The Role of Kaiso and p120 in EMT

Studying the effects of p120 and Kaiso-mediated gene regulation on epithelial-to-mesenchymal-transition

By

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Abstract

Downregulation of E-cadherin is a frequent event in epithelial cancers and it correlates with weakened cell-cell adhesion and the induction of an epithelial-to-mesenchymal transition (EMT). It is postulated that E-cadherin downregulation liberates the catenin p120 and allows p120's translocation to the nucleus where it interacts with and functionally regulates the novel BTB/POZ transcription factor, Kaiso. Kaiso mediates transcriptional repression of various tumourigenesis-associated genes via methylated CpG dinucleotides or a sequence-specific Kaiso binding site (KBS). The Kaiso/p120 interaction has been detected in E-cadherin expressing cells of various origins, but is seldom detected in N-cadherin expressing cells or cells that have undergone EMT. We hypothesize that p120 and Kaiso play a role in EMT by modulating the expression of EMT-associated genes. We demonstrated that TGF-β-induced EMT occurs in a dose- and time-dependent manner in NMuMG cells but not in FHL-124 cells. In both cells lines, the Kaiso/p120 interaction occurred irrelevant of EMT induction by TGF-β. In NMuMG cells, the expression of p120 increased with EMT induction, while the expression of Kaiso remained unchanged. Finally, misexpression of Kaiso and p120 in mammary epithelial cells affected TGF-β-mediated EMT induction by delaying the upregulation of the positive mesenchymal markers, N-cadherin and α-SMA.

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Abbreviations:

α-SMA	α-smooth muscle actin
AR	Acidic region
APC	Adenomatous Polyposis Coli
AJ	*Adherens junctions*
BTB/POZ	Broad complex, Tramtrak, Bric ā brac/Pox virus
ChIP	Chromatin immunoprecipitation
CSF-1	Colony stimulating factor 1
Dsh	Dishevelled
EGF	Epidermal growth factor
EMT	Epithelial-to-mesenchymal-transition
EC	Extracellular
Fz	Frizzled
GSK	Glycogen synthase kinase
GEF	Guanine nucleotide exchange factor
HGF	Hepatocyte growth factor
HDAC	Histone deacetylase
JMD	Juxtamembrane domain
KBS	Kaiso binding site
MDCK	Madin-Darby canine kidney
MMP	Matrix metalloproteinase
NES	Nuclear export signal

NLS	Nuclear localization signal
PDGF	Platelet derived growth factor
pRS	pRetroSuper
RTK	Receptor tyrosine kinase
qRT-PCR	Quantitative real time-polymerase chain reaction
siRNA	short interfering RNA
TCF/LEF	T-cell factor/lymphoid enhancing factor
TGF	Transforming growth factor
ZF	Zinc Finger

1.0 Background

1.1 Cadherin-mediated cellular adhesion

Adhesion of neighboring cells is integral for tissue morphogenesis and essential cellular functions such as growth, differentiation, movement and survival. Epithelial cell-cell adhesion is mediated by cadherin-based *adherens junctions* (AJ), which play an important role in maintaining cell polarity and overall tissue architecture [Christofori and Semb, 1999]. The cadherin superfamily includes: classical cadherins, protocadherins, desmosomal cadherins and other cadherin-like molecules. The most well studied cadherins are the classical cadherins, which were first identified and named according to their tissue-specific type expression, for example epithelial (E-), neural (N-), placental (P-) and vascular endothelial (VE-) cadherins [Christofori and Semb, 1999; Gumbiner, 2005; Yap *et al.*, 1997]. Classical cadherins contain five extracellular domains (EC1-5), each made of 110 amino acids [Takeichi, 1995]. The EC domains are integral for mediating homotypic interactions between cadherins on adjacent cells and organizing cells into different tissues. When cells expressing different types of cadherins are mixed in culture, they sort themselves by adhering only to those cells that express the same cadherin type [Nose *et al.*, 1988; Takeichi, 1991]. Furthermore, the separation of cells into distinct tissue during development is associated with altering the types of cadherins expressed by cells in the different tissues [Takeichi, 1991]. In addition to their EC domains, cadherins also contain a conserved cytoplasmic tail which associates with other cytoplasmic proteins known as catenins [Takeichi, 1995]. The catenins function to link cadherin molecules to the actin cytoskeleton [Ozawa *et al.*, 1990; Rimm *et al.*, 1995] as

well as to regulate cadherin stability and turnover [Davis *et al.*, 2003; Ireton *et al.*, 2002; Thoreson and Reynolds, 2002].

When the integrity of AJ is compromised in carcinomas, it leads to weakened cell adhesion and cells become metastasic. The decrease of cellular adhesion between adjacent cells results in a loss of cell differentiation where cells convert to a mesenchymal type, in the same manner that epithelial cells become mesenchymal during development. Consequently the tumour cells display increased motility and become invasive [Birchmeier and Behrens, 1994].

1.2 The E-cadherin-catenin complex

E-cadherin, the major adhesion molecule in epithelial cells [Christofori and Semb, 1999], is the prototype for a superfamily of transmembrane adhesion molecules that mediate cell-cell adhesion via extracellular, calcium-dependent, homotypic interactions [Kemler, 1992; Takeichi, 1990]. Seminal studies demonstrated E-cadherin to be a tumour suppressor by virtue of its ability to promote tight *adherens junctions* and prevent metastasis [Hazan *et al.*, 2004; Thiery, 2002]. Conversely, downregulation of E-cadherin is frequently observed in epithelial cancers [Christofori and Semb, 1999], often leading to AJ deficiency, tumour progression and metastasis [Vleminckx *et al.*, 1991].

The E-cadherin extracellular domain binds to other E-cadherin molecules on adjacent cells, while its intracellular domain binds to the Armadillo proteins β-, γ- and p120-catenin (p120) to form the cadherin-catenin complex [Reynolds *et al.*, 1994; Shibamoto *et al.*, 1995; Staddon *et al.*, 1995] (Figure 1). Each of these proteins contains

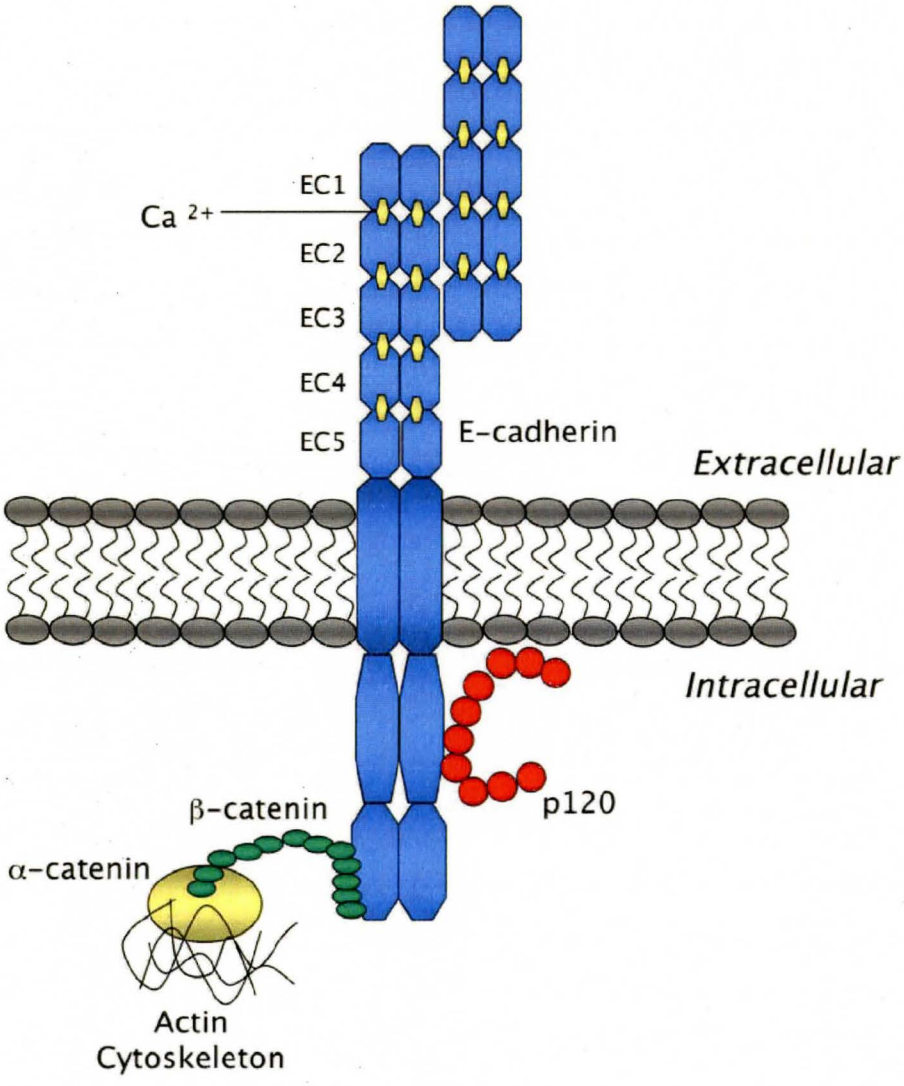


Figure 1: The E-cadherin-catenin complex. Epithelial cells are held together through the extracellular domain of E-cadherin molecules of neighboring cells. The extracellular domain of E-cadherin is composed of five repeats (EC1-5) which are held together through Calcium-dependent interactions. The cytoplasmic domain of E-cadherin is made of the juxtamembrane domain which is bound to the catenin p120 and the carboxy terminal domain which is bound to β-catenin and anchors E-cadherin to the actin cytoskeleton via α-catenin.

an Armadillo domain containing ten or more tandem Armadillo repeats of 42 amino acids each [Peifer *et al.*, 1994]. This domain exhibits high sequence homology with the *Drosophila* segment polarity protein Armadillo after which it was named [McCrea *et al.*, 1991]. The catenins β– or γ–catenin bind to E-cadherin at the carboxy-terminal region, anchoring E-cadherin to the actin cytoskeleton via α–catenin, whereas p120 binds at the juxtamembrane domain (JMD) of E-cadherin and does not bind to α–catenin [Daniel and Reynolds, 1995; Thoreson *et al.*, 2000].

1.3 Malfunctioning of the E-cadherin-catenin complex in cancer

The formation of stable and functional AJ relies heavily on the formation of the E-cadherin-catenin complexes and anchorage to the actin cytoskeleton [Nagafuchi, 2001]. This has been proven to be true in many carcinomas where malfunction or downregulation of one of the proteins in the complex compromises AJ integrity, thereby leading to tumours with high metastatic potential. There have been many reports of catenin mutations that prevent E-cadherin from being anchored to the cytoskeleton in several different cancers. In one ovarian carcinoma cell line, a mutation in the α–catenin gene that inhibits its interaction with β–catenin prevents AJ formation. The introduction of wild-type α-catenin restores AJ, and results in decreased tumourigenecity of the cells [Bullions *et al.*, 1997]. In another study which analyzed tumour tissues from 90 primary breast carcinomas, it was found that a reduction in any one of E-cadherin, α-, β-, or γ-catenin reduces the ability of the other AJ components to suppress tumour metastasis [Bukholm *et al.*, 1998].

Moreover, E-cadherin is important for catenin localization to the membrane; knockdown of E-cadherin using small interfering RNA (siRNA) in Madin-Darby canine kidney (MDCK) epithelial cells causes a delay in the correct localization of α- and β-catenin [Capaldo and Macara, 2007]. The p120 catenin is also important for the formation of stable AJ. Unstable AJ are seen in mammalian cells with reduced p120 levels or a mutated form of p120 that is unable to localize to the membrane and in mammalian cells artificially depleted of p120 using siRNA [Davis *et al.*, 2003; Thoreson *et al.*, 2000; Xiao *et al.*, 2003]. In conclusion, the cadherin-catenin interaction is vital for the integrity of adherens junctions, which in turn, is essential for preventing the development of metastasic tumours.

1.4 p120, a catenin with roles in regulating cadherin function

The catenin p120, is the prototypical member of a conserved family of Armadillo proteins, which includes ARVCF, δ-catenin, and p0071 [Behrens, 1999]. Originally, p120 was discovered as a major substrate of the Src tyrosine kinase and was found to be tyrosine phosphorylated in cells stimulated by epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and colony stimulating factor 1 (CSF-1) [Downing and Reynolds, 1991; Kanner *et al.*, 1991]. Subsequent co-immunoprecipitation experiments demonstrated that p120 is a component of the E-cadherin-catenin cell adhesion complex [Reynolds *et al.*, 1994; Shibamoto *et al.*, 1995; Staddon *et al.*, 1995].

There are several p120 isoforms that are differentially expressed in different cell types as a result of alternative splicing and post-translational modifications. At the 5' end of the gene, four alternative ATG start sites result in four protein isoforms known as p120

isofom 1 through 4. Moreover, at the C-terminus, there are four internal exons, A, B, C and D, which also contribute to making different p120 isoforms [Aho *et al.*, 1999; Keirsebilck *et al.*, 1998; Mo and Reynolds, 1996] (Figure 2). Since different cell types express specific isoforms of p120, it has been postulated that the various p120 isoforms perform different functions in a cell-type-specific manner. Cells expressing N-cadherin for instance preferentially express p120 isoform 1, while epithelial cells expressing E-cadherin preferentially express p120 isoform 3 [Anastasiadis and Reynolds, 2000].

Unlike α- and β-catenin whose stability is dependent on the physical association with cadherins [Nagafuchi *et al.*, 1991], p120 stability is not affected by the loss of E-cadherin. Instead, in the absence of E-cadherin, p120 localizes to the cytoplasm or to the nucleus [Thoreson *et al.*, 1997; van Hengel *et al.*, 1999]. In cells that lack cadherins such as A431D, MDA-231 or L-cells, p120 is stranded in the cytoplasm [Thoreson *et al.*, 1997]. Expression of wild type E-cadherin that is able to bind p120 in E-cadherin deficient cells relocolizes p120 to the membrane [Thoreson *et al.*, 1997]. However, expression of a mutated E-cadherin that is unable to bind p120, results in the retention of p120 in the cytoplasm. This suggests that E-cadherin is both sufficient and required for p120 localization to cell-cell contacts [Thoreson *et al.*, 2000; Thoreson *et al.*, 1997].

Intriguingly however, cadherin stability at cell-cell contacts is dependent on the interaction of p120 with the cytoplasmic tail of cadherins via the JMD. This was demonstrated by the expression of E-cadherins with different cytoplasmic domains and mutation analysis of the JMD, which confirmed that E-cadherin/p120 binding is essential for E-cadherin lateral clustering and increased adhesive strength [Thoreson *et al.*, 2000;

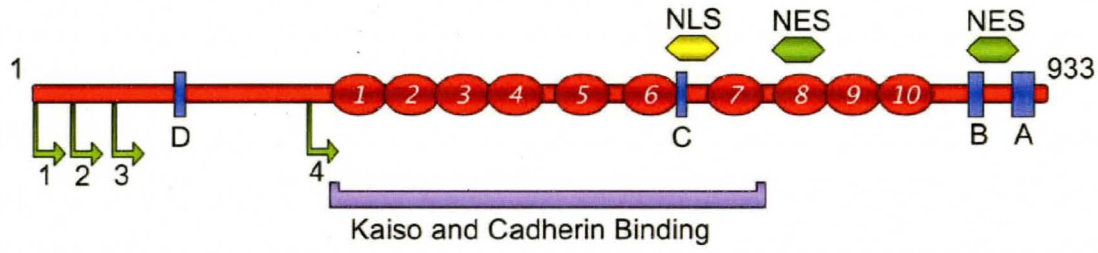


Figure 2: Schematic representation of the catenin p120. The p120 protein is 933 amino acids in length. It contains 10 Armadillo repeats which consist of 42 amino acids and are named due to their sequence homology with the *Drosophila* segment polarity protein Armadillo. The green arrows indicate the four alternative ATG start site which give rise to four different p120 isoforms 1 through 4, while the blue boxes represent three exons which are spliced to produce three additional isoforms A, B, C and D. A nuclear localization signal (NLS) and two nuclear export signals (NES) are also indicated in this diagram. The binding site for Cadherins as well as the p120 nuclear binding partner Kaiso is indicated in lavender. It spans Armadillo repeats 1 through 7.

Yap *et al.*, 1998]. Furthermore, a direct correlation between p120 and cadherin function was observed in the colon carcinoma cell line, SW48, which expresses negligible levels of p120 protein due to mutations in the p120 gene (*CTNND1*) [Ireton *et al.*, 2002]. The absence of p120 in SW48 cells led to failure in the development of compact adherent colonies. Moreover, although E-cadherin mRNA levels were unaffected, E-cadherin protein was unstable and its levels were dramatically reduced [Ireton *et al.*, 2002]. Re-expression of p120 in SW48 cells resulted in stable E-cadherin protein and formation of compact adherent colonies. This reinforced the idea that p120 interaction with the E-cadherin JMD domain regulates cadherin expression by regulating cadherin stability and turnover rates [Thoreson and Reynolds, 2002]. A possible mechanism by which p120 may regulate cadherins at the cell surface is through its interaction with the microtubule motor, kinesin. Kinesin regulates the localization of p120 in the cell and allows it to facilitate the transport of cadherin-catenin complexes to intercellular junctions [Chen *et al.*, 2003; Yanagisawa *et al.*, 2004]. Another possibility is through p120 regulation of Rho-GTPase signaling [Anastasiadis and Reynolds, 2001].

1.5 p120, a regulator of cytoskeleton dynamics

In addition to being found at sites of cell-cell contact in association with E-cadherin, p120 exists in a soluble cytoplasmic pool [Mehta, 2004]. In the cytoplasm, p120 plays a key role in regulating the activity of Rho-GTPases (RhoA, Rac and Cdc42) which are important regulators of cytoskeletal organization [Anastasiadis and Reynolds, 2000; Grosheva *et al.*, 2001; Noren *et al.*, 2000]. A role for p120 in regulating Rho-GTPases was first postulated when ectopic expression of p120 resulted in striking

"branching" phenotype in fibroblasts, and increased formation of lamellipodia in epithelial cells [Anastasiadis *et al.*, 2000; Reynolds *et al.*, 1996]. Additionally, this phenotype was seen when p120 was expressed in cadherin-deficient cells, suggesting that cytoplasmic p120 was responsible for the phenotype [Anastasiadis *et al.*, 2000]. Overexpression of a constitutively active mutant of RhoA blocked p120's ability to form the branching phenotype; thus it was suggested that the branching phenotype was caused by inhibition of RhoA activity by p120 [Anastasiadis *et al.*, 2000]. Later studies proved that in addition to inhibiting RhoA activation, p120 inhibits stress fiber-mediated contractility and maturation of focal contacts [Grosheva *et al.*, 2001]. Moreover, a study by Noren *et al.* showed that p120 directly interacts with a Rho-guanine nucleotide exchange factor (GEF) called Vav2 which enables p120 to activate two other Rho-GTPases Rac1 and Cdc42 [Noren *et al.*, 2000].

The mechanisms by which p120 and Rho-GTPase signaling regulate cadherin stability are still unknown. One proposed model suggests that p120 mediates the activation of Rho-GTPases via its interaction with GEFs. RhoA Activation promotes myosin II-dependent clustering of cadherin complexes, while Rac1 and Cdc42 activation promotes actin cytoskeleton reorganization at emerging cell-cell contacts, intercellular junctions maturation, and increase cell-cell adhesion [Anastasiadis, 2007] (Figure 3). Thus, both cytoplasmic and membrane-associated p120 may regulate cell-cell adhesion by modulating E-cadherin stability and turnover [Reynolds and Carnahan, 2004].

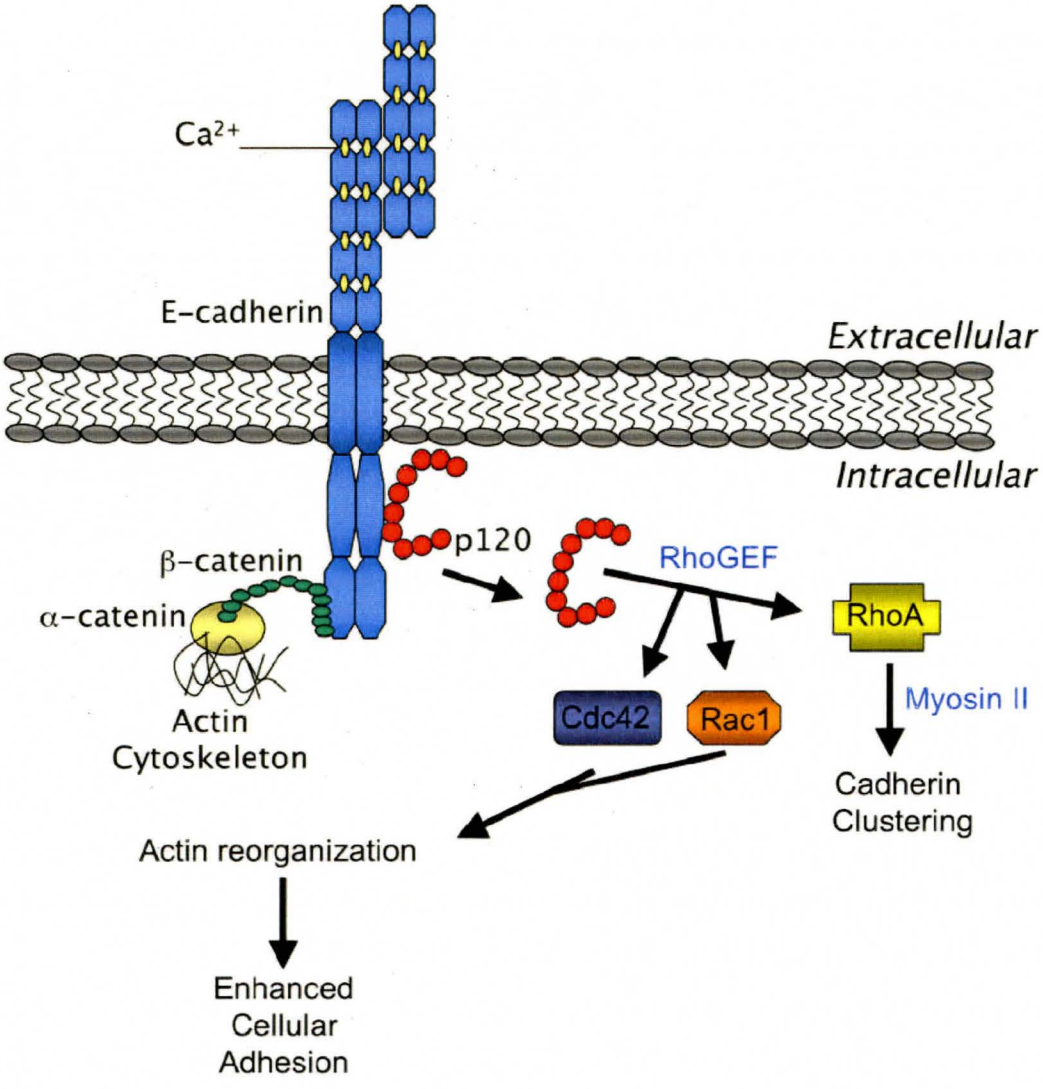


Figure 3: Model of p120 regulation of Cadherin stability through Rho-GTPases. A model proposed by Anastasiadis (2007) suggests that p120 mediates the activation of Rho-GTPases via its interaction with Rho-guanine nucleotide exchange factors (GEFs). RhoA Activation promotes myosin II-dependent clustering of cadherin complexes, while Rac1 and Cdc42 activation promotes actin cytoskeleton reorganization at emerging cell-cell contacts, intercellular junctions maturation, and thus increase cell-cell adhesion.

