# THE TRANSCRIPTION FACTOR KAISO IN BREAST TUMOURIGENESIS

# ANALYZING THE BIOLOGICAL ROLE OF THE TRANSCRIPTION FACTOR KAISO IN BREAST TUMOURIGENESIS

**BY**
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## ABSTRACT

Kaiso is a member of the BTB/POZ-ZF (hereafter POZ-ZF), family of transcription factors that have significant roles in development and cancer. Kaiso was identified as a $p120^{ctn}$ interaction partner, and has a highly conserved protein-protein interaction POZ domain at its N-terminus and three DNA binding $C_2H_2$ zinc fingers (ZF) at its C-terminus. Kaiso is a bi-modal transcriptional regulator; it recognizes and binds both a sequence specific Kaiso binding site (KBS), TCCTGCNA, and methylated CpG-dinucleotides. Since Kaiso was initially identified, several tumourigenesis-associated Wnt signalling target genes such as *matrilysin, cyclin D1, metastasin, MTA2, Wnt11* and *siamois* have been found to be negatively regulated by Kaiso. These data implicated Kaiso in Wnt signalling and in fact, the rescue of the β-catenin induced double-headed phenotype in *Xenopus laevis* by Kaiso overexpression provided the first strong experimental evidence that Kaiso was linked to Wnt signalling. Since then, Kaiso has been implicated in tumourigenesis both as a potential tumour suppressor (decreased Kaiso expression in 30% of human breast tumours and 50% of human ovarian tumours) and as an oncoprotein (Kaiso-null mice being viable, fertile and displaying delayed tumour onset when crossed with $APC^{Min/+}$ tumour susceptible mice). Interestingly, Kaiso protein expression varies in human tumour tissue and Kaiso's subcellular localization varied according to the tissue microenvironment.

The goal of this research project was to elucidate the biological role of Kaiso in breast tumourigenic processes, and to correlate the expression patterns of *kaiso* and its target genes (*cyclinD1, matrilysin and mta2*) in human breast normal and tumour tissue. Focus formation assays, showed that Kaiso over-expression increased focus formation in Rat-1 rodent non-transformed cells, suggesting that Kaiso promotes oncogenic transformation. Kaiso misexpression

tumourigenesis-associated processes of cell proliferation, motility and invasion were also examined on MCF7 human breast adenocarcinoma cells. Kaiso misexpression did not have a significant effect on cell proliferation but Kaiso depletion appeared to hinder cell motility and invasion in breast tumour cells, thus suggesting that Kaiso displays tumour promoting properties.

We also examined the expression patterns of Kaiso's target genes in matched pairs of breast lobular and ductal carcinoma tissues, and detected increased *matrilysin* levels relative to *kaiso* in all patient samples analyzed by qRT-PCR. There was no noticeable trend in *mta2* levels relative to Kaiso in the ductal carcinomas, but there was a positive correlation between *mta2* and *kaiso* in the lobular carcinomas. In contrast, *cyclinD1* levels were varied across all tissues examined.

This trend continued in the immunohistochemical analysis performed on the ductal and lobular breast carcinomas, with variable CyclinD1 staining occurring in all tissues examined. However, there was an increase in nuclear and cytoplasmic Kaiso in the lobular breast carcinomas, coinciding with decreased, cytoplasmic $p120^{ctn}$ and significantly decreased E-cadherin in the tumour tissue relative to normal. Our ductal carcinomas displayed negligible Kaiso staining, along with decreased $p120^{ctn}$ and increased cytoplasmic E-cadherin in the tumour tissue relative to normal, although $p120^{ctn}$ and E-cadherin did not display the expected localization in normal tissue. The expression and sub-cellular localization of these proteins are heavily dependent on the tumour microenvironment and external factors such as patient age, disease progression and type of carcinoma. In conclusion, data suggests that Kaiso plays a tumour promoting role in the processes of transformation, cell motility and migration in MCF7 breast tumour cells.

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## CONTRIBUTIONS BY OTHERS

The MCF7 pcDNA3-Empty and pCDNA3-Kaiso cell lines were generated by Michelle Anstey while the MCF7 pRS-Scrambled Kaiso and pRS-Kaiso cell lines were generated by Dr. Kevin Kelly; both former members of the Daniel Lab.

Paraffin embedding and sectioning of mouse tissue was performed by Mary Jo Smith and Mary Bruni of the McMaster University Centre for Gene Therapeutics Central Histology Facility.

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## ABBREVIATIONS

| | |
|---|---|
| **α-SMA** | smooth muscle actin |
| **APC** | adenomatosis polyposis coli |
| **$APC^{Min/+}$** | adenomatosis polyposis coli, multiple intestinal neoplasia |
| **Bcl-6** | B cell lymphoma 6 |
| **BTB** | broad complex, tramtrak, bric a brac |
| **CBD** | catenin binding domain |
| **CXCL12** | stromal-cell-derived factor 1 |
| **Dsh** | Dishevelled |
| **E-cadherin** | epithelial cadherin |
| **ECL** | enhanced chemiluminescence |
| **ECM** | extracellular matrix |
| **EGF** | epidermal growth factor |
| **EMT** | epithelial to mesenchymal transition |
| **FAZF** | Fanconi anemia zinc finger |
| **FBI-1** | factor binding to IST-1 |
| **FGF-2** | fibroblast growth factor-2 |
| **Frp1** | frizzled-related protein 1 |
| **Fz** | Frizzled |
| **GSK3** | glycogen synthase kinase-3 |
| **HDAC** | histone deacetylase |
| **HIC-1** | hypermethylated in cancer-1 |
| **IHC** | Immunohistochemistry |
| **JMD** | juxtamembrane domain |
| **KBS** | Kaiso binding site |
| **LRF** | leukemia/lymphoma related factor |
| **LRP** | lipoprotein receptor-related protein |
| **MET** | mesenchymal to epithelial transition |
| **MMP7** | matrix metalloproteinase-7 |
| **MTA2** | metastatis tumour antigen family 1, member 2 |
| **mTOR** | mammalian target of rapamycin |
| **N-cadherin** | neuronal cadherin |
| **NLS** | nuclear localization signal |
| **OCZF** | osteoclast derived zinc finger protein |
| **PLZF** | promyelocytic leukemia zinc finger |
| **POZ-ZF** | pox virus and zinc finger |
| **qRT-PCR** | quantitative reverse transcriptase-polymerase chain reaction |
| **RTK** | receptor tyrosine kinase |
| **siRNA** | short interfering RNA |
| **TBS** | tris-buffered saline |
| **TCF/LEF** | T-cell factor/lymphoid enhancing factor |

| | |
|---|---|
| **TGF-β** | **t**ransforming **g**rowth **f**actor-β |
| **TZFP** | **t**estis **z**inc **f**inger **p**rotein |
| **VEGF** | **v**ascular **e**ndothelial **g**rowth **f**actor |
| **ZBTB33** | Gene name: Human Kaiso |

# INTRODUCTION

## 1.1 Breast Cancer progression and Metastasis

Breast cancer is a complex disease arising in the ducts and lobules of the breast, and it is the most common cancer amongst Canadian women and the second leading cause of cancer death in women worldwide. According to the Canadian Cancer Society, there were 23,200 estimated new cases of breast cancer among women in Canada in 2009, constituting 28% of detected cancers from all anatomical sites. Although breast cancer has been a health concern for many decades, statistics show that the incidence rate of breast cancer has been steadily increasing; from 1 in 20 women in the 1960's to 1 in 8 women today [22]. However, the overall breast cancer mortality rate has decreased steadily since 1990 (about 37%), partly due to increased screening (which results in early detection) and new advances in adjuvant therapies (which result in improved treatment and survival) [56]. In most cases, mortality is caused not by the primary tumour but by secondary tumours that develop in other vital organs (e.g. lung, liver, brain) as a result of tumour cell metastasis.

Metastasis is a complex multi-step process that begins with the dissemination of tumour cells from the primary site and ends with proliferation and survival at distant sites [22]. The first step in tumour metastasis is the loss of local constraints; both physical, e.g. loss of cell-cell adhesion [6, 16], and regulatory, e.g. abnormal cell-cell signalling activity [12]. The second step occurs when tumour cells enter the blood or

lymphatic circulatory system by a process called intravasation. The third stage involves tumour cell extravasation from blood and lymphatic systems followed by proliferation and survival in hostile ectopic environments. Metastasis culminates with the colonization of distant vital organs, which is the last stage [22, 112]. Cancer is therefore a multi-factorial disease promoted by a variety of tumourigenic processes that confer the tumour cells with enhanced survival abilities compared to normal cells.

## 1.2 Tumourigenic processes in the context of Breast Cancer

The initial development of a malignant breast tumour requires six essential alterations in cell physiology. Oncogenic transformation induces a complete cellular re-programming, stimulating these six alterations: self-sufficiency in growth-promoting factors, insensitivity to growth-inhibitory factors, evasion of apoptosis, limitless cell proliferation, sustained angiogenesis and tissue invasion & metastasis [46]. Mammary oncogenesis is characterized by the dysregulation of a multitude of signalling pathways, including the Notch, Hedgehog and Wnt pathways [104]. Recent evidence suggests that Wnt induces oncogenic transformation via downstream activation of β-catenin and mTOR pathways [70].

One of the key initial steps in the physical process of metastasis during the latter stages of breast cancer progression, is the loss of local physical constraints as a result of dysregulated cell-cell adhesion [22]. Epithelial cells that line the breast ducts and lobules are organized as single or multilayered sheets of structured cells that are highly

polarized along an apical-basal axis [6]. This highly cohesive assembly is maintained by laterally associated adhesive structures such as adherens junctions, tight junctions and desmosomes [16]. In fact, disruption of adherens junctions caused by loss of the integral transmembrane protein E-cadherin is sufficient to bestow metastatic ability to non-metastatic cells [5]. More specifically, it is the start of a complex, tightly regulated process known as the **e**pithelial to **m**esenchymal **t**ransition (EMT), that causes stationary, contact-dependent epithelial cells to become motile, invasive cells with a fibroblastic phenotype [22].

### 1.3 EMT

EMT is an important cellular process during embryogenesis when global cellular changes and movements occur, driven by changes in the expression of genes that control cell-cell adhesion, polarity, viability, motility and **e**xtra**c**ellular **m**atrix (ECM) degradation (reviewed in [6]). Specifically, a down regulation of epithelial markers such as E-cadherin, Occludin, Claudin and Desmoplakin occurs in conjunction with an up regulation of mesenchymal markers such as Snail, Slug, **n**euronal cadherin (N-cadherin), Vimentin and Twist (reviewed in [6, 22, 53]. Incidentally, several of these mesenchymal markers (Snail, Slug) are negative regulators of E-cadherin expression and it is believed that they are the main mechanism of E-cadherin down regulation in tumour cell metastasis [80, 112]. EMT can also be induced by a variety of cytokines in the context of tumour initiation and metastasis; the TGF-β ligands are the most often used as inducers

of EMT. In fact the TGF-β signalling pathway is perturbed in many human cancers including mammary carcinomas [29, 69]. Following EMT induction, a rearrangement of the actin cytoskeleton occurs and this results in a switch from the apical-basal polarity of epithelial cells to the anterior-posterior polarity of fibroblastic cells. Disruption of cell-cell adhesion complexes contribute to the motility of cells and may be responsible for the preliminary dissemination of cells from the primary tumour. **M**atrix **m**etallo**p**roteinases (MMP's) and integrins function to enhance matrix and cell junction degradation and assist in steering cells through collagenous and endothelial barriers in response to growth factor receptor signalling [85, 111]. Stimulated **r**eceptor **t**yrosine **k**inases (RTK's) and G-protein coupled receptors also function to augment cell migration and intravasation into circulatory systems via rearrangement of the actin cytoskeleton [106]. Once tumour cells extravasate out of the circulatory systems and invade hostile ectopic tissue (e.g. lung, liver, bone), they can undergo a **m**esenchymal to **e**pithelial **t**ransition (MET) to colonize secondary tissue and initiate angiogenesis and cell survival pathways to form secondary tumours [53].

### 1.4 The adherens junction E-cadherin-catenin complex

E-cadherin is connected to the actin cytoskeleton via interactions with β-catenin, γ-catenin (plakoglobin) and δ-catenin at its cytoplasmic domain. β-catenin and γ-catenin bind E-cadherin at its carboxy terminal **c**atenin **b**inding **d**omain (CBD) in a mutually exclusive manner, and anchor E-cadherin to the actin cytoskeleton via interactions with

α-catenin [11]. In contrast, the Armadillo catenin $p120^{ctn}$ binds E-cadherin at its juxtamembrane domain (JMD) **(Figure 1)** [25, 87]. Aside from their roles in cell-cell adhesion, the catenins are also important components of many signalling pathways and have significantly altered levels in many cancers [45].There is often a loss of catenin expression at adherens junctions in E-cadherin negative breast cancers, and altered catenin phosphorylation affects adhesion in E-cadherin positive cancers [11]. For example, α-catenin is downregulated more than E-cadherin in some types of breast cancers [90].

### 1.5 The transmembrane cell adhesion protein E-cadherin in Breast Cancer

E-cadherin is an important cell-adhesion protein that first homodimerizes, and then interacts in a $Ca^{2+}$ dependent, homophilic manner to Cadherin dimers in neighbouring cells to form adherens junctions. These adherens junctions play a role in the maintenance of tissue structure and cell-cell adhesion in epithelial cells. The E-cadherin protein is comprised of 5 extracellular cadherin repeats, a transmembrane region and a highly conserved cytoplasmic tail [113]. E-cadherin activity is crucial for normal tissue structure development and knockout of the E-cadherin gene *CDH1* in mice results in embryonic lethality [68]. E-cadherin downregulation and misexpression is often implicated in tumourigenesis and defects in catenin expression and function play a significant role in metastatic cancers. In fact, E-cadherin is down-regulated in about 50% of human metastatic carcinomas [45, 48]. Hence, E-cadherin is often used as a

prognostic marker in clinical settings for breast cancer [50]. In several studies, ectopic E-cadherin expression has been shown to reduce mammary tumour progression, invasion and the formation of metastasis ([109], reviewed in [5]). Immunohistochemical (IHC) analysis has also shown that E-cadherin expression was frequently decreased in invasive ductal carcinomas and their distant metastases [67]. However E-cadherin expression was not detected in invasive lobular carcinomas suggesting its role maybe different in that type of breast cancer [67]. E-cadherin gene and protein expression is often reduced or absent in primary breast tumours and cell lines, and allelic losses were often observed at the E-cadherin locus in lobular carcinomas [10]. Several E-box elements in the E-cadherin promoter have also been identified and they appear to be responsible for E-cadherin's transcriptional repression in mesenchymal cells that do not express the E-cadherin protein [39]. This E-cadherin transcriptional repression in mesenchymal cells is mediated by several zinc finger transcription factors that are specifically expressed in mesenchymal cells and directly bind to E-boxes in the E-cadherin promoter. These transcription factors include Snail 1, Slug, ZEB1 (also known as δEF1) and ZEB2 (also known as SIP1) [18, 21, 30, 44]. As a result of E-cadherin loss, the actin cytoskeleton is rearranged via its association with the cytoplasmic catenins and cell-cell adhesion is disrupted.

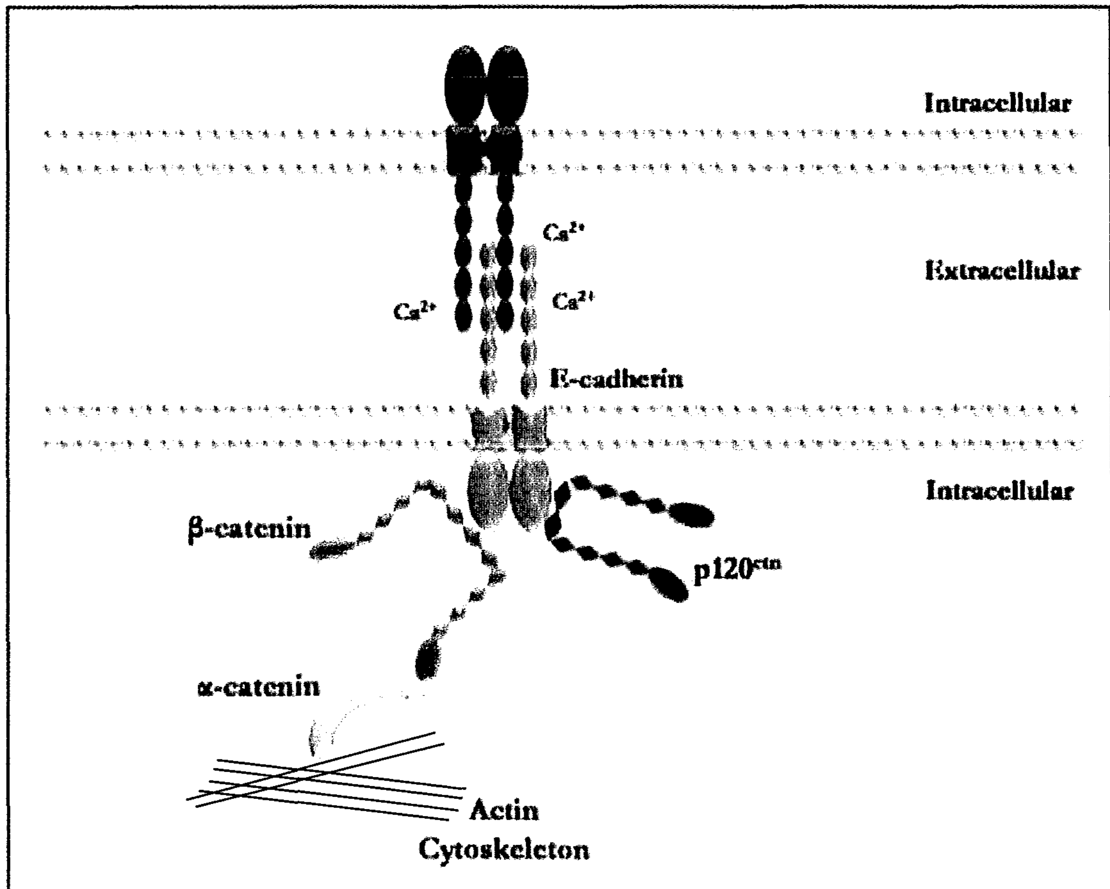



**Figure 1: The Adherens Junction E-cadherin-Catenin complex.** The transmembrane tumour suppressor protein E-cadherin mediates cell-cell adhesion in a $Ca^{2+}$ dependent manner via homophilic interactions with E-cadherin molecules on adjacent cells at adherens junctions. β-catenin and γ-catenin (not depicted) bind E-cadherin in a mutually exclusive manner at its carboxy terminal catenin binding domain (CBD) and anchor E-cadherin to the actin cytoskeleton via interactions with α-catenin. $p120^{ctn}$ binds E-cadherin at its juxtamembrane domain (JMD) and regulates E-cadherin stability and turnover.

