool of total β-catenin in either EPH4 or AC2M2 cells (Figure 17A&B), demonstrating that the elimination of nuclear β-catenin observed by confocal imaging in the AC2M2 cells was due to relocalization of β-catenin out of the nucleus rather than its degradation. β-catenin has been shown to associate with Met; HGF-induced activation of Met on the other hand, can induce dose-dependent nuclear localization of β-catenin [210]. As mentioned earlier, an activated Src may also lead to the phosphorylation of β-catenin at the C terminus on Tyr-654, which in turn serves as a signal for disruption of the adherens junctions [143,211]. In addition, β-catenin target genes have been shown to be upregulated in a majority of invasive breast carcinomas [203-205]. For example, cyclin D1, is upregulated in approximately 40% of breast cancers [203]. As shown in Figure 17, our results demonstrate that CPA7 treatments (both alone and in combination with Dasatinib) inhibited cyclin D1 expression in both cell lines but more dramatically in the AC2M2
cells. Considering that Stat3 is a known transcription activator of cyclin D1 expression [154], this result shows that β-catenin might act as a transcriptional coactivator of Stat3.

4.2 Activated Stat3 induced transformation in the EPH4 cells

Overexpression or constitutive activation of proteins can reveal their contributions to cell morphology. In this study, we showed that activated Stat3 induces transformation in EPH4 cells as demonstrated by nuclear translocation of activated Stat3, spindular morphology and increased filopodial extensions. Once in the nucleus, Stat3 can activate the transcription of growth factor genes (e.g., HGF, VEGF) [145,152] and cell growth regulatory genes including myc [153], cyclin D1 [154] and bcl-xL [155]. Moreover, FN was identified recently as a Stat3 target gene because of its increased expression in Stat3C expressing cells, and its ability to enhance cell migration [242]. Expression of Stat3C in fibroblasts [154], lung epithelium [243] cardiac myocytes [244] and keratinocytes [245] followed by subsequent injection of these cells into mice has been shown to induce tumour formation, inhibition of immune responses, and angiogenesis. In addition, Bromberg et al. have shown that Stat3C-derived tumours exhibit a spindle-like morphology and grow slower than those derived from v-Src expressing fibroblasts [154]. However, metastases to distal sites have not yet been confirmed in these models [242]. These results may suggest that additional tumourigenic effects may be required for activated Stat3 to induce full malignancy in vivo.
4.3 Activated Src induced transformation in the EPH4 cells

Constitutively active Src has been shown to transform cells *in vitro* by stimulating cell scattering, spreading, and migration as well as deregulation of cell-cell contacts (reviewed in [130]). Other characteristic morphological events attributed to Src activation include integrin-mediated cell spreading, stress fiber formation and pseudopodia formation [141]. In this study, we showed that activated Src induced transformation in EPH4 cells as demonstrated by marked cell spreading and a greater number of filopodial extensions and focal adhesions. Src and Stat3 therefore have somewhat distinct, though symbiotic, contributions to the EMT phenotype. As mentioned earlier in section 1.6.3, Diaz *et al.* [175] have shown in a prospective study of patients undergoing chemotherapy that increased expression of nuclear pYStat3 and pYSrc is associated with regions of invasive breast cancer compared to matched non-invasive tissues. Furthermore, pYStat3 levels were higher in partial than complete pathologic responders to treatment. On the other hand, Dolled-Filhart *et al.* [176] have shown in a retrospective study that the activation of Stat3 signaling is an independent marker of better overall survival of node-negative breast cancer patients. Therefore, tumours that become highly aggressive may engage additional signaling molecules (e.g. Src) that act with Stat3 to stimulate an invasive phenotype. Thus, it would be of interest to co-transduce both activated Src and Stat3 into the EPH4 cells and assess morphology of the cells as well as expression of HGF-Met signaling in these cells. A similar transforming effect of activated Src and Stat3 on HGF expression in a pre-malignant epithelial cell line HC11 was previously observed [177].
4.4 HGF shRNA induced a reversal of the transformed phenotype in the carcinoma cell line, AC2M2.