1.6 p120: shuttling in and out of the nucleus

Several studies demonstrated that upon downregulation of cadherins (a frequent event in human cancers), p120 localizes to the cytoplasm and in some cases, also to the nucleus [Lo Muzio *et al.*, 2002; Mayerle *et al.*, 2003; Valizadeh *et al.*, 1997]. This indicated that p120 might have a functional role in the nucleus. Earlier studies identified a putative nuclear export signal (NES) located in the carboxy terminus of p120 [van Hengel *et al.*, 1999]. Further analysis in our lab demonstrated that p120 has a nuclear localization signal (NLS) situated between Armadillo repeats 6 and 7 [Kelly *et al.*, 2004b] (Figure 2), suggesting that p120 can shuttle in and out of the nucleus. A point mutation in the NLS abolishes nuclear localization of p120. Intriguingly, the same mutation also inhibits the branching phenotype that is induced by p120-overexpression, indicating that it may also play a role in p120's function in regulating Rho-GTPases [Kelly *et al.*, 2004b]. To date, there is no known signaling event that induces the nuclear translocation of p120, however, nuclear-localized p120 has a recently discovered gene regulatory role through its interaction with the DNA-binding protein Kaiso [Kelly *et al.*, 2004b].

1.7 Characterization of Kaiso, the nuclear p120 binding partner

Kaiso was first discovered as a p120 binding partner using p120 as bait in yeast two-hybrid experiments [Daniel and Reynolds, 1999]. Kaiso is a member of the BTB/POZ-ZF (Broad complex, Tramtrak, Bric ā brac/Pox virus and zinc finger) family of zinc finger transcription factors (hereafter POZ-ZF proteins) [Albagli *et al.*, 1995;

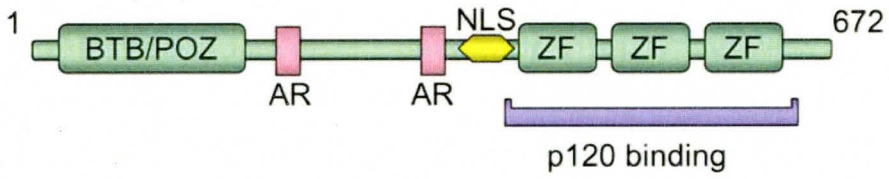


Figure 4: Schematic representation of the p120 nuclear binding partner Kaiso. Kaiso is a 672 amino acid protein that contains a BTB/POZ domain at its N-terminus and three C_2H_2 type zinc finger (ZF) domains at its C-terminus. Kaiso also contains two acidic regions (AR), shown in pink and one nuclear localization signal (NLS) shown in yellow. Finally, the p120 binding region encompasses the ZF DNA-binding domain.

Bardwell and Treisman, 1994; Davis *et al.*, 2003]. Characterization of Kaiso revealed that it contains three C_2H_2 type zinc finger (ZF) motifs at its carboxy-terminus and a conserved POZ domain at its extreme amino-terminus [Daniel *et al.*, 2002] (Figure 4). The POZ domain consists of ~120 hydrophobic amino acids that regulate homodimerization or heterodimerization of Kaiso with other POZ-ZF proteins such as Znf131 [Daniel and Reynolds, 1999], the vertebrate insulator protein CTCF [Defossez *et al.*, 2005] and the histone deacetylase (HDAC) coreporessor NCoR [Yoon *et al.*, 2003]. Moreover, Kaiso zinc fingers 2 and 3 have been shown to bind DNA directly: through these zinc fingers Kaiso is able to bind to methylated CpG dinucleotides or to a specific Kaiso binding site (KBS) TCCTGCNA, where N is any nucleotide. Electrophoretic mobility shift assays further demonstrated that Kaiso has a higher affinity for the KBS-derived oligonucleotides than for methyl-CpG dinucleotides [Daniel *et al.*, 2002]. In fact, Kaiso was the first POZ-ZF protein to display bimodal activity, i.e. binding to both a conserved consensus sequence and methylated DNA. Interestingly, a recent study characterized another POZ-ZF protein, ZBTB4, with Kruppel-like C_2H_2 zinc fingers. Similar to Kaiso, ZBTB4 is able to bind methylated DNA and repress transcription in a methyl-dependent manner. However, unlike Kaiso which binds to dinucleotide methylated CpGs, ZBTB4 binds to single methylated CpG. Finally, like Kaiso, ZBTB4 is able to bind to the KBS [Filion *et al.*, 2006]. In addition, Kaiso possesses two highly acidic regions (AR) situated between the POZ and ZF domains; AR regions are usually associated with transcriptional activation [Cowell, 1994; Mitchell and Tjian, 1989]. As expected for a protein displaying DNA-binding activity, Kaiso functions as a

transcription factor. Kaiso possesses transcriptional repression and activation capabilities and like other POZ-ZF proteins, Kaiso is postulated to control the expression of genes involved in development and cancer, reviewed in [Daniel, 2007].

In normal cells, Kaiso is found to be predominantly nuclear, as expected for a transcription factor. However, immunohistochemical analysis of normal and tumour human tissue revealed that Kaiso was predominantly cytoplasmic or completely absent [Soubry *et al.*, 2005]. Intriguingly, cytoplasmic Kaiso is always seen with cytoplasmic p120, further, Kaiso's localization in the cell appears to be controlled by the tumour microenvironment [Soubry *et al.*, 2005]. Studies in our lab identified a conserved highly autonomous NLS that utilizes the classical Importin-α/β pathway for nuclear import [Kelly *et al.*, 2004a] (Figure 4).

1.8 Kaiso mechanisms of gene regulation

Putative Kaiso target genes include the tumourigenesis-associated genes *matrilysin* [Spring *et al.*, 2005], *metastasin* [Prokhortchouk *et al.*, 2001], *MTA2* [Yoon *et al.*, 2003], the acetylcholine-receptor-clustering gene *raspyn* [Rodova *et al.*, 2004], the non-canonical Wnt signaling gene *Wnt11* [Kim *et al.*, 2004], and the canonical Wnt signaling target genes *siamois* and *cyclinD1* [Park *et al.*, 2005]. To date, *rapsyn* is the only target gene activated by Kaiso [Rodova *et al.*, 2004]; this is consistent with the presence of two AR regions that are implicated in transcription activation [Cowell, 1994; Mitchell and Tjian, 1989]. On the other hand, Kaiso is more commonly seen as a transcriptional repressor since it negatively regulates the expression of the other putative target genes mentioned above. Kaiso-mediated repression occurs via an HDAC-

dependent mechanism. Kaiso recruits HDAC complexes, which mediate repression by catalyzing the deacetyalation of histone tails, leading to a compact chromatin confirmation that inhibits transcription [Daniel, 2007].

Additionally, studies demonstrated that the interaction between p120 and Kaiso is mediated by p120 Armadillo repeats 1 through 7 and a noncontiguous region of Kaiso that encompasses the three ZF motifs [Daniel and Reynolds, 1999]. Since Kaiso binds to DNA through ZF 2 and 3, it is not surprising that p120 binding to Kaiso abrogates Kaiso's DNA-binding ability. Studies from our lab showed that binding of p120 to Kaiso negatively regulates the interaction of Kaiso with target gene promoters and is reminiscent of the TCF/β-catenin interaction [Kelly *et al.*, 2004b; Spring *et al.*, 2005]. Thus, the interaction between Kaiso and p120 has raised the possibility of a previously uncharacterized signaling pathway affecting the expression of genes involved in tumourigenesis and cell-cell adhesion.

1.9 The common thread between Kaiso/p120 and TCF/β-catenin signaling

The catenin p120 has many common features with β-catenin, which plays a major role in the Wnt pathway (Figure 5). The canonical Wnt pathway is initiated by the release of a Wnt signaling molecule that subsequently binds to a Frizzled (Fz) receptor at the cell surface. The signal is then passed into the cell via a Dishevelled (Dsh) molecule. This leads to the inhibition of glycogen synthase kinase-3β (GSK-3β), which marks β-catenin for degradation and is found in complex with the Adenomatous Polyposis Coli protein (APC) and Axin. In the absence of a Wnt signal, the protein complex mediates the

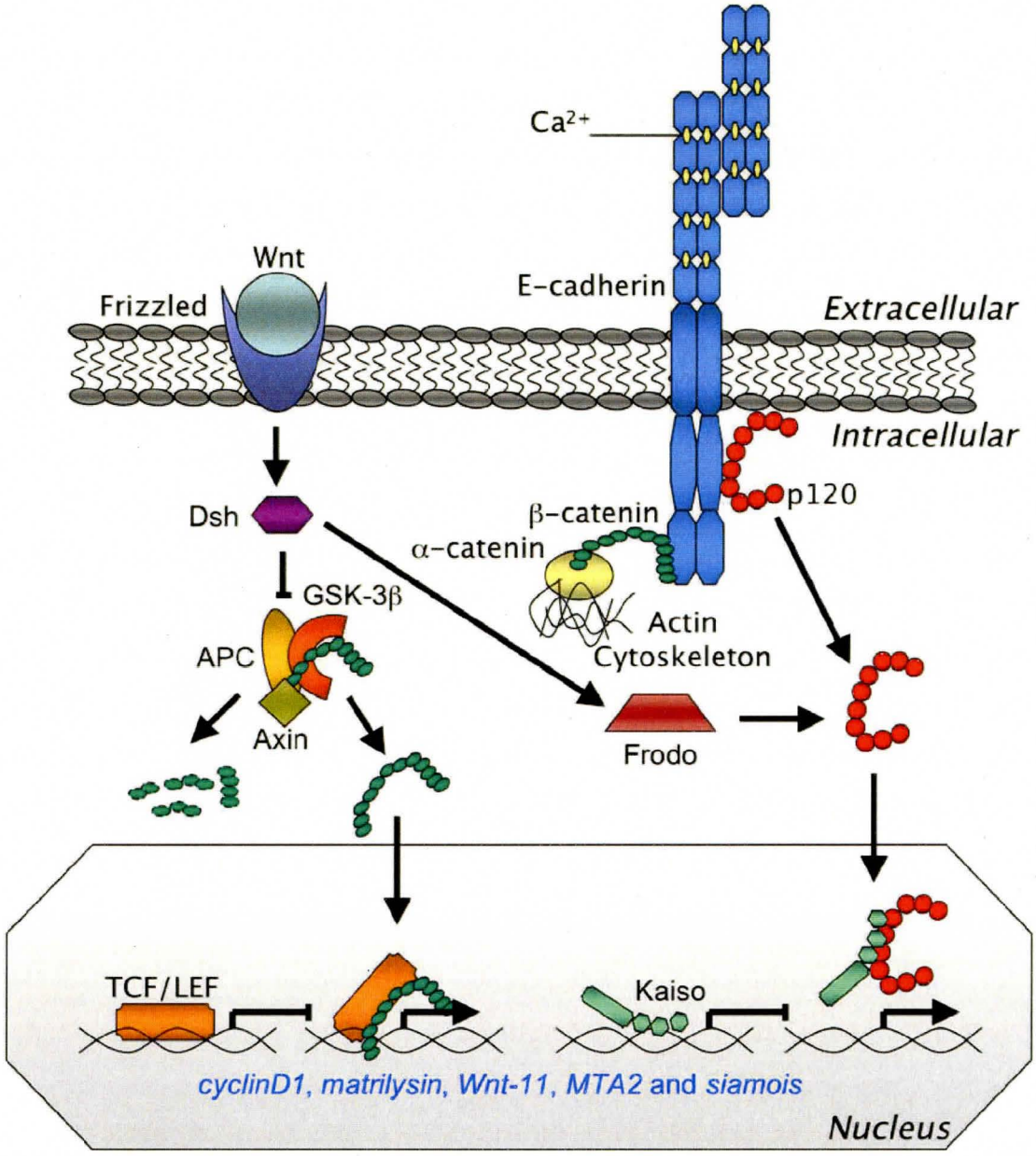


Figure 5: The Wnt signaling pathway and p120/Kaiso signaling. Gene regulation by p120 and Kaiso share similar steps with β-catenin and TCF/LEF gene regulation. In the absence of E-cadherin, β-catenin and p120 are released into the cytoplasm from the cadherin-catenin complex at cell-cell contacts after which they translocate to the nucleus. There, β-catenin and p120 bind to TCF/LEF and Kaiso respectively to relieve repression of target genes (*cyclinD1*, *matrilysin*, *Wnt-11*, *MTA2* and *siamois*).

degradation of the transcriptional regulator β-catenin by continuously targeting it to the proteosome. However, when the GSK-3/APC/Axin complex is inhibited, β-catenin accumulates in the cytoplasm and the nucleus. In the nucleus, β-catenin interacts with the transcriptional repressor T cell-specific transcription factor/lymphoid enhancing factor (TCF/LEF) to activate target genes [Logan and Nusse, 2004]. Like β-catenin, p120 is found at the membrane bound to cadherins or it may be translocated to the nucleus where it interacts with the transcription factor Kaiso. Moreover, a subset of the genes which have been identified as putative Kaiso target genes are also targets of TCF/β-catenin signaling (*cyclinD1*, *matrilysin*, *Wnt-11*, *MTA2* and *siamois*), suggesting that Kaiso and p120 indirectly regulate the Wnt signaling pathways [Daniel, 2007].

Perhaps the strongest link between Kaiso/p120 and Wnt signaling came about with the discovery that in *Xenopus* p120 binds Frodo, a Dsh interacting protein [Park *et al.*, 2006]. Dsh and Frodo were shown to be upstream regulators that act together to stabilize p120 and its association with Kaiso, thus relieving Kaiso-mediated repression of its target genes. This study postulates that Wnt signals act through Dsh to regulate Kaiso transcriptional regulation, similar to the way that Dsh stabilizes β-catenin and enhances expression of TCF target genes [Park *et al.*, 2006]. Finally, in certain epithelial cancers, the presence of β-catenin and p120 in the nucleus is associated with E-cadherin downregulation, and the induction of an epithelial-to-mesenchymal transition (hereafter EMT), which is an important step in metastasis [Lee *et al.*, 2006].

1.10 Epithelial-to-mesenchymal transition in development and cancer

The induction of EMT is a process where cellular adhesion between adjacent cells is compromised, leading to a loss of cell differentiation where cells convert from an epithelial to a mesenchymal type. EMT is observed in normal physiological processes during development [Camenisch *et al.*, 2002; Savagner, 2001] as well as pathological processes such as metastasis [Birchmeier and Behrens, 1994; Moustakas *et al.*, 2002; Piek *et al.*, 1999] and renal fibrosis [Eddy, 2000; Fan *et al.*, 1999; Rastaldi *et al.*, 2002]. During early development, EMT is important for gastrulation where the three germinal layers are formed. EMT is also essential for a variety of morphogenesis stages, in particular, the formation of heart and somites [Thiery, 2002; Thiery, 2003]. Moreover, during metastasis, cells at the edge of primary tumours undergo EMT and become invasive and motile. Subsequently, cells that undergo EMT break off and travel through the blood/lymphatic system where they relocalize at distant sites and form secondary tumours [Yang *et al.*, 2006]. The movement of cells undergoing EMT during development is subtle and regulated, however, during metastasis, the process is aggressive and unregulated [Hay, 2005].

Epithelial and mesenchymal cells differ not only morphologically but functionally as well. Epithelial cells are found next to each other in a regularly shaped array; they are polarized, non-motile and tightly associated with each other. They exhibit tight *adherens junctions*, desmosomes, and they express E-cadherin, Cytokeratin and actin. On the other hand, mesenchymal cells are elongated, non-polar and have no junctions. They form focal adhesions, are loosely associated, motile, invasive, surrounded by extracellular

matrix, and express Vimentin and α-smooth muscle actin (hereafter α-SMA) [Hynes, 1992; Lee *et al.*, 2006]. Thus, during epithelial-to-mesenchymal-transition, a switch from polarized, epithelial cells to dynamic mesenchymal cells is required. A seminal discovery in the EMT field came when Trocker and Perryman discovered that a type of polarized epithelial cells (MDCK) can be converted into migratory fibroblasts when incubated with fibroblast culture supernatant [Stoker *et al.*, 1987; Stoker and Perryman, 1985]. Subsequent studies identified hepatocyte growth factor (HGF) as the agent responsible for the observed scattering of MDCK cells [Nakamura *et al.*, 1989; Naldini *et al.*, 1991; Weidner *et al.*, 1993].

1.11 Developmental pathways involved in EMT induction

More recently, developmental pathways such as Wnt/β-catenin pathway, as well as, Notch and Hedgehog signaling were also shown to affect EMT in the context of development and cancer progression [Huber *et al.*, 2005; Thiery, 2003]. During EMT, E-cadherin protein is proteolytically disrupted by Presenilin-1 which controls a gamma-secretase-like cleavage of E-cadherin [Marambaud, 2002] or it is downregulated via transcription factors that inhibit E-cadherin expression (Snail superfamily of ZF proteins) [Grunert *et al.*, 2003; Nelson and Nusse, 2004]. The reduction of E-cadherin frees β-catenin into the cytoplasm and its levels become enhanced since GSK-3β activity also becomes suppressed by the activation of PI3K, a downstream regulator of Receptor tyrosine kinases (RTK)/Ras signaling [Bachelder *et al.*, 2005; Nelson and Nusse, 2004; Zhou *et al.*, 2004] (Figure 6). Thus, β-catenin levels and stability, and its interaction with

TCF/LEF is enhanced, leading to transcriptional activation of EMT-related genes [Bachelder *et al.*, 2005; Liebner *et al.*, 2004; Zhou *et al.*, 2004]. Moreover, although Notch signaling is known to have tumour suppressor roles, it has been implicated as a tumour promoter when it cooperates with RTK/Ras signaling and other oncogenic events [Grego-Bessa *et al.*, 2004; Radtke and Raj, 2003]. For instance, the induction of Notch signaling by RTK/Ras signaling or Smads leads to the upregulation of E-cadherin repressors (Snail, Hey1) [Timmerman *et al.*, 2004; Zavadil *et al.*, 2004], thereby leading to E-cadherin downregulation and eventually EMT induction. Finally, the Hedgehog signaling pathway also contributes to EMT. In certain basal cell carcinomas (gastrointestinal, lung and prostate tumours), the Hedgehog pathway becomes hyperactive and participates with RTK and Wnt signaling to upregulate the Snail family of E-cadherin suppressors, again, leading to E-cadherin downregulation and consequently EMT [Huber *et al.*, 2005].

1.12 TGF-β as an EMT-inducing molecule

In addition to developmental pathways, which lead to EMT induction, one intensely studied molecule in cancer-related EMT is transforming growth factor-β (hereafter TGF-β). TGF-β is the prototype of a large family of signaling molecules with over 40 members. It signals through type1 and II receptors (TβRI and II), Smad transcription factors, and other signaling pathways [Yue and Mulder, 2001]. In normal epithelial cells, TGF-β functions as a growth suppressor by way of its ability to induce apoptosis, angiogenic repressors and replicative senescence, preserve genomic stability, and hinder

cell immortalization [Derynck *et al.*, 2001; Wakefield and Roberts, 2002]. However, the *TGF-β* gene is genetically disrupted in some cancers possibly leading to unregulated cell growth [Brattain *et al.*, 1996; de Caestecker *et al.*, 2000; Massague *et al.*, 2000; Waite and Eng, 2003]. It has been shown to switch from a tumour suppressor to a prometastatic factor in breast cancer [Seton-Rogers *et al.*, 2004; Tang *et al.*, 2003]. In tumour cells, the anti-proliferative response of TGF-β is bypassed with the cooperation of oncogenic Ras or RTKs to trigger EMT [Huber *et al.*, 2005]. In these cells, TGF-β signaling induces changes to the cytoskeleton through the action of RhoA, and it increases expression of the E-box binding transcription factor that inhibits E-cadherin expression (Snail, Slug, and Sip1). Cytoskeletal reorganization and downregulation of E-cadherin are events which ultimately lead to EMT [Thiery, 2002] (Figure 6).