## 1.6 The Armadillo catenin $p120^{ctn}$

$p120^{ctn}$ is a key member of the E-cadherin/catenin complex that is now being recognized for its important roles in cell-adhesion and tumourigenesis among others. $p120^{ctn}$ was first identified as a Src kinase substrate [88] and was later found to associate directly with E-cadherin via its protein-protein interaction Armadillo (Arm) domain [25, 86]. $p120^{ctn}$ also interacts with the BTB/POZ-ZF (**B**road complex, **T**ramtrak, **B**ric a brac/**Po**x virus and **z**inc finger) transcriptional repressor Kaiso; the interaction occurs via Kaiso's C-terminal zinc finger domain and $p120^{ctn}$'s Arm domain [24]. Hence, the interaction of $p120^{ctn}$ with Kaiso and E-cadherin is mutually exclusive. $p120^{ctn}$ is 991 amino acids in length, has a molecular weight of 120 kDa and has 10 Armadillo repeats [2]. Due to alternate splicing events and multiple start codons, there are over 20 different isoforms of $p120^{ctn}$ that are differentially expressed in various tissues [61].

In addition to its presence in the E-cadherin/catenin membrane complex, $p120^{ctn}$ also localizes to the cytoplasm and the nucleus. Cytoplasmic $p120^{ctn}$ plays a role in cell motility and cytoskeletal organization via regulation of the activity of Rho-GTPases; $p120^{ctn}$ inhibits RhoA and activates Rac and Cdc42 [2, 3]. Within the nucleus, $p120^{ctn}$'s interaction with Kaiso prevents Kaiso-mediated repression of target genes by inhibiting Kaiso's DNA binding ability both *in vitro* and *in vivo* [26]. Interestingly, this is the first evidence of a gene regulatory role for nuclear $p120^{ctn}$. In breast cancer, levels of membrane-localized $p120^{ctn}$ were reduced in many ductal carcinomas, and $p120^{ctn}$ was

found to be predominantly cytoplasmic in the majority of invasive lobular carcinomas [95]. This was found to correlate with loss of E-cadherin expression in those tumours. p120$^{ctn}$ isoform 1 which is associated with mesenchymal cells was found to increase upon loss of E-cadherin and increase in N-cadherin [95].

**1.7 β-catenin and the canonical Wnt signalling pathway**

The Armadillo protein β-catenin is the key downstream regulator of the canonical Wnt signalling cascade, and was first studied as a key cytoplasmic co-factor for the transmembrane tumour suppressor E-cadherin [9]. The Wnt signalling cascade is an important regulator of many important cellular processes. These include cell proliferation, differentiation and apoptosis in embryogenesis and the self-renewal process in adult mammalian tissue and mammalian tumourigenesis [20, 31, 41]. The Wnt genes are a conserved family of at least 19 secreted growth factors that participate in a variety of signalling pathways. The canonical Wnt pathway, which involves β-catenin, is the most extensively studied due to its important role in embryogenesis and tumourigenesis (reviewed in [9]). Recent evidence shows that β-catenin is downregulated or mislocalized from the cell-surface adhesion complex to the nucleus in various cancers including those of the breast, colon and bladder [7, 17].

In the absence of the Wnt ligand, cytoplasmic β-catenin is serine/threonine phosphorylated and subsequently targeted for ubiquitin-mediated proteosomal degradation by a large macromolecular complex consisting of the serine-threonine

kinase Glycogen Synthase Kinase-3 (GSK3), the scaffolding protein Axin, and the tumour suppressor Adenomatous Polyposis Coli (APC) [8, 15, 43]. The Wnt cascade is activated when lipid-modified Wnt proteins are released from the surfaces of signalling cells and these ligands bind to their heterodimeric receptors, Frizzled and its co-receptor low-density lipoprotein receptor-related protein (LRP-5/6), on receiving cells [71]. Activation of the dimeric Frizzled/LRP-5/6 receptors then enables Dishevelled to bind Axin, thus preventing the formation of the GSK3/Axin/APC 'destruction complex'. As a result, unphosphorylated β-catenin accumulates in the cytoplasm and translocates to the nucleus where it interacts with the T-cell factor/lymphoid enhancing factor (TCF/LEF) family of transcription factors **(Figure 2).** This interaction activates various tumourigenesis-associated target genes including *cyclin D1*, *c-myc*, *matrilysin* and *Id2* [14, 23, 49, 91, 98]. Mutations occurring in Wnt signalling proteins such as APC or β-catenin, can render the Wnt signalling cascade constitutively active and ultimately contribute to tumourigenesis [38, 107]. It is now known that there are several signalling pathways that result in a negative or positive change in Wnt signalling and thus affect its normal activation pattern. Understanding and characterizing these various cascades, as well as the targeted inhibition of excessive β-catenin activity have been identified as two important steps towards preventing Wnt- mediated tumourigenesis.

The aberrant activity of Wnt pathway components, i.e. the inactivation of negative Wnt regulators, or amplification of positive Wnt regulators, has been reported in many human breast cancers. The *DVL-1* gene, encoding the positive Wnt activator

Disheveled (Dsh), is amplified and upregulated in 50% of ductal breast carcinomas [76]. The gene encoding secreted **f**rizzled-**r**elated **p**rotein 1 (sFrp1), a negative Wnt regulator, is repressed or absent in 80% of breast carcinomas [103]. Axin, a component of the destruction complex is downregulated in some breast cancers [93], and the *APC* gene is mutated or hypermethylated in many breast tumours, resulting in loss of expression [59].

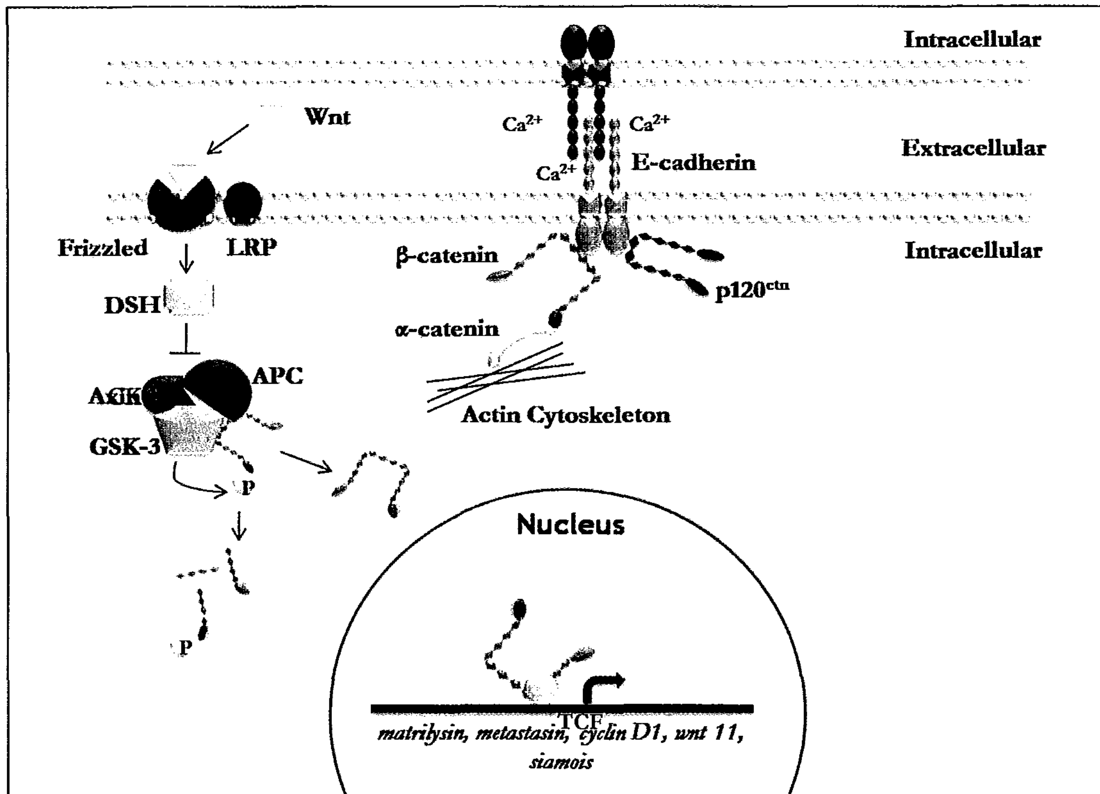



**Figure 2: The canonical Wnt signalling pathway.** β-catenin is phosphorylated and targeted for ubiquitin-mediated degradation by a `destruction complex' consisting minimally of Axin, APC, GSK-3 and CK1. Upon activation of the Fz/LRP-5/6 co-receptors by the extracellular Wnt ligand, Dsh is activated and inhibits the destruction complex, allowing β-catenin to accumulate in the cytoplasm. Stabilized β-catenin translocates to the nucleus where it activates transcription of Wnt target genes via its association with LEF/TCF transcription factors.

## 1.8 Kaiso

Kaiso (also known as the zinc finger and **BTB**-domain-containing **33** gene, *ZBTB-33*) was first identified as a novel BTB/POZ zinc finger protein (hereafter POZ-ZF), following a Yeast-Two-Hybrid Screen using the p120$^{ctn}$ Armadillo domain as bait [24]. Kaiso is a 671 amino acid protein with an approximate molecular weight of 100 kDa. Like other POZ-ZF transcription factors, Kaiso has a unique, highly conserved protein-protein interaction POZ domain at its amino terminus and three DNA-binding $C_2H_2$ zinc fingers (ZF) at its carboxy terminus **(Figure 3).** In addition, Kaiso also has a nuclear localization signal (NLS) and two acidic regions between the POZ and ZF domains [63]. Given that acidic regions are implicated in transcriptional activation, and that Kaiso activates transcription of the neuromuscular gene *rapsyn* [92], it is possible that Kaiso mediates this activation via its acidic domains.

Kaiso is a dual-specificity transcription factor that recognizes and binds a sequence specific Kaiso binding site (KBS), TCCTGCNA, (also known as the Kaiso Binding Site (KBS)), and methylated CpG-dinucleotides [26]. Since Kaiso was initially identified, several tumourigenesis-associated genes such as *matrilysin, cyclin D1, metastasin, MTA2, Wnt11* and *siamois* have been found to contain copies of the KBS in their promoter sequences, and are negatively regulated by Kaiso [64, 79, 82, 92, 100]. Although previously thought to have no involvement in the canonical Wnt pathway, the rescue of the β-catenin induced double-headed phenotype in *Xenopus laevis* by Kaiso

over-expression provided the first strong experimental evidence that Kaiso was linked to Wnt signalling [79]. This link was further substantiated by the findings that (i) Kaiso repressed a subset of Wnt target genes (*cyclinD1, matrilysin, siamois*) [79, 100], (ii) Kaiso inhibited β-catenin mediated activation of these target genes [64, 79, 82, 92, 100], and (iii) p120$^{ctn}$ over-expression alleviates Kaiso-mediated transcriptional repression and enhances Wnt target gene transcription **(Figure 4)** [64].

### 1.9 The role of POZ-ZF proteins in Cancer

Since their original identification and characterization, there is mounting evidence that links POZ-ZF proteins to cancer. Approximately sixty POZ-ZF proteins are encoded in the human genome, many of which are associated with tumourigenesis. For example, the promyelocytic leukemia zinc finger protein (PLZF) is involved in promyelocytic leukemia and B cell lymphoma 6 (Bcl-6) is involved in B cell fate determination and is deregulated in non-Hodgkin's B-cell lymphoma [77, 78]. Other POZ-ZF proteins include ZBTB7 (or leukemia/lymphoma related factor (LRF)), osteoclast derived zinc finger protein (OCZF), factor binding to IST-1 (FBI-1), hypermethylated in cancer 1 (HIC-1), Fanconi anemia zinc finger (FAZF), testis zinc finger protein (TZFP) and Kaiso, reviewed in [62]. These proteins play important roles in DNA damage responses and cell cycle progression in cancer; most behave as transcriptional repressors while some play dual roles as a repressor and activator (e.g Miz-1, Kaiso) [1, 64, 92, 96, 101].

Since POZ-ZF proteins heterodimerize via their POZ domains, the possibility exists that they may regulate each other's transcriptional activity or play redundant roles in cells. The tissue specific expression of different POZ-ZF proteins may result in tissue-specific or cancer-specific functional behaviour. The mechanisms that regulate the activity of Kaiso are currently unknown, but it has been suggested that the Kaiso-like proteins ZBTB4 and ZBTB38 may play functionally redundant roles in cells in the absence or depletion of Kaiso [62]. These two proteins contain Kaiso-like zinc fingers and repress methylated DNA *in vitro*[33].

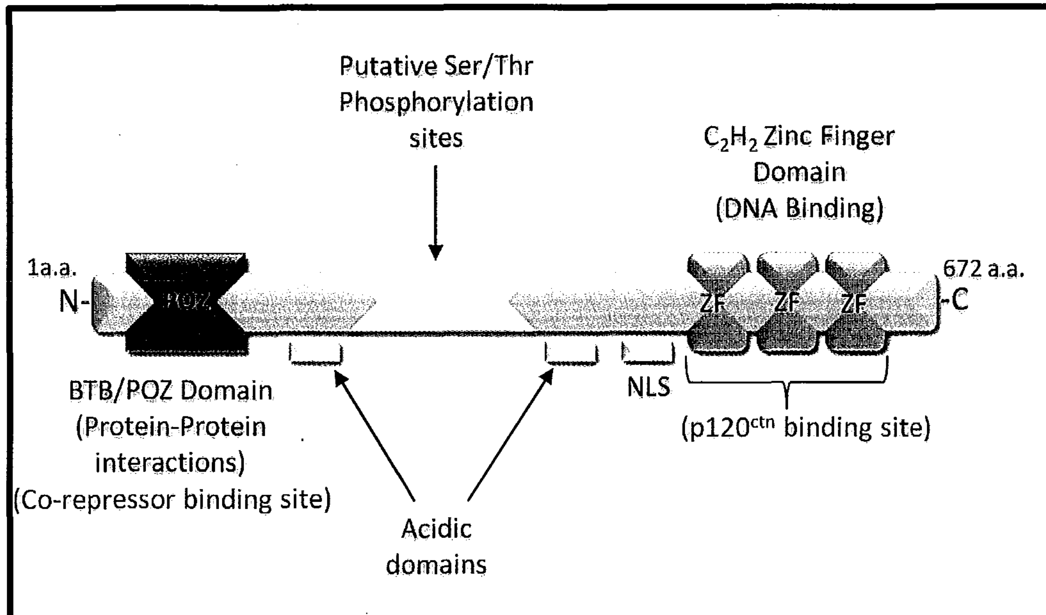



**Figure 3: Schematic representation of Kaiso** Kaiso is a 671 amino acid protein that possesses an amino terminal protein-protein interaction POZ domain, and a carboxy terminal DNA binding domain with three $C_2H_2$ zinc fingers. Between the POZ and ZF domains, Kaiso also has a nuclear localization signal (NLS) and two highly acidic regions that may play a role in transcriptional activation. Kaiso also contains several putative Ser/Thr phosphorylation sites that may regulate its activity.

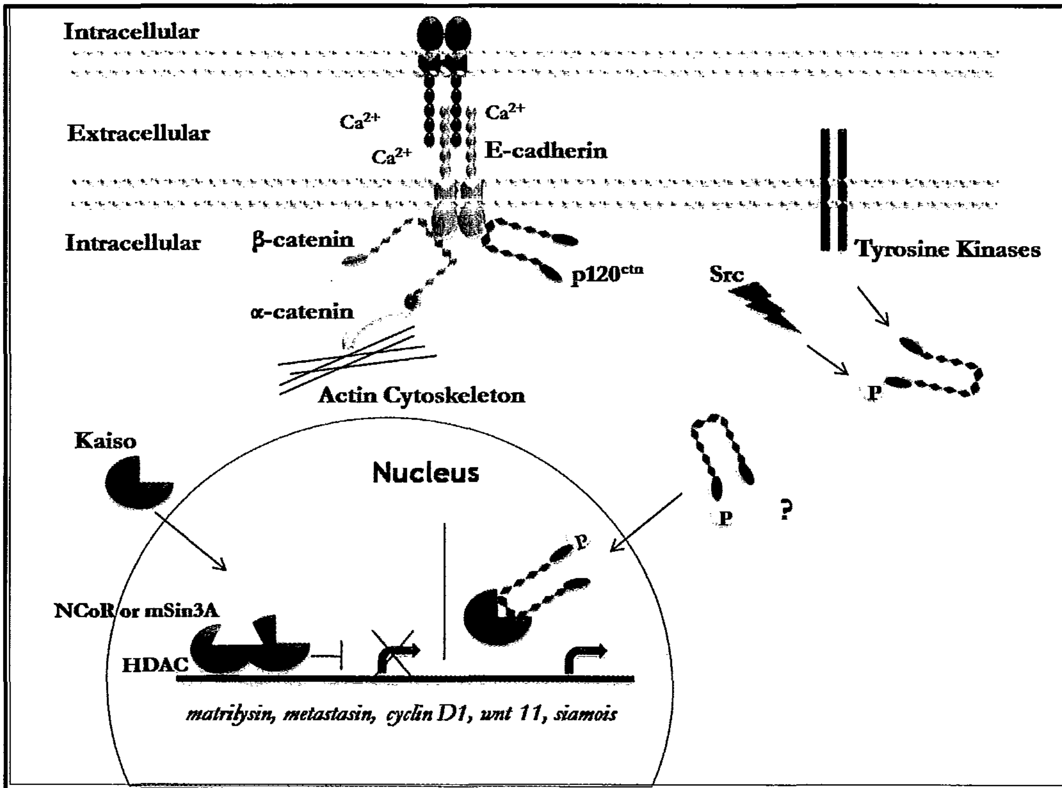



**Figure 4: Kaiso/p120$^{ctn}$ regulation of Wnt target genes.** p120$^{ctn}$ is phosphorylated by Src or other unknown kinases as a result of RTK activation. It then translocates to the nucleus, binds to Kaiso's DNA-binding ZF motif and inhibits Kaiso-DNA binding. p120$^{ctn}$ thereby derepresses or inhibits Kaiso's repression of Wnt target genes. Within, the nucleus, Kaiso regulates *matrilysin, siamois* etc. via recruitment of HDAC complexes containing HDAC, mSin3A and NCor.