RNA interference technology has been used to knock down genes *in vitro* [246] and *in vivo* [247]. This effect can be achieved in two ways: using synthetic double stranded 21- to 23-nucleotide long small interfering RNA (siRNA) or (b) using plasmid-based expressed short hairpin RNA (shRNA) [248]. Although both methods have been proven to be quite effective, shRNA seems to be advantageous over siRNA since it leads to more stable gene knock downs [248]. Therefore, we decided to use shRNA interference to silence HGF expression *in vitro* to examine the impact of HGF expression in AC2M2 cells. Since on average, more than four shRNA clones target each gene, five shRNA clones targeting our gene of interest (HGF) were chosen [249]. This redundancy is desirable since RNAi-mediated knock-down is variable, and any one clone may be more or less effective than others [249]. Transduction with vectors 3 and 4 proved to be effective (see Table 2 for description) in inducing a loss of colocalization of HGF and pY_{1234/1235}Met at the membrane, which was followed by a morphological reversion to a non-transformed phenotype as indicated by increased cell rounding and cell-cell contacts and reduced filopodial extensions. Vector 5, on the other hand, was not as effective in blocking either HGF or pY_{1234/1235}Met expression. These findings suggest that targeting autocrine HGF expression directly might suppress breast cancer progression with minimal effect on normal tissues. Experiments are currently underway in our lab to validate the effect of shRNA on HGF mRNA production by using RT-PCR and HGF protein expression using Western blotting.
4.5 The phenotypes of both EPH4 cells and AC2M2 cells are better maintained in 3D

In terms of providing a quasi-imitation of *in vivo* cell growth, the 3D technique seems to be more efficient when compared to the 2D technique, although the latter is easier to use and more practical during the initial testing of therapeutics. Accordingly, we sought to better understand our observations of cell signaling events in this more physiologically relevant system. Thus, we first compared the behaviour of EPH4 and AC2M2 cells on laminin-rich 3D cultures. As expected, when cultured in 3D on Matrigel, EPH4 cells formed uniform, well-organized structures that resembled differentiated alveoli. AC2M2 cells, on the other hand, formed large, disorganized aggregates with numerous irregular elongated protrusions (Figure 21). In contrast to 2D, EPH4 cells grown in 3D did not express activated Src and Stat3, while pY1234/1235Met remained at a very low level (compare Figures 12 & 22). Also in contrast to 2D, EPH4 in 3D can grow and differentiate in serum-free conditions (see Appendix), which helped eliminate any possibility of the confounding Met activation observed in 2D. Decreasing Stat3 and increasing Stat5 levels correlated with transient expression of cyclin D1 and increasing expression of the milk protein, β-casein, in EPH4 cells. Since EPH4 cells were able to produce milk proteins in 3D, they are representative of an *in vivo* lactating mammary gland. A recent report has demonstrated that the increased expression of Stat5 prevents invasion of human breast cancer cells *in vitro* [250]. Also upregulation of Stat5 in breast cancer has been shown to correlate with better prognosis [251]. Taken together these findings suggest that a negative regulatory effect of Stat5 on Stat3 function may occur during tumourigenesis.
AC2M2 cells, on the other hand, expressed relatively constant levels of the various signaling proteins throughout the 3D growth period, similar to 2D growth at various cell densities (compare Figures 12 & 22). These findings indicate that AC2M2 cells exhibit constitutive activation of Src, Stat3, and Met in both 2D and 3D culture, characteristic of their malignant phenotype. It is important to note that AC2M2 cells expressed higher levels of pY$_{1234/1235}$Met, HGF, pY$_{705}$Stat3 and pY$_{418}$Src, compared to non-malignant EPH4 cells based on equally loaded protein amounts for both cell lines (Figure 22). As expected, AC2M2 did not express β-casein, but did express low levels of Stat5. Overall, we concluded that the phenotypes of both non-neoplastic (EPH4) cells, and malignant (AC2M2) cells are better maintained in 3D (Figure 22). Thus, in vitro 3D models seem to provide the missing link between growing cells in monolayers (2D) and in vivo animal systems. Future studies are needed to compare and contrast activation of Stat5 and Stat3 in the EPH4 vs AC2M2 cells using appropriate phospho-specific antibodies. Other studies may also investigate the role of Stat5 in the regulation of the HGF promoter by cotransfection of Stat5 expression vector in the presence or absence of a Stat3 vector into our cell lines.