In addition to inducing EMT in tumour cells, TGF-β was also shown to induce EMT in various epithelial cell lines; non-transformed murine mammary epithelial cells (NMuMG) [Maeda *et al.*, 2005; Piek *et al.*, 1999; Xie *et al.*, 2003], lens epithelial cells (FHL-124) [Dawes *et al.*, 2007], porcine kidney cells (LLC-PK1 CL4) [Fan *et al.*, 2007; Masszi *et al.*, 2004], and human mammary cells (MCF-10A) [Tang *et al.*, 2003]. The induction of EMT in these cells is characterized by many molecular changes (Table 1). A loss of epithelial markers such as Desmoplakin, Cytokeratin and Occludin is coupled with an increase in mesenchymal markers Vimentin and Fibronectin [Lee *et al.*, 2006]. Furthermore, E-cadherin is downregulated while N-cadherin is upregulated [Bhowmick *et al.*, 2001; Maeda *et al.*, 2005; Piek *et al.*, 1999], causing the formation of weak, less stable cell-cell adhesions and the promotion of cell motility [Bixby, 1999];

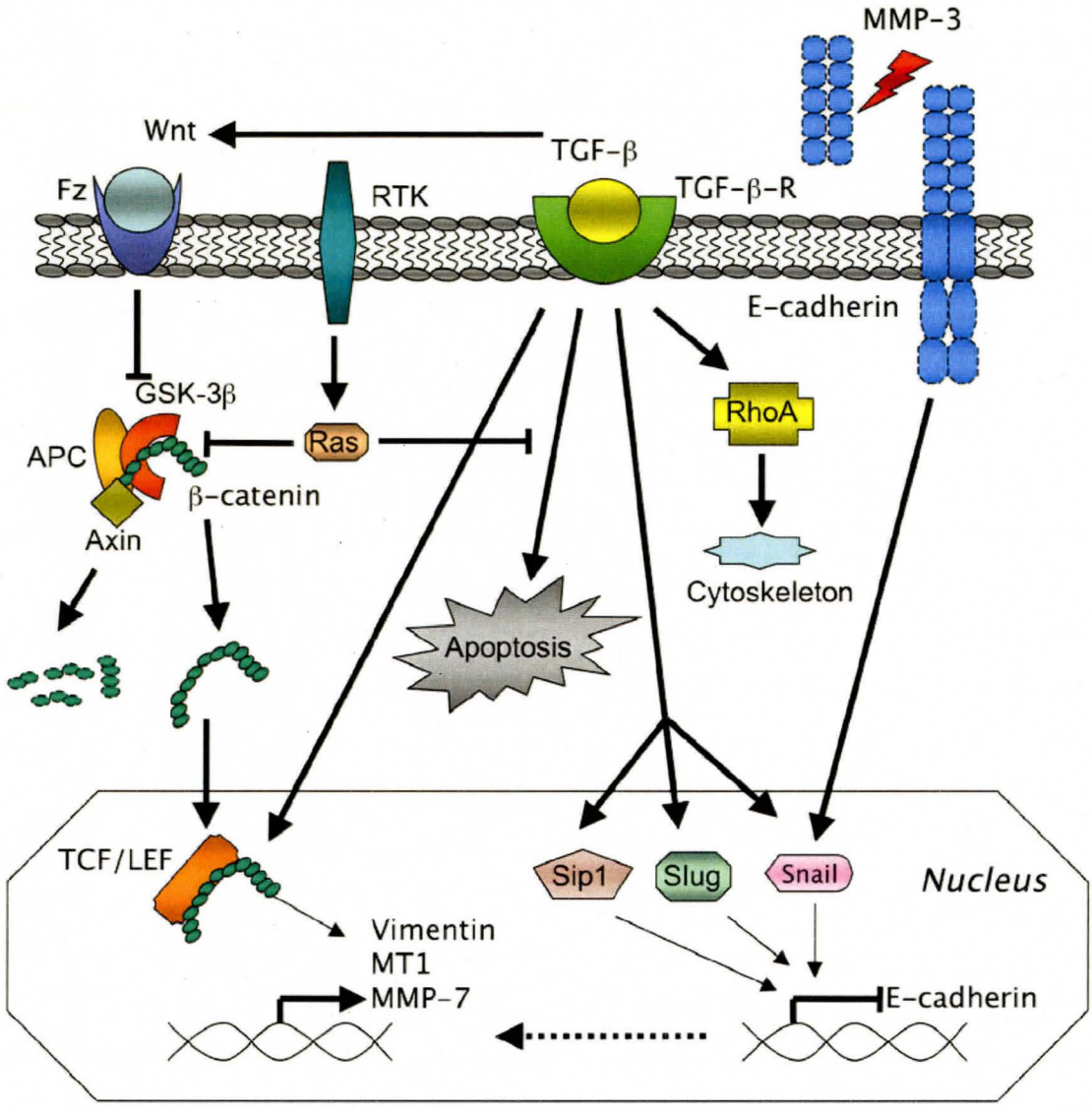


Figure 6: Molecular Pathways leading to EMT. Induction of EMT is a complex process that occurs in response to many signaling pathways some of which are indicated here. Ultimately, these pathways cooperate to lead to increased expression of E-cadherin repressors (Sip1, Slug, and Snail) along with EMT-related genes (Vimentin, MT1, and MMP-7) as well as cytoskeletal changes that lead to motile mesenchymal cells.

this event is termed 'cadherin switching' [Thiery, 2002; Thiery, 2003]. The above changes are also concurrent with amplification of α-SMA expression and increased production and deposition of extracellular matrix components which destroy cell-cell adhesion [Eddy, 2000]. Furthermore, cells exhibit increased migration, invasion and scattering, and they become elongated. Finally cells also become resistant to apoptosis caused by inadequate cell-matrix interaction (anoikis) [Lee *et al.*, 2006]. Thus, TGF-β-induced EMT in normal epithelial cells provides us with a suitable model to study epithelial-to-mesenchymal transition.

Changes due to EMT	***Affected proteins/process***
Increase in abundance	N-cadherin, Vimentin, Fibronectin, Snail, Slug, Twist, α-SMA, MMP's.
Decrease in abundance	E-cadherin, Desmoplakin, Occludin, Cytokeratin
Activity increases	Rho
Accumulate in nucleus	β-catenin, Snail, Slug, Twist
Functional Markers	Increased migration, invasion, scattering, cell elongation, weakened cell-cell adhesion, and anoikis resistance.

Table 1: Protein and biological changes which occur due to TGF-β-mediated EMT induction [Lee et al., 2006]

2.0 Rationale and Hypothesis

Downregulation of E-cadherin is postulated to free p120 from cell junction complexes and allow its localization into the cytoplasm, and the nucleus [Lo Muzio *et al.*, 2002; Mayerle *et al.*, 2003; Valizadeh *et al.*, 1997]. Nuclear-localized p120 may inhibit Kaiso-mediated transcriptional repression of tumourigenesis-associated genes, thus activating oncogenes and possibly genes involved in metastasis. Although the Kaiso/p120 interaction was detected in E-cadherin expressing cells of various origins, the interaction has not been studied in cells that have undergone EMT and thus cadherin switching. Moreover, although the localization of p120 was shifted from the cell membrane to the cytoplasm in colon carcinoma cells stimulated to undergo EMT [Bellovin *et al.*, 2005], the exact roles of p120 and its nuclear binding partner Kaiso in EMT remain unstudied. **Therefore, we investigated a) whether the induction of EMT affects with the expression and interaction between p120 and Kaiso, and b) whether changes in p120 and Kaiso expression levels impact EMT in cultured cells.**

Hypothesis

p120 and Kaiso-mediated signaling may play a role in epithelial-mesenchymal-transition by controlling the expression of EMT-associated genes.

3.0 Materials and Methods

3.1 Cell Lines and Tissue Culture

The cell lines used for this thesis include NMuMG (murine mammary epithelial, kindly provided by Dr. Margaret J. Wheelock, University of Nebraska Medical Center) and FHL-124 (human lens epithelial, a generous gift from Dr. Judith West-Mays, McMaster University). All cells were propagated in 1X high-glucose Dulbecco's Modified Eagle Medium (DMEM) (HyClone/MediaTech) and supplemented with 10% Fetal Bovine Serum (FBS) (HyClone/PPA), 0.5 μg/ml Fungizone, 100 μg/ml Streptomycin and 100 units/ml Penicillin (Invitrogen). NMuMG cells were also supplemented with 10 μg/ml Insulin (Sigma). To induce EMT in the NMuMG cell line, cells were treated with 5 ng/ml of TGF-β (R&D Systems). Culture medium was changed every three days and all cells were grown at 37 °C in a humidified incubator (5% CO_2). The human embryonic kidney cell line Phoenix-ampho was used for retroviral infections. These cells were grown in DMEM medium supplemented with 0.5 μg/ml Fungizone, 100 μg/ml Streptomycin and 100 Units/ml Penicillin (Invitrogen), 10% heat inactivated FBS (HyClone/PPA).

3.2 Co-Immunoprecipitation and Western Blots

Cells were grown until ~90% confluent on 100 mm culture dishes. Each dish was washed with 5 ml of 1X cold PBS (/PPA) then lysed with 0.5% Nonidet P40 (NP40) lysis buffer containing 0.5% NP40, 50 mM Tris, 150 mM NaCl and a Complete Mini protease inhibitor tablet without EDTA (Roche). Cells were harvested using a cell scraper and cell

debris was pelleted by centrifugation at 14,000 rpm for 5 minutes at 4 °C. The supernatants were transferred to new 1.5 ml tubes and protein concentrations were determined using Bradford assays. Equivalent amounts of protein were subsequently used for immunoprecipitations. 4 μg of the indicated antibodies were added to each sample and rotated end-over-end at 4 °C for one hour. The following antibodies were used for immunoprecipitation: anti-p120 15D2 monoclonal antibody, anti-Kaiso 6F monoclonal antibody, and finally anti-KT3 monoclonal antibody (a negative control).

Protein-A-sepharose beads (Amersham Biosciences) were incubated with rabbit anti-IgG (Jackson ImmunoResearch Laboratories), and 0.5% NP40 lysis buffer for one hour at 4 °C, then washed 3X times with NP40, added to each lysate sample and incubated end over end at 4°C for one hour to capture antibody-protein-complexes. Beads were then washed three times with 0.5% NP40 lysis buffer, and proteins were released from the beads and denatured in 3X Laemmli Sample Buffer (0.1 M Tris pH 6.8, 2% SDS, 10% sucrose, 0.008% Bromophenol blue, 0.24 M β-mercaptoethanol) by boiling for 4 minutes.

Proteins were then resolved on a 7% SDS-PAGE gel (unless otherwise specified) for 3.5 hours at 40 mAMPs and transferred onto a nitrocellulose membrane (Fisher Scientific) for 2 hours at 125 mAMPs. The membrane was blocked with 3% TBS-milk for five minutes and immunoblotted with the appropriate primary antibody diluted in 3% TBS-milk at 4 °C overnight. The following antibodies were used at the specific concentrations for Western blotting: anti-p120 15D2 mouse monoclonal antibody (1:5,000), anti-p1201A 2B12 (1:1,000), anti-Kaiso rabbit polyclonal antibody (1:12,000),

anti-E-cadherin mouse monoclonal antibody (1:5,000; BD Transduction Laboratories), anti-N-cadherin mouse monoclonal antibody (1:2,500; Zymed), anti-α-Smooth Muscle Actin 14A antibody (1:1,000; Sigma), anti-β-tubulin mouse monoclonal antibody (1:40,000; Sigma), and anti-GAPDH mouse monoclonal antibody (1:1,000; Sigma).

The following day, blots were washed five times with 1X TBS pH 7.4 for five minutes and incubated at room temperature for 2 hours with the appropriate peroxidase-conjugated secondary antibody diluted at 1:40,000 in 3% TBS-milk. The secondary antibodies used were, donkey-anti-mouse or goat-anti-rabbit (Jackson ImmunoResearch Laboratories) antibody. Finally, membranes were washed again as described above, and processed with Western Lightning Chemiluminescence reagent (PerkinElmer LAS, Inc.) according to the manufacturer's protocol. Blots were visualized on Kodak XAR film (Amersham).

3.3 Immunofluorescence

Cells were grown on 22 mm^2 cover slips (Fisher Scientific) in 6-well dishes until 60-80% confluent. Cells were then washed twice with 2 ml of cold 1X PBS and fixed with 1 ml of 100% cold methanol at -20 °C for 6 minutes. Fixed cells were immediately washed 2 times with 2 ml of cold 1X PBS and blocked with 3% PBS-milk solution for 12 minutes at room temperature. Cells were then incubated with the desired primary antibody diluted in 3% PBS-milk for 30 minutes at room temperature. The following antibodies were used at the specific concentrations: p120 F1 rabbit polyclonal antibody (1:600), Kaiso 6F mouse monoclonal antibody (1:200), α-Smooth Muscle Actin 14A (1:300, Sigma). After incubation with the primary antibody, cells were washed three times with 2 ml cold PBS

times with 2 ml cold PBS and incubated with the appropriate secondary antibody diluted 1:300 and 10 μg/ml Hoechst DNA staining reagent (Sigma) diluted 1:20 in 3% PBS-milk for 30 minutes at room temperature in the dark. The secondary antibodies used were Alexa594-conjugated anti-mouse IgG (1:300; Molecular probes) and Alexa488-conjugated anti-rabbit IgG (1:300; Molecular Probes). Next, the cover slips were washed three times with cold PBS and mounted onto slides with Poly-Aquamount (PolyScience) and the cells were imaged using a Carl Zeiss Axiovert 200, inverted fluorescent microscope.

3.4 Kaiso and p120 RNAi

Kaiso and p120 knockdown was achieved using the previously characterized Phoenix cell retroviral packaging line and the pRetroSuper (pRS) vector, into which 63-bp shRNA inserts were cloned as in Davis *et al.*, 2003. To achieve Kaiso knockdown, the following short hairpin RNA (shRNA) sequence was used to make pRS-mKaiso; GCA GAA ATT CTG AAC TAT A (bp 195-214). For p120 silencing we used an siRNA sequence previously shown to successfully deplete mouse p120 (pRS-mp120) [Davis *et al.*, 2003]. We also used a scrambled murine Kaiso siRNA (GAA CAA CTG GTT ATT ATC T) as a negative control (pRS-Scrambled).

The pRS vectors were transfected into the Phoenix cells as follows. Cells were grown to 70% confluency on 100 mm plates and transfected with 5 μg of DNA using ExGen500 (Fermentas) as per the manufacturer's protocol. Cells stably incorporating the plasmid were selected with 4 μg/ml Puromycin (CalBiochem/InvivoGen) for approximately 7 days, changing the medium every two days.

24 hours prior to viral harvest and infection of NMuMG cells, the Phoenix packaging lines were seeded to approximately 50-75% confluency on 100 mm plates in 5 ml of DMEM supplemented with 10% heat inactivated FBS and lacking puromycin. These cells were grown at 37 °C for 24 hours, after which time the viral supernatant was collected from the 100 mm plate and filtered through a 0.45μm syringe filter (Pall Corporation). Polybrene (Sigma) was subsequently added to the viral supernatant at a final concentration of 4 μg/ml. Before infection, NMuMG cells were seeded on 100 mm dishes such that, at the time of infection the next day, they would be 30-50% confluent and not in contact with neighboring cells. Culture medium was removed from cells to be infected and the Virus/Polybrene-containing medium was added. Cells were then incubated at 37 °C for 3-6 hours, after which time the virus-containing medium was removed and infected cells were incubated in standard growth medium for up to 24 hours at 37 °C. To select for cells that stably incorporated the pRetroSuper vector, cells were grown in 4 μg/ml Puromycin (CalBiochem/InvivoGen) for approximately 7 days.

3.5 Quantitative RT-PCR

Cells were grown until ~90% confluent on a 100 mm dish, then harvested into 1.5 ml microfuge tubes using a cell scraper. RNA was isolated using the RNEasy Kit (Qiagen) according to the manufacturer's protocol. The isolated RNA samples were diluted to 0.5 μg/ml and 2 μl of RNA from each sample was treated with DNaseI (Invitrogen) prior to cDNA synthesis. Synthesis of cDNA was carried out using SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen) according to

manufacturer's protocol. Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) was carried out using 12.5 µl of SybrGreen (BioRad), 1 µl of forward and reverse primers at 10 µM each, 5 µl of cDNA and 5.5 µl of dH_2O (Ultra Pure Distilled Water DNase, RNase Free; Invitrogen) for a total volume of 25 µl.

Primers from Table 1 were used to amplify the cDNA using a Stratagene Mx3000P real-time PCR system as per the following program: an initial denaturation period of 3 minutes, followed by 40 cycles of a 30 second denaturing step at 94 °C, a 30 second annealing step at 60 °C and a 30 second elongation step at 72 °C. A final cycle, consisting of 1 minute at 95 °C, 30 seconds at 60 °C and 30 seconds at 74 °C, was added to construct a dissociation curve and to ensure that non-specific amplification did not occur. Data were then analyzed using SigmaStat by performing a one-way ANOVA test using followed by pairwise comparison analysis of variance (Tukey test).

Mobix ID	***Primer***	***Sequence (5' – 3')***
ML16282	mKaiso Forward	CATGGAAAAATCAAAAGATGAAGC
ML16283	mKaiso Reverse	AAATAGCAACAGAGGCTGGGTTAG
ML181871	p120 Forward	GACGGGCTGCCCGTGGACGCC
ML181872	p120 Reverse	AGCTAAAGGAGCCCAATAGTA
ML070304	mE-cadherin Forward	ATGGGAGCCCGGTGCCGCAG
ML072883	mE-cadherin Reverse	CGCGGACGAGGAAACTGGTC
ML072884	mN-cadherin Forward	GGAGCTGACCAGCCTCCAAC
ML072839	mN-cadherin Reverse	CTGTACCGCAGCATTCCATT
ML072840	α-SMA Forward	AGACATCAGGGAGTAATGGT
ML070304	α-SMA Reverse	ATGGCATGAGGCAGGGCATA
ML16283	GAPDH Forward	GAAGGTCGGTGTGAACGGATT
ML16284	GAPDH Reverse	CCTGGAAGATGGTGATGGGC

Table 2: Primers used in qRT-PCR experiments

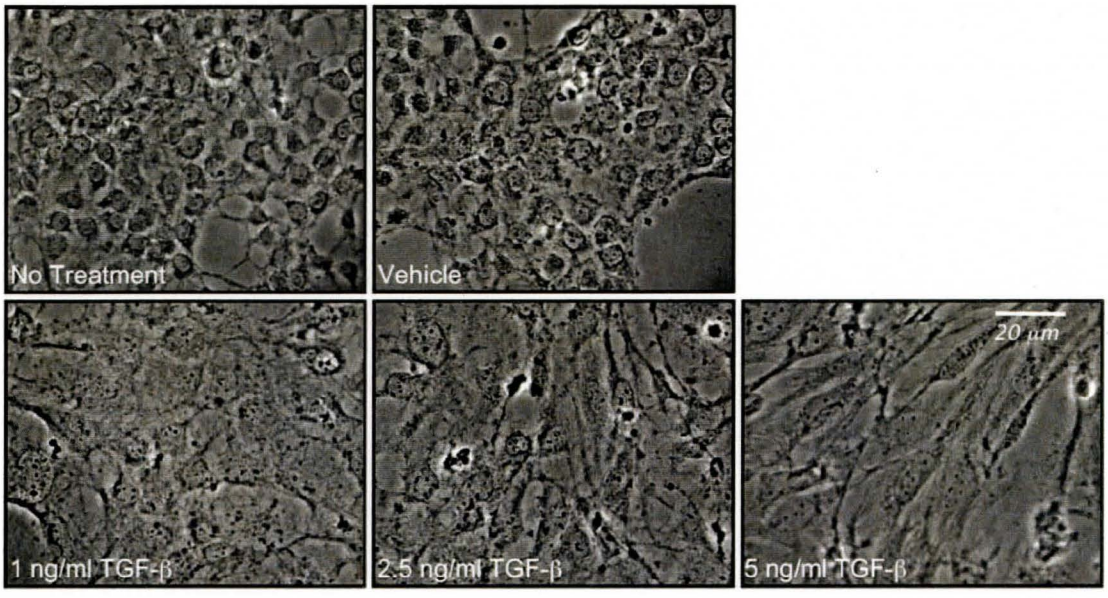
4.0 Results

4.1 TGF-β induces α-SMA expression in a dose-dependent manner in NMuMG cells but not in FHL-124 cells

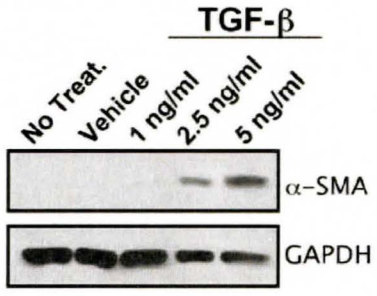
The mouse mammary epithelial cell line NMuMG has been shown to undergo changes associated with EMT following treatment with TGF-β [Maeda *et al.*, 2005; Piek *et al.*, 1999]. Furthermore, FHL-124 human epithelial cells have also been shown to express α-SMA, a major marker of EMT [Darby *et al.*, 1990; Maeda *et al.*, 2005; Xie *et al.*, 2003], following TGF-β treatment [Dawes *et al.*, 2007]. Before determining the effect of EMT on Kaiso and p120 levels, we set out to determine whether TGF-β induces EMT and the expression of α-SMA in NMuMG and FHL-124 cells in a dose-dependent manner. We treated cells with 0, 1.0, 2.5 or 5.0 ng/ml TGF-β. Changes in cell morphology were monitored by phase-contrast microscopy, and protein expression levels were monitored by Western blotting and densitometry analyses using the ImageJ software. In NMuMG cells TGF-β treatment was initiated when cells reached 50% confluency for 48 hours, whereas the FHL-124 cells were treated at 100% confluency for a period of 24 hours. FHL-124 cells exhibit small amounts of α-SMA expression when subconfluent thus, cells were grown to full confluency to eliminate residual α-SMA protein (West-Mays lab, McMaster University, personal communication).

Vehicle-treated control NMuMG cells exhibited no discernable morphological changes relative to untreated or vehicle-treated cells (Figure 7A, top panel). Both the vehicle and untreated cells were tightly packed together and exhibited a 'cobblestone'

A.


No Treatment
Vehicle
20 μm
1 ng/ml TGF-β
2.5 ng/ml TGF-β
5 ng/ml TGF-β

B.


TGF-β
No Treat.
Vehicle
1 ng/ml
2.5 ng/ml
5 ng/ml
α-SMA
GAPDH

C.

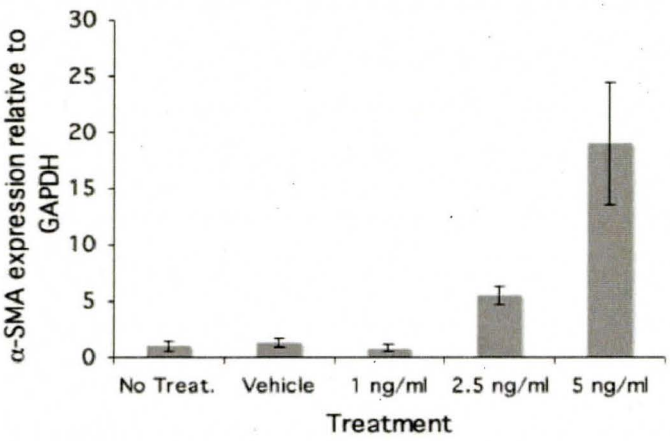
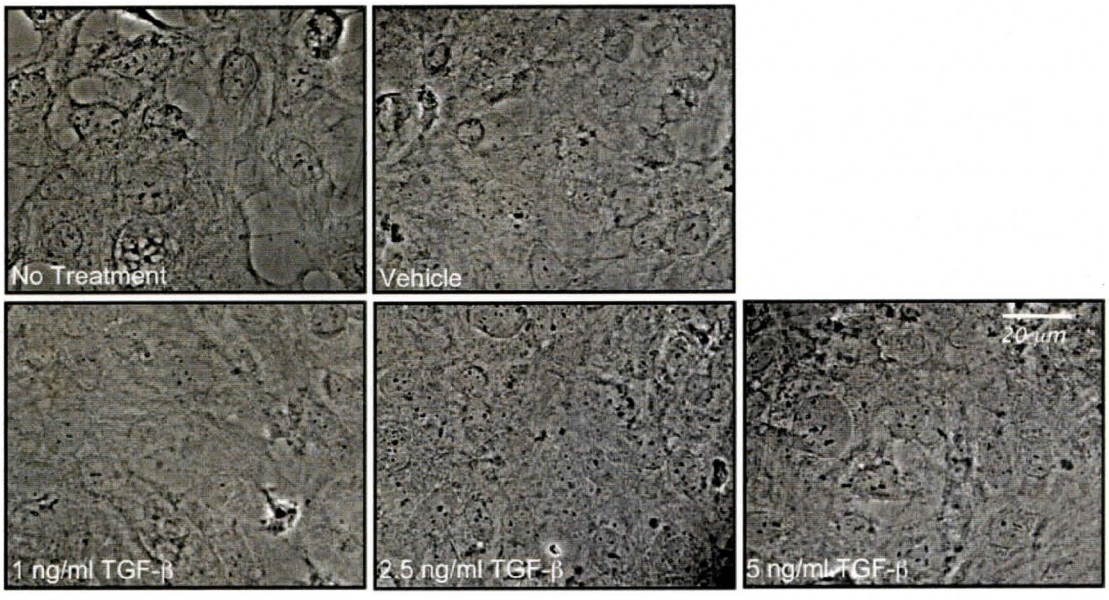

α-SMA expression relative to GAPDH
30
25
20
15
10
5
0
No Treat.
Vehicle
1 ng/ml
2.5 ng/ml
5 ng/ml
Treatment

Figure 7: TGF-β treatment induces phenotypic changes and α-SMA expression in a dose-dependent manner in NMuMG cells. When cells reached 30% confluency, they were either left untreated, exposed to a vehicle solution (4 mM HCL/1 mg/ml BSA), or treated with TGF-β at concentrations of 1.0, 2.5 and 5.0 ng/ml for 48 hours. **A**. After cells were fixed on 20 mm cover slips, cell morphology was observed via phase-contrast microscopy using a Carl Zeiss Axiovert 200 microscope. Images were taken at 400X magnification (bar = 20 μm). Vehicle and untreated cells showed an epithelial morphology while TGF-β treated cells showed a gradual transition to a mesenchymal phenotype with increasing TGF-β concentration. **B**. Protein lysates were collected and 50 μg of protein was resolved on a 12% SDS gel, then immunoblotted for α-SMA and GAPDH. Protein expression of α-SMA is only detected when cells were treated with 2.5 ng/ml or more of TGF-β. To quantify α-SMA expression relative to GAPDH, densitometry analysis was carried out using ImageJ **C**. Values were normalized to the basal levels measured in untreated control cells, standard errors bars are included. NMuMG cells treated with 2.5 and 5.0 ng/ml of TGF-β showed a 5.50 ± 0.81 and 19.1 ± 5.47 (n = 2) fold increase of α-SMA expression respectively.