## 1.10 Kaiso function in mammalian tumourigenesis

In addition to Kaiso's negative regulation of several tumourigenesis-associated genes (e.g. *cyclin D1* and *matrilysin*), several studies have implicated Kaiso in tumourigenesis. A Clontech Cancer Profiling Array representing 12 different human tissue types was screened by our laboratory using a Kaiso-specific cDNA. Decreased Kaiso expression was detected in 30% of human breast tumour tissue and 50% of human ovarian tumour tissue (our unpublished data). Another study examined Kaiso protein expression in various human tumour tissue and found that Kaiso's subcellular localization varied according to the tissue microenvironment; in some tissues it was mainly nuclear or cytoplasmic, in others it was both and in others it was completely absent [99]. This was in stark contrast to cultured mammalian tumour cells where Kaiso is primarily nuclear. Interestingly however, when cultured cells with nuclear Kaiso were xenografted onto nude mice, Kaiso subcellular localization shifted from the nucleus to the cytoplasm [99]. Previously, increased Kaiso expression was seen in human colon cancers and was upregulated in murine intestinal tumours; Kaiso-null mice were viable, fertile and displayed delayed tumour onset when crossed with $APC^{Min/+}$ (**m**ultiple **i**ntestinal **n**eoplasia) tumour susceptible mice [83].

These studies imply that Kaiso's subcellular localization is dynamic and possibly tissue specific, and hence it is important to elucidate the upstream signalling events that regulate the function and interactions of Kaiso and $p120^{ctn}$. The delay in tumour onset

caused by lack of Kaiso suggests that Kaiso facilitates tumourigenesis, and Kaiso's cytoplasmic localization in tumour tissue likely means it is unable to repress its genes.

### 1.11 Rationale

In recent years, several lines of evidence have hinted at the involvement of $p120^{ctn}$ and Kaiso in a novel signalling pathway that converges on and negatively regulates the canonical Wnt pathway. Previous evidence has shown a controversy in how levels of Kaiso affect its endogenous regulation of target genes. Loss of function of Kaiso has been shown to abrogate Wnt-mediated activity of a reporter; whereas gain of function analysis of Kaiso showed synergistic or suppressive effects in a dose-dependent manner [54]. Another unanswered question is whether Kaiso plays on oncogenic or tumour suppressor role in tumour cells. While some evidence implicates Kaiso as a tumour suppressor (decreased expression in breast, ovarian and some colon tumours), other studies implicate Kaiso in tumour initiation or anti-apoptosis (Kaiso-deficient mice showing a resistance to intestinal cancer) [83]. These contradicting findings suggest that Kaiso's role may be cell or tissue type dependent, and that it plays both a tumour suppressing and initiating role in different tissues. The **main objective** of my thesis is to elucidate the biological role of Kaiso in tumourigenic processes and to correlate the expression patterns of Kaiso and its target genes (*cyclinD1, matrilysin* and *MTA2*) in human breast normal and tumour tissue.

## Hypothesis

**Kaiso over-expression will have tumour promoting effects and Kaiso depletion will have tumour suppressing effects on breast tumour cells.**

## MATERIALS AND METHODS

### 2.1 Cell culture

The human breast adenocarcinoma cell line MCF7 was used for these studies and was purchased from ATCC (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, California) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone, Utah), 0.1 mg/ml penicillin/streptomycin and 0.25 μg/ml Fungizone (Invitrogen, California). The cells were cultured in a 5% $CO_2$ humidified incubator at 37°C. The MCF7 Kaiso over-expression cell line (pcDNA3-Kaiso) and its control (pcDNA3-empty) were previously made and were grown in G418 (Geneticin) (GIBCO, Cat #11811-031) selective media, once every 4-5 passages. The MCF7 Kaiso-depletion cell line (pRS-Kaiso) and its control (pRS-scrambled Kaiso) were grown in Puromycin (Invivogen) selective media, once every 4-5 passages.

### 2.2 Western blot Analysis

Cells were cultured until they reached 90% confluency in 100 mm dishes and then washed twice with 5 ml ice-cold PBS. The cells were then lysed with 450 ml Nonidet P-40 lysis buffer (0.5 % NP-40, 50 mM Tris, 150 mM NaCl) containing protease and phophatase inhibitors (1 mM PMSF, 10 μg/mL Leupeptin, 10 μg/mL Aprotinin, 1 mM Sodium orthovanadate, and 25 mM EDTA) for 7 minutes on ice. The lysates were then harvested with a cell scraper and transferred to a 1.5 mL Eppendorf tube. Cell debris was

pelleted by centrifugation at 4°C for 5 minutes at 14,000 r.p.m. and lysates were transferred to a new tube. A Bradford Assay (BIO-Rad,CA) was performed to determine the protein concentration of the lysates. Equal amounts of protein (100 μg) were re-suspended in 2X Laemmli Sample Buffer (0.1 M Tris [pH 6.8], 2 % SDS, 10 % sucrose, 0.008 % Bromophenol blue, 0.24 M β-mercaptoethanol), boiled for 5 minutes, and run on a 7 % SDS-polyacrylamide gel for 4 hours at 35 mA. The resolved gel was then transferred to a nitrocellulose membrane (Perkin Elmer) using the Hoeffer semi-dry transfer apparatus (Amersham/Pharmacia, California). The membrane was then blocked for 15 minutes at room temperature in 3 % milk in **T**ris-**B**uffered **S**aline (TBS) [pH 7.4]. The blocked membrane was then incubated overnight at 4°C with primary antibodies in 3 % milk-TBS. Antibodies were used at these specified concentrations for Western blotting: Kaiso rabbit polyclonal (1:12,000) (Daniel lab) and β-tubulin mouse monoclonal antibody (1:40,000)(Sigma, MO, Cat#076K4770). The membranes were washed five times for five minutes each with TBS the following day to remove any unbound primary antibody and residual milk. Membranes were then incubated for 2 hours at room temperature with horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibody (1:40,000 in 3 % milk-TBS) (Jackson ImmunoResearch, PA). The membranes were again washed with TBS five times for five minutes each before incubation with the **E**nhanced **C**hemi**L**uminescence system (ECL) (Amersham/Pharmacia) according to the manufacturer's protocol. Membranes were developed on XAR film

(KODAK BioMax, NY) using the developer in the Department of Biology, Life Science Building at McMaster University.

**2.3 Focus formation assay using Rat-1 fibroblasts**

The focus formation assay is used to examine the oncogenic or tumour-initiating potential of a gene of interest. The focus formation assay was performed using the non-transformed Rat-1 rodent fibroblast cell line. Briefly, the Rat-1 fibroblasts were grown to a confluency of 60-70 % in 6-well dishes in DMEM supplemented with 10 % FBS and an antibiotic-antimycotic cocktail containing penicillin, streptomycin and fungizone (PS/F) prior to transfection. Three wells of a 6-well plate were transfected with 2 µg test DNA diluted in 150 mM NaCl and 30 µl ExGen 500 *in vitro* transfection reagents (Fermentas, Canada). The mixture was gently vortexed and incubated without disturbing for 15 mins at RT to allow for reagent/DNA complex formation. The complexes were then added dropwise to the cells in fresh serum-supplemented DMEM medium with the antibiotics/antimycotic. The over-expression plasmids used were pcDNA3-Kaiso and pcDNA3-empty as a control, whereas the siRNA knockdown plasmids were pRS-Kaiso, pRS-scrambled-Kaiso and pRS-empty, of which the latter two were controls. A vector containing the Neu oncogene with a mutation resulting in a constitutively activated Neu receptor (pJ4Ω-NeuNT6/YC) (Warner Lab) was used as a positive control. The cells were also co-transfected with the pGk-puro vector (phospho glycerate kinase) to calculate transfection efficiency. The cells with transfection complexes were incubated at 37°C

with 5 % $CO_2$ for 7 hrs, following which the reagent was aspirated and supplemented with fresh DMEM growth medium. The cells were allowed to grow until 90 % confluency after which they were trypsinized and the cells from three wells were pooled together, before culturing in 6 individual 100 mm tissue culture plates. Three plates were cultured in low-serum containing DMEM supplemented with 2 % FBS and PS/F and three plates were cultured in low-serum DMEM supplemented with 2 % FBS, PS/F and 2.2 mg/ml puromycin. The cells were then cultured and monitored routinely over a period of 4 weeks, with the media replaced every 3 days. Once the foci in the low-serum media containing plates were visible to the human eye, and the puromycin-resistant colonies in the three antibiotic-containing media plates were at a diameter of 3 mm, the plates were ready to be stained. The cells were fixed with 5 ml 10 % formalin buffered phosphate and left at RT for 1 hour, before being washed with 5 ml PBS. The cells were then stained with a solution of 2 % Giemsa stain in PBS for a period of 12 hours. The following day, the cells were washed with 5 ml PBS and left overnight to dry. The number of foci in each triplicate plate was counted and an average of all three plates was taken per trial. The number of puromycin colonies was also counted in a similar manner, and used to calculate transfection efficiency of each vector. Relative number of foci was generated by dividing the average number of foci by the average number of puromycin colonies per vector transfected. The experiment was performed three times and an unpaired, two-tailed, two sample T-test was performed in order to determine statistical significance.

## 2.4 Cell Proliferation Curves

Cell proliferation curves were generated over a six day period using the MCF7 Kaiso-misexpressing cells. Briefly, MCF7 cell lines were grown in 100 mm tissue culture plates to a confluency of ~80 %. The media was aspirated and the cells were washed with 5 ml PBS followed by trypsinization with 1 ml of Trypsin-Versene (EDTA). Once the cells were no longer adherent, 9 ml of fresh supplemented DMEM was added to resuspend the cells. 0.5 ml of resuspended cells were added to a blood cell counting vial (VWR) and the number of cells in each plate was calculated using the Beckman-Coulter Cell and Particle Counter according to the manufacturer's protocol. 5,000 cells of each cell line were seeded into a 24-well tissue culture plate treated with vacuum gas plasma (Becton Dickinson) containing 1 ml of DMEM supplemented with FBS and PS/F. The cells were grown in a 37 °C incubator with 5 % $CO_2$ overnight. On the following day, the media was aspirated carefully from the first four wells (labelled Day 1) so as not to disrupt the cells. The cells were trypsinized with 0.5 ml Trypsin-Versene and added to a blood cell counting vial with 9.5 ml of Isoton II solution and cell numbers were calculated using a Beckman-Coulter Cell and Particle Counter. This procedure was performed at the same time everyday for the following five days, thereby providing cell numbers in four wells over a period of 6 days. The cell numbers were then averaged across the wells to get an average cell density each day for 6 days. The experiments were repeated in triplicate and Graphpad PRISM software was used to perform a non-linear regression analysis using the exponential growth curve equation.

## 2.5 Wound Healing Assay

Prior to the wound healing assay, MCF7 wild type and Kaiso misexpressing cell lines were cultured in 60 mm tissue culture plates until a confluency of 90-100% was reached. Using a marker, a line was drawn under each plate in areas of similar confluence. Using a p200 pipette tip, the cells were scratched in a straight line perpendicular to the marked line. The culture media was aspirated and cells were washed with 5 ml 1 x PBS to remove the dislodged cells away from the plate surface. The cells were then supplemented with 5 ml of fresh culture media, added gently to the side of the plate to avoid disruption of cells. The scratch was then imaged with 5x and 10 x objectives using a Zeiss AxioVision microscope (Germany) under phase contrast as the cells filled in the damaged region at 0, 12 and 24 hours post scratching. Due to cell death at the latter time points which resulted in a lot of cell debris, the media was removed and centrifuged to pellet the dead cells and debris. The old media was then re-applied to the cells so as to prevent re-stimulation of the cells that could be caused by fresh growth factors.

## 2.6 Matrigel Invasion Assay

BD Biocoat matrigel invasion chambers with 8 µm pores in 24 well Tissue culture dishes were thawed from -20 °C to RT and rehydrated for two hours with serum free warm (37 °C) bicarbonate based culture medium in a 37 °C incubator with 5 % $CO_2$. Control chambers lacking matrigel were also treated in the same manner. Following

rehydration, the medium was removed and cell suspensions containing 250,000 cells of each cell line were prepared in serum free media. Chemoattractant (DMEM supplemented with 10 % FBS) was added in amounts of 0.75 ml to the wells of the BD Falcon 24-well tissue culture plates, and the chambers and control inserts were transferred to the wells containing the chemoattractant using sterile forceps. The cell suspensions in serum free media were immediately added into the invasion chambers in 0.5 ml volumes. The chambers were then incubated for 22 hours in a humidified tissue culture incubator at 37 °C with 5 % $CO_2$. The following day, cells that failed to migrate from the upper surface of the membrane were removed by 'scrubbing'. In brief, serum-free media and any remaining cells were aspirated from the inserts, and a sterile cotton swab dipped in serum free media was used to remove cells from the upper membrane surface. The inserts were then washed twice with serum free media to dislodge all remaining non-migratory cells. Matrigel and control inserts were then fixed for 10 minutes in ice cold 100 % Methanol and subsequent staining was performed. The inserts were incubated in wells containing 0.5 ml of Hoechst solution in 1x PBS at a dilution of 1:300 for 1 hour in the dark. The inserts were then briefly rinsed with a solution of 1x PBS, followed by a brief rinse in tap water to prevent formation of PBS crystals. Inserts were then laid upside down to dry in a fume hood. The membranes were then removed from the insert housing by inverting the insert and inserting the tip of a scalpel blade through the membrane at the edge adjacent to the housing wall. The inserts were then rotated against the stationary blade, such that the membrane was released. The inserts

were removed with sterile forceps and mounted using Prolong-Gold Antifade Reagent (Invitrogen, CA). Fluorescent images for the DAPI stain were acquired using a Zeiss Axiovert 200 microscope, and the number of cells invading each insert was counted by examining five individual fields of view per insert. The 63x objective was use to perform fluorescent microscopy and therefore, I was not able to image the entire insert. Therefore I picked five fields of view and imaged those fields in all the inserts. Three inserts were analyzed per cell line, per experiment, and the experiment was performed three times or in triplicate.

### 2.7 RNA isolation from human breast tissue

Human breast matched normal and tumour tissue were purchased and received from the Ontario Tumour Bank in February 2009. RNA was isolated from each sample in two separate tissue sections for subsequent qRT-PCR experiments. Briefly, the tissue was retrieved from storage in the vapour phase of the -196 °C liquid nitrogen freezer, and kept on ice. A Magna #22 sterile surgical blade and forceps were used to quickly slice a 3 mm cube of the tissue in a sterile bio-safety cabinet environment. The tissue was placed in a microcentrifuge tube containing Buffer RLT (Qiagen RNeasy Kit, Qiagen). The remaining tissue was immediately returned to storage in the liquid nitrogen freezer. The sliced tissue was weighed and the weight recorded before the tissue was chopped in a tissue culture dish and transferred to a glass homogenizer. The tissue was lysed by gently rotating the homogenizer in a circular motion until the tissue was completely

homogenized. The solution was then poured into a microcentrifuge tube and re-homogenized with a 3 ml syringe using an 18 gauge needle. The lysate was gently pipetted up and down approximately 20 times to ensure complete homogenization of the tissue. The solution was then topped up to 900 µl with RNAse-free water (Qiagen) and the lysate was incubated with 5 µl Proteinase K solution at a concentration of 20 µg/µl at 50 °C for 10 mins. The cells were then centrifuged for 3 mins at 10,000 rpm to pellet debris and the supernatant was transferred to a new microcentrifuge tube. Following this, the RNA isolation procedure was performed according to the manufacturer's protocol. The concentration and amount of RNA isolated was recorded and the RNA was temporarily stored at -20 °C. To eliminate contamination by genomic DNA, a subsequent DNAse I digest was performed on the RNA using the Deoxyribonuclease I, Amplification Grade kit (Invitrogen).