4.6 HGF stimulates tubulogenesis of epithelial mammospheres in culture.

HGF is primarily expressed by mesenchymal/stromal cells, whereas its receptor, Met, is expressed selectively by epithelial cells, thereby creating a paracrine regulatory system [79]. In normal mammary development, this paracrine loop is tightly controlled and plays an important role in mammary morphogenesis [78]. Previous studies in our laboratory have exclusively used 2D techniques to study the effect of HGF on cell
transformation [100-102]. However, since 3D models provide a more physiologically relevant system to study cell behaviour, we decided to establish such models in our lab. In this study, we show that HGF induces the formation of tubulogenesis without lumen filling in EPH4 cells cultured on Matrigel. This process in vivo involves an HGF-induced partial EMT transition associated with side branching of ductal epithelium [21]. Moreover, HGF has been shown to inhibit the expression of β-casein, which is consistent with inhibition of milk production observed during ductal morphogenesis in vivo [252].

4.7 Fibronectin and HGF in combination stimulate tubulogenesis and lumen filling of mammospheres.

In normal mammary development, HGF produced by mesenchymal cells is a potent inducer of partial EMT-induced tubulogenesis [28]. However, overexpression of HGF and Met [98] and cross talk with integrin signaling [104] has been shown to be linked to many types of invasive cancers. Stromal interactions have been shown to play a key role in metastasis. ECM proteins involved in this process mainly include integrin-ligands [17]. For instance, the overexpression of integrin α5β1 and its receptor FN, have been shown to elicit EMT in mammary epithelial cells [14]. Both Met and α5β1 integrin are expressed by AC2M2 cells [90,253]. This study showed that co-stimulation of mammary epithelial cells with HGF and FN might have a co-operative effect on induction of lumen filling of spheroids – a pre-malignant progression – presumably through simultaneous stimulation of Met and α5β1 integrin. Previous studies have shown that Src catalytic activity is required for α5β1-stimulated cell motility [254]. Thus, a Src-adhesion-dependent mechanism may act as a modulator of HGF overexpression and the
establishment of an autocrine loop. However, further studies will be required to casually link α5β1-integrin engagement with Src-Stat3-induced HGF autocrine loop and malignancy. Future studies will also compare the effect of FN and HGF alone on EPH4 mammospheres to the co-stimulatory effect seen when they were used in unison.

4.8 A proposed model for the role of Src-Stat3 in HGF-Met autocrine loop

The present study demonstrates the importance of the Src-Stat3 signaling axis on the induction of an HGF-autocrine-loop and transformation of breast epithelial cells and that cell adhesion to FN switches the epithelial phenotype towards transformation. Figure 25 proposes a model based on the results discussed in this thesis and related literature. The HGF autocrine loop occurs when epithelial cells, which already express the receptor Met, begin to express HGF thereby shifting the paracrine system to an autocrine loop. Sustained hyper-activation of Src and Stat3, which occurs in breast cancer by a variety of mechanisms, such as increased stimulation by upstream transducer molecules like integrins, may lead to the development of this autocrine loop. In an attempt to extrapolate our model to a 3D model, we expect that constitutive activation of α5β1 integrin will lead to sustained hyper-activation of Src and Stat3 which will in turn induce the autocrine HGF loop and promote lumen filling, invasion and disruption of mammospheres. In addition to over-expression of Met and HGF by AC2M2 cells, our laboratory has previously found that AC2M2 cells strongly express FN [41]. Others have also demonstrated increased FN expression by ductal breast carcinomas [255]. This finding suggests that there are multiple contributors to the co-operative cell transformation effect of HGF and FN. At first, FN-integrin adhesion may function as a modulator of HGF-
Figure 25. A proposed model of HGF autocrine loop activation in 3D