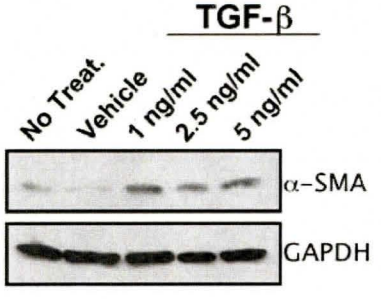
morphology, characteristic of epithelial cells. In contrast, TGF-β-treated cells became elongated and lost contact with each other (Figure 7B, bottom panel). Furthermore, the treated cells became larger when compared to the vehicle-treated and untreated cells. As the TGF-β concentration increased, the treated cells appeared progressively more elongated, appearing more mesenchymal. The TGF-β-induced mesenchymal morphology developed gradually, in a dose-dependent manner, and the effect was most distinct in cells treated with 5.0 ng/ml of TGF-β (Figure 7A). Moreover, Western blot analysis demonstrated that α-SMA protein expression increased in NMuMG cells as the TGF-β concentration increased. Similar to the untreated cells, the vehicle control cells did not express any α-SMA protein. The expression of α-SMA was only detectable when cells were treated with at least 2.5 ng/ml of TGF-β (Figure 7B). Densitometry analysis of the Western blots showed that NMuMG cells treated with 2.5 and 5.0 ng/ml of TGF-β exhibited a 5.50 ± 0.81 (n = 2) and 19.1 ± 5.47 (n = 2) fold increase of α-SMA expression, respectively (Figure 7C).

In the FHL cell line, no discernable morphological changes occurred in the TGF-β-treated cells compared to untreated, vehicle-treated cells. When compared to the untreated cells, in both vehicle- and TGF-β-treated samples, the cells appeared to have characteristics of epithelial morphology where cells are tightly adherent to each other (Figure 8A). Western blot analysis of lysates from the human lens epithelial cells showed an increase of α-SMA protein expression when cells were treated with TGF-β compared to untreated cells or cells treated with vehicle alone (Figure 8B). Interestingly, the observed increase in α-SMA protein levels was not gradual, and did not exhibit the

A.


No Treatment
Vehicle
1 ng/ml TGF-β
2.5 ng/ml TGF-β
5 ng/ml TGF-β
20 μm

B.


TGF-β
No Treat.
Vehicle
1 ng/ml
2.5 ng/ml
5 ng/ml
α-SMA
GAPDH

C.

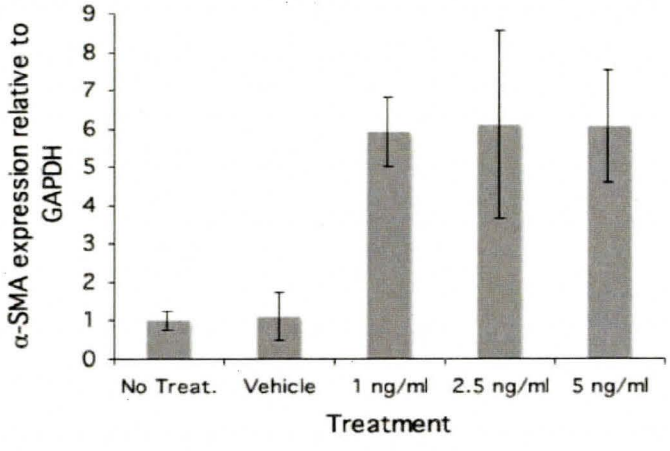

α-SMA expression relative to GAPDH
0
1
2
3
4
5
6
7
8
9
No Treat.
Vehicle
1 ng/ml
2.5 ng/ml
5 ng/ml
Treatment

Figure 8: TGF-β treatment has little effect on FHL-124 phenotype but induces α-SMA protein expression. When cells reached 100% confluency, they were either left untreated, exposed to a vehicle solution (4 mM HCL/1 mg/ml BSA), or treated with TGF-β at concentrations of 1.0, 2.5 and 5.0 ng/ml for 24 hours. **A**. After cells were fixed on 20 mm cover slips, cell morphology was observed via phase-contrast microscopy using a Carl Zeiss Axiovert 200 microscope. Images were taken at 400X magnification (bar = 20 μm). TGF-β treated, vehicle- and untreated control cells exhibited epithelial morphology with no discernable differences despite increasing TGF-β concentration. **B**. Protein lysates were collected and 50 μg of protein was resolved on a 12% SDS gel, then immunoblotted for α-SMA and GAPDH. Low levels of α-SMA expression were detected in untreated and vehicle treated control cells, higher expression was seen when treated with TGF-β treatment, but expression did not correlate with the increase of TGF-β concentration. To quantify α-SMA expression relative to GAPDH, densitometry analysis was carried out using ImageJ **C**. FHL-124 cells treated with 1.0, 2.5 and 5.0 ng/ml of TGF-β showed a 5.90 ± 0.90 , 6.11 ± 2.45 and 6.07 ± 1.46 (n = 2) fold increase of α-SMA expression respectively. Values were normalized to the basal levels measured in untreated control cells, standard errors bars are included.

same pattern observed with the NMuMG cells. In the FHL-124 cells, the increase was consistent between the three TGF-β concentration treatments, 1.0, 2.5 and 5.0 ng/ml (Figure 8B). Densitometry analysis of the blots confirmed the results; cells treated with 1.0, 2.5 and 5.0 ng/ml of TGF-β showed a 5.90 ± 0.90 (n = 2), 6.11 ± 2.45 (n = 2) and 6.07 ± 1.46 (n = 2) fold increase of α-SMA expression respectively (Figure 8C).

4.2 TGF-β-mediated EMT induction does not affect Kaiso/p120 co-precipitation

Kaiso was previously shown to interact with p120 in epithelial cell lines using co-immunoprecipitation experiments [Daniel and Reynolds, 1999]. This interaction has been well characterized in epithelial cell lines [Daniel and Reynolds, 1999]; however, it is yet to be characterized in mesenchymal cells. To determine whether EMT affects the Kaiso/p120 interaction, co-immunoprecipitation experiments were performed using normal NMuMG cells (epithelial) and TGF-β-induced cells (mesenchymal). NMuMG cells were seeded onto 100 mm plates and when the cells reached 30% confluence, they were either left untreated, exposed to a vehicle solution, or treated with 5 ng/ml of TGF-β for 2 and 4 day (2D and 4D) time periods. FHL-124 cells were seeded onto 100 mm plates and treated with vehicle solution or 5 ng/ml of TGF-β when cells were 100% confluent for a period of 24 hours. A concentration of 5 ng/ml of TGF-β was chosen, since it was shown to induce EMT efficiently in NMuMG cells (Figure 7) in relation to morphological changes and α-SMA expression, and to keep experimental conditions consistent, the same concentration was also used for FHL-124 cells.

When NMuMG cell lysates were immunoprecipitated and Western blotted with Kaiso specific antibodies (Figure 9, top panel), a prominent band migrating at approximately 100 kDa was observed in both vehicle-treated and TGF-β-treated cells. Kaiso also co-precipitated with p120 in lysates of both vehicle and TGF-β treated cells. Interestingly, more Kaiso co-precipitated with p120 with prolonged TGF-β treatment. Lysates that were immunoprecipitated and Western blotted with pan-specific p120 antibodies (15D2), detected at least four different isoforms of the protein, with the upper isoforms being more abundant than the lower isoforms (Figure 9, bottom panel). Consistent with previous reports [Daniel and Reynolds, 1999], p120 was not detected in Kaiso immunoprecipitates.

FHL-124 cell lysates were immunoprecipitated with Kaiso- or p120-specific antibodies and Western blotted for Kaiso. Bands corresponding to Kaiso appeared as a 100-kDa doublet (Figure 10, top panel). Previous studies performed in our laboratory found that the slower-migrating band represents phosphorylated Kaiso [Daniel lab, unpublished data]. Again, Kaiso and p120 co-precipitated from lysates of both epithelial cell lines, both before and after TGF-β-mediated EMT induction. Interestingly, two isoforms of p120 were detected from lysates that were immunoprecipitated and Western blotted with p120-specific antibodies. The more abundant, slower-migrating band likely represents isoform 1A, whereas the faster-migrating band likely represents isoform 3A (Figure 10, bottom panel).

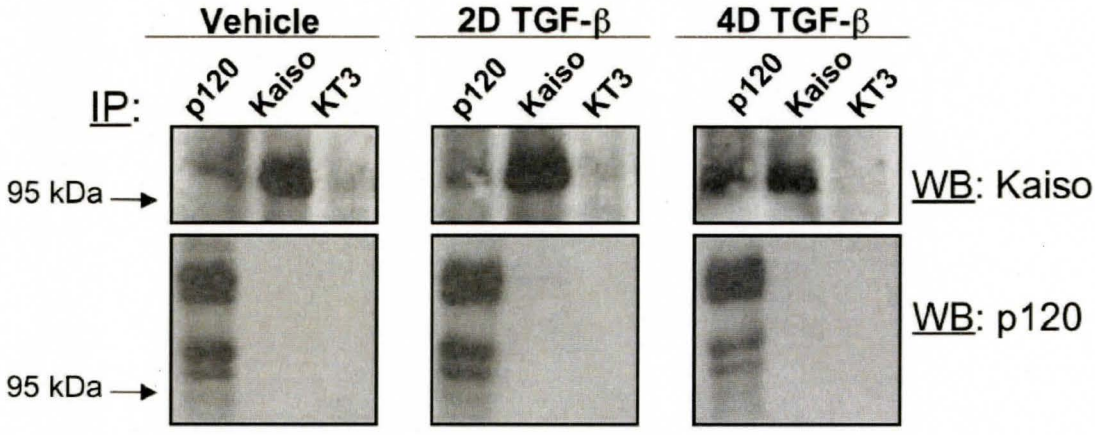


Figure 9: The Kaiso/p120 interaction is detected in NMuMG cells when treated with vehicle and TGF-β for 2-4 days. When NMuMG cells reached 30% confluence, they were treated with a Vehicle solution (4 mM HCL/1 mg/ml BSA) or TGF-β (5 ng/ml) for 2 or 4 days (2D and 4D). Protein lysates were extracted and immunoprecipitated with monoclonal antibodies against Kaiso (6F), p120 (15D2) and KT3 (a negative control). Lysates were standardized to equal protein content, resolved on a 7% SDS-PAGE then immunoblotted for Kaiso and p120. A Kaiso band was detected when proteins were immunoprecipitated using Kaiso and p120 antibody. The catenin p120 was only detected when lysates were immunoprecipitated using p120 antibody. More Kaiso co-precipitated with p120 in lysates from 2 and 4 day-treated cells when compared to vehicle-treated cells. Note: The interaction is detectable in one direction only, Kaiso is co-precipitated with p120 but not vice-versa.

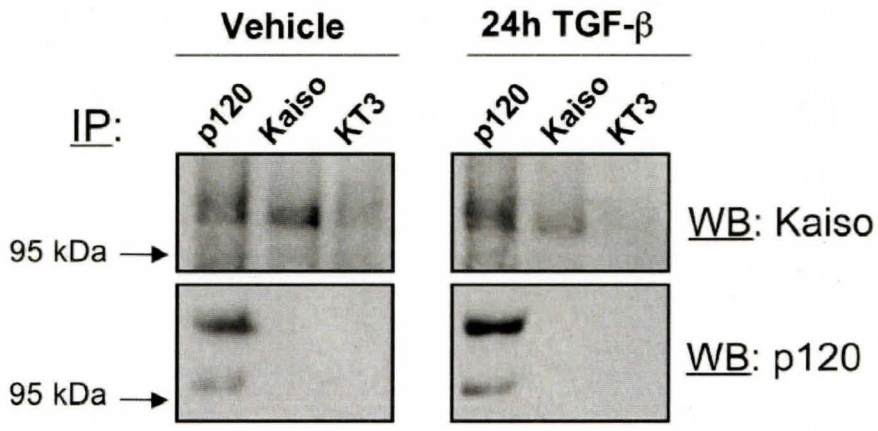


Figure 10: The Kaiso/p120 interaction is detected in FHL-124 cells in both Vehicle and TGF-β treated cells. FHL-124 cells were treated with TGF-β (5 ng/ml) or a Vehicle solution (4 mM HCL/1 mg/ml BSA) for 24 hours at 100% confluency. Protein lysates were extracted and immunoprecipitated with monoclonal antibodies against Kaiso (6F), p120 (15D2) and KT3 (a negative control). Lysates were standardized to equal protein content, resolved on a 7% SDS-PAGE then immunoblotted for p120 and Kaiso. A Kaiso band is detected when proteins were immunoprecipitated using Kaiso and p120 antibody. The catenin p120 is only detected in lysates immunoprecipitated using p120 antibody.

4.3 The expression of α-SMA protein is augmented as a result of longer TGF-β treatments in NMuMG cells

It was determined that the induction of EMT in NMuMG cells, as determined by α-SMA expression and cell morphology changes, is directly related to TGF-β concentration (Figure 7). To determine whether EMT induction correlates with the length of TGF-β treatment, immunofluorescence experiments were carried out to test for α-SMA expression. When NMuMG cells became 30% confluent, they were either left untreated, treated with a vehicle solution or with 5 ng/ml TGF-β for 2 or 4 day periods. Cells were fixed and stained with α-SMA antibody (red) to detect protein expression levels. Hoechst staining was used to visualize nuclei (blue). Images were captured via fluorescence microscopy using a Carl Zeiss Axiovert 200 inverted microscope. Untreated cells or cells treated with vehicle solution showed negligible amounts of α-SMA staining, which was expected since α-SMA expression is a hallmark of mesenchymal and not epithelial cells [Darby *et al.*, 1999]. On the other hand, TGF-β treated cells showed elevated α-SMA staining, indicating that EMT induction was underway in these cells. More cells exhibited α-SMA staining after four days of treatment (approximately 90% of cells) relative to cells treated for two days only (50-60% staining) (Figure 11A).

Similarly, the cell morphology of untreated and vehicle-treated cells was epithelial like. The cells appeared tightly packed, exhibiting the cobblestone morphology seen in cells with strong intercellular adhesion. After TGF-β treatment however, cells became more mesenchymal in appearance, exhibiting long extensions and lesser adhesion

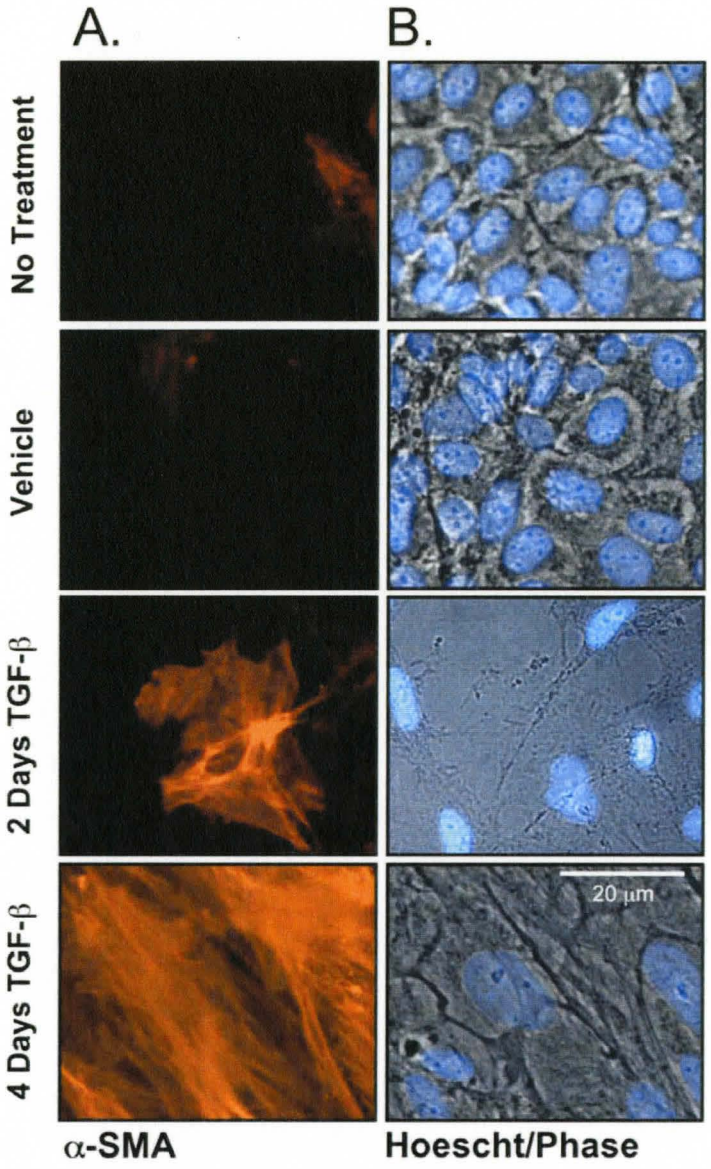


Figure 11: Expression of α-SMA increased with prolonged TGF-β treatment in NMuMG cells. When NMuMG cells became 30% confluent, they were either left untreated, treated with a vehicle solution or with 5 ng/ml TGF-β for 2 and 4 days. Cells were fixed and stained with **A**. α-SMA antibody (red) to detect its expression or **B**. Hoescht staining was used to visualize nuclei (blue). Images were captured using phase-contrast microscopy using a Carl Zeiss Axiovert 200 inverted microscope (400X magnification, bar = 20 μm). α-SMA expression increased as cells were exposed to longer treatments of TGF-β. Cells also became more elongated with time.

to neighboring cells. Again, this effect was more prominent in cells that were treated with TGF-β for four days than cells treated for two days (Figure 11B). Thus, EMT induction appears to correlate directly with TGF-β concentration and the duration of treatment.

4.4 Effect of TGF-β-mediated EMT induction on Kaiso and p120 subcellular localization in NMuMG cells

In normal epithelial cells, Kaiso is found to be predominantly nuclear while p120 localizes at cell-cell contacts bound to E-cadherin. Immunofluorescence experiments were carried out to determine whether the subcellular localization of Kaiso and p120 in NMuMG cells changes following TGF-β-induced EMT. Cells were seeded until they reached 30% confluence. Cells were then subjected to three experimental conditions: untreated, vehicle or TGF-β for 2 or 4 days. Next, the cells were fixed and doubly immunostained with Kaiso mouse, monoclonal antibodies 6F (red) and p120 rabbit polyclonal antibodies (green) that recognizes all p120 isoforms. Nuclear visualization was achieved using Hoescht dye (blue). Images were obtained via an inverted, phase-contrast Carl Zeiss Axiovert 200 microscope. All cell images were obtained at 400 times magnification.

As expected, Kaiso staining was found to be mainly nuclear in epithelial cells (untreated and vehicle-treated) and cells that were induced to become mesenchymal using TGF-β. However, in cells that were induced to undergo EMT for two days with TGF-β, Kaiso staining was also seen at focal adhesion of cells that became elongated (Figure 12; first column).