## 2.8 Quantitative Reverse-Transcriptase PCR

| Gene | Mobix Accession No. | Primer Sequence | Amplicon length |
|---|---|---|---|
| β-Actin | Fwd. ML-09-517<br>Rev. ML-09-518 | 5'-CTC TTC CAG CCT TCC TTC CT-3'<br>5'-AGC ACT GTG TTG GCG TAC AG-3' | ~100 bp |
| Kaiso | Fwd. ML-16-839<br>Rev. ML-16-840 | 5'-TGC CTA TTA TAA CAG AGT CTT T-3'<br>5'-AGT AGG TGT GAT ATT TGT TAA AG-3' | ~248 bp |
| CyclinD1 | Fwd. ML-08-3222<br>Rev. ML-08-3223 | 5'-CCG TCC ATG CGG AAG ATC -3'<br>5'-GAA GAC CTC CTC CTC GCA CT-3' | ~150 bp |
| Matrilysin /MMP7 | Fwd. ML-18-078<br>Rev. ML-18-079 | 5'-TCT TTG GCC TAC CTA TAA CTG G-3'<br>5'-CTA GAC TGC TAC CAT CCG TC-3' | ~400 bp |
| MTA2 | Fwd. ML-16-941<br>Rev. ML-16-976 | 5'-CCA AGC TAC TGG CTA AGA ACA GAC AAA CTT-3'<br>5'-TTG ATC GCA TTG GCA TTG ATA GGA GCA TAA-3' | ~150 bp |

Using the DNAse I-treated RNA from the breast tissue, a one step quantitative reverse-transcriptase PCR (qRT-PCR) was performed using the Superscript III Platinum One-Step Quantitative RT-PCR System (Invitrogen). The primer sets used were ordered by previous members of the Daniel lab and optimized by myself for the use of qRT-PCR. The primers used were designed to detect *kaiso*, *cyclinD1*, *matrilysin*, *mta2* and *β-Actin* as a control. Each experiment was performed in triplicate using gene-specific primers, SYBR Green Dye, Superscript RT/Taq, ROX reference dye and RNA from each sample, for all the available patients. Each 96-well qRT-PCR plate consisted of two sets of samples, one for the control gene (*β-Actin*) and one for the gene of interest. Due to the number of samples being tested, each experimental trial could only assess one gene of interest at a time. A 'No Template control', a 'no Reverse-Transcriptase control' and a 'No-Primer

control' were performed as negative controls during each experimental trial. The experiments were performed using the ABI Prism 7900HT sequence detection system from Applied Biosystems Inc. The change in CT values obtained from each experiment was used to calculate the mRNA expression of each gene of interest relative to endogenous β-Actin. The results were calculated as a fold change of expression in tumour tissue, relative to the normal matched tissue.

**2.9 Immunohistochemistry**

Normal and tumour breast tissues were thawed, sliced into 5 mm sections, fixed in 10 % neutral-buffered formalin for 48 hours and stored in 70 % Ethanol for further processing. The paraffin embedding, tissue sectioning and H&E staining were performed by the Core Histology Facility at the Centre for Gene Therapeutics (McMaster University). Once slides were obtained, they were de-waxed in xylene twice for 5 minutes each, and hydrated in an ethanol gradient from 100 % to 70 %. The tissue was boiled in a sodium citrate buffer (pH 6.0) for 20 minutes and cooled down at RT for 10 minutes for antigen retrieval. The tissue was then permeabilized with TBS-T (0.05 % Tween-20 in Tris-buffered Saline). Endogenous peroxidase activity was quenched with a solution of 3 % Hydrogen peroxide in TBS for 10 minutes followed by two washes in TBS. Tissue was then incubated for 1 hour in a blocking solution consisting of 1 % bovine serum albumin, normal donkey serum (Jackson Immunoreasearch Inc., NY) and an avidin solution (Vector Labs, Burlingame, CA) to prevent non-specific epitope and avidin

binding. After the blocking step, the primary antibodies were applied at varying dilutions for each protein. The Kaiso rabbit polyclonal antibody was used at 1:1000 (Daniel Lab), Cyclin D1 mouse monoclonal antibody (BD Biosciences) was used at 1:500 (BD Biosciences, Cat# 556470), p120 catenin rabbit polyclonal antibody (Santa Cruz, Cat #sc1101) was used at 1:500; and E-cadherin mouse monocolonal antibody (BD Biosciences, Cat# 610182) was used at 1:400 dilutions. A biotin blocking solution (Vector Labs) was added to the mixture of each antibody to block endogenous biotin sites. The primary antibody incubations occurred overnight in a humidified chamber at 4 °C. A biotin blocking solution without antibody was also used every time as a negative control to test antibody specificity. After overnight incubation, slides were washed with TBS-T and a biotin-conjugated donkey anti-rabbit or goat anti-mouse antibody (Jackson Immunoresearch) was applied at a dilution of 1:1000 for 2 hours. Excess secondary antibody was washed off using TBS-T and the Vectastain Elite ABC (Avidin-Biotin horseradish peroxidase complex) (Vector Labs) reagent was applied to the tissue for 30 minutes. After a brief TBS wash, Vectastain DAB substrate (Vector Labs) was applied for 3 minutes and washed with tap water for 5 minutes after satisfactory color development. The tissue was then counterstained using Harris' modified hematoxylin (Sigma, St. Louis, MO), differentiated in acid ethanol, blued in Scott's tap water substitute and dehydrated in a gradient of Ethanol. The slides were then cleared in Xylene and mounted using Polymount (Polysciences Inc, Warrington, PA). Images were captured using a Zeiss Axiovert 200 microscope.

# RESULTS

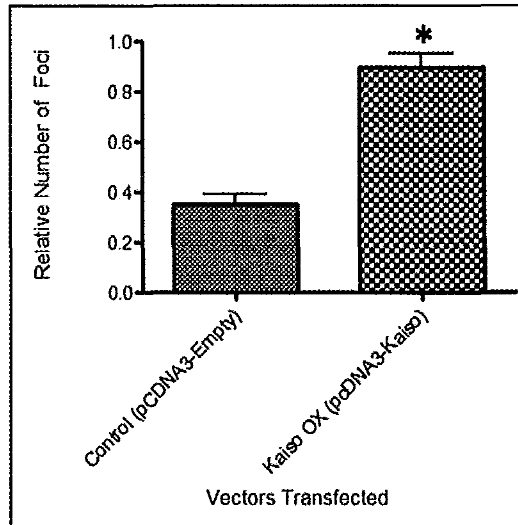
## 3.1 Kaiso over-expression promotes oncogenic transformation in Rat-1 rodent non-transformed cell lines.

The focus formation assay using Rat-1 rat embryo fibroblast cells, is commonly used as a model for *in vitro* transformation and oncogenesis [89]. Rat-1 cells are immortalized, non-tumourigenic cells that have a low incidence of focus formation *in vitro* and fail to form tumours in animals [89]. When transformed, Rat-1 cells will display loss of contact-inhibition and grow as foci (colonies or clumps of cells) [55, 89]. Therefore, transformed Rat-1 colonies are considered to represent tumours *in vitro* and are generally caused by over-expression of an oncogene or knockdown of a tumour suppressor.

To determine Kaiso's potential oncogenic or tumour-suppressing role in cellular transformation, we assessed the effects of Kaiso over-expression or depletion on Rat-1 cells. The cells were also transfected with the respective control vectors for each condition. Our results obtained over three independent focus formation experiments were subjected to an unpaired two-tailed two sample t-test, which showed that Kaiso over-expression enhanced relative focus formation by 2.55 fold (p=0.0013), when compared to cells transfected with the pcDNA3-Empty vector control **(Figures 5A & 7B).**

Kaiso knock-down had the opposite effect on these cells, showing a 2.57 fold decrease in transformation when compared to the control scrambled-Kaiso sequence containing vector **(Figures 5B & 6B).** The activated Her2/Neu oncogene was used as a positive control for focus formation, and it produced a significantly high rate of transformation as expected, causing more foci to be formed on average (~200 foci more) **(Figure 6).** The relative number of foci was calculated by dividing the number of foci counted by the number of puromycin colonies generated by the transfection of each vector. Our results show a statistically significant increase in oncogenic transformation corresponding to the over-expression of Kaiso, suggesting that Kaiso displays oncogenic properties *in vitro* in Rat-1 non-transformed cells.

A.




B.

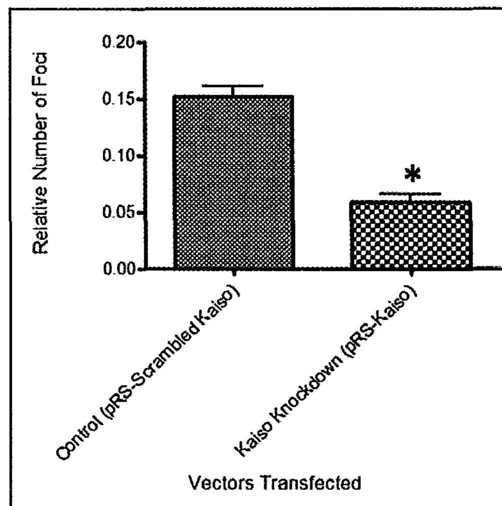



**Figure 5. Kaiso misexpression effects on oncogenic transformation in Rat-1 rodent non-transformed cells. A.** Focus formation experiments indicate that Kaiso over-expression significantly increased oncogenic transformation in these cells, as shown by two sample t-test results (p=0.0013). **B.** Conversely, Kaiso depletion decreases oncogenic transformation relative to the scrambled-Kaiso control (p=0.0013).

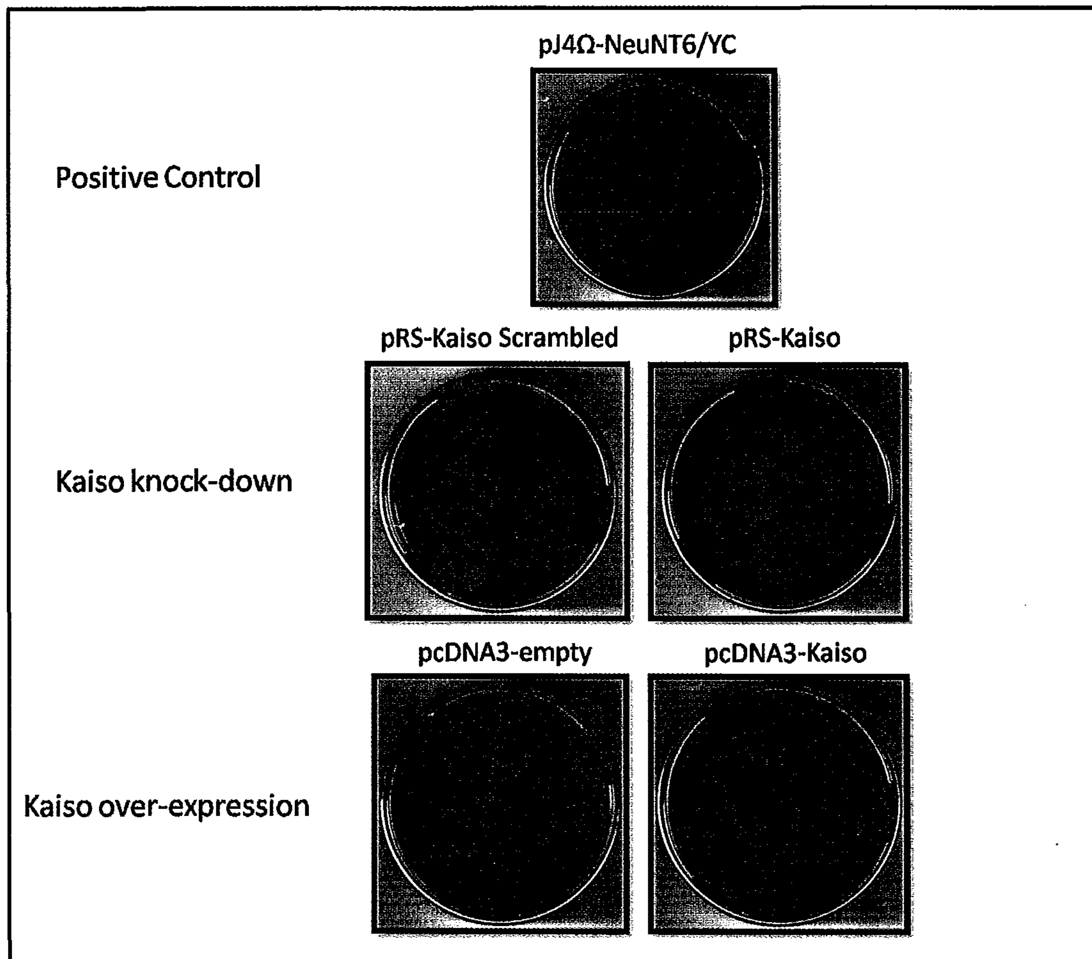



**Figure 6. Focus formation in Kaiso misexpressing Rat-1 cells.** Images of focus formation plates used show differences in foci numbers between the individual vectors transfected. The activated Her2/Neu oncogene, used as a positive control, yields the highest number of foci.

A.

| Vectors Transfected | Average No. of Foci | Average No. of puromycin colonies |
|---|---|---|
| Kaiso Over-expression | | |
| pcDNA3-Empty | 8 | 22.77 |
| pcDNA3-Kaiso | 11.11 | 12.77 |
| Kaiso Knockdown | | |
| pRS-Scrambled Kaiso | 12.77 | 88.66 |
| pRS-Kaiso | 12.55 | 214.77 |
| Control | 184.44 | 129.33 |

B.

| Kaiso Over-expression Vectors | Relative No. Foci ±SE | Two-sample t-test p Value |
|---|---|---|
| pCDNA3-Empty | 0.3514 ± 0.04103 N=3 | 0.0013 |
| pCDNA3-Kaiso | 0.8959 ± 0.05382 N=3 | |

| Kaiso Knock-down Vectors | Relative No. Foci ±SE | Two-sample t-test p Value |
|---|---|---|
| pRS-Scrambled Kaiso | 0.1517 ± 0.009403 N=3 | 0.0013 |
| pRS Kaiso | 0.05903 ± 0.006810 N=3 | |

**Figure 7. Relative number of Foci in Kaiso misexpressing Rat-1 cells and statistical analysis. A.** Average number of foci and puromycin colonies generated from each vector transfected. Relative foci number was calculated as number of foci/number of puromycin colonies. **B.** Statistical analysis of Relative foci numbers in Kaiso over-expression and knockdown vectors show statistical significant changes, analyzed by unpaired, two-tailed, two sample t-test results.

### 3.2 Kaiso misexpression does not affect rates of cell proliferation in MCF7 cell lines.

Cells have autonomous signalling pathways that regulate cell proliferation and inhibit the growth of abnormal cells. An example is the p53 tumour suppressor pathway, that inhibits abnormal cell proliferation by initiating DNA repair, cell cycle arrest or apoptosis [105]. However, deregulation of these proliferative pathways is often a key hallmark of tumour progression. For example, inactivation of the p53 and pRb tumour suppressor proteins causes cells to escape regulatory control with the consequence of uncontrolled proliferation [36, 46]. Potential therapeutic agents aimed at reversing tumour malignancy are often directed towards inhibition of the proliferative ability of cells [35].

In order to determine Kaiso`s role in this process, cell proliferation curves were generated to assess the effects of Kaiso misexpression on cell proliferation rates in MCF7 breast adenocarcinoma cells. As seen by invasion assays, the MCF7 cell line is epithelial, and generally less metastatic than other breast cancer cells such as the MDA-MB-231 cell line [102]. It is therefore a suitable model system to study changes in metastasis-associated processes such as proliferation, invasion and motility, which are caused by the misexpression of oncoproteins or tumour suppressors. Our lab has generated stable MCF7 Kaiso over-expressing and depleted cell lines, which were used for the majority of the experiments in this project **(Figure 8).**

In these experiments, MCF7 cells were trypsinized and cell numbers were counted each day for six days to provide data for the proliferation curves **(Figure 9).** Results obtained from three independent experimental trials indicate that Kaiso over-expressing cells grow slightly slower (growth constant *k* value of 0.6759 compared to 0.7319 for the pcDNA3-Empty control cell line) and have a lower final cell density than the control pcDNA3-Empty vector containing cell line. Following a lag period for the first four days, Kaiso over-expressing cells proliferated exponentially from day 4-6, with a mean population doubling time of approximately 24 hours **(Table 1)**. Although cell numbers continued to increase until a maximum cell density of 2.2 $x10^5$ cells per well was reached, the final cell numbers were lower than those of the control cell line by $3x10^4$ cells.

Consistent with results from Kaiso over-expressing cells, Kaiso-depleted cells proliferated slightly faster (growth constant *k* value of 0.7456 compared to 0.6325 in the pRS-Scrambled Kaiso control cell line), with higher cell numbers at the end of six days. Following a preliminary lag phase over the first two days, the cells grew exponentially from day 2-6 and had a mean population doubling time of approximately 22 hours. Maximum cell density was at 2.6 $x10^5$ cells per well, with final cell numbers being higher than the control cell line by 2.4 $x10^5$ cells **(Table 1)**. These results show slight variations in the growth curves caused by misexpression of Kaiso, however they do not show a significant change in the rates of proliferation. Therefore, this suggests that the misexpression of Kaiso has no effect on cell proliferation in MCF7 cells.

