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The role of the Snail2 transcription factor in Twist1- induced EMT and metastasis

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The Role of the Snail2 Transcription Factor in Twist1-induced EMT and Metastasis

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences

by

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2011
The dissertation of Esmeralda Casas Ruiz is approved, and it is acceptable in quality and form for publication in microfilm and electronically:

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Chair

University of California, San Diego

2011
DEDICATION

To my dad, mom, Grace, Sara, Dona, and Sergio, who have never let the “impossible” stop them.

You are my inspiration everyday.

I love you.
Every sickness is a musical problem.

The healing, therefore, is a musical resolution

-Novalis
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The ability of cancer cells to successfully metastasize depends on a myriad of factors that allow cells to leave their tumor of origin, travel within the body and successfully establish themselves in remote locations. Recent evidence suggests that cancer cells activate a conserved
developmental program termed Epithelial Mesenchymal Transition (EMT) to facilitate their metastatic dissemination [1]. EMT is activated during development in epithelial cells to facilitate their movement and survival enabling the formation of new tissues and organs [2]. One of the first and most important steps in EMT progression is the loss of cell-cell adhesion. E-cadherin, a key mediator of cell-cell adhesion [3], has been shown to be directly and indirectly downregulated via many transcription factors expressed during EMT, these include Twist1 [4], Twist2 [5], ZEB1 [6], ZEB2 [7], Snail1 [8] and Snail2 [9]. In cancer, E-cadherin loss is associated with increased invasiveness [10]. It remains largely unknown however, whether transcription factors induced after EMT function independently or in concert to inhibit E-cadherin expression and promote EMT and metastasis.

Twist1 is a basic-helix-loop helix transcription factor and a potent activator of EMT [11]. Twist1 was found to be necessary for the metastatic spread of highly invasive mouse mammary epithelial cells in a mouse model of breast cancer [11]. Additionally, it has been shown to correlate with invasiveness in many cancers [12], [13], [14], [15]. My studies were aimed at determining how Twist1 downregulates E-cadherin expression and promotes metastasis. I show that the Snail2 zinc-finger transcription factor is a direct transcriptional target of Twist1 and a vital downstream mediator of Twist1-induced E-cadherin suppression and EMT. Furthermore,
my studies indicate that Snail2 is essential for the metastatic spread of breast cancer cells to the lung in a mouse mammary tumor model and that Snail2 and Twist1 expression are highly correlated in a large number of breast cancer clinical samples.

Overall, my results demonstrate an important conserved relationship between Twist1 and Snail2 in the regulation of cell-cell adhesion and EMT. This important relationship also appears to be exploited by cancer cells during cancer progression.
CHAPTER I:

INTRODUCTION
1.1 Epithelial Mesenchymal Transition (EMT)

Epithelial Mesenchymal Transitions (EMT) are conserved developmental programs whereby a cascade of molecular and physiological changes within a cell results in the transformation of the epithelial cell into a phenotypically and genetically distinct mesenchymal cell. EMTs allow epithelial cells to dissociate from their neighboring epithelium and travel to different locations within the body. They were first characterized during embryonic development where they facilitate tissue morphogenesis. Since then, they have been identified in wound repair [16] and implicated in pathologies such as fibrosis and carcinogenesis [1],[17].

The epithelial cell is characterized at the cellular level by the expression of cell-cell and cell-extracellular matrix adhesion proteins, apical basal polarity and restricted motility. In contrast, the mesenchymal cell is a phenotypically fibroblast-like cell with decreased cell-cell adhesions, increased migratory behavior and increased ability to degrade extracellular matrix (ECM) [2]. Biochemically, epithelial cells can be characterized by their expression of epithelial markers such as E-cadherin and membrane-localized p120, α and β-catenins [3] and cytokeratins. Mesenchymal cells can be characterized by their expression
of vimentin, fibronectin, N-cadherin, α-smooth muscle actin (α-SMA), ZEB1, ZEB2, Snail1, Snail2 and Twist1 [18],[2].

One of the first and most important phenotypic changes observed during EMT progression is the downregulation of cell-cell adhesion. Adherens junctions are major cell-cell adhesion complexes whose central component is E-cadherin. Structurally, E-cadherin is a single transmembrane glycoprotein that complexes calcium ions on its extracellular domain to mediate homophilic dimerization with E-cadherin proteins on adjacent epithelial cells [19]. E-cadherin’s cytoplasmic domain is complexed to catenin proteins including β-catenin, α-catenin and p120 [3]. These can act as signaling proteins when dissociated from adherens junctions and also function to link E-cadherin to the actin cytoskeleton and can modulate actin assembly [20].

E-cadherin expression is critical in maintaining epithelial morphology. Indeed, E-cadherin has been described as the guardian of epithelial phenotype. Blocking E-cadherin via shRNA or E-cadherin antibodies has been shown to induce a mesenchymal phenotype and expression of EMT markers [21],[22] and its re-expression can revert EMT phenotype [10]. Many transcription factors that drive EMT progression do so through direct inhibition of E-cadherin transcription. Twist1 [4], ZEB1 [6], ZEB2 [7], Snail1 [8] and Snail2 [9], have all been shown to bind specific E-
boxes on the E-cadherin promoter to silence its transcription and promote EMT.

A mechanism