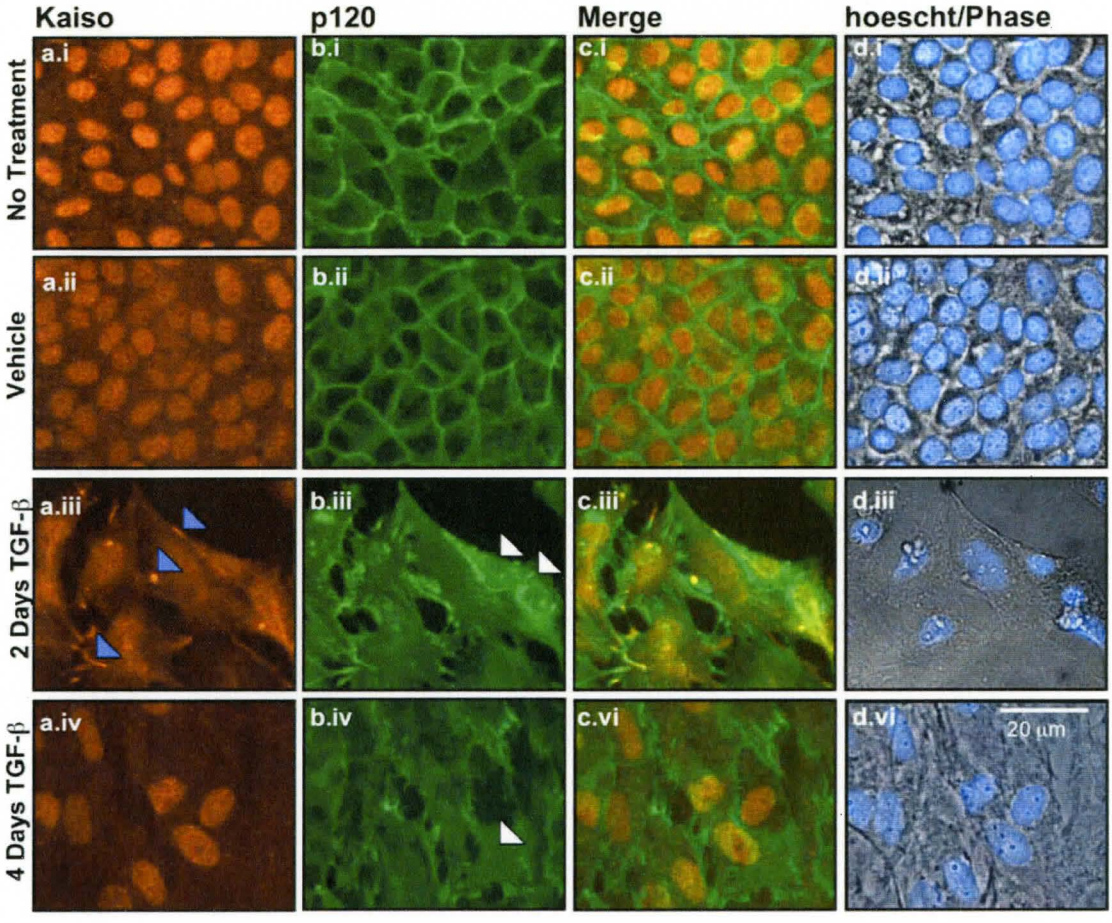


Figure 12: Correlation of subcellular localization of Kaiso and p120 with TGF-β-induced EMT in NMuMG cells. When cells became 30% confluent, they were left untreated, treated with a vehicle solution or 5 ng/ml TGF-β for 2 and 4 days. To detect changes in protein localization, cells were fixed and stained with 6F monoclonal Kaiso antibody (red, a.i-a.iv) or polyclonal p120 antibody (green, bi-b.iv). Nuclei were stained using hoescht (blue, di-div). Images were captured with a Carl Zeiss Axiovert 200, inverted phase-contrast microscope (400X magnification, bar = 20 μm). Kaiso staining remained predominantly nuclear in cells under all four conditions, however, some Kaiso staining is detected at what appears to be focal adhesions (blue arrows) in cells that underwent two days of treatment. Staining of p120 is mainly at cell-cell contacts in control cells (untreated and vehicle). p120 is seen at cell junctions as well as in the cytoplasm and possibly perinucleus (white arrow heads) of EMT-induced cells.

In the control cells, i.e. cells that were left untreated or treated with a vehicle solution; p120 staining was predominantly seen at cell-cell contacts, which was expected. On the other hand, following 2 days of TGF-β-induced EMT, cells obtained a mesenchymal phenotype, and although some p120 staining remained at cell-cell contacts, the protein also appeared to localize to the cytoplasm and perinuclear regions (white arrow heads). Similarly, after 4 days of TGF-β treatment, p120 was also found in the cytoplasm (white arrow heads) (Figure 12; second column).

4.5 Expression profiling of Kaiso, p120 and EMT markers in NMuMG cells

To determine whether protein expression of Kaiso, p120 and some EMT markers differs between normal (epithelial) and EMT-induced NMuMG cells (mesenchymal), Western blot analysis was carried out. Cells were induced to undergo EMT using 5 ng/ml of TGF-β, alongside control cells that were left untreated or treated with a vehicle solution. Whole cell lysates were extracted and 50 μg of protein (100 μg for Kaiso) was resolved on an 8% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and immunoblotted with the following antibodies: E-cadherin (an epithelial marker), N-cadherin and α-SMA (mesenchymal markers), Kaiso, p120 (15D2 antibody to recognize all isoforms, and 2B12 antibody to recognize p1201A isoform) and β-tubulin (loading control).

Compared to control cells (untreated and vehicle), protein expression levels of the epithelial marker, E-cadherin, gradually reduced with prolonged TGF-β treatment. On the other hand, protein levels of the mesenchymal markers, N-cadherin and α-SMA, were

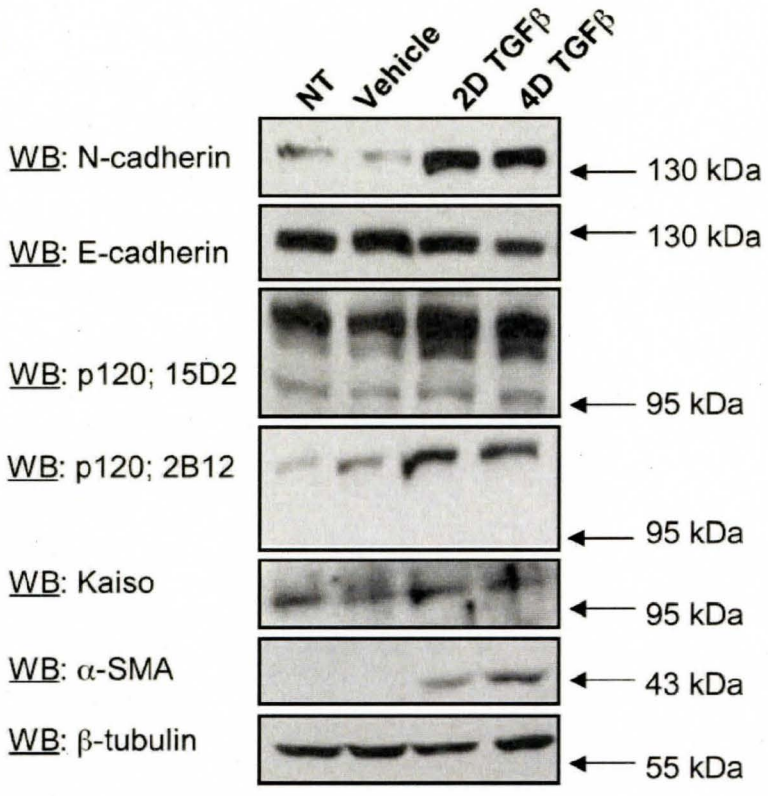


Figure 13: Expression profiling of Kaiso, p120 and EMT markers in NMuMG cells. NMuMG cells were either left untreated, treated with a vehicle solution or treated with TGF-β (5 ng/ml) for 2 or 4 days (2D or 4D) respectively. 50 μg of protein extracts (except for Kaiso 100 μg) were ran on an 8% SDS-PAGE followed by Western blot analysis. Antibodies for N-cadherin (mesenchyme marker), E-cadherin (epithelial marker), p120 (15D2 to recognize all isoforms, 12B2 specific to isoform 1A), Kaiso, and β-tubulin (a loading control) were used to detect protein expression changes. E-, N-cadherin and α-SMA levels showed the expected trend where E-cadherin decreased while N-cadherin and α-SMA increased after TGF-β treatment. Levels of the upper isoforms of p120 are augmented after TGF-β treatment while Kaiso protein levels remain constant.

augmented in a steady manner after 2 and 4 days of TGF-β-induced EMT. This result was expected and supports findings that have been previously observed by other groups [Maeda *et al.*, 2005, Piek *et al.*, 2003]. Kaiso expression levels appeared to be unaffected regardless of EMT induction. However, p120 levels appeared to increase, especially the slower migrating upper isoforms while the smaller isoforms appeared to be unchanged between control cells and cells that were induced to undergo EMT. Interestingly, a previous study showed that following EMT induction, an isoform switch from the smaller isoforms to the larger isoforms in mouse mammary epithelial cells [Eger *et al.*, 2000]. To determine whether this increase affects the larger p1201A isoform in particular, an isoform-specific antibody (2B12) was used for immunoblotting. Levels of p1201A are low in the control untreated cell and those exposed to a vehicle solution, nevertheless, an increase in protein expression was observed following TGF-β treatment (Figure 13).

4.6 Retroviral knockdown of Kaiso and p120 in NMuMG cells

To test the effects of Kaiso and p120 misexpression on EMT induction in NMuMG cells, Kaiso and p120 expression was silenced using Kaiso- and p120-specific siRNA-producing retroviruses. To achieve knockdown, we used the previously characterized Phoenix cell retroviral packaging system and the pRetroSuper (pRS) vector, into which 63-bp shRNA insert was placed. Cells were infected with retrovirus expressing a mouse Kaiso siRNA sequence (pRS-mKaiso), an siRNA sequence previously shown to successfully deplete mouse p120 (pRS-mp120) [Davis *et al.*, 2003], and finally, a negative control scrambled siRNA sequence (pRS-Scrambled). Following infection with virus, cells that were infected with the virus and were producing siRNA

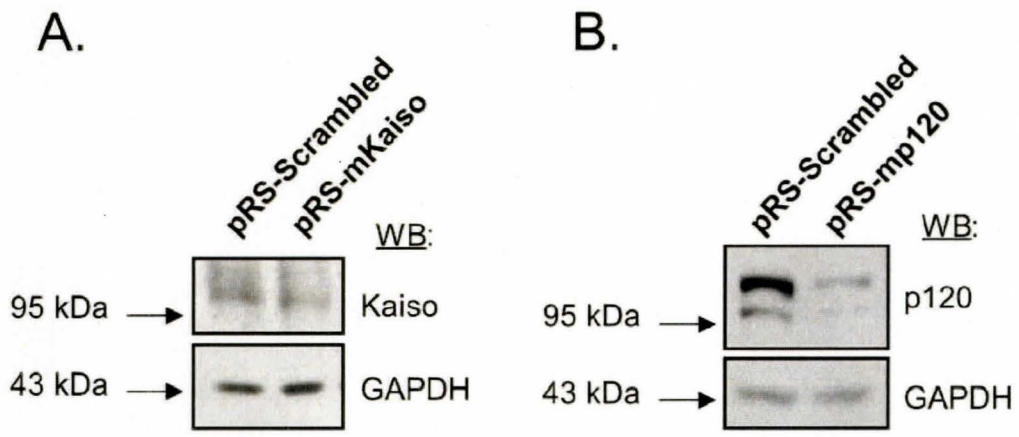


Figure 14: p120 and Kaiso siRNA-mediated knockdown in NMuMG cells. Cells were infected with MoMuL retroviruses carrying pRS-mKaiso, pRS-mp120 and pRS-Scrambled vectors. Following puromycin selection, protein lysate was extracted and immunoblotted with antibodies against Kaiso, p120, GAPDH (loading control). Knockdown of Kaiso and p120 was achieved using the pRS-mKaiso and pRS-mp120 respectively in relation to the pRS-Scrambled negative control.

were selected using puromycin. Protein lysates were then extracted, separated on an 8% SDS-PAGE then transferred onto a nitrocellulose membrane, and immunoblotted using Kaiso and p120 antibodies. Cells infected with retroviruses carrying pRS-Kaiso and pRS-p120, showed lower expression levels of Kaiso and p120 respectively in comparison to control cells infected with pRS-Scrambled-containing retrovirus (Figure 14A and B).

In NMuMG Kaiso-depleted cells, expression of E-cadherin gradually decreased with prolonged TGF-β treatment, conversely, N-cadherin and α-SMA protein levels increased steadily following 2 and 4 days of EMT induction. Kaiso expression appeared to be unchanged despite of TGF-β-induced EMT while the expression of p120 became greater in cells that were exposed to TGF-β, especially the slower migrating upper isoforms. The p1201A isoform was not detected in control cells, but its levels also increased as a result of EMT (Figure 15).

Interestingly, in p120 knockdown NMuMG cells, the expression of E-cadherin remained the same regardless of prolonged TGF-β treatment and did not decrease as was observed in wild type and Kaiso-depleted cells. Furthermore, expression of the mesenchymal markers, N-cadherin and α-SMA, was greatly hindered. N-cadherin levels are not detected in control cell lysates (untreated and vehicle) or in 2- and 4-day TGF-β treated cell lysates. As for α-SMA expression, its expression is delayed and not seen until 4 days post treatment, while it is detected after 2 days in wild type and Kaiso knockdown cells. However, similar to wild type and Kaiso-depleted cells, p120 protein levels were amplified after 2 and 4 days of EMT induction when compared to the control vehicle and untreated cells. Although, the p1201A isoform was not detected in any of the

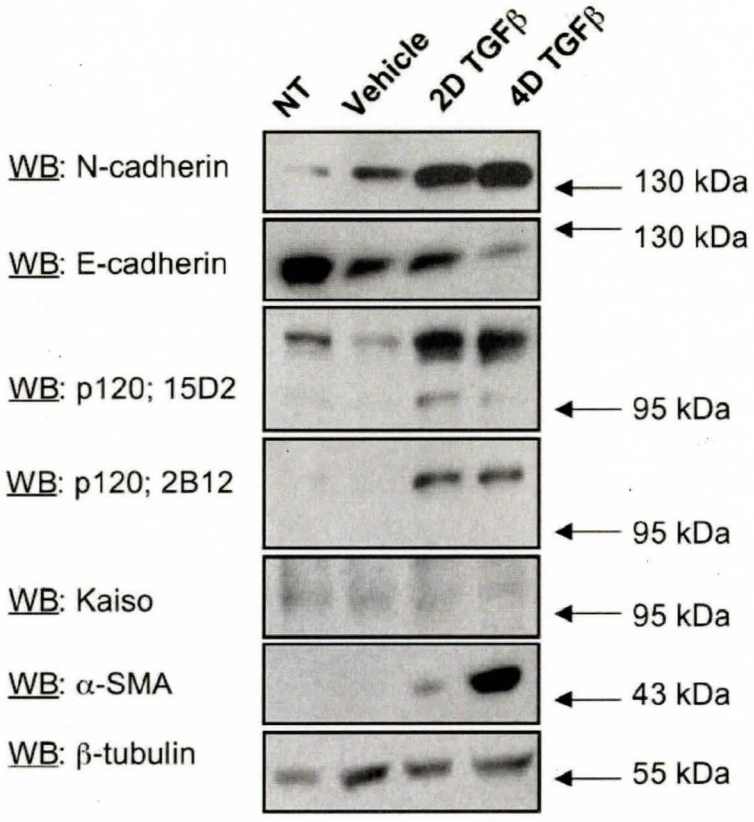


Figure 15: Expression profiling of Kaiso, p120 and EMT markers in Kaiso knockdown NMuMG cells. NMuMG cells with Kaiso knockdown were either left untreated, treated with a vehicle solution or treated with TGF-β (5 ng/ml) for 2 or 4 days (2D or 4D) respectively. 50 μg of protein extracts (except for Kaiso 100 μg) were ran on a 8% SDS-PAGE followed by Western blot analysis. Antibodies for N-cadherin and α-SMA (mesenchyme markers), E-cadherin (epithelial marker), p120 (15D2 to recognize all isoforms; 12B2 specific to isoform 1A), Kaiso, and β-tubulin (a loading control) were used to detect protein expression changes. E-, N-cadherin and α-SMA levels showed the expected trend where E-cadherin decreased while N-cadherin and α-SMA increased while after TGF-β treatment. Levels of the upper isoforms of p120 are augmented after TGF-β treatment while Kaiso protein levels remain constant.

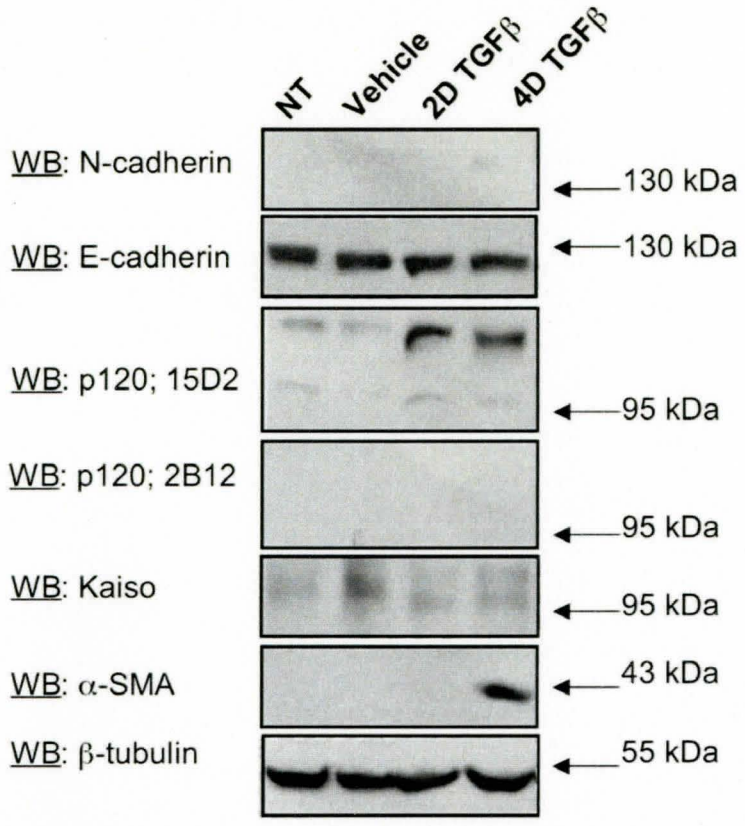


Figure 16: Expression profiling of Kaiso, p120 and EMT markers in p120 knockdown NMuMG cells. NMuMG cells with p120 knockdown were either left untreated, treated with a vehicle solution or treated with TGF-β (5 ng/ml) for 2 or 4 days (2D or 4D) respectively. 50 μg of protein extracts (except for Kaiso 100 μg) were ran on a 8% SDS-PAGE followed by Western blot analysis. Antibodies for N-cadherin (mesenchyme marker), E-cadherin (epithelial marker), p120 (15D2 to recognize all isoforms, 12B2 specific to isoform 1A), Kaiso, and β-tubulin (a loading control) were used to detect protein expression changes. E-cadherin levels did not decrease in response to EMT induction while N-cadherin were not detected in lysates from any of the treated cells. α-SMA expression was not detected until four days post treatment. Upper isoforms of p120 are augmented with TGF-β treatment, however, isoform 1A was not detected in any of the treatments. Kaiso levels were unchanged.

treated and control cells. Indicating the increase seen in the upper isoforms is not occurring in isoform p1201A specifically, and that knockdown is greatly affecting the expression of this isoform (Figure 16).

4.7 Transcription level profiling of Kaiso and p120 in wild type, Kaiso- and p120-knockdown NMuMG cells

To determine whether the transcriptional expression pattern of Kaiso and p120 is altered before and after EMT induction in wild type, Kaiso- and p120-depleted cells, qRT-PCR experiments were utilized. Three independent experiments were performed using cells induced to undergo EMT using 5 ng/ml of TGF-β, treated with a vehicle solution or left untreated. RNA was then extracted and used to make cDNA followed by real time-PCR amplification; each of the three samples was amplified in duplicate.

In wild type cells, Kaiso levels showed little variation between cells exposed to TGF-β and those cells that were exposed to vehicle solution or were left untreated. One-way analysis of variance confirmed this observation ($P = 0.336$); the difference was not statistically significant (Figure 17; Wild Type). Similarly, no change occurred in Kaiso expression levels among cells that were induced to undergo EMT and those that were not induced in p120-depleted cells ($P = 0.115$) (Figure 17; pRS-p120). On the other hand, in Kaiso knockdown cells a small increase in Kaiso levels was seen after four days of treatment when compared to the control cells (vehicle and untreated), this overall change was statistically significant ($P = 0.044$). Nevertheless, a pairwise multiple comparison procedures test (Tukey Test) which was utilized to detect where the

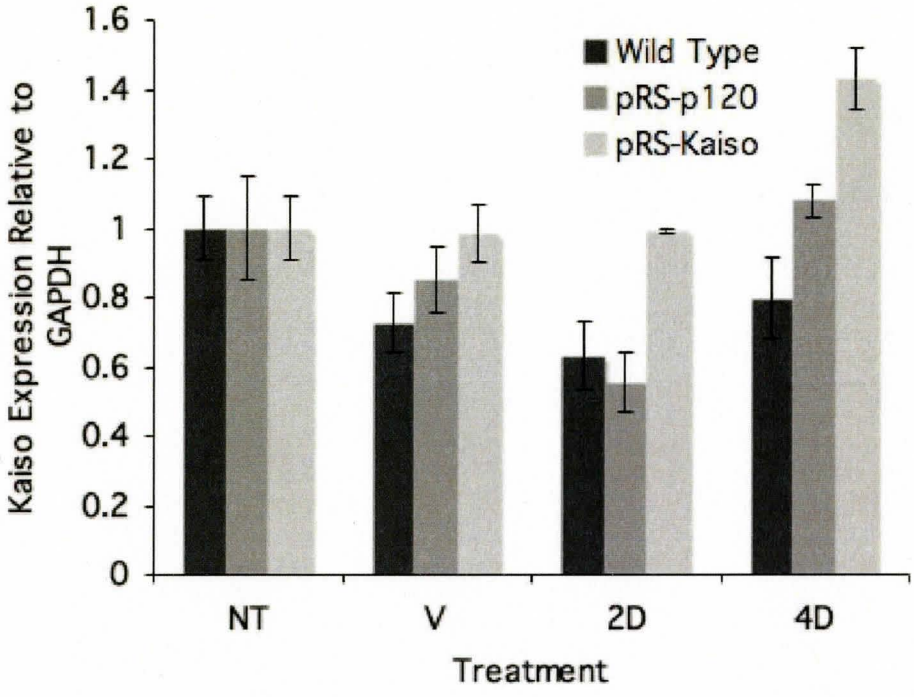


Figure 17: Expression profiling of Kaiso in wild type, Kaiso- and p120-depleted NMuMG cells as a result of EMT induction. Cells were either left untreated (NT), treated with a vehicle solution (V) or treated with 5 ng/ml TGF-β for 2 or 4 days (2D or 4D) respectively. RNA was extracted and used to make cDNA followed by qRT-PCR. Values were normalized to the basal levels measured in untreated control cells (n = 3, standard errors bars are included). Kaiso levels remained constant in all treatments in both wild type and p120 knockdown cell. However, in Kaiso knockdown cells a small increase in Kaiso expression was seen in 2D cells in relation to NT and V controls. The overall change was statistically significant (P = 0.044) but a pairwise multiple comparison procedures test (Tukey Test) suggested that no statistical significance existed between any of the treated cell groups.

difference between the groups lies, showed no statistical significance among any of the treated cell samples (Figure 17; pRS-Kaiso).

Additionally, in wild type NMuMG cells, p120 transcription levels showed a gradual increase following TGF-β treatment as opposed to vehicle- and untreated cells. In relation to untreated cells, p120 levels were 2.66 ± 0.22 and 3.56 ± 0.68 fold higher after 2 and 4 days of TGF-β treatment respectively. Although a change in p120 levels was detected, the differences were not great enough to exclude the possibility that it occurred as a result of random sampling variability; thus, the values were not statistically significant. A small increase was also seen in vehicle-treated cells compared to untreated cells (1.76 ± 0.19 folds), but this change was not found to be statistically significant (Figure 18; Wild Type).