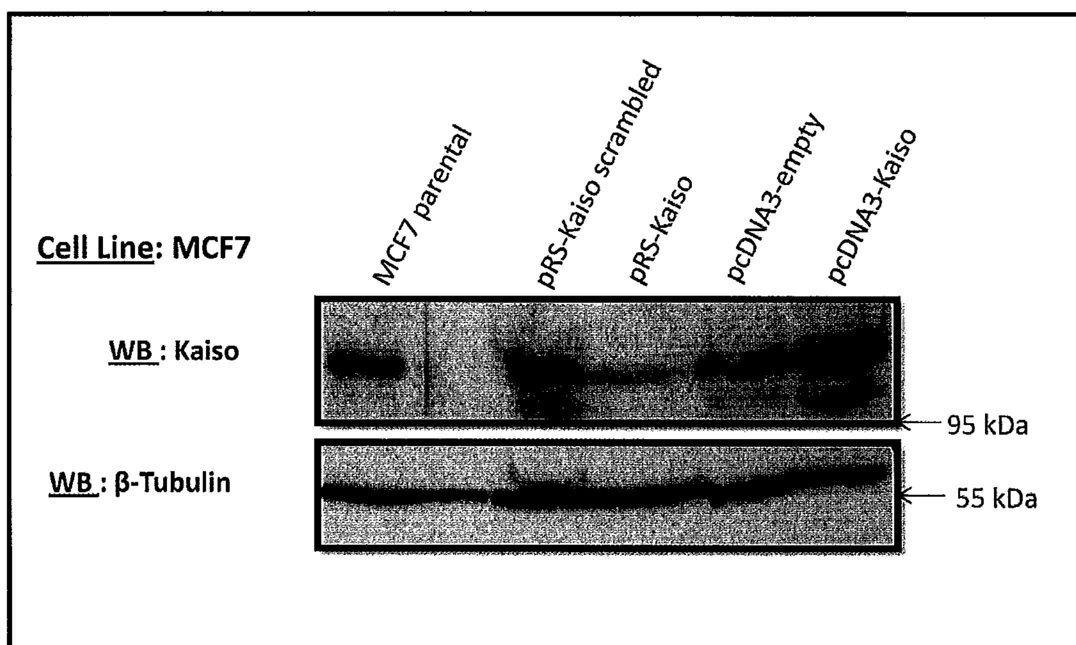
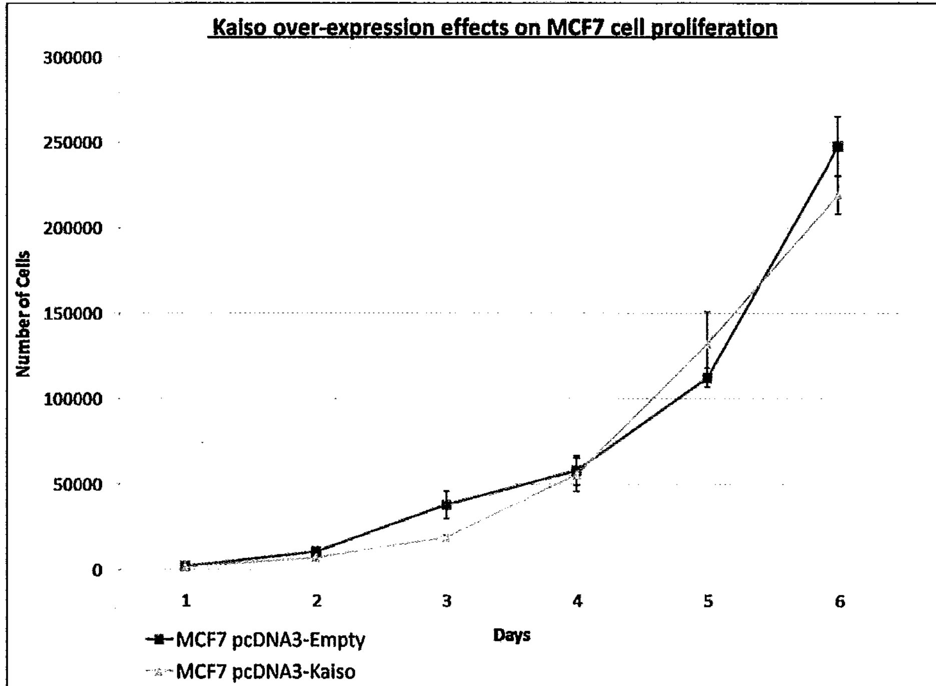



**Figure 8. Kaiso misexpression in MCF7 human breast adenocarcinoma cell lines.** Western Blot analysis of Kaiso expression levels in the Kaiso over-expressing (MCF7 pcDNA3-Kaiso) and Kaiso depleted (MCF7 pRS-Kaiso) cells, indicates that Kaiso is over-expressed ~ 3 fold and depleted respectively when compared to MCF7 parental, MCF7 pcDNA3-empty and MCF7 RNAi scrambled controls. Some protein spillover from the MCF7 parental cells is seen in lane two where no protein was added. Fold change was determined via densitometry analysis.

**Figure 9. Kaiso misexpression does not affect rates of cell proliferation in MCF7 human breast adenocarcinoma cells.** Proliferation curves generated over a period of six days showed a slight decrease in proliferation in the Kaiso over-expression (pcDNA3-Kaiso) cell line and a slight increase in cell proliferation in the Kaiso depleted (pRS-Kaiso) cells compared to controls; however they do not show a significant change in the rates of cell proliferation. The experiment was repeated in quadruplicate wells in three independent trials.

A.




B.

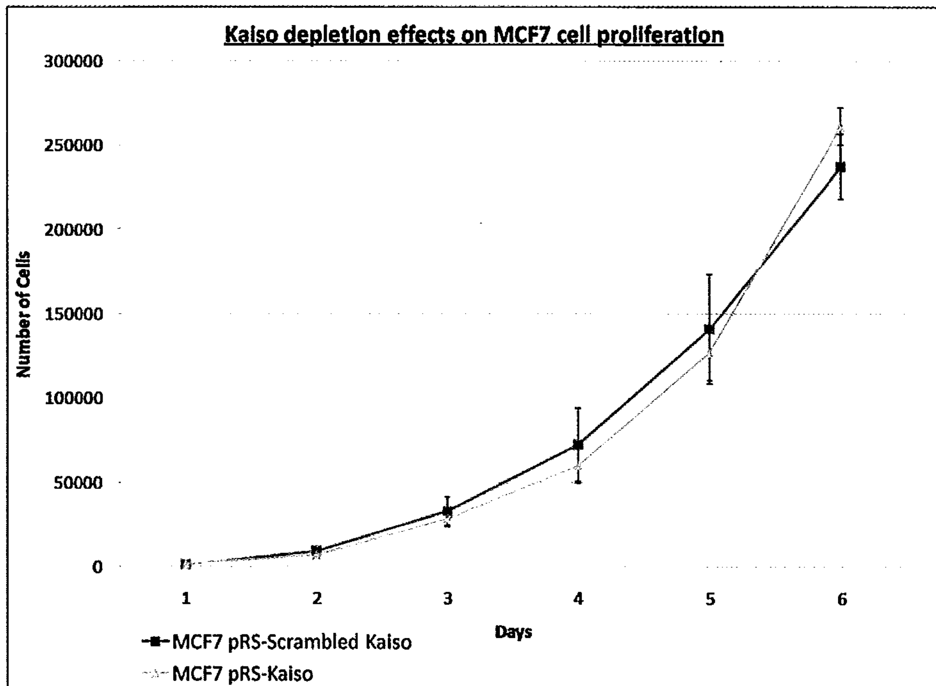


**Table 1: Statistical analysis of Kaiso misexpression effects on MCF7 cell proliferation rates.** A non-linear regression analysis was performed using the exponential growth equation of the cell proliferation curves. The growth constant (k) and population doubling time was calculated using cell density values obtained. Final cell density of each of the Kaiso misexpression cell lines was also compared.

| Cell Type | Growth constant (*k*) | Population doubling time (hrs) | Final Cell density |
|---|---|---|---|
| Kaiso Over-expression | | | |
| MCF7 pcDNA3-Empty | 0.7319 ± 0.09064 | 22.728 | 247586.6 |
| MCF7 pcDNA3-Kaiso | 0.6759 ± 0.1017 | 24.6 | 219348.3 |
| Kaiso Knockdown | | | |
| MCF7 pRS-Scrambled Kaiso | 0.6235 ± 0.1493 | 26.688 | 237265 |
| MCF7 pRS-Kaiso | 0.7456 ± 0.08986 | 22.3104 | 261165.8 |

### 3.3 Kaiso over-expression may enhance cell motility in MCF7 cell lines

The migratory ability of a tumour cell is an important characteristic during metastasis. Primarily, the tumour cell undergoes EMT, characterized by the downregulation of epithelial proteins and expression of mesenchymal-associated proteins [6]. Cellular motility also depends on localized actin polymerization at the leading edge of the cells, and dynamic polymerization and re-polymerization of these actin filaments [19]. These cellular changes that induce motility and migration are often triggered by the misexpression of tumour suppressing or tumour-promoting proteins.

To assess for changes in cell motility and migration caused by the misexpression of Kaiso, wound healing assays were performed. This assay examines cell motility *in vitro* during closure of a scratch made with a pipette tip. The process of wound healing is an example of epithelial tissue regeneration, where a small population of stem cells give rise to differentiated cells that replace the damaged tissue [34]. Although the wound healing assay does not exactly reflect the process of wound healing *in vivo, t*he epithelial cells of a growing tumour display characteristics of accelerated epithelial tissue regeneration [34]. This makes the wound healing assay a suitable experimental tool to investigate the tumourigenic processes of migration and motility in tumour cells.

Experiments were performed over a 24-hour period, with images taken at 12-hour time points. Overall, our results show that Kaiso over-expression increases the motility of the cells, making them appear more fibroblast-like, and causing the wound to

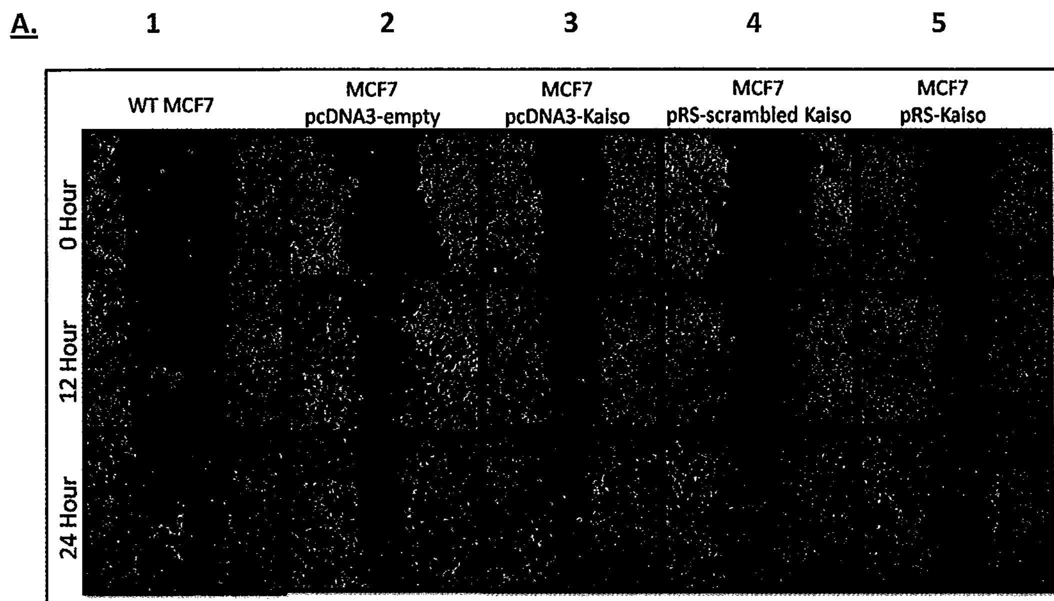
close approximately twice as fast (2.235 fold) compared to the pcDNA3-Empty control MCF7 cell line **(Figure 10A, panel 2-3).** The MCF7 Kaiso over-expressing cells were also more fibroblast-like at the leading edge of the wound, compared to the other cell lines at 24-hours **(Figure 10.B).**

In contrast, the Kaiso-depleted cell line showed slower closure of the wound (approximately 1.2 fold) in comparison to the control MCF7 pRS-Scrambled Kaiso cell line **(Figure 10.A, panel 4-5).** In addition, the Kaiso-depleted MCF7 cells displayed a sheet-like migration at the leading edge as opposed to individual motile cells migrating towards the centre of the damaged region **(Figure 10B).** The distances between the leading edges of the wound i.e. the scratch, were quantified using Image J software and fold migration was calculated for each experimental condition relative to its control. Our results suggest that Kaiso may enhance cell motility in MCF7 cells.

**Table 2: Fold migration of MCF7 Kaiso-misexpressing cells calculated over 24 hours.** Image J software was used to analyze the distances migrated by the leading edges of the wound during the 24 hour period. Fold migration was calculated in the Kaiso over-expressing and depleted cells relative to their respective control cell lines.

| Cell type | Distance migrated | Fold migration |
|---|---|---|
| Kaiso Over-expression | | |
| pcDNA3-Empty | 17 | |
| pcDNA3-Kaiso | 38 | 2.235 |
| | | |
| Kaiso Knockdown | | |
| pRS-Scrambled Kaiso | 24 | |
| pRS-Kaiso | 20 | -1.2 |

A. 1 2 3 4 5




B.

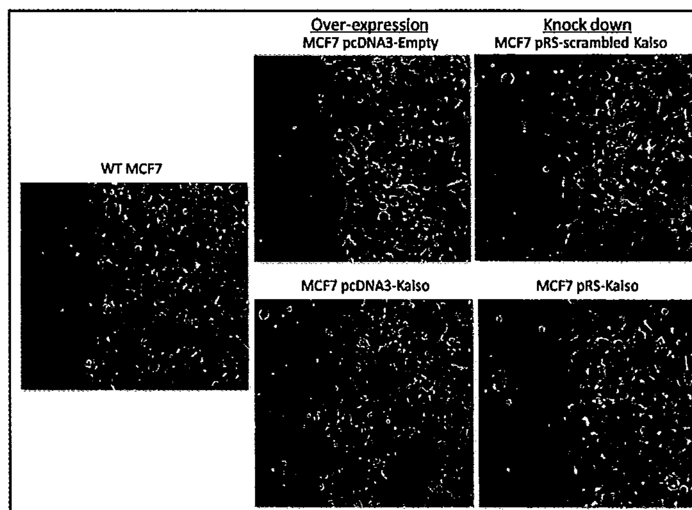



**Figure 10. Kaiso over-expression may enhance motility and may induce morphological changes in MCF7 cells.** Wound Healing assays were performed to study the effects of Kaiso misexpression on cell motility. A. Kaiso over-expression appears to increase cell motility and migration compared to wild type and control cells (panel 3 vs. panels 1&2 at 24 hrs). In contrast, Kaiso-depleted cells appear to move slower and migrate less when 'healing' the wound (panel 5 vs. panels 1&4 at 24 hrs). B. Higher magnification images show that Kaiso over-expressing cells have a more fibroblastic appearance at the leading edge of the wound at 24hrs. Kaiso-depleted cells mostly retained their epithelial-like morphology and migrate in a sheet-like formation.

### 3.4 Kaiso depletion decreases cell invasion in MCF7 adenocarcinoma cells

The invasiveness and capacity of tumour cells to undergo metastasis enables them to leave the primary tumour mass and travel to other regions in the body [46]. We performed matrigel invasion assays to study whether Kaiso misexpression had an effect on cell migration and invasion in breast cancer. This assay determines the invasive potential of tumour cells by examining their capacity to invade a basement membrane [66]. It involves the migration of cells through a matrigel layer which represents the extracellular matrix (ECM). Cells that invade and migrate through the matrigel would mimic cells that destroy the ECM in order to penetrate tissue endothelial barriers and metastasize.

Our results show that Kaiso depletion results in decreased cell invasion in MCF7 cells, displaying approximately 60% lower cell invasion in the pRS-Kaiso cells compared to the control pRS-scrambled Kaiso cells in an unpaired, two-tailed, two sample T-test ($p=0.0269$). Our Kaiso over-expression cells did not yield statistically significant results ($p = 0.2232$), generating varied cell counts across the three experimental trials (Figure 12, Table 3). However, Kaiso over-expression data shows a similar trend as the depletion, appearing to decrease cell invasion. This variation in cell counts may be due to the fact that cell counts were generated using five fields of view at 63x magnification. Therefore, it may be possible to improve cell counts and error bars by examining the entire insert

using a low-magnification fluorescent microscope. This preliminary analysis of MCF7 cell invasion suggests that Kaiso may positively regulate cell invasion through matrigel.

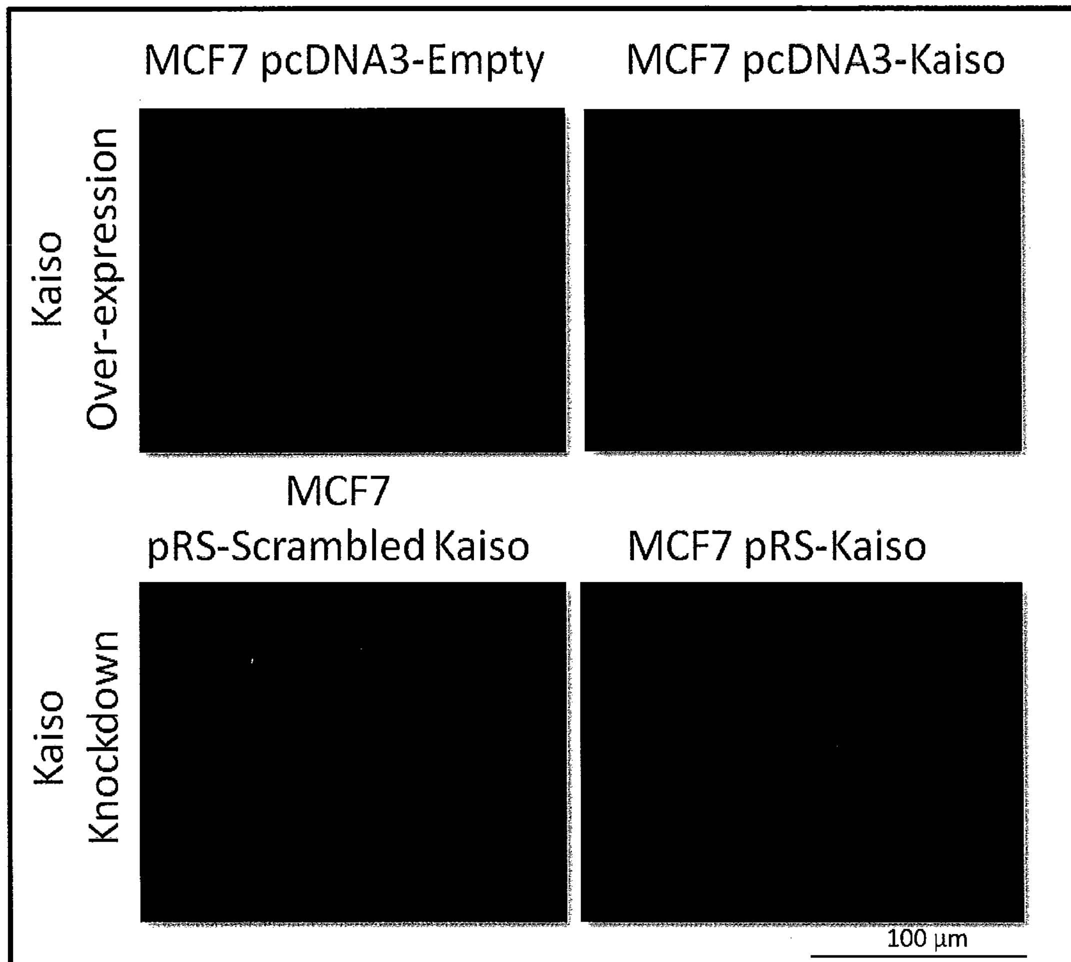
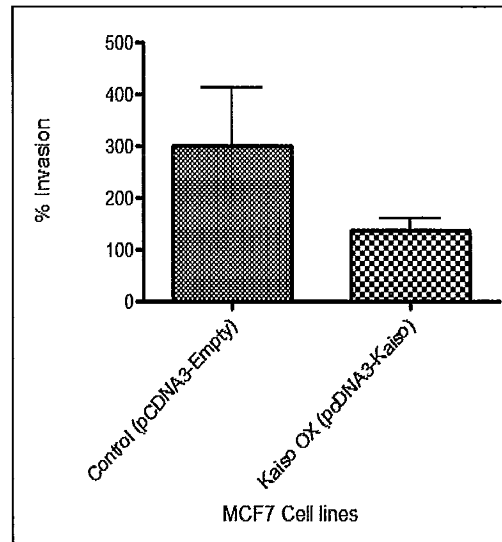



**Figure 11. MCF7 Kaiso-misexpressing cells imaged following invasion through matrigel coated membranes.** Matrigel invasion assays performed on Kaiso misexpressing cells were fixed with methanol and DAPI-stained to count 'live' cells that had invaded through the matrigel coated pores. Inserts were washed with PBS and imaged at 60x magnification. Scale bar= 100µm

**A.**




**B.**

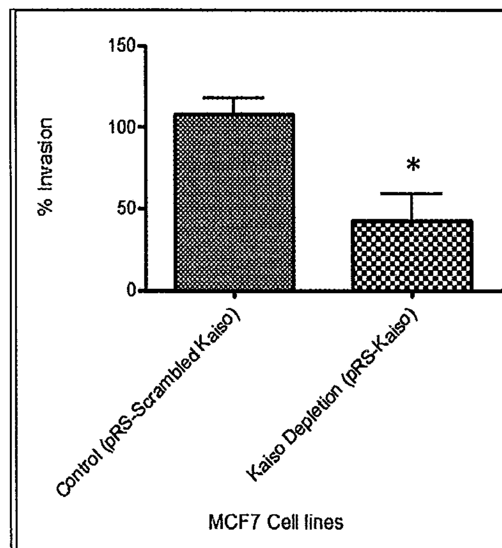



**Figure 12. Kaiso depletion decreases cell invasion in MCF7 cells.** Matrigel invasion assays performed on Kaiso misexpressing MCF7 cells showed 60% decreased cell invasion in the Kaiso depleted cells when compared to the control pRS-Kaiso scrambled cells (p= 0.0269). Kaiso over-expressing cells failed to generate reproducible and statistically significant results. OX= overexpression.