The HGF autocrine loop occurs when epithelial cells, which already express the receptor Met begin to express HGF thereby shifting the paracrine system to an autocrine loop. Sustained hyper-activation of Src and Stat3, which may lead to this autocrine loop development, occurs in breast cancer by a variety of mechanisms, such as increased stimulation by upstream transducer molecules like integrins. Therefore, we expect that in a 3D environment, constitutive activation of $\alpha_5\beta_1$ integrin will lead to sustained hyper-activation of Src and Stat3 which will in turn induce the autocrine HGF loop and promote disruption of mammospheres. Thus, blocking $\alpha_5\beta_1$ integrin/Src/Stat3-dependent HGF transcriptional activity by specific inhibitors would be expected to neutralize autocrine HGF-Met activation in breast carcinoma cell lines and revert the transformed phenotype.
induced Met activation. Alternatively, an HGF-autocrine loop may represent a cell-matrix independent mechanism of FN production, important in promoting later stages of adhesion-independent survival. Thus, blocking \(\alpha5\beta1\) integrin/Src/Stat3-dependent HGF transcriptional activity with specific inhibitors would be expected to neutralize autocrine HGF-Met activation in breast carcinoma cell lines and revert the transformed phenotype.

4.9 Future Directions

There are several interesting implications to the findings of this thesis, as well as considerations for possible future directions:

1) As metastatic potential can also be assessed using motility, an invasion assay would also be a quantifiable way to evaluate phenotypic changes associated with Src/Stat3 inhibition and HGF knockdown in our cell models. Increased cell growth is another indicator of the malignant phenotype; thus, assessing the growth rate of EPH4 and AC2M2 cells could also provide quantitative information on the changing phenotype [257].

2) Most of the work so far with the Src and Stat3 inhibitors has been done in 2D. It would be of great relevance if we could extrapolate this established model in 2D to a 3D model. However, it has to be noted that cells growing in 3D are more resistant to pharmacological agents than cells grown in 2D [258]. To overcome this challenge higher drug concentrations would need to be used, but assays done with this approach may still confirm that 3D culture is a more relevant model for testing therapeutic agents in vitro.

3) Further elucidation of the transforming properties of the Src-Stat3 axis could also be explored by transducing both constitutively active Src and Stat3 into
non-malignant EPH4 cells in an effort to induce a malignant morphology resembling the AC2M2 phenotype in 3D. The formed aggregates should be characterized in terms of morphology as well as expression and localization of various signaling molecules, including HGF and Met/pY1234/1235Met.

4) Since knockdown of HGF inhibits the transformed phenotype in vitro, then it would be of interest to further assess the efficacy of HGF blockade in inhibiting tumourigenesis of these cells (AC2M2) following transplantation into cleared mammary fat pads. Since HGF knockdown would not block paracrine or ligand-dependent Met activation, a parallel approach to directly target Met with shRNA or dominant negative (kinase dead) Met can be used.

5) Ultimately, we will want to determine whether integrin adhesion is linked to transformation of epithelial cells through activation of Src/Stat3 and autocrine HGF/Met signaling in 3D. First, we have to start by probing or staining for Src/Stat3 and HGF/Met expression in EPH4 cells stimulated with FN. To further assess integrin involvement, we will use anti-β1 integrin function-blocking antibodies, previously shown to revert malignant breast epithelial cells to a differentiated state, manifested as formation of polarized mammospheres in 3D culture [256].
4.10 Clinical relevance of this study.