Similarly, an increase in p120 expression was also observed in Kaiso- and p120-depleted cells that were induced to undergo EMT compared to those that were not induced. In Kaiso-depleted cells p120 levels increased by 1.98 ± 0.50 and 2.23 ± 0.09 folds in 2 and 4 TGF-β-treated cells in comparison to untreated cells (Figure 18; pRS-Kaiso). While in p120-depleted cells the increase was less subtle, only 1.26 ± 0.37 and 1.21 ± 0.40 in 2 and 4 day EMT induced cells compared uninduced cells (Figure 18; pRS-p120). Again, a one-way analysis of variance did not find the change to be statistically significant. However, in all three of the cell lines, the power of the performed test was lower than the desired value (0.800) indicating that we are more likely to not detect a difference when one actually exists. In both depleted cell lines, a decrease in p120 expression was observed in vehicle-treated versus untreated cells; nevertheless, this

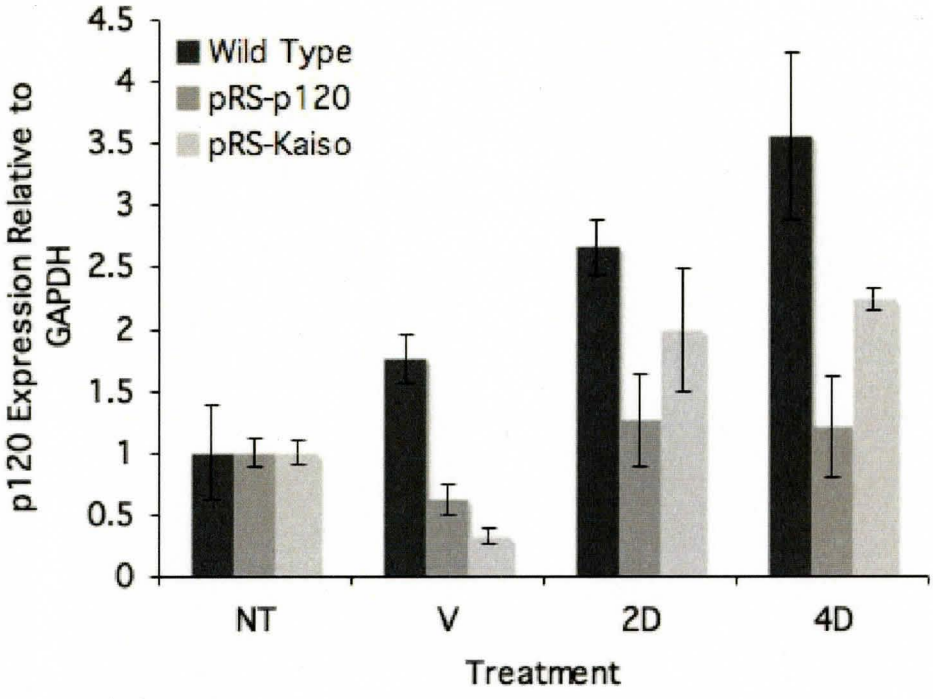


Figure 18: Expression profiling of p120 in wild type, Kaiso- and p120-depleted NMuMG cells as a result of EMT induction. Cells were either left untreated (NT), treated with a vehicle solution (V) or treated with 5 ng/ml TGF-β for 2 or 4 days (2D or 4D) respectively. RNA was extracted and used to make cDNA followed by qRT-PCR. Values were normalized to the basal levels measured in untreated control cells (n = 3, standard errors bars are included). In relation to untreated cells, p120 expression levels were higher in 2D- and 4D- treated cells in all three cell lines. While the change was gradual in wild type cells, it plateaued in Kaiso- and p120-depleted cells. The difference in p120 expression was not great enough to exclude the possibility that it occurred as a result of random sampling variability and the values were not statistically significant. However, the power of the performed test was below the desired value (0.800) indicating that we are more likely to not detect a difference when one actually exists. In Kaiso and p120 knockdown cell lines, a decrease in p120 expression was observed in vehicle treated versus untreated cells; nevertheless, an ANOVA test suggested that the change was not statistically significant.

change was not statistically significant. To ensure whether the changes in gene expression are real and not a result of random sampling, additional independent experiments must be carried out so that the sample number is higher and statistical analysis is more reliable.

4.8 Effects of Kaiso and p120 misexpression on E-cadherin transcription regulation

To determine whether Kaiso and p120 knockdown affect the regulation of E-cadherin, an epithelial marker whose expression is reduced as a result of TGF-β-mediated EMT induction in NMuMG cells [Maeda *et al.*, 2005], qRT-PCR experiments were performed. Wild type, Kaiso and p120 knockdown NMuMG cells were induced to undergo EMT using 5 ng/ml of TGF-β, treated with a vehicle solution or left untreated. RNA was then extracted, used to make cDNA and real-time PCR was performed. Again, all three independent experiments were carried out for each cell line, and each qRT-PCR reaction was carried out in duplicate.

In wild type NMuMG cells, the vehicle-treated cells showed a slight decrease in E-cadherin expression (0.62 ± 0.06 folds) when compared to the untreated cells, however, this decrease is negligible since there is overlap between the error bars. Moreover, E-cadherin transcription levels gradually reduced as a result of prolonged TGF-β-treatment; after 2 and 4 days of treatment, E-cadherin levels were 0.32 ± 0.02 folds and 0.18 ± 0.02 lower than in the untreated cells. While this difference was not found to be statistically significant ($p = 0.133$), the power of the performed test (0.542) was below the desired value (0.800) indicating that we are more likely not to detect a difference when one

actually exists (Figure 19, Wild Type). Again, to improve this experiment, a larger sample number is needed and thus more independent qRT-PCR experiments must be carried out.

Interestingly, in Kaiso knockdown NMuMG cells, vehicle-treated cells showed a decrease in E-cadherin expression when compared to the untreated cells (0.43 ± .10). There was also a change in E-cadherin expression after 2 and 4 days of treatment with TGF-β, 0.45 ± 0.14 and 0.19 ± 0.04 folds lower respectively. This overall the change was found to be statistically significant (P = 0.039) using a one-way ANOVA test. A pairwise multiple comparison procedures test (Tukey Test) was performed and found that the difference lies between the 4 day TGF-β-treated cells and the untreated cells (Figure 19, pRS-Kaiso).

Finally, in p120 knockdown NMuMG cells, an overall gradual decrease in E-cadherin expression was also observed in treated cells versus the vehicle control and the no treatment control cells. Compared to the untreated cells, expression of E-cadherin decreased by 0.32 ± 0.02 and 0.18 ± 0.09 folds in cells exposed to TGF-β for 2 and 4 days respectively. A one-way ANOVA comparison test showed that this overall change was found to be statistically significant (P = 0.041). A Tukey test revealed that no statistical significance exists between any of TGF-β-, vehicle-, or untreated cells (Figure 19, pRS-p120).

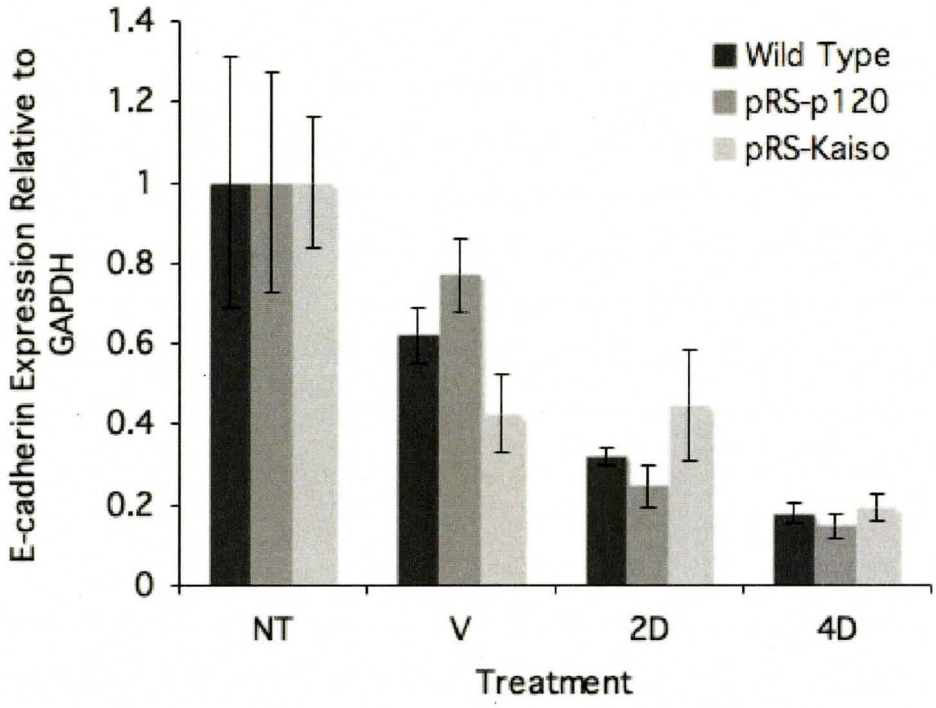


Figure 19: The effects of Kaiso and p120 misexpression on E-cadherin mRNA transcript levels. Cells were either left untreated (NT), treated with a vehicle solution (V) or treated with 5 ng/ml TGF-β for 2 or 4 days (2D or 4D) respectively. RNA was extracted and used to make cDNA followed by qRT-PCR. Values were normalized to the basal levels measured in untreated control cells (n = 3, standard errors bars are included). In relation to untreated cells, a gradual decrease of E-cadherin expression was seen in all three cell lines. In wild type cells, the differences were not great enough to exclude the possibility that it occurred as a result of random sampling variability and the values were not statistically significant (P = 0.133). The power of the performed test was was below the desired value (0.800) indicating that we are more likely to not detect a difference when one actually exists. On the other hand, in Kaiso and p120 knockdown cell lines, the change was found to be statistically significant (P = 0.039, P = 0.041 respectively). A Tukey test showed no statistical significance between any of the treatment groups in all three cell lines.

4.9 Effects of Kaiso and p120 misexpression on transcript levels of mesenchymal markers, N-cadherin and α-SMA

To determine whether Kaiso and p120 knockdown affects the mRNA levels of two mesenchymal markers whose protein expression is augmented as a result of TGF-β-mediated EMT induction, N-cadherin [Maeda *et al.*, 2005] and α-SMA [Darby *et al.*, 1999], expression were analyzed by qRT-PCR. Three independent experiments were carried out where wild type, Kaiso and p120 knockdown NMuMG cells were left without treatment, treated with vehicle solution, or 5 ng/ml of TGF-β for 2 or 4 days. RNA was extracted, then used for cDNA synthesis and real-time PCR was performed in duplicate. In wild type NMuMG cells, vehicle treated cells show no change in N-cadherin expression when compared to the untreated cells. However, N-cadherin transcription gradually increased (2.70 ± 0.10 and 4.07 ± 0.53 folds) in cells induced to undergo EMT for 2 and 4 days respectively. The overall increase in N-cadherin expression was found to be statistically significant ($P = 0.009$) across the four treatments. A pairwise multiple comparison procedures (Tukey Test) showed that the statistically significant difference was between the 4 day TGF-β-treated cells and both the untreated cells ($P = 0.012$) and between the 4 day TGF-β-treated cells the vehicle-treated cells ($P = 0.020$) (Figure 20, Wild Type).

Similarly, NMuMG Kaiso knockdown cells, also exhibited an increase in N-cadherin expression in response to EMT induction. However, compared to wild type cells, the increase does not appear in a gradual manner and it peaked following 2 days of

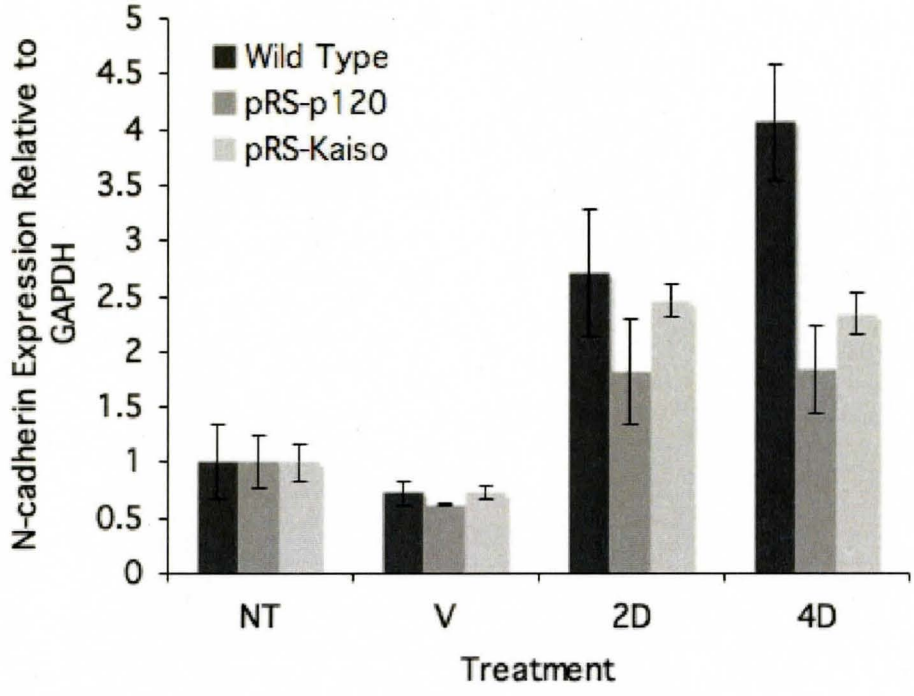


Figure 20: The effects of Kaiso and p120 misexpression on N-cadherin mRNA transcript levels. Cells were either left untreated (NT), treated with a vehicle solution (V) or treated with TGF-β (5 ng/ml) for 2 or 4 days (2D or 4D) respectively. RNA was extracted and used to make cDNA followed by real time-PCR amplification. Values were normalized to the basal levels measured in untreated control cells (n = 3, standard errors bars are included). In comparison to untreated cells, a gradual increase of N-cadherin expression was seen in wild type cells. Similarly, an increase in expression was also seen in 2D and 4D treatments but the increase was not gradual. A one-way ANOVA suggested that the increase was statistically significant in wild type ($P = 0.009$) and Kaiso-depleted cells ($P = 0.006$) but not in p120-depleted cells ($P = 0.209$). A Tukey test showed that difference was between 4D cells and NT ($P = 0.012$) as well as V ($P = 0.020$) control cells in wild type cells, and between 4D and NT ($P = 0.005$) as well as 4D and V ($P = 0.002$) cells in Kaiso-depleted cells.

TGF-β induction. Relative to wild type cells; N-cadherin transcript levels in 2 and 4 day-treated cells were 2.46 ± 0.15 and 2.34 ± 0.19 folds higher respectively. The increase was found to be statistically significant (P = 0.006). A Tukey test revealed that the change in expression was statistically significant between cells exposed to TGF-β for 2 days and cells that were left untreated (P = 0. 008) and vehicle cells (P = 0.003). A statistical significance was also detected between EMT induced cells for 4 days and control cells that were not treated (P = 0.005) or treated with vehicle solution (P = 0.002). (Figure 20, pRS-Kaiso).

Moreover, in NMuMG p120 knockdown cells, amplification of N-cadherin showed the same trend seen in Kaiso knockdown cells. Although expression levels were augmented following EMT induction when compared to control cells, N-cadherin levels hit a plateau. More interestingly, the increase in N-cadherin fold expression was not as robust in p120-depleted cells when compared to wild type and Kaiso knockdown cells. The fold increase was only 1.82 ± 0.48 in cells exposed to TGF-β for 2 and 1.84 ± 0.39 in cells exposed to TGF-β for 4 days respectively in relation to untreated cells. This increase in N-cadherin expression was not found to be statistically significant (P = 0.209) (Figure 20, pRS-p120).

Additionally, the overall expression of α-SMA was also amplified as a result of EMT induction, it increased by 48.30 ± 6.76 fold and 229.79 ± 17.76 fold in 2 and 4 day TGF-β-treated cells respectively. The increase in α-SMA expression was found to be highly statistically significant (P < 0.001) using a one-way ANOVA test. A Tukey pairwise multiple comparison procedures test showed a significant difference between the

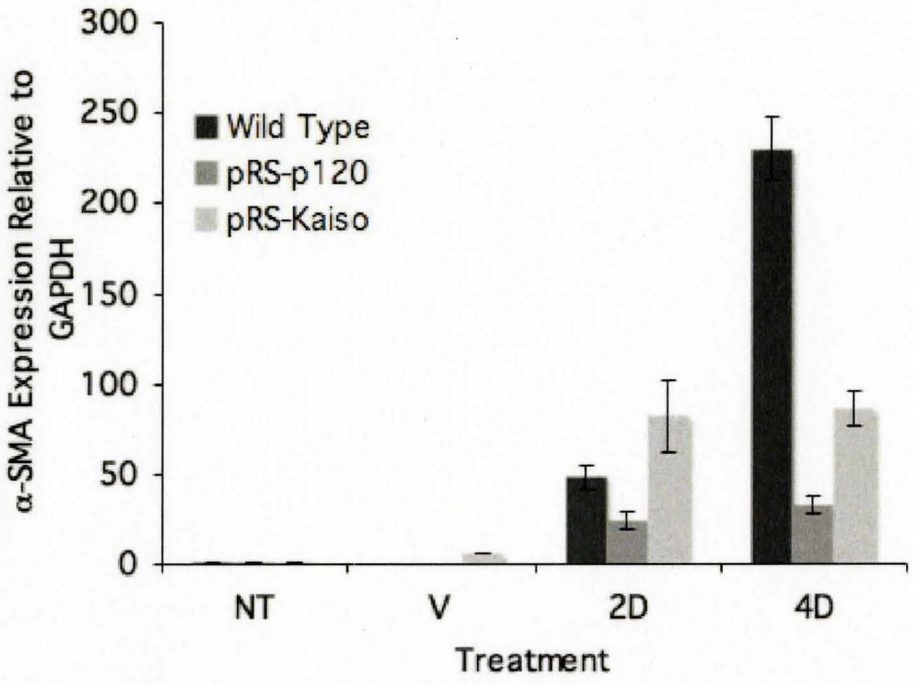


Figure 21: The effects of Kaiso and p120 misexpression on α-SMA mRNA transcript levels. Cells were either left untreated (NT), treated with a vehicle solution (V) or treated with TGF-β (5 ng/ml) for 2 or 4 days (2D or 4D) respectively. RNA was extracted and used to make cDNA followed by real time-PCR amplification. Values were normalized to the basal levels measured in untreated control cells (n = 3, standard errors bars are included). In comparison to NT cells, a gradual increase of α-SMA expression was seen in wild type cells. Similarly, an increase in expression was also seen in 2D and 4D treatments but the increase was not gradual and it was not as robust. A one-way ANOVA suggested that the increase was statistically significant in wild type ($P < 0.001$), Kaiso ($P = 0.006$) and p120 ($P = 0.002$) knockdown cells. In wild type cells, a Tukey test showed that difference was between 4D and both NT and V treatments, as well as between 2D and 4D treatment ($P < 0.001$). In Kaiso knockdown cells the difference was between 4D and NT ($P = 0.020$) as well as V ($P = 0.027$) treated cells. The same was also true for p120 treated cells ($P = 0.006$).

4 day TGF-β-treated cells and both control cells (untreated and vehicle), as well as, between the 4 and 2 day TGF-β-treated cells ($P < 0.001$) (Figure 21, Wild Type).

Intriguingly, in NMuMG Kaiso knockdown cells, an increase in α-SMA transcriptional expression was also observed as a result of EMT induction mediated by TGF-β compared to non-induced cells. Although, compared to wild type cells, the increase was not gradual; rather it remained constant after 2 days of TGF-β treatment. Transcript levels were 82.12 ± 20.28 and 85.96 ± 9.70 fold higher in 2- and 4-day TGF-β-treated cells. The overall increase was found to be statistically significant ($P = 0.006$) using a one-way ANOVA comparison of variance. A Tukey test revealed the change in α-SMA expression was found to be statistically significant between cells treated for 2 days with TGF-β and cells that were left untreated ($P = 0.025$) and vehicle-treated cells ($P = 0.034$). Likewise, the Tukey test showed that the change in α-SMA expression was statistically significant between cells that were exposed to TGF-β for four days and control cells that were not treated ($P = 0.020$) or treated with the vehicle solution ($P = 0.027$) (Figure 21, pRS-Kaiso).

Finally, also interesting, in p120 knockdown NMuMG cells the amplification of α-SMA expression was hindered in comparison to wild type cells. Although α-SMA expression increased in EMT-induced cells compared to control cells, the fold increase was only 22.23 ± 5.02 in cells exposed to TGF-β for 2 days and 32.84 ± 4.57 in cells exposed to TGF-β for 4 days in relation to cells that were left untreated. This increase in α-SMA expression was found to be statistically significant ($P = 0.002$) using a one-way ANOVA test. Pairwise multiple comparison procedures (Tukey test) suggested that

statistical significance of the change in expression lies between the 2 day TGF-β-treated cells and the untreated (P = 0.035) and between the 2 day treated cells and vehicle-treated cells (P = 0.029. Likewise, the increase in α-SMA expression was suggested to be statistically significant between cells treated for 4 days with TGF-β and control cells left untreated and between cells treated for 4 days with TGF-β and vehicle-treated cells (P = 0.006) (Figure 21, pRS-p120).

5.0 Discussion

Cell-cell adhesion is integral for various biological processes including tissue morphogenesis, cell growth and differentiation. Maintenance of epithelial cell polarity and overall tissue architecture is mediated by the adhesion molecule and tumour suppressor, E-cadherin [Christofori and Semb, 1999]. At cell-cell contacts, E-cadherin forms complexes with the catenins, β–/γ– and p120-catenin, which serve to enhance cell adhesion strength and to regulate E-cadherin-mediated cell adhesion, respectively [Valizadeh *et al.*, 1997]. Downregulation of E-cadherin is a frequent event in human cancers and it is postulated that this liberates p120 and allows its translocation to the nucleus [Mayerle *et al.*, 2003]. Nuclear p120 interacts with, and functionally regulates, the novel BTB/POZ transcription factor, Kaiso, which mediates transcriptional repression of various tumourigenesis-associated genes via methylated CpG dinucleotides or a sequence-specific Kaiso binding site [Kelly *et al.*, 2004b; Spring *et al.*, 2005]. Therefore p120 and Kaiso may be components of a signaling pathway that affects the expression of genes involved in tumourigenesis and cell adhesion.

In colorectal cancers, cytoplasmic and nuclear p120 correlate with E-cadherin down-regulation, weakened cell-cell adhesion, and the induction of an epithelial-to-mesenchymal transition [Thoreson *et al.*, 2000; Thoreson *et al.*, 1997; van Hengel *et al.*, 1999]. In addition, one independent study found that upon treatment of a human colon carcinoma cell line with the EMT-inducing factor TGF-β, p120 localization shifted from the plasma membrane to the cytoplasm and nucleus [Bellovin *et al.*, 2005]. These findings led to our hypothesis that altered p120 subcellular localization may promote the

Kaiso/p120 interaction and inhibit Kaiso-mediated regulation of genes involved in tumourigenesis and EMT. Thus our goal was to determine whether Kaiso and p120 regulate the expression of EMT-related genes such as α-SMA [Darby *et al.*, 1990], E- and N-cadherin [Hazan *et al.*, 2004].