**Table 3: Statistical analysis of Kaiso-misexpressing MCF7 cell invasion over 22 hours.** Graphpad PRISM software was used to perform an unpaired two-tailed two sample t-test on cell numbers obtained from the invaded MCF7 Kaiso-misexpressing cells. % invasion was calculated using cells that migrated through a matrigel layer compared to control uncoated inserts. Kaiso depleted cells displayed a 60% lower invasion relative to the control cells (p= 0.0269).

| Cell Type | % Invasion | Relative to control | p Value | Invasion Index |
|---|---|---|---|---|
| Kaiso Over-expression | | | | |
| pcDNA3-Empty | 301.9 ± 111.7 N=3 | 100% | 0.2232 | 0.457 |
| pcDNA3-Kaiso | 137.9 ± 22.11 N=3 | 54.3% | | |
| Kaiso Knockdown | | | | |
| pRS-Scrambled Kaiso | 108.1 ± 9.669 N=3 | 100% | 0.0269 | 0.394 |
| pRS-Kaiso | 42.55 ± 16.57 N=3 | 60.6% | | |

### 3.5 Correlation between transcript levels of Kaiso and selected target genes (*cyclinD1, matrilysin, mta2*) in human breast carcinomas

Quantitative Reverse Transcription PCR (qRT-PCR) analysis was performed to analyze the expression of Kaiso and three known Kaiso target genes (*cyclinD1, matrilysin, mta2*) in human breast tumour and normal tissue. The results obtained were analyzed by type of carcinoma; the expression levels of Kaiso and its target genes were displayed in bar graphs as seen in **Figure 13.** Each gene was normalized relative to β-Actin, which was used as an endogenous control. Results are shown as fold change of mRNA expression in tumour tissue, relative to matched normal tissue.

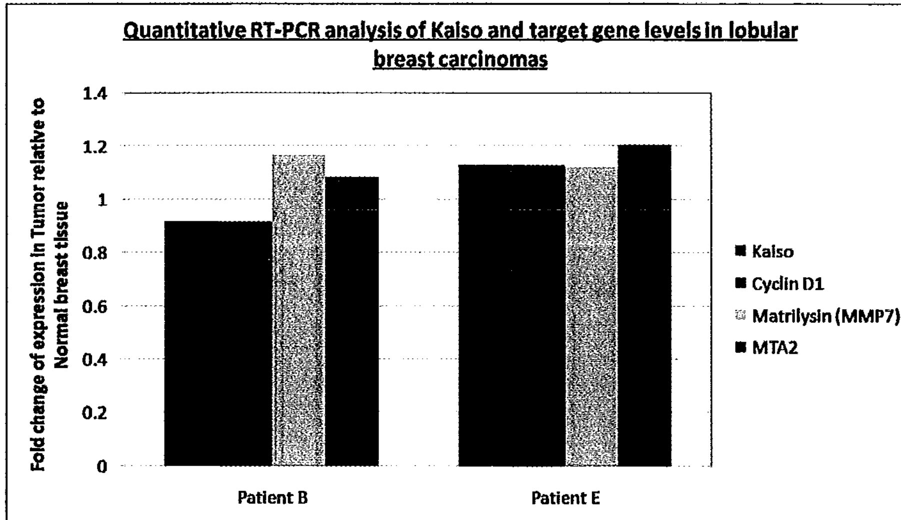
Our qRT-PCR analysis of the infiltrating lobular breast carcinomas (Patients B and E) showed an increase in Matrilysin and MTA2 levels when the tumour was larger than 5 cm, and when vascular and lymphatic invasion had occurred. Kaiso levels negatively correlated with Matrilysin and MTA2, particularly in patient B; however Kaiso and CyclinD1 levels were approximately the same in both patients **(Figure 13A).**

In our ductal breast carcinomas, Matrilysin levels were higher than Kaiso and the other genes in all patients tested (A, C and D). Similar to the lobular carcinomas, the highest expression of Matrilysin was seen in large scale-tumours (10 cm and 11 cm), where vascular and extensive lymphatic invasion had occurred. CyclinD1 levels were either slightly down-regulated or at similar levels to Kaiso in the ductal carcinomas

tested, and our MTA2 expression levels were also varied, showing no specific trend **(Figures 13B & 14B).**

We were unable to perform a matched pair analysis for tumours from patients F and G since we were unable to obtain the normal tissue from the Ontario Tumour Bank. The varied expression levels obtained can be attributed to various factors affecting tumour development, such as the microenvironment, type of breast carcinoma and the metastatic potential of these tumours. However, it is important to note that due to the fact that we were only able to obtain one sample of tissue per patient, these data are not biologically significant. The magnitude of all changes is extremely small as well and likely hints towards biological insignificance. This data would be greatly enhanced by an analysis of microarray data of breast tumours that is publicly available.

A.




B.

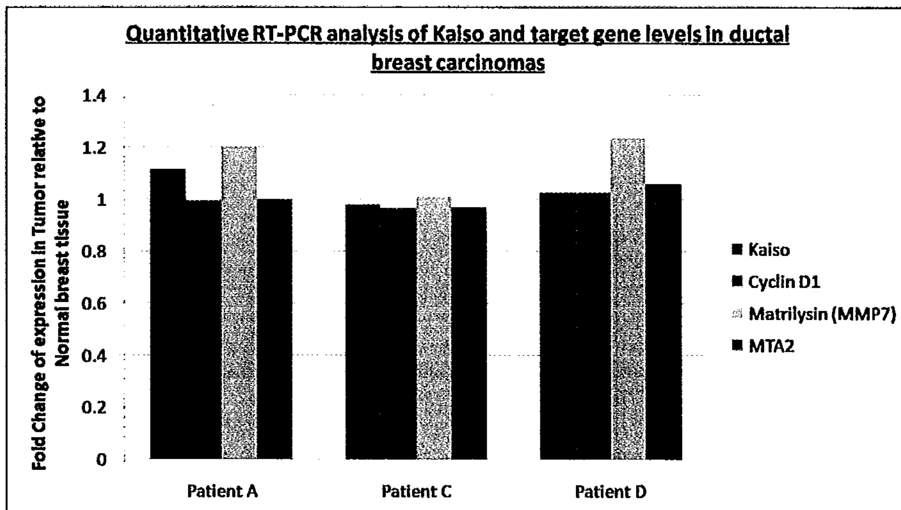



**Figure 13. Quantitative RT-PCR analysis of *kaiso* and target genes on human breast normal and matched tumour tissue.** *kaiso, cyclinD1, matrilysin* (MMP7) and *mta2* mRNA expression levels were examined in patients with ductal and lobular breast carcinomas. **A.** There were similar levels of *cyclinD1* and increased levels of *matrilysin* and *mta2* relative to *kaiso* in the lobular breast carcinomas. Higher *matrilysin* and *mta2* levels corresponded with increased tumour size (11 cm) in patient B. **B.** Ductal carcinomas display varied expression of *cyclinD1* and *mta2* but increased levels of *matrilysin* relative to *kaiso* in all patients.

A.

| Patient | Age | Tumour Size (cm) | Histology | Lymphatic Invasion | Vascular Invasion |
|---|---|---|---|---|---|
| A - A00299 | 41 | 11.00 | Infiltrating ductal, NOS | Y EXT | YES |
| B - A00341 | 34 | 11.00 | Infiltrating lobular, pleomorphic | Y NOS | YES |
| C - A00455 | 46 | 3.50 | Infiltrating ductal, NOS | Y NOS | YES |
| D - D01409 | 56 | 10.00 | Invasive ductal carcinoma | Y EXT | YES |
| E - D01560 | 79 | 1.70 | Infiltrating lobular, pleomorphic | N | NO |
| F - B01019 | 52 | 3.00 | Invasive mammary, NOS | N | NO |
| G - B01698 | 40 | 4.00 | Invasive mammary, NOS | Y NOS | NO |

EXT – Extensive
NOS – Not Otherwise specified

B.

| Type of Carcinoma | Tumour Size (cm) | Lymphatic Invasion | Vascular Invasion | Levels of proteins relative to Kaiso | | |
|---|---|---|---|---|---|---|
| | | | | CyclinD1 | Matrilysin (MMP7) | MTA2 |
| Lobular<br>Patient B | 11 | Y | Y | Similar | Increased | Increased |
| Patient E | 1.7 | N | N | Similar | Similar | Increased |
| Ductal<br>Patient A | 11 | Y, Extensive | Y | Decreased | Increased | Decreased |
| Patient C | 3.5 | Y | Y | Slightly decreased | Slightly increased | Similar |
| Patient D | 10 | Y, Extensive | Y | Similar | Increased | Increased |

**Figure 14. Quantitative RT-PCR analysis results grouped by patient number and disease type. A.** Clinical data of the age, tumour size, histology and presence of lymphatic and vascular invasion in all carcinoma tissues tested, listed by patient number. **B.** Summary of qRT-PCR analysis performed of *cyclinD1*, *matrilysin* and *mta2* levels relative to *kaiso*, grouped by disease type. Y= Yes, N= No.

### 3.6 Subcellular localization of Kaiso and selected target genes in human breast normal and tumour tissue

We performed immunostaining analysis to examine the subcellular localization of Kaiso, Cyclin D1, $p120^{ctn}$ and E-cadherin in our breast normal and carcinoma tissues. H&E staining was also performed to give an overview of breast tissue structure and composition. Images were obtained at 40x objective and grouped by type of carcinoma to examine the localization of each of these proteins in the same tissue section.

In the ductal breast carcinomas, there was negligible Kaiso staining in the tissues from patients C and D **(Figure 15 and 16).** Both samples displayed strong $p120^{ctn}$ staining throughout the cell in the normal tissue, with a decrease in cytoplasmic $p120^{ctn}$ in the tumours. The tissues also displayed strong E-cadherin staining, with an increase in cytoplasmic E-cadherin in the tumour tissue relative to normal. However, it is important to note that E-cadherin was mislocalized in the normal tissue, where it is not membrane-bound but cytoplasmic instead. Finally, CyclinD1 expression varied between the two patients studied; there was negligible CyclinD1 staining in the tissue from patient C, whereas patient D showed that CyclinD1 localized only to the nucleus in the normal tissue, and was significantly higher and localized only to the cytoplasm of the tumour tissue. We were unable to perform IHC analysis on the tissue from patient A since the tissue was degraded due to freeze-thawing and its structure was not intact.

The lobular breast carcinomas show a different trend, wherein there is an increase in both cytoplasmic and nuclear Kaiso staining in the tumour relative to normal.

The normal tissue from both patients (B and E) displayed strong nuclear and cytoplasmic $p120^{ctn}$ and E-cadherin staining, with a significant decrease in both proteins in the tumour tissue **(Figures 17 and 18).** Specifically, $p120^{ctn}$ staining was retained in the nucleus and was significantly decreased in the cytoplasm of both patients. Likewise, E-cadherin displayed strong staining in the normal tissue, but was negligible in the tumour tissue of both patients. However, it must be noted that $p120^{ctn}$ and E-cadherin staining is mislocalized in the normal tissue, where membrane-staining is expected for E-cadherin; and membrane and cytoplasmic staining is expected for $p120^{ctn}$.

The mislocalization in normal tissue seen by these proteins can be attributed to the poor sample quality obtained. However, if similar subcellular localization patterns of these proteins can be attained from fresh-frozen tumour tissue, then it could be attributed to the difference in type of carcinomas and different stages of disease progression of each patient.

**Figure 15: Immunohistochemistry analysis of Kaiso, Cyclin D1, p120$^{ctn}$ and E-cadherin in human ductal breast carcinoma tissue (Patient C).** Results show negligible Kaiso staining in the normal and tumour tissue. The tissue also displays a slight decrease in cytoplasmic p120$^{ctn}$ staining, along with an increase in E-cadherin localization in the cytoplasm. E-cadherin and p120$^{ctn}$ are mislocalized in the normal tissue. Cyclin D1 staining is almost negligible in both the normal and tumour tissue. Scale bar= 100µm

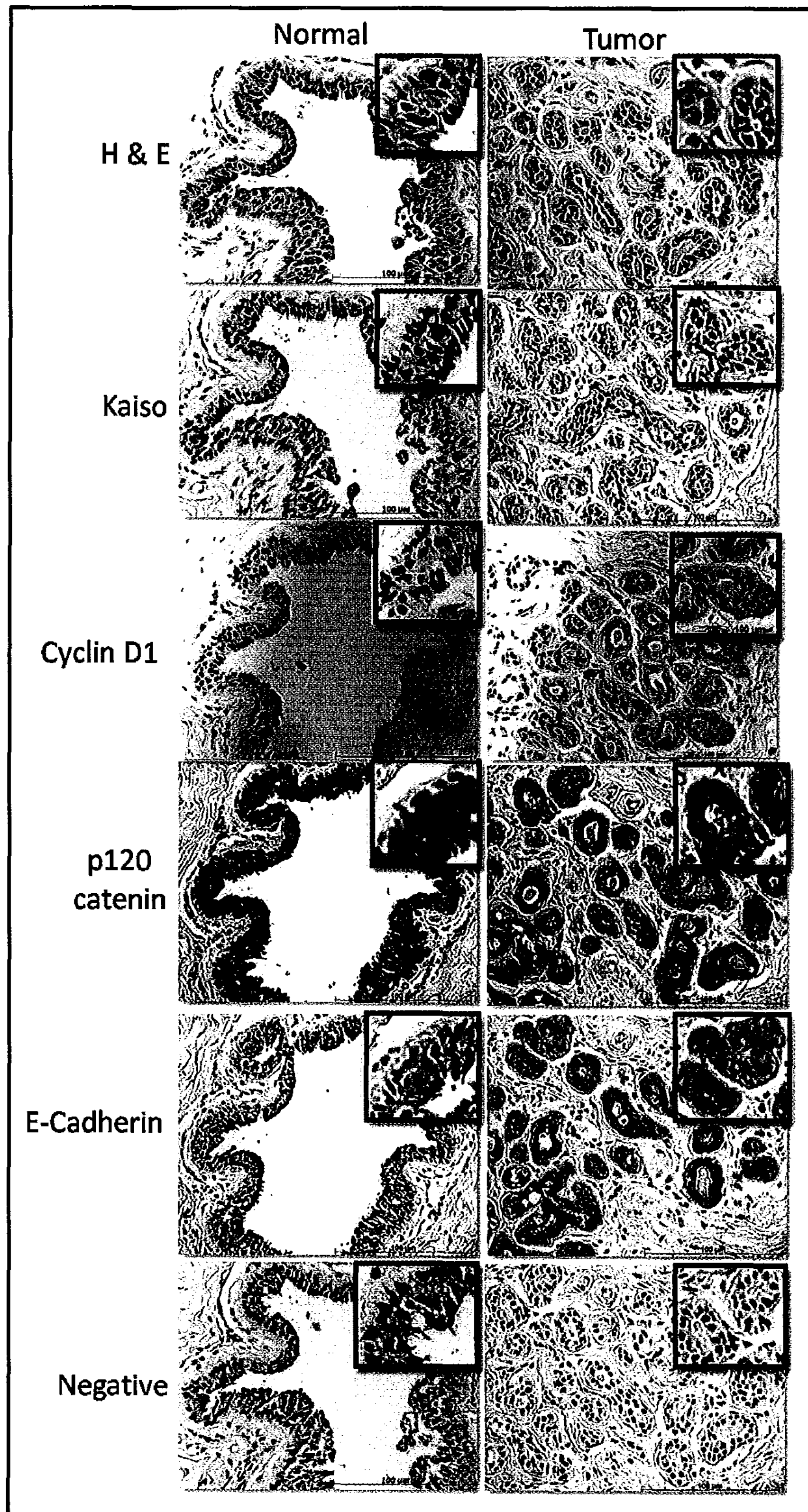

Normal
Tumor
H & E
Kaiso
Cyclin D1
p120 catenin
E-Cadherin
Negative

**Figure 16: Immunohistochemistry analysis of Kaiso, Cyclin D1, p120$^{ctn}$ and E-cadherin in human ductal breast carcinoma tissue (Patient D).** Kaiso staining is negligible in both normal and tumour tissue, p120$^{ctn}$ decreases and there is an increase in cytoplasmic E-cadherin in the tumour tissue relative to normal. E-cadherin and p120$^{ctn}$ are mislocalized in the normal tissue. CyclinD1 is localized primarily in the nucleus in the normal tissue, but increases significantly in the cytoplasm of the tumour tissue. Scale bar= 100µm