HGF/Met, Src and Stat3 have all been strongly implicated in cancer progression to metastasis in a number of major cancers, including breast. Each molecule has been studied as a potential therapeutic target in a variety of cancer types since HGF/Met signaling is involved in different steps of tumour formation, growth and spreading. Although many advances have been made in the search for inhibitors of Met and Src \textit{in vitro}, targeting of tyrosine kinases has proven difficult due to the high homologies among proteins in this complex superfamily. Stat3 may therefore present a unique opportunity to attenuate signaling via both Src and Met in a tumour-specific manner. The concurrent inhibition of Src and Stat3 has not yet been investigated in depth, as reflected by the minimal amount of published findings. The synergistic effect observed with concurrent use of the two inhibitors, Dasatinib and CPA7, particularly for pY$_{705}$Stat3 inhibition and the reversal of the malignant morphology, is clinically relevant and advantageous. Inhibition via two separate mechanisms producing synergy allows required drug doses to be reduced, thereby minimizing the adverse effects and toxicity of each drug therapy. If the synergistic effects observed \textit{in vitro} can be reproduced in an \textit{in vivo} model, these findings could be applied to drug design. Dasatinib is currently in clinical trials as an anti-cancer drug [224], perhaps its efficacy could be improved by inclusion of a pY$_{705}$Stat3 inhibitor, such as CPA7.

Stat3 activation has been shown to up-regulate expression of vascular endothelial VEGF through a binding site in its promoter [152]. In addition, Src also plays a role in VEGF production and angiogenesis [259,260]. Therefore, Src and Stat3 may be useful therapeutic targets for inhibition of angiogenesis (reviewed in [209]). Together, these
findings suggest that disrupting the Src-Stat3 signaling axis might have a potential clinical relevance in the treatment of invasive human breast carcinomas.

HGF mRNA is usually weakly expressed in normal mammary epithelium, although epithelial expression of HGF mRNA has been observed in cases of hyperplasia [100]. Several studies [98,261,86] have reported a similar gradient of HGF and Met expression in the mammary gland ranging from benign hyperplasia (lowest), to DCIS (higher), to invasive carcinoma (highest). Together, these findings raise the possibility that an HGF-Met autocrine loop in breast carcinoma cells can promote increased EMT, invasion, and metastasis. In addition to indirect blocking of HGF-induced Met signaling through Src and Stat3 inhibition, targeting HGF expression directly might be of clinical relevance. A possible, but more futuristic, approach is the use of ligand-targeted HGF-specific siRNA, which might achieve a good uptake of RNAi into tumour cells [262].

This study has provided some insight into how normal breast development deteriorates to produce pre-cancerous and eventually fully cancerous cells. It will also likely lead to novel strategies for targeting HGF expression in tumour cells with minimal effects on normal tissues as a potential new treatment of invasive breast cancer.
REFERENCES


61. Koch, A., A. Mancini, O. El Bounkari, and T. Tamura, *The SH2-domain-containing inositol 5-phosphatase (SHIP)-2 binds to c-Met directly via tyrosine residue 1356 and involves hepatocyte growth factor (HGF)-induced*


(Stat3) and phospho-Stat3 (Tyr705) in node-negative breast cancer shows nuclear localization is associated with a better prognosis. Clin Cancer Res. 2003. 9(2): p. 594-600.


235. Bigelow, R.L.H. and J.A. Cardelli. The green tea catechins, (-)-Epigallocatechin-3-gallate (EGCG) and (-)-Epicatechin-3-gallate (ECG), inhibit HGF/Met


APPENDIX– MATERIALS/RECIPES/PROTOCOLS

General Use

*Phosphate Buffered Saline (PBS)*

- 137 mM NaCl
- 2.68 mM KCl
- 21.7 mM Na$_2$HPO$_4$.H$_2$O
- 1.47 mM KH$_2$PO$_4$
Adjust to volume with ddH$_2$O and pH 7.3

*Phosphate Buffered Saline* (PBS*)

- PBS
- 0.1 mM CaCl$_2$
- 0.1 mM MgCl$_2$

Cell Maintenance

*EPH4 maintenance media*

50:50 DMEM/F12 Media, supplemented with:
- 5% FBS (Gibco 12483-020)
- 5ug/ml Insulin (Sigma I5500)
- 50ug/ml Gentamycin (Sigma G1264)