5.1 α-SMA expression in response to TGF-β-treatment in the epithelial cell lines NMuMG and FHL-124

The non-transformed mouse mammary epithelial cell line, NMuMG, was chosen as a model to study epithelial-to-mesenchymal-transition because several studies have proven it responds to TGF-β-treatment by exhibiting EMT-associated changes [Maeda *et al.*, 2005; Piek *et al.*, 1999; Xie *et al.*, 2003]. Some of the hallmark changes that occur during EMT are a loss of cell-cell contacts through the downregulation of E-cadherin [Savagner, 2001], increased cell motility, degradation of basement membrane through matrix metalloproteinases [Yang and Liu, 2001], and cytoskeletal rearrangement with increased expression of α-SMA [Kalluri and Neilson, 2003; Tomasek *et al.*, 2002]. However, to our knowledge, NMuMG cells have not been shown to express α-SMA upon exposure to TGF-β. As a result, before determining the role of Kaiso and p120 during EMT in these cells, we set out to determine whether α-SMA, a major marker of mesenchymal cells [Darby *et al.*, 1990], is upregulated in NMuMG cells as a result of TGF-β-treatment. Our experiments were the first to demonstrate that NMuMG cells express α-SMA in response to TGF-β exposure. In addition, α-SMA expression occurs in a TGF-β dose-dependent manner and NMuMG cells also exhibit a TGF-β dose-

dependent response when it comes to morphological changes associated with EMT (Figure 7). Additionally, the cells became more elongated and were no longer closely associated with each other. This observation is consistent with other studies, which showed that in NMuMG cells, epithelial markers such as E-cadherin, ZO-1 and Paxillin are downregulated, while extracellular components such as Fibronectin are upregulated in response to TGF-β-treatment [Maeda *et al.*, 2005; Xie *et al.*, 2003]. Thus, NMuMG cells are induced to undergo EMT in response to TGF-β treatment, and EMT induction occurs in a TGF-β dose-dependent manner.

The human lens epithelial cell line, FHL-124, was also chosen for the experiments in this thesis since it was shown to respond to TGF-β exposure by upregulating EMT-related genes. A recent microarray study of the cell line indicated that α-SMA transcript levels are greatly amplified in response to 24 hours of 10 ng/ml TGF-β-treatment [Dawes *et al.*, 2007]. Before determining whether the FHL-124 cell line was a suitable model to study p120 and Kaiso during EMT, we examined the effects of different TGF-β dosages on α-SMA protein expression and cell morphology. We found that FHL-124 cells expressed minimal amounts of α-SMA before EMT induction, however, the cells respond to TGF-β by producing additional α-SMA protein (Figure 8). This observation was consistent with that seen in the West-Mays lab, McMaster University. They too detect basal levels of α-SMA protein when the cells are subconfluent. In contrast, when the cells reach 100% confluency, expression of α-SMA is abolished (West-Mays lab, personal communication). However, our attempt to eliminate α-SMA expression failed, even when permitting the cells to become fully confluent before exposing them to TGF-

β. Furthermore, the induction of α-SMA expression was not dependent on the concentration of TGF-β. Moreover, no morphological changes were observed despite increasing amounts of TGF-β treatment (Figure 8). In conclusion, in our studies FHL-124 cells displayed a less robust response to TGF-β-mediated EMT induction than NMuMG cells, indicating that the NMuMG cell line is a more suitable model to use for our studies.

5.2 The Kaiso/p120 interaction in epithelial cells before and after TGF-β-mediated EMT induction

The interaction between Kaiso and p120 was first detected *in vitro* via a yeast-two-hybrid assay and the association was then confirmed in epithelial cultured cell lines (HCT116 and MDCK) using co-immunoprecipitation experiments [Daniel and Reynolds, 1999]. While the interaction has been well characterized by our lab using epithelial cell lysates, it has yet to be studied using mesenchymal cells or cells that have undergone EMT. Therefore, utilizing co-immunoprecipitation experiments, we analyzed the correlation between Kaiso/p120 co-precipitation and EMT induction in two epithelial cell lines (NMuMG and FHL-124) that have been shown to undergo EMT in response to TGF-β-treatment. Using both cell lines, we were able to detect Kaiso with p120 complexes in cell lysates that were treated with a vehicle solution (not induced to undergo EMT), and in cell lysates that were induced to undergo EMT using TGF-β (Figures 9 and 10). The interaction in cells that did not undergo EMT was expected and confirms previous results, which demonstrated the Kaiso/p120 interaction in epithelial cell lines [Daniel and Reynolds, 1999].

There are several lines of evidence suggesting that the Kaiso/p120 interaction is preferentially detected between Kaiso and p120 isoform-3 in comparison to Kaiso and p120 isoform-1. In MDCK and HCT116 cell lysates, Kaiso did not co-precipitate with p120 when the 2B12 antibody, which only recognizes p120 isoform-1, was used [Daniel and Reynolds, 1999]. This is likely because p120 isoform-1 is poorly expressed in epithelial cell lines [Daniel and Reynolds, 1999]. Therefore, the interaction in both NMuMG and FHL-124 cell lines is intriguing since unlike other epithelial cell lines we have studied, both NMuMG and FHL-124 cell lines show higher levels of p120 isoform-1 than of p120 isofrom-3. Furthermore, studies in our lab have shown that Kaiso exhibits a more robust interaction with p120 isoform-3 isoform than to p120 isoform-1 and p120 isoform-4, but it is still able to bind to all three isoforms (Appendix, Figure A.1). Another study showed that nuclear localization occurred in a p120 isoform-dependent manner. The p120 Isoform-3 was more likely to be observed in the nucleus while p120 isoform-1 was rarely observed in nuclear components [Roczniak-Ferguson and Reynolds, 2003]. Since Kaiso is predominantly nuclear in normal epithelial cells [Daniel *et al.*, 2001], we would expect the interaction to take place in the nucleus. However, there is evidence suggesting that p120 sequesters Kaiso from the nucleus to the cytoplasm to relieve Kaiso-mediated repression of its target promoter. While it was previously hypothesized that p120 sequesters Kaiso into the cytoplasm, it was only recently demonstrated that the overexpression of xp120 led to the translocation of xKaiso from the nucleus to the cytoplasm [Park *et al.*, 2006]. It is therefore possible that the interaction between Kaiso and p120 is taking place in the cytoplasm between Kaiso and p120 isoform-1 since it is

more abundant than p120 isoform-3 in NMuMG and FHL-124 cells. To confirm this result, further co-immunoprecipitation studies should be carried out using lysates from cytoplasmic and nuclear fractions of NMuMG cells and with antibodies that are p120 isoform-specific. If Kaiso co-precipitates with p120 in cytoplasmic fractions and in lysates using p120 isoform-1-specific antibodies, then the results would confirm that p120 sequesters Kaiso into the cytoplasm to relieve repression of its target genes, and the Kaiso interaction occurs with p120 isoform-1.

What is more, in NMuMG cells, the Kaiso/p120 interaction was more robust in lysates from the two and four day TGF-β-treated cells, than in cells that were treated with a vehicle solution and thus did not undergo EMT (Figure 9). This finding suggests that p120 regulation of Kaiso-mediated repression may be increased when cells are undergoing EMT, and supports our hypothesis that p120 interacts with Kaiso to activate the expression of genes that drive EMT induction.

5.3 EMT induction in NMuMG cells is dependent on TGF-β time treatment

We concluded that for our experiments, the NMuMG cell line was a better-suited model to study EMT progression than the FHL-124 cell line since it showed a greater response to TGF-β-treatment. We also determined that NMuMG cells undergo EMT in a TGF-β-dose dependent manner. Next we attempted to examine whether time of treatment also affects EMT induction. Immunofluorescence analysis showed that more cells exhibited α-SMA staining after four days of TGF-β exposure (90-95% of cells) than cells that were only exposed to TGF-β for two days (50-60% of cells) (Figure 11). This finding

was supportive of a previous microarray study where researchers observed a progressively greater alteration in the expression of EMT-related genes over time as a result of TGF-β treatment using NMuMG cells [Xie *et al.*, 2003]. The alteration in the expression of EMT-associated genes took place at least 6-24 hours post TGF-β-treatment, whereas in the first hour, the genes whose expression was altered were cell-cycle control genes [Xie *et al.*, 2003]. This supported previous studies suggesting that TGF-β loses its tumour suppressing property [Derynck *et al.*, 2001; Wakefield and Roberts, 2002] and acquires a pro-metastatic promoting property byway of its ability to induce EMT [Seton-Rogers *et al.*, 2004; Tang *et al.*, 2003]. Interestingly, the microarray study did not identify any changes in the expression of the α-SMA gene (*actn2*) in response to TGF-β treatment [Xie *et al.*, 2003]. It is possible that cells were not exposed to TGF-β for long enough to observe changes in α-SMA expression. The maximum amount of TGF-β-treatment was 24 hours; in contrast, we observed changes at least 48 hours post exposure. Our data suggests that EMT induction as determined by the expression of α-SMA is dependent on TGF-β length of treatment.

5.4 Kaiso and p120 localization in EMT-induced NMuMG cells

In normal epithelial cells, p120 localizes to cell-cell contacts as a part of the E-cadherin-catenin cell adhesion complex [Reynolds *et al.*, 1994; Shibamoto *et al.*, 1995; Staddon *et al.*, 1995]. Hence, the downregulation of E-cadherin in human cancers has been hypothesized to liberate p120 from AJ and into the cytoplasm [Roczniak-Ferguson and Reynolds, 2003; van Hengel *et al.*, 1999]. Nuclear-localized p120 has also been

observed in pancreatic [Mayerle *et al.*, 2003] and mouth [Lo Muzio *et al.*, 2002] cancers, as well as in colorectal polyps [Valizadeh *et al.*, 1997]. The catenin p120 was also observed to localize to the cytoplasm in colon carcinoma cells stimulated to undergo EMT using TGF-β and TNF-α [Bellovin *et al.*, 2005]. Moreover, in *Xenopus*, when xp120 was overexpressed it exhibited perinuclear localization and it also sequestered Kaiso to the cytoplasm [Park *et al.*, 2006]. Kaiso localization was predominantly nuclear in normal cells, however, in some breast carcinomas, it was also seen to be cytoplasmic [Soubry *et al.*, 2005]. We hypothesized that as a result of E-cadherin downregulation during EMT induction, p120 will be released from cell-cell contacts, and it will accumulate in the cytoplasm and the nucleus where it will interact with Kaiso to allow for the expression of EMT-associated genes. We found that in normal (untreated) NMuMG epithelial cells and in vehicle control cells, Kaiso and p120 exhibited the expected localization in the nucleus and cell contacts respectively. Interestingly, following two days of TGF-β-mediated EMT induction, p120 was localized to the cytoplasm and perinuclear region (Figure 12), consistent with previous observations in colon carcinoma cells that undergo TGF-β mediated EMT [Bellovin *et al.*, 2005] and in *Xenopus* when xp120 was overexpressed [Park *et al.*, 2006]. Park *et al.* also observed that xp120 sequestered xKaiso into the cytoplasm. However, we were unable to conclude whether Kaiso was co-localized with p120 in NMuMG cells since these images were taken using a phase-contrast microscope. To determine whether Kaiso and p120 co-localize in cells following EMT induction, confocal microscopy must be performed.

Moreover, following 4 days of TGF-β-treatment in NMuMG cells, p120 was localized in the cytoplasm, supporting our hypothesis that as a consequence of EMT induction, p120 will be freed into the cytoplasm as a result of E-cadherin downregulation. Cytoplasmic p120 has been shown to regulate Rho-GTPases, which play a major role in cytoskeletal dynamics [Anastasiadis, 2007]. In particular, p120 inhibits RhoA which has been shown to regulate cytoskeletal remodeling and the positive regulation of the α-SMA promoter during EMT [Mack *et al.*, 2001; Masszi *et al.*, 2003]. These results suggest a role for cytoplasmic p120 in activating α-SMA through RhoA, and consequently regulating the induction of EMT.

Remarkably, after two days of exposure to TGF-β, some Kaiso staining appeared to localize at cell-cell contacts at the extension terminals of NMuMG cells (Figure 12). A previous study by Daniel *et al.* showed that while Kaiso staining is predominantly nuclear, when another Kaiso monoclonal antibody, 12G, was used, Kaiso exhibited cell-cell junction staining in epithelial MDCK cells, and cell-extension tip staining in fibroblast NIH3T3 cells [Daniel *et al.* 2001]. However, the researchers postulated that non-nuclear staining is probably due to cross reactivity with proteins other than Kaiso. It is unknown why Kaiso, a transcription factor, would localize near the plasma membrane, and the possibility exists that this observation is an artifact. Further Kaiso staining studies with other Kaiso-specific antibodies should be carried out in NMuMG cells to verify whether Kaiso is indeed localized at cell extensions.

5.5 Induction of EMT in NMuMG cells

The induction of EMT in NMuMG epithelial cells using TGF-β was previously shown to result in the downregulation of E-cadherin and loss of cell-cell contacts and the upregulation of N-cadherin [Maeda *et al.*, 2005; Piek *et al.*, 1999; Xie *et al.*, 2003]. Our Western blot and qRT-PCR experiments support these findings. We found that E-cadherin protein and mRNA progressively decreased in cells that were treated with TGF-β for 2 and 4 days versus untreated cells (Figures 13 and 19). In addition, we observed that N-cadherin levels progressively increased (Figures 13 and 20) in cells that were induced to undergo EMT versus uninduced cells.

While the expression of E- and N-cadherin has been extensively studied in NMuMG cells treated with TGF-β [Maeda *et al.*, 2005], expression profiling of Kaiso, p120 and α-SMA in correlation with TGF-β-mediated EMT induction had not been previously addressed. As expected, protein and mRNA expression levels of α-SMA increased with EMT induction. The level of α-SMA expression was progressively higher in 2-day and 4-day TGF-β-treated cells than in untreated cells (Figures 13 and 21). While many studies have showed that TGF-β treatment leads to increased expression of α-SMA in cells that undergo EMT (e.g. FHL-124 [Dawes *et al.*, 2007], LLC-PK CL4 [Fan *et al.*, 2007; Masszi *et al.*, 2003], and MCF10A [Tang *et al.*, 2003]) we were the first to report an increase in α-SMA expression in NMuMG cells.

Interestingly, we also observed an increase in the expression of p120 in cells that were treated with TGF-β for 2 and 4 days in comparison to control cells that were untreated or treated with vehicle solution alone (Figures 13 and 18). Western blot

analysis suggests that the increase in expression appeared to be isoform specific; only p120 isoform 1 levels increased while the p120 isoform 3 were unaltered (Figure 13). Intriguingly, in another mouse mammary epithelial cell line, EMT induction causes a switch from p120 isoform 3 to p120 isofrom 1 [Eger *et al.*, 2000]. Also, a study by Siedel *et al.* indicated that N-cadherin binds preferentially to the larger p120 isoform 1, whereas E-cadherin binds to smaller p120 isoform 3 [Seidel *et al.*, 2004]. Thus, a change in p120 isoform expression may be taking place to accommodate the cadherin switch resulting from EMT induction.

Another recent study showed that p120 was necessary for the downregulation of E-cadherin and the upregulation of N-cadherin during EMT [Yanagisawa and Anastasiadis, 2006]. Also, microarray analysis of NMuMG cells showed that Kinesin expression levels increased in cells that were treated with TGF-β for 24 hours [Xie *et al.*, 2003]. Kinesin microtubules have been shown to interact with p120 and it is thought that the interaction plays a role in shuttling cadherins to cell-cell contacts [Yanagisawa *et al.*, 2004]. Wahl *et al.* suggested a mechanism for p120 regulation of N-cadherin shuttling to the membrane by way of a microtubule-dependent mechanism. They postulated that following N-cadherin synthesis; pro-N-cadherin is phosphorylated and bound to p120 [Wahl *et al.*, 2003]. Next, β- and γ-catenins also bind to N-cadherin in the endoplasmic reticulum or Golgi compartments and the complex is transferred to the cell membrane where it is anchored to the cytoskeleton. The transfer of the cadherin-catenin complex is thought to take place in a microtubule-dependent manner and it is mediated via the interaction of p120 with Kinesin [Wahl *et al.*, 2003; Yanagisawa *et al.*, 2004]. This

therefore suggests a mechanism of how p120 regulates the up-regulation of N-cadherin in NMuMG cells.

In normal epithelial cells p120 is thought to promote E-cadherin stability through Rho-GTPases. A mechanism has been suggested whereby p120 regulates Rho-GTPases through GEFs which activate RhoA, Rac1 and Cdc42, promoting stronger adhesion [Anastasiadis, 2007]. However, new roles for p120 in regulating Rho-GTPases have been suggested in mesenchymal cells and cells that have undergone EMT. In colon carcinoma cells, p120 localizes to the cytoplasm where it inhibits RhoA activity, consequently affecting AJ integrity in negative manner [Bellovin *et al.*, 2005]. In addition, p120 was shown to promote invasiveness and cell migration in EMT-induced cells by increasing Rac1 activity and inhibiting RhoA activity [Yanagisawa and Anastasiadis, 2006]. Also, p120 may play a role in regulating α-SMA expression indirectly through the inhibition of RhoA activity which leads to the activation of α-SMA promoter [Fan *et al.*, 2007]. Since p120 has been implicated as a key regulator of EMT induction, then it is not surprising that its protein and mRNA levels increase in NMuMG cells following TGF-β treatment.

Finally, the expression of Kaiso does not appear to change in cells that did not undergo EMT (epithelial cells) versus cells that were induced to undergo EMT using TGF-β (mesenchymal cells) (Figures 13 and 17). We hypothesized that Kaiso regulates EMT-related genes. Since Kaiso both represses and activates transcription, we expected Kaiso levels to decrease if it acts as repressor to relieve repression of its target genes, or increase if it activates EMT-related genes. However, it is still possible that Kaiso regulates EMT-promoting genes through its interaction with p120. Repression of Kaiso

target genes is relieved when Kaiso is bound to p120 [Kelly *et al.*, 2004b]. The levels of p120 increase in EMT induced cells, and we proposed that the interaction between Kaiso and p120 is more abundant in EMT induced cells compared to control cells that did not undergo EMT (Figure 9). The mechanism by which Kaiso relieves silencing of genes involved in EMT induction may be through its interaction with and modulation by p120, rather than Kaiso's downregulation. However, further experiments are needed to support this hypothesis.

5.6 The role of Kaiso in EMT induction

Studies in *Xenopus* showed that xKaiso is essential during amphibian development. XKaiso is a genome-wide repressor whose depletion causes premature zygotic gene expression subsequently leading to developmental arrest and apoptosis [Kim *et al.*, 2002; Ruzov *et al.*, 2004]. On the other hand, in mammalian systems, the depletion of Kaiso has no discernable phenotype. Kaiso-deficient mice are fertile and viable, but when these mice were crossed with tumour-susceptible $Apc^{min/+}$ mice, Kaiso-null mice exhibited delayed onset of intestinal tumours [Prokhortchouk *et al.*, 2006]. The lack of an obvious phenotype in Kaiso-null mice was later attributed to the discovery of another Kaiso-like POZ-ZF protein (ZBTB4) that is able to bind to the KBS as well as methylated DNA [Filion *et al.*, 2006]. It was suggested that perhaps there was some redundancy in the target genes of these two transcription factors. We set out to determine whether misexpression of Kaiso affects EMT, to do this we repressed Kaiso expression in NMuMG through the use of retrovirally-delivered shRNAs (Figure 14A).

Not surprisingly, Western blot analysis showed no effects of Kaiso depletion on EMT induction (Figure 15). The expression of mesenchymal markers (N-cadherin and α-SMA) as well as p120 increased as result of EMT induction, while the expression of the epithelial marker (E-cadherin) decreased. Kaiso levels were very low and unaffected by EMT induction. It appears that Kaiso-depleted NMuMG cells show a similar trend as that seen in the wild type NMuMG cells.

Intriguingly however, qRT-PCR analysis indicated that during EMT, Kaiso silencing affects the transcript expression of N-cadherin (Figure 20) and α-SMA (Figure 21) but not E-cadherin (Figure 19). The expression of the two mesenchymal markers was not greatly affected after 2 days of EMT induction using TGF-β-treatment, however, EMT induction appeared to be retarded because the expression of the two genes hit a plateau and did not increase further as was seen in wild type NMuMG cells. To determine whether E- and N-cadherin and α-SMA are potential Kaiso target genes, we performed a visual analysis of the three promoters (-2000 to 500 bp from start site). We detected three core KBS (CTGCNA, where N is any nucleotide) sequences in the E-cadherin promoter and two core sequences in each of the α-SMA and N-cadherin promoters.

While the discovery of core KBS's in the E-, N-cadherin and α-SMA promoters does sound promising, additional experiments are needed to verify that these are *bona fide* Kaiso target genes. Therefore, chromatin immunoprecipitation (ChIP) experiments must be carried out. Also, ChIP analysis must also be performed using NMuMG cells that are not treated with TGF-β and NMuMG cells that are treated with TGF-β. If

Kaiso binding is relieved as a result TGF-β-mediated EMT induction, it would support the idea that Kaiso controls EMT progression by regulating EMT-related genes.

5.7 The role of p120 in EMT induction

A new study suggested that p120 is necessary for the downregulation of E-cadherin and the upregulation of N-cadherin during EMT [Yanagisawa and Anastasiadis, 2006]. To determine the role of p120 in TGF-β-mediated EMT induction in NMuMG cells, we performed p120 knockdown using retroviral siRNA infection (Figure 14B). Using p120-depleted NMuMG cells, qRT-PCR experiments revealed that E-cadherin expression is downregulated in cells that are treated with TGF-β-treatment as compared with untreated cells. The same trend was seen in wild type and Kaiso-depleted NMuMG cells (Figure 19). However, we were surprised to find that the expression of E-cadherin remained constant and did not appear to decrease as a result of p120 depletion or as a result of EMT induction (Figure 16). Several experiments have highlighted the importance of p120 in E-cadherin regulation and stability in normal epithelial cells and in cells undergoing EMT [Thoreson *et al.*, 2000; Yanagisawa and Anastasiadis, 2006]. However, another study found that E-cadherin was not downregulated following p120 depletion using siRNA in colon carcinoma cells [Bellovin *et al.*, 2005]. The researchers suggested that when cells were selected after infection with the siRNA carrying retrovirus, the selection process eliminated cells that did not express siRNA as well as cells that express p120-siRNA but do not express stable E-cadherin. Cells that do not express E-cadherin were thought to be unable to form cell-cell contacts and thus did not survive [Bellovin *et al.*, 2005]. It is possible that we are observing the same effect here.