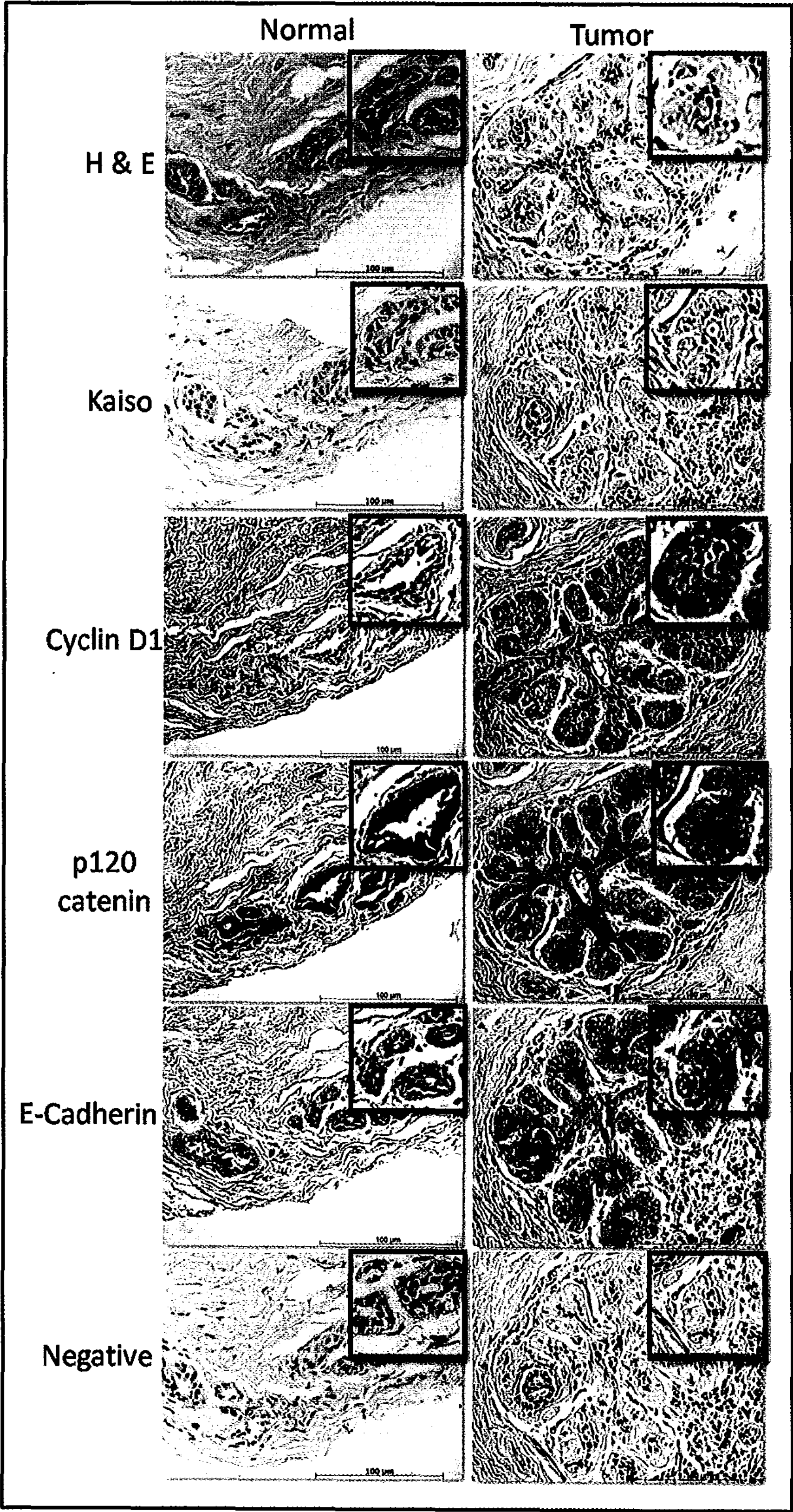

Normal
Tumor
H & E
Kaiso
Cyclin D1
p120
catenin
E-Cadherin
Negative

**Figure 17: Immunohistochemistry analysis of Kaiso, Cyclin D1, $p120^{ctn}$ and E-cadherin in human lobular breast carcinoma tissue (Patient B).** Kaiso staining is stronger than in the ductal carcinoma tissues, with a significant increase in cytoplasmic and nuclear Kaiso staining in the tumour tissue, relative to normal. Conversely, there is a significant decrease in $p120^{ctn}$ staining, both nuclear and cytoplasmic, and significantly decreased E-cadherin staining in the tumour tissue relative to normal. Once again, E-cadherin and $p120^{ctn}$ are mislocalized in the normal tissue. CyclinD1 levels are negligible in both tissues. Scale bar= 100μm

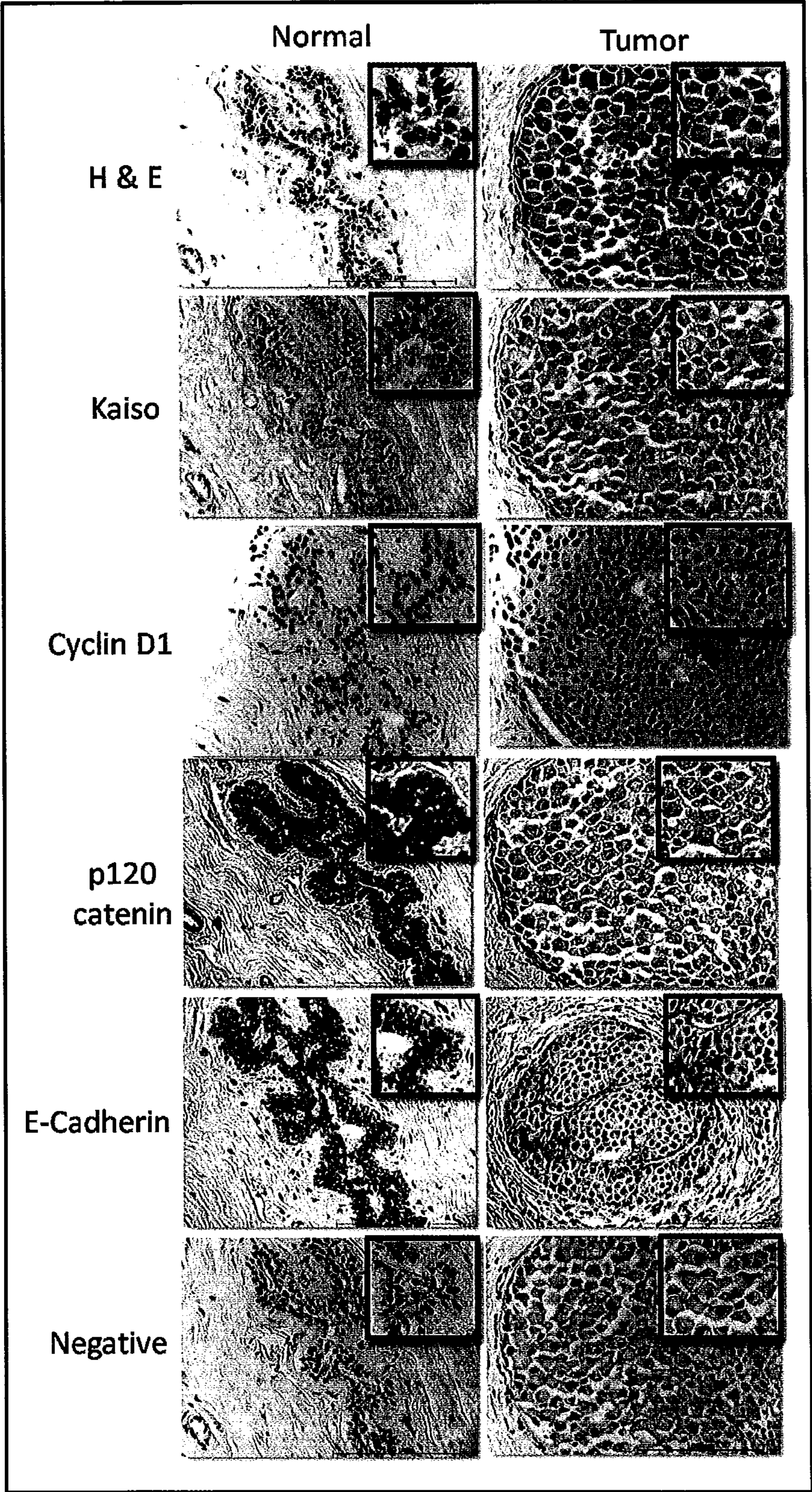
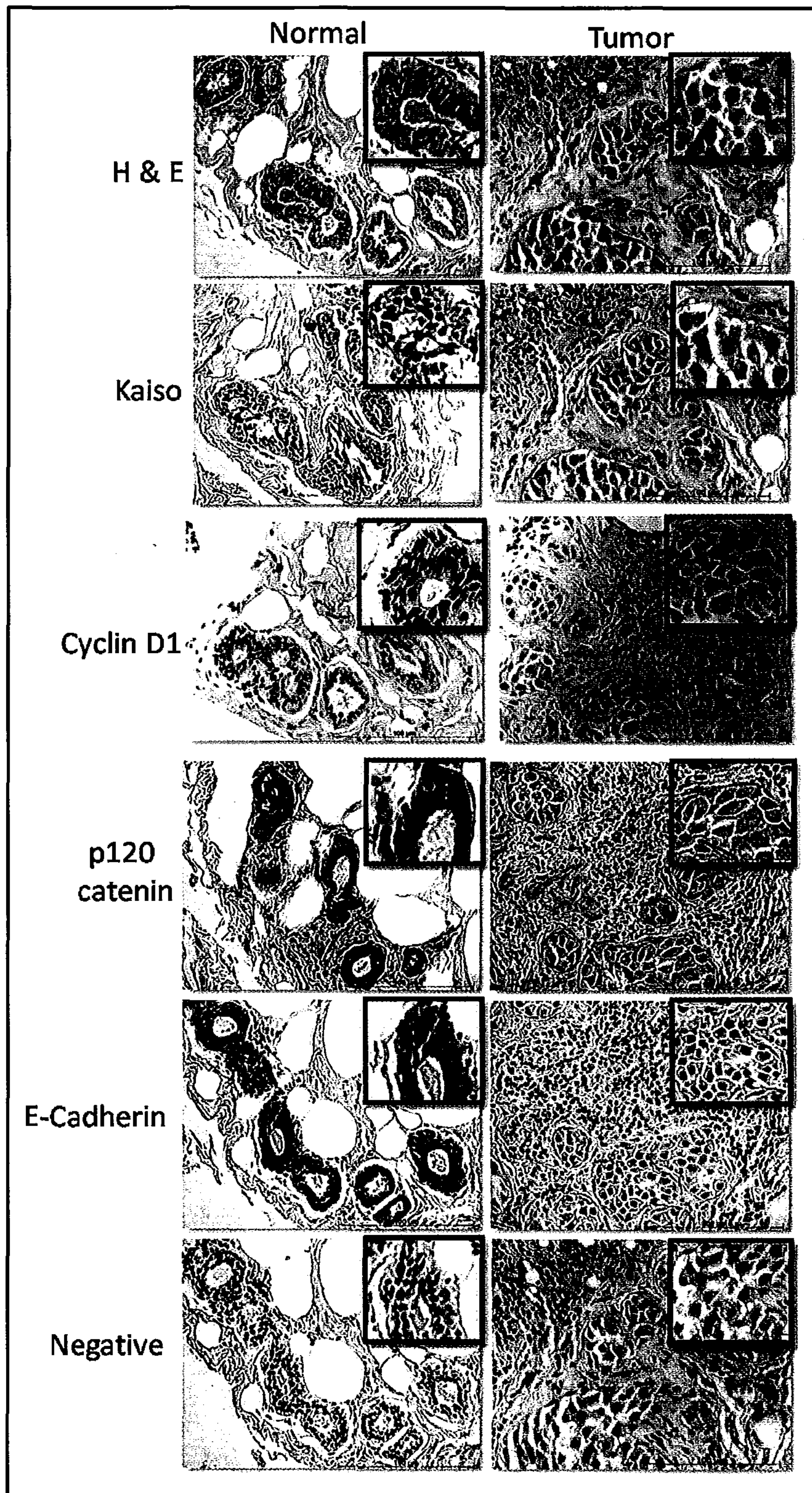

Normal
Tumor
H & E
Kaiso
Cyclin D1
p120
catenin
E-Cadherin
Negative

**Figure 18: Immunohistochemistry analysis of Kaiso, Cyclin D1, p120$^{ctn}$ and E-cadherin in human lobular breast carcinoma tissue (Patient E).** Kaiso staining is again stronger than in the ductal carcinoma tissues, with a significant increase in cytoplasmic and nuclear Kaiso staining in the tumour tissue, relative to normal.Conversely, there is a significant decrease in p120$^{ctn}$ staining, both nuclear and cytoplasmic, and significantly decreased E-cadherin staining in the tumour tissue relative to normal. E-cadherin and p120$^{ctn}$ are mislocalized in the normal tissue. CyclinD1 staining is slightly increased in the cytoplasm as well as the nucleus of the tumour tissue. Scale bar= 100μm


Normal
Tumor
H & E
Kaiso
Cyclin D1
p120
catenin
E-Cadherin
Negative

**Discussion:**

The POZ-ZF family of transcription factors have roles in a variety of processes in development and tumourigenesis [62]. Kaiso, a member of the POZ-ZF family, has been implicated in tumourigenesis through several independent studies. First, a Clontech cancer profiling array representing 12 different human tissue types indicated that Kaiso's expression pattern was decreased in 30% of human breast tumour tissue and 50% of human ovarian tumour tissue (Daniel Lab, unpublished data). While this evidence implicates Kaiso as a tumour suppressor, other studies using Kaiso-null mice crossed with APC $^{Min/+}$ tumour-susceptible mice resulted in mice having decreased susceptibility to intestinal cancer, suggesting a tumour-supportive role for Kaiso [83]. Hence, it is possible that Kaiso plays tumour suppressing <u>and</u> tumour initiating role depending on the cell or tissue type. In fact, there are proteins that display both oncogenic and tumour suppressor roles; for instance, the transcription factor E2F1 [58, 110] and the POZ-ZF protein Miz-1 [1, 101] display both tumour suppressing and oncogenic properties. Hence, the objective of my research thesis was to determine and clarify Kaiso's role as an oncogene or tumour suppressor in breast tumourigenesis by examining its effect on cellular processes such as transformation, cell proliferation, motility and invasion. To this end, we used the human breast adenocarcinoma cell line MCF7 and human breast normal and tumour tissue to correlate the expression patterns of Kaiso and some of its target genes such as *cyclinD1*, *matrilysin* and *mta2*.

**4.1 The effects of Kaiso on oncogenic transformation.**

Tumourigenesis in humans is a multi-step process involving genetic alterations that drive the transformation of normal cells into malignant cells [46]. It is believed that cellular 'transformation' requires six essential alterations in cell physiology to successfully result in malignant growth [46]. These changes include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, unlimited cell proliferation, sustained angiogenesis and tissue invasion and metastasis [46]. These characteristics are common in most types of human cancers and together they represent a complete breach of the anti-tumour defence mechanisms found in cells. As a first step in analyzing Kaiso's role in tumourigenesis, we examined the effect of Kaiso misexpression on oncogenic transformation. Focus formation assays carried out in Rat-1 cells showed that Kaiso over-expression significantly enhanced focus formation (~ 2.55 fold) when compared to the control empty vector, consistently producing more foci in each of the three experimental trials **(Figures 5,6 and 7).** In contrast, Kaiso depletion significantly decreased focus formation (~ 2.57 fold) when compared to the scrambled-Kaiso control vector. These results implicate Kaiso as a promoter of oncogenic transformation in these cells. It has been reported that transformation in immortalized fibroblasts can be caused by deregulation of the Ras/Raf/Mek/Erk and the Ras/PTEN/PI3K/AKT pathways, which co-interact with each other and the p53 pathway to regulate growth, the cell cycle and apoptosis [55, 75, 116]. It is possible that Kaiso's

effect on cellular transformation may be occurring due to the regulation of yet unknown target proteins that are present in these signalling pathways.

Interestingly, the constitutively activated Her2/Neu oncogene, which was used as a positive control, yielded a much higher number of foci (~200) compared to the Kaiso-over expression vectors (~12). This suggests that while Kaiso may have some oncogenic effects on breast carcinoma cells, Kaiso's effect is not as strong or penetrant as Her2/Neu. While some of Kaiso's identified target genes are activated by the Wnt signalling pathway, it is also possible that alternate mechanisms exist that regulate Kaiso's target genes, thereby minimizing any strong effects caused by Kaiso over-expression. However, another explanation for the vast difference in foci yield could be that the transfection efficiency of the Kaiso-containing vectors was lower than the efficiency of the Her2/Neu-containing vector. Therefore, the focus formation assay should be repeated using the same vector as that for Her2/Neu to over-express Kaiso.

Additional studies to further validate these findings include anchorage independent growth assays and *in vivo* tumourigenicity assays. Soft agar colony formation assays could be performed with clonal populations of the Kaiso over-expressing and Kaiso depleted Rat-1 cells to study anchorage-independent growth. The tumourigenic potency of these cells, specifically their ability to form *in vivo* metastases, can be studied using immuno-compromised nude mice.