* Passage with Trypsin/EDTA
**Best to passage EPH4 cells before they are fully confluent (~90%)**
1. 3x10 minute washes with 10ml Ca$^{2+}$ Free media at 37°C (DME/F12 no Calcium Chloride)
2. 1x 2 minute with 2ml 0.05% trypsin (aspirate off)→ room temp
3. 1x 5-6 minute wash with 2ml 0.05% trypsin→ 37°C

**important to use low concentration trypsin, standard 0.25% trypsin can cause loss of differentiation capacity**
4. Add 4mls of growth media to dilute out trypsin
5. Pellet cells at 1500rpm for 5 minutes
6. Resuspend in 10ml Growth media
7. Plate cells at 1:4-1:10 dilution

* Freeze in 10% DMSO + 15% Serum

*EPH4 3D media*

50:50 DMEM/F12 Media, supplemented with
- 50ug/ml Gentamycin
- 5ug/ml Insulin
- 1ug/ml Hydrocortisone
- 3ug/ml Prolactin
- 1% FBS on first day, 0% after
**AC2M2 maintenance media**
DMEM Media, supplemented with:
- 10% FBS
- 5μg/ml Insulin

**AC2M2 3D media**
50:50 DMEM/F12 Media, supplemented with:
- 5% FBS
- 5μg/ml Insulin
- 5% Matrigel

**Cell Lysis**

*Radioimmunoprecipitation (RIPA) Buffer (used for cells grown in 3D) - 100ml composition*

- 0.79 g Tris Base
- 0.9 g NaCl
- 10 ml 10% NP-40
- 2.5 ml Sodium deoxycholate
- 1 ml (1mM) EDTA
- 100 ml ddH2O
- 1 μg/ml Trasylol*
- 1 μg/ml Leu/Pep*
- 1 mM DTT*
- 1 mM PMSF*
- 1 mM NaF*
- 1 mM Na3VO4*
- 20mM β-Glycerophosphate

* Protease and phosphotase inhibitors added prior to use

**2X SDS-PAGE Sample Buffer (used for cells grown in 2D)**

- 20% glycerol
- 4.6% SDS
- 1% Bromophenol Blue
- 0.15% Tris

Adjust to volume with ddH2O and pH 6.8
Add β-mercaptoethanol to 3% of total sample volume

**SDS-PAGE and Western Blotting**

*Resolving Gel (makes one mini Bio-Rad 8% gel)*

- 5.15 ml ddH2O
- 2.60 ml 4x Lower Gel Buffer (LGB)
- 2.85 ml 29:1 Acrylamide
- 60 μl 10% Ammonium Persulfate (APS)
- 6 μl TEMED
Stacking Gel (makes one mini Bio-Rad gel)
2.4 ml ddH2O
1.0 ml 4x Upper Gel Buffer (UGB)
0.6 ml 29:1 Acrylamide
24 μl 10% Ammonium Persulfate (APS)
8 μl TEMED

4x Lower Gel Buffer (LGB)
1.5 M Tris
0.4% sodium dodecyl sulfate (SDS)
Adjust to volume with ddH2O and pH 8.8

4x Upper Gel Buffer (UGB)
0.5 M Tris
0.4% SDS
Adjust to volume with ddH2O and pH 6.8

Acrylamide
29% Acrylamide
1% Bis-acrylamide
Adjust to volume with ddH2O and pass through 45 μm nitrocellulose filter

SDS-PAGE Running Buffer (10X)
0.25 M Tris
1.92 M Glycine
1% SDS
Adjust to 1L with ddH2O and dilute to 1X prior to use

Semi-dry Transfer Buffer
50 mM Tris
40 mM Glycine
0.0375% SDS
20% Methanol
Adjust to 1L with ddH2O and pH between 9.0 and 9.4

TBST Buffer
1 M Tris
150 mM NaCl
0.1% Tween-20
Adjust volume with ddH2O and pH 8.0

Stripping Buffer
78 mM Tris
2% SDS
Adjust to volume with ddH2O and pH 6.7
Add 0.7% β-mercaptoethanol just before use
**Confocal Microscopy**

*Wash Reagent*
- 0.01% Triton X-100
- 0.3% BSA
- Adjust to volume with PBS*