Western blot analysis of p120-depleted cell lysates failed to detect N-cadherin in cells that did not undergo EMT, or in cells that were induced to undergo EMT (Figure 16). On the other hand, similar to the observation in Kaiso-depleted cells, qRT-PCR analysis suggests that N-cadherin transcription levels went up after 2 days of EMT induction using TGF-β-treatment. However, N-cadherin levels did not increase any further following 4 days of TGF-β-treatment as was observed in wild type cells. While there is a discrepancy between the Western blot data and the qRT-PCR data, this result is not surprising since p120 regulates N-cadherin in a post-translational manner [Peifer and Yap, 2003]. Our Western blot data supports the previous finding that p120 is necessary for the upregulation of N-cadherin during EMT [Yanagisawa and Anastasiadis, 2006]. The delay in the increase of N-cadherin transcriptional levels in response to EMT induction is quite striking however, and it exhibits the same trend as in Kaiso knockdown cells. This data suggests that the two proteins may be working in the same signaling pathway to regulate N-cadherin expression. To confirm this hypothesis, double knockdown of Kaiso and p120 should be performed in NMuMG cells. If the delay in N-cadherin upregulation is exacerbated in the absence of both proteins, then it is very likely that p120 and Kaiso work in conjunction to relieve repression of EMT-associated genes.

Moreover, the expression of α-SMA in response to EMT induction was greatly inhibited at the protein and mRNA levels in p120-depleted cells. The expression of α-SMA protein is not detected until four days post TGF-β-treatment in p120 knockdown cells, while it is detected after only 2 days in wild type cells. Additionally, qRT-PCR analysis suggested that α-SMA expression as a result of TGF-β-mediated EMT induction

was greatly inhibited when compared to wild type cells. This data suggests that p120 positively regulates the expression of α-SMA, p120 may lead to activation of the α-SMA promoter by relieving Kaiso-mediated repression. This is supported by the observation that the upregulation of the α-SMA is delayed in response to TGF-β when either p120 or Kaiso are depleted. Suggesting that the two proteins are co-operating in the same signaling pathway to activate α-SMA expression. Again, further double knockdown studies are needed to confirm this hypothesis.

5.8 A possible mechanism for Kaiso and p120 signaling in EMT

Our data suggest that NMuMG cells undergo EMT in a TGF-β dose- and time-dependent manner. Following treatment with TGF-β, cells lose their 'cobblestone' epithelial morphology, become loosely adherent, acquire an elongated morphology and they express α-SMA. Moreover, our data supports previous findings that show that E-cadherin levels are downregulated and N-cadherin levels are upregulated as a result of TGF-β-mediated EMT induction in NMuMG cells. Furthermore, siRNA-mediated knockdown of Kaiso and p120 in NMuMG cells suggest that Kaiso and p120 are positive regulators of EMT. Kaiso and p120 are necessary for the appropriate transcriptional upregulation of N-cadherin and α-SMA following EMT induction with exposure to TGF-β. In addition, p120 is required for the upregulation of N-cadherin and α-SMA protein levels following TGF-β-mediated EMT induction.

Here we suggest a possible mechanism for Kaiso/p120 signaling in EMT induction as a response to TGF-β exposure (Figure 22). Following EMT induction, E-

cadherin is downregulated and p120 is freed from cell-cell contacts into the cytoplasm. In the cytoplasm p120 may activate Rac1 and Cdc42 and inhibit RhoA, which leads to promoting cytoskeletal changes that lead cell invasion and motility. Furthermore, p120 may translocate into the nucleus to interact with Kaiso or sequester Kaiso from the nucleus into the cytoplasm to relieve Kaiso-mediated repression of Kaiso target genes that promote EMT and metastasis such as N-cadherin, α-SMA, MMP-7, MTA-2 and others.

5.9 Future Experiments and Overall Significance:

More evidence is necessary to confirm a role for Kaiso and p120 signaling in EMT induction. First, further analysis of whether Kaiso and p120 interact in the nucleus or in the cytoplasm is necessary. Thus, co-immunoprecipitation experiments using cytoplasmic and nuclear fractions will allow us to determine where the interaction takes place in the cell. This data would give us further insight on whether p120 regulates Kaiso target genes by relieving Kaiso binding to the promoters in the nucleus, or whether it sequesters Kaiso to the cytoplasm. Secondly, E-cadherin, N-cadherin and α-SMA must be confirmed as Kaiso target genes. To do this, ChIP experiments may be carried out to determine whether Kaiso binds to these promoters directly. Furthermore, to determine whether Kaiso target gene recognition correlates with EMT induction, ChIP analysis of several putative Kaiso target genes (e.g. *matrilysin*, *metastasin*, and *MTA2*) must be carried out in NMuMG cells in which EMT has been induced using TGF-β in comparison to cells that are not induced to undergo EMT. These studies may reveal the

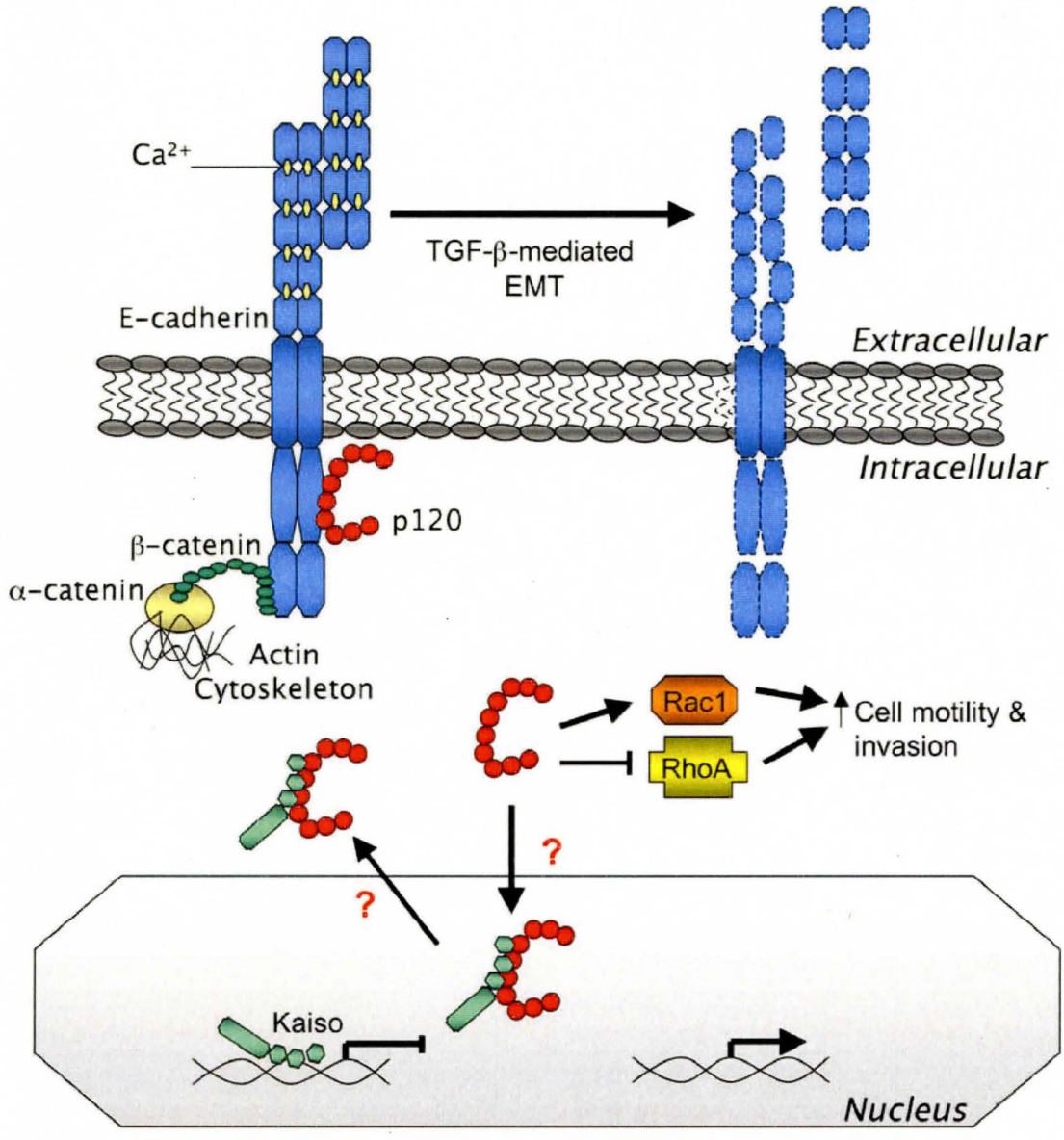


Figure 22: The mechanism of Kaiso/p120 signaling during EMT. Following TGF-β -mediated EMT in NMuMG cells, E-cadherin is downregulated causing p120 to localize to the cytoplasm. Cytoplasmic p120 activates Rac1 and inhibits RhoA activity leading to an increase in cell invasion and motility. p120 is also thought to sequester Kaiso into the cytoplasm where it relieves expression of α-SMA, N-cadherin and other EMT-associated genes.

importance of Kaiso in the regulation of target genes that are involved in EMT induction. Moreover, we can overexpress p120 in conjunction with ChIP analysis to determine the effects of p120 on Kaiso's ability to bind to promoters during EMT induction. We anticipate that p120 will inhibit transcriptional repression by Kaiso. Our analysis of whether p120 and Kaiso affects gene regulation during the induction of EMT, may allow us to more easily decipher the physiological mechanism by which Kaiso regulates genes during tumourigenesis and metastasis and their role in EMT.

To determine whether Kaiso and p120 cooperate in the same pathway, or whether they work separately during the induction of EMT, double knockdown of Kaiso and p120 in NMuMG cells must be carried out in NMuMG cells. If double knockdown cells exhibit the same trends seen in Kaiso-depleted cells and p120-depleted cells, it would suggest that they are working in the same signaling pathway. Finally, future experiments to determine whether Kaiso and p120 cooperate with other EMT-related pathways must also be done. Previous studies have suggested a role for Kaiso/p120 signaling in Wnt signaling, one pathway that is involved in EMT induction, thus there could be other uncharacterized pathways that collaborate with Kaiso and p120 to perform their physiological functions.

Unraveling the mechanism of Kaiso and p120 signaling and identifying potential target genes will help us to learn more about the physiological role of Kaiso and p120 and the processes that they regulate. If Kaiso/p120 signaling contributes to the regulation of EMT then it may provide novel avenues for therapeutic intervention of metastasis.

Apendix: Control and Supplemental Data

A. Materials and Methods:

A.1 GST Pull-Down Experiments:

Large-scale Kaiso-GST fusion and GST protein preps from pGEX vectors were performed as follows: 12 ml of Luberia Broth (LB) media was inoculated with the suitable bacteria and grown overnight at 37 °C, for 16-18 hours. Next 10 ml of the culture was used to inoculate 90 ml of LB containing 50 μg/ml Carbenicillin (Carbenicillin Direct) and was incubated at 30 °C for 90 minutes. The culture was next induced with 0.1 M IPTG for 3 hours after which the cells were collected and resuspended with 10 ml of cold PBS/0.1% NP40. Cells were lysed using a probe sonicator for 3 minutes (40% output and 40% duty cycle) on ice and cell lysates were cleared by centrifugation at 10,000 rpm for 20 minutes at 4°C. Lysates were then incubated with 800 μl of pre-prepared glutathione-Sepharose 4B beads (Amersham Pharmacia) equilibrated in PBS/0.1 % NP40 and mixed end over end for 1 hour at room temperature. The beads-protein complexes were washed twice with 1X binding buffer (25 mM HEPES pH 7.5, 100 mM KCl, 1 mM EDTA, 10 mM $MgCl_2$, 0.1% NP40, 5 % Glycerol, 1 mM DTT). Next the beads were resuspended in 250 ml of 1X binding buffer and stored at 4 °C. 10-20 μl of protein was then separated on an 7% SDS-PAGE gel to check for protein purity and concentration.

In vitro transcription and translation (IVTT) of proteins was carried out for all T7 promoter-containing constructs (pMS-p1201A, pMS-p1203A, and pMS-p1204A;

generously provided by Dr. Albert Reynolds) with the TnT-coupled Transcription and Translation kit as per the manufacturer's protocol (Promega). Five μl of each IVTT sample was analyzed by 7% SDS-PAGE and fluorography, and translated proteins were stored at -20 °C.

Equal amounts of indicated GST-fusion proteins immobilized on glutathione-Sepharose 4B beads (Amersham Pharmacia) were incubated with 10 ml of ^{35}S-IVTT products in binding buffer (25 mM HEPES pH 7.6, 100 mM NaCl, 10% glycerol, 2 μM $ZnCl_2$, 3 mM β-mercaptoethanol, 0.1% NP40, 3.4 μg/ml phenylmethylsulfonyl fluoride and 10 μg/ml leupeptin) to a final volume of 100 μl. The samples were incubated for 2 hours at 4 °C followed by the addition of 400 μl of binding buffer. Next the beads were centrifuged at 4 °C for 2 minutes at 1000 rpm and washed three times with 500 ml of binding buffer. Proteins were eluted by boiling samples in 20 μl of 3X Laemmli Sample buffer and ran on a 7% SDS-PAGE gel along 1.5 μl of IVTT product (15% input). Next, gels were fixed in fixing solution (25% isopropanol, 65% water, 10% acetic acid) for 20 minutes at room temperature followed by treatment in Amplify solution (Amersham Pharmacia) for 10-15 minutes at room temperature. The gels were then dried and exposed to Kodac X-AR film for 24-48 hours at -80 °C.

A.2 Yeast-two-Hybrid Assays

A panel of p120 Arm deletion mutants (p120ΔR1-10) cDNA were subcloned in frame into the pGBKT9 DNA-binding domain vector (Clontech) and used as bait, while murine Kaiso cDNA was subcloned into the activation domain vector pGADT7

(MATCHMAKER Two Hybrid System; Clontech) and used as prey. To test for protein-protein interactions, the appropriate pair of bait and prey was co-transformed into the yeast strain AH109 according to the Clontech MATCHMAKER protocol. Transformants were grown selective drop out media (-LT) to determine whether co-transfection occurred and quadruple drop out media (QDO) to determine whether an interaction exists.

B. Results and Discussion:

B.1 In vitro analysis of the Kaiso interaction with different p120 isoforms

Several lines of evidence suggest that the Kaiso preferentially interacts with p120 isoform-3 in comparison to p120 isoform-1. For instance in MDCK and HCT116 cells, Kaiso did not co-precipitate with p120 when p120 isoform-1-specific antibodies were used. However it was detected in immunocomplexes with p120 when antibodies that recognize p120 isoform-3 were utilized [Daniel and Reynolds, 1999]. Moreover, another study showed that nuclear localization of p120 occurs in an isoform-dependent manner. p120 isoform-3 is more likely to be observed in the nucleus while p120 isoform-1 is seldom found there [Roczniak-Ferguson and Reynolds, 2003]. However, we found that we were still able to detect an interaction between Kaiso and p120 despite higher levels of the p120 isoform-1 in NMuMG cells. We also detected an increase in the expression of the p120 isoform-1 following EMT induction, thus *in vitro* analysis was conducted to test the relative affinity of Kaiso for different p120 isoforms (p120-1A, p120-3A and p120

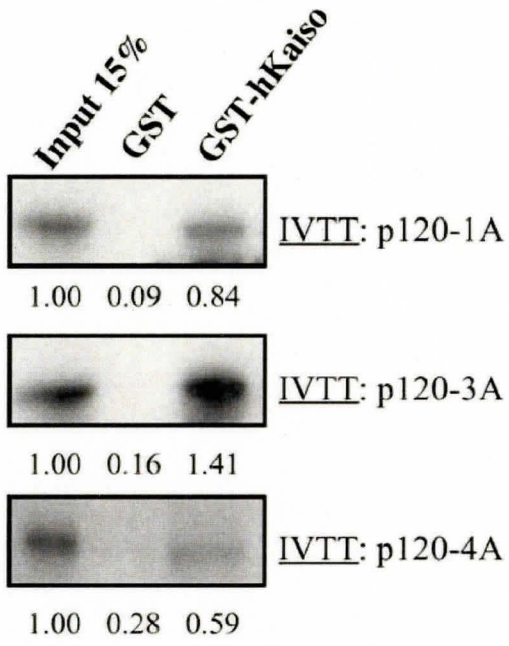


Figure A.1: Analysis of the Kaiso/p120 interaction in vitro. An in vitro transcription and translation (IVTT) reaction was carried out to produce different isoforms of p120. A GST-pulldown assay using GST-hKaiso and GST (as a negative control) was carried out to determined whether Kaiso binds to different isoforms of p120 at different affinities. Kaiso interacts with isoform 3A to a higher degree than both the 1A and 4A isoforms respectively.

4A). GST pull down assays showed that when compared to the 15% input Kaiso had the highest affinity to p1203A (1.41), followed by p1201A (0.84), the largest isoform, and finally the lowest affinity to the smallest isoform p1204A (0.59) (**Figure A.1**). This supports previous experiments that Kaiso appears to co-precipitate with the smaller p120 isoform 3 preferentially to the larger p120 isoform 1 [Daniel and Reynolds, 1999], however, it is still able to bind to other p120 isoforms albeit with a lower affinity.

B.2 Fine mapping the Kaiso/p120 interaction

Previously, the Kaiso/p120 interaction shown to take place between the p120 Armadillo repeats 1 through 7 and a region flanking the Kaiso ZF domain [Daniel and Reynolds, 1999]. In order to fine map the Kaiso p120 interaction more specifically and to determine which Armadillo repeats were required for the interaction, a yeast-two-hybrid analysis was carried out between Kaiso and 10 mutant forms of p120, each with a deletion in one of the Armadillo repeats (p120ΔR1-10). The yeast-two-hybrid screen suggests that Armadillo repeats 1-3 and 5 of p120 are required for its interaction with Kaiso (Figure A.2). This data supports that previous finding that showed that Kaiso binding spans Armadillo repeats 1 to 7. Further fine mapping is required to allow us to determine the exact residues that are required for the Kaiso/p120 interaction. Knowing the exact domain that is required for p120 to bind Kaiso will allow us to possibly make functional p120 mutants that are able to function in the cytoplasm and at the cell-cell membrane, but are unable to interact with Kais. Such mutants would allow us to determine the exact role of the Kaiso/p120 interaction and provide us with further insight to their physiological role in the cell.

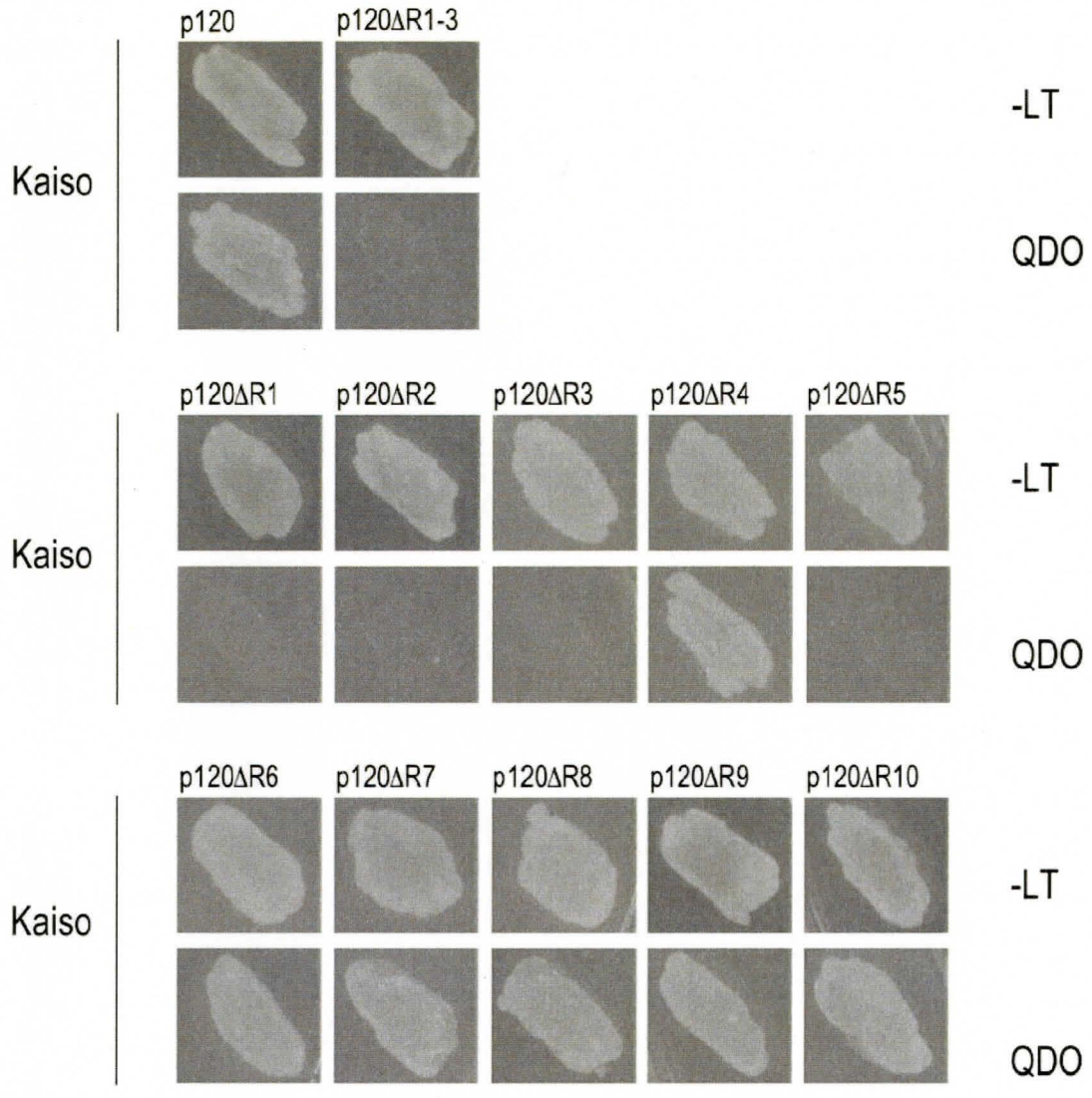


Figure A.2: Analysis of the Kaiso/p120 interaction in vitro. To test for protein-protein interactions, the appropriate pair of bait and prey was cotransformed into the yeast strain AH109. Transformants were grown selective drop out media (-LT) to determine whether co-transfection occurred and quadruple drop out media (QDO) to determine whether an interaction exists. The yeast-two-hybrid screen suggests that Armadillo repeats 1-3 and 5 of p120 are required for its interaction with Kaiso. Full length p120 and Kaiso was used as a positive control while p120ΔR1-3 and Kaiso was used as a negative control.

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