### 4.2 Kaiso misexpression effects on MCF7cell proliferation.

Another signature of cancer and tumour development is uncontrolled cell proliferation. Normal cells have a finite replicative potential that causes them to senesce or stop growing once they progress through a certain number of cell divisions [47]. The ability of most tumour cells to replicate in an uncontrolled and infinite manner is a phenotype acquired during tumour progression and is essential for their growth and malignancy [46]. Cell proliferation assays were performed on Kaiso misexpressing MCF7 human breast tumour cells to determine the role of Kaiso in breast tumour proliferation. Kaiso over-expression slightly decreased the rate of cell proliferation whereas Kaiso depletion produced the opposite effect, i.e. slightly increased cell proliferation **(Figure 8 and Table 1)**; however these changes were not large enough to result in significant changes in cell proliferation rates. The cell proliferation curves of the control cell lines and Kaiso misexpression cell lines were similar and had small variations in overall cell number. Therefore in this assay, Kaiso does not affect cell proliferation in MCF7 cells. These results can be confirmed by performing an MTT Cell proliferation assay and BrdU DNA incorporation studies.

### 4.3 Kaiso misexpression effects on MCF7cell motility and migration.

During the progression of most cancers, tumour cells leave the primary tumour site, invade adjacent tissues, enter the blood stream or lymphatic system and travel to distant sites where they can establish secondary tumours. This process, known as

calculate the rate of wound closure for several reasons. First, since the wound/scratch was manually made, there were variations in wound size. Second, we were unable to perform a time-lapse study of the wound closure. Third, transporting the cells between the humidified chamber and the microscope room may have dislodged some cells from the leading edge of the wound. Since the images were quantified via ImageJ, it is also possible that slight variations occurred when measuring the gap closure over time. It is therefore necessary to conduct time-lapse experiments where the scratch is made via an automated instrument, and the cells are imaged within a sterile, incubated environment at shorter time intervals (e.g. every hour), without being disturbed. The leading edges of the wound would be automatically calculated and a more accurate and quantitative analysis of wound closure could be obtained.

Western blot analysis was performed on the Kaiso-misexpressing cells for changes in the levels of proteins associated with an epithelial-mesenchymal transition. This may explain the fibroblastic nature of the Kaiso over-expressing cells at the leading edge of the wound. Since MCF7 cells do not express Vimentin, α-SMA (smooth muscle actin), fibronectin and other mesenchymal-associated markers, Twist and Snail were the only proteins used as mesenchymal markers [27, 60, 81]. Western blot assays were performed to confirm the lack of Vimentin and N-cadherin expression in the MCF7 cells. E-cadherin levels were also analyzed as a marker for epithelial cells. We were unable to obtain reproducible results due to difficulties in antibody optimization, and these experiments must be repeated to determine if the Kaiso over-expressing cells are

undergoing epithelial-mesenchymal transition. It remains possible that while Kaiso over-expression may cause some morphological changes in the cells, it does not alter protein expression levels substantially. Examining the expression levels of metastasis-associated genes that are also Kaiso targets (e.g. MTA2 and Matrilysin/MMP7) would help confirm this theory. To that end, western blot analysis should be performed to examine the levels of MTA2, and gelatin zymography assays could be performed to analyze the activity of Matrilysin, a secreted zymogen.

**4.4 Kaiso misexpression effects on cell invasion in breast cancer.**

The invasiveness of a tumour cell is an important characteristic that describes its capacity to migrate through the extracellular matrix. For successful tumour cell invasion, the expression of extracellular proteases and unique integrins (e.g. $\alpha3\beta1$ and $\alpha V\beta3$) occurs along with the expression of matrix metalloproteinases [46]. These proteins enhance matrix and cell junction degradation and assist in steering cells through collagenous and endothelial barriers in response to growth factor receptors [22]. The matrigel invasion assay, commonly used as an *in vitro* analysis of tumour cell invasion, was performed using the stable MCF7 Kaiso misexpressing cells. We found that Kaiso depletion significantly decreased cell invasion; Kaiso depleted cells invaded the matrigel 60% less than the control MCF7 pRS-scrambled Kaiso cells **(Figures 11 and 12, Table 3)**. These results are consistent with those seen in the preliminary wound-healing analyses, where Kaiso depletion appears to slow wound closure and motility. Unfortunately, we

were unable to obtain consistent and statistically significant results from the Kaiso over-expressing cells, although they showed the same trend as the Kaiso depleted cells. An analysis of the entire field of view of each insert would provide the total number of cells migrated and improve the error bars. Interestingly, we have often observed that it is difficult to create stable Kaiso over-expressing cell lines, and we postulate that Kaiso over-expression may be toxic to the cells. An inducible system could be used as an alternative strategy to circumvent this problem. Apoptosis assays would therefore be essential to determine if Kaiso over-expression results in increased apoptosis in our stable Kaiso over-expressing MCF7 cells.

Since the processes of tumour cell motility and invasion are associated with a major re-organization of the actin cytoskeleton, it may be useful to visualize the actin cytoskeletal organization in the Kaiso misexpressing cells. Actin is a major cytoskeletal protein that occurs either as globular (G-actin) or filamentous (F-actin) proteins [37]. Filamentous actin is the major component of microfilaments that facilitate cell migration via the formation of filipodia and lamellipodia [73]. Fluorophore conjugated-phalloidin, which specifically binds to polymerized actin, can be used to stain cells for confocal microscopy for visualization of filament structure and density. The staining can be performed at different time points during a wound-closure experiment, where the wound was made at the same time in all the plates being stained. These experiments could provide insight into whether the misexpression of Kaiso indirectly causes any changes in the polymerization dynamics of these proteins.

### 4.5 Quantitative Reverse Transcription PCR analysis of Kaiso and its target genes.

To correlate the expression levels of Kaiso and its target genes in breast tumours, we performed qRT-PCR analysis on matched pairs of human breast ductal and lobular carcinomas. We analyzed the expression of *kaiso* and its target genes, *cyclinD1, matrilysin (MMP7)* and *MTA2*. In general, *kaiso* was expressed at similar levels in matched normal and breast carcinoma tissues **(Figures 13 and 14).** There was also no difference between *kaiso* levels in the lobular carcinomas and in the ductal carcinomas, a result that could be attributed to the small sample size (5 tumours) and a wide variation in patient characteristics and their stages of cancer progression. To obtain a more definitive analysis of *kaiso* levels, it will be important to perform tissue microarray studies of larger sample sizes (~100-200 tumours), with the tissues grouped by patient age, disease progression and prognosis markers such as estrogen receptor-α and Her2/Neu. Data mining and bioinformatic analysis can also be performed on existing breast tumour databases for the ZBTB33 gene (Kaiso), in order to determine if Kaiso misexpression is a common phenomenon in breast tumours.

*CyclinD1* levels were also examined and they were found to be slightly decreased or at a similar level when compared to Kaiso in both the human ductal and lobular breast carcinomas **(Figure 13 and 14).** A previous tissue microarray study analyzing 880 invasive breast tumours, showed strong CyclinD1 expression in 43.6% of the tumours and an amplification in 9.6% of the tumours, therefore its expression pattern was varied

across the tumours [32]. Although *cyclinD1* appears to be transcriptionally repressed by Kaiso, it is also a known oncogene and cell cycle effector and is regulated by many cellular proteins and pathways. The fact that there is not a significant inverse relationship between *cyclinD1* and *Kaiso* expression levels may be attributed to these multiple regulatory mechanisms.

*Matrilysin* (MMP7) levels were consistently upregulated in all the tumour tissues relative to the normal, with the highest levels being in the invasive ductal carcinomas. This is consistent with previous independent studies which found that high expression levels of *matrilysin* were observed in ductal *in situ* carcinomas of the breast [40]. Additionally, Matrilysin over-expression is known to be an early event in the carcinogenic cascade [51], and has been linked to the invasiveness of tumour cells *in vitro*[108]. Interestingly, Kaiso is a known repressor of *matrilysin* [100] and *matrilysin* levels were higher in all the breast carcinoma tissues corresponding to lower *kaiso* levels **(Figure 13).** We postulate that this increase in *matrilysin* is due to regulation by other cellular mechanisms since *matrilysin* is known to be regulated by various proteins including β-catenin [23], Fibroblast growth factor-2 (FGF-2) [52] and Interleukin-1β[65]. Tumours of a larger scale (approximately 10 cm – 11 cm), in patients where vascular and extensive lymphatic invasion had occurred, displayed the highest expression of *matrilysin*, thus signifying the importance of matrix metalloproteinases in advanced tumour progression and metastasis. This evidence correlates with previous independent

evidence showing that Matrilysin-depleted cells display a significant reduction in tumour growth in mice [57].

Finally, we also examined the expression levels of MTA2, another Kaiso target gene [114]. The MTA proteins are transcriptional co-repressors that function in histone deacetylation and are involved in NuRD complex nucleosome remodelling [115]. MTA protein expression correlates with tumour formation in the mammary gland and the transition of breast tumour cells to a more aggressive phenotype. We found that *mta2* levels were upregulated in the lobular breast carcinomas relative to *kaiso*, with no observable trend in the ductal carcinomas **(Figures 13 and 14).** There is currently limited literature on the expression of *mta2* in breast cancer, however it has been shown to be highly expressed in cervical cancer tissue and this expression correlates with rapid cell division [74]. It has also been proposed that MTA2 interacts with p53 and in this way, plays an important role in tumour progression [72]. However, this does not account for the higher *mta2* levels seen in our lobular breast carcinomas, since lobular carcinomas are expected to be p53 negative [4]. It is therefore possible that *mta2* promotes tumour progression via another, currently unknown mechanism. It is possible that Kaiso is downregulated via an upstream mechanism, thereby relieving its repression of MTA2, or it is possible that over-expression of MTA2 occurs in lobular carcinomas via aberrant activity of other signalling pathways, e.g. the Wnt signalling pathway.

### 4.6 Kaiso, Cyclin D1, p120 catenin and E-cadherin expression and subcellular localization in breast normal and carcinoma tissue.

Immunostaining analysis showed that the expression of all proteins varied between the normal and tumour tissues and between patients; however a common trend exists between tissues of a similar type of carcinoma. For example, the ductal breast carcinomas showed negligible Kaiso staining, decreased p120 catenin staining and increased E-cadherin staining in the tumour tissue relative to normal **(Figures 15 and16).**

When individually analyzing Kaiso staining, we found that it was much weaker in the ductal carcinomas compared to the lobular carcinomas, which show high Kaiso expression. Negligible Kaiso staining was a common trend in the tumour tissue relative to normal in the ductal carcinomas. **(Figures 15 and 16, panel 2)**. This trend is also observed in the lobular breast carcinomas, where the normal tissue lacks nuclear Kaiso staining, but the tumour tissue displays significant increases in cytoplasmic and nuclear Kaiso staining **(Figures 17 and 18, panel 2).** This finding correlates with previous studies performed by Soubry *et al.* in which they found that 8/32 inflammatory breast carcinoma samples displayed weak cytoplasmic Kaiso and no nuclear Kaiso, and 24/32 carcinomas were strongly positive for both nuclear and cytoplasmic Kaiso [99]. However, it was unclear whether the tissues were ductal or lobular carcinomas. Kaiso's mislocalization in our tumour tissues supports the theory that Kaiso's subcellular localization is dependent on the tumour microenvironment. Additionally, the difference in Kaiso staining could be attributed to the difference in tissue structure of the ductal

and lobular carcinomas. Furthermore, the increased Kaiso staining observed in the tumour tissues would implicate Kaiso to be important in tumour development, which supplements our *in vitro* assays that suggest a tumour promoting role for Kaiso.

Interestingly, the levels of Kaiso's interaction partner, $p120^{ctn}$, were strong in the nucleus and cytoplasm of both normal and tumour tissue of both types of carcinomas, with decreased cytoplasmic $p120^{ctn}$ staining in the tumour tissue. In lobular carcinomas specifically, $p120^{ctn}$ expression was decreased in the tumour relative to normal tissue, corresponding with an increase in Kaiso staining **(Figures 17 and 18, panel 2).** Previous studies have shown frequent cytoplasmic $p120^{ctn}$ in lobular carcinomas and rare expression of cytoplasmic $p120^{ctn}$ in ductal tumours [95, 97]. Our analysis of the lobular carcinomas remains consistent with these studies, however our analysis of the ductal carcinomas do not. Although our results show a very significant decrease in cytoplasmic $p120^{ctn}$ in the ductal tumours, it is not completely absent **(Figures 15 and 16, panel 2).** We would need to analyze a larger sample size of ductal carcinomas in order to see if this trend remains consistent.

E-cadherin is a known tumour suppressor protein that is an important cell-cell adhesion molecule and is significantly downregulated in epithelial cancers [45]. Our immunohistochemistry analysis of E-cadherin shows significantly decreased E-cadherin staining in the lobular carcinomas in the tumour tissues relative to normal, with an increase in cytoplasmic E-cadherin in the ductal carcinomas **(Figures 15-18).** A previous

independent analysis of E-cadherin subcellular localization and expression in breast tumours showed that 55% of invasive ductal carcinomas displayed normal E-cadherin expression at the cell membrane and 45% had aberrant E-cadherin expression (decreased and cytoplasmic) [67]. Our findings show that E-cadherin is mislocalized in the normal tissue and is not membrane-bound. In our ductal carcinoma tissues however, E-cadherin levels localizing to the cytoplasm in the tumour tissue relative to normal **(Figures 15 and 16, panel 2)** similar to the aberrant expression seen in [67]. In the same study performed by Kowalski *et al.*, E-cadherin levels were decreased and seen in the cytoplasm in 78% of the lobular carcinomas analyzed [67]. Once again, immunohistochemical analysis of the lobular carcinomas remains consistent, with a significant decrease in E-cadherin levels in tumour tissue relative to normal **(Figures 17 and 18).** The role of E-cadherin has been studied extensively in the progression of invasive lobular carcinomas, and it was found to be frequently downregulated [28] or mutated in these carcinomas. Additionally, allelic losses were often observed at the E-cadherin locus [10]. E-cadherin inactivation in lobular carcinomas has also been attributed to promoter hypermethylation [94], and several studies have shown that 84% of invasive lobular carcinomas completely lack membranous staining but have cytoplasmic localization of E-cadherin in tumour cells [28]. In light of this evidence and our findings, we postulate that the different patterns of E-cadherin expression in the invasive ductal and lobular carcinomas may be indicative of different roles of this protein in tumour development. Previous studies have suggested that the absence of

membranous E-cadherin expression in invasive lobular carcinomas may cause changes in morphological features such as the cellular non-cohesion which is characteristic of lobular carcinoma cells. Invasive lobular carcinomas are characterized by tumour cells that generally migrate as single cells or rows of cells [67]. It has also been postulated that decreased E-cadherin expression may correlate with the pattern of stromal invasion in these carcinomas [67]. The mislocalization of $p120^{ctn}$ and E-cadherin in the normal tissue could be attributed to the poor sample quality and degradation caused by improper preparation of tissue. Generally, human tissue used in IHC experiments is frozen immediately after surgery to maintain tissue architecture and cell integrity. The tissue used in these IHC experiments was not fresh-frozen and was obtained from tissue samples used for the qRT-PCR experiments. Therefore, due to the freeze-thaw time, the tissue could have been degraded and therefore not suitable for analysis. This suggests that the trends seen of all the proteins analyzed may change if the tissue were to be fresh-frozen following acquisition.

Finally, our analysis of Cyclin D1 subcellular localization revealed no noticeable trend, with varied expression across patients. In some cases, CyclinD1 levels were upregulated in the tumour cytoplasm and nucleus **(Figure 16)**, in other cases CyclinD1 levels were upregulated only in the nucleus and were weakly present in the cytoplasm **(Figure 18).** In some tissues, CyclinD1 levels were negligible in both normal and tumour tissue **(Figures 15 and 17)**. Our findings are consistent with previous evidence that show

a wide range of CyclinD1 expression in breast cancer, varying between 35-81% with an average of 50% [13].

Overall, these results suggest that breast cancer progression, as well as tumour type, strongly affect the localization of these proteins. To date, the mechanisms involved in Kaiso regulation are unknown. Deciphering these mechanisms may lead to a more cohesive understanding of Kaiso's role during breast tumourigenesis.

**Conclusions and Significance**

This study was the first to assess the role of the transcription factor Kaiso, in breast tumourigenesis, i.e. in processes such as cell transformation, proliferation, motility and invasion. Our transformation assays suggest a tumour promoting role for Kaiso in Rat-1 rodent non-transformed cells. Our cell proliferation, wound healing and invasion assays suggest that while Kaiso does not seem to affect cell proliferation, it may play a role in promoting motility and invasion in MCF7 breast adenocarcinoma cells. Kaiso may play an important role in breast tumourigenesis depending on the context, be it cell type or tumourigenic process. It is necessary to perform apoptosis assays to determine if Kaiso over-expression increases the rate of apoptosis in these cells.

qRT-PCR analysis on breast ductal and lobular carcinomas showed that *matrilysin* levels were upregulated compared to *kaiso* in all samples tested, with increased levels correlating with large tumour size. *mta2* levels showed no specific trend in ductal carcinomas, however was upregulated in lobular carcinomas. *cyclinD1* levels varied

across all tissue, showing no specific trend. These fold changes were not large in all the genes studied. Immunohistochemistry analysis showed varied expression levels in all proteins tested as well. Kaiso levels were negligible in the ductal breast carcinomas, and upregulated in the nucleus and cytoplasm of the lobular breast carcinomas. CyclinD1 staining varied across the different carcinomas, whereas $p120^{ctn}$ was significantly decreased in all the tumour tissue examined. Finally, E-cadherin staining was increased in the cytoplasm of the ductal carcinomas, and almost negligible in the tumour tissue of the lobular carcinomas. Our results suggested that level of breast cancer progression and the tumour type strongly affect the localization of these proteins; however our data may be inaccurate due to poor preparation of tissue samples. Based on these findings, it is possible that Kaiso may function in conjunction with other proteins to induce these tumourigenic processes, thereby participating in a multi-oncogenic model of carcinogenesis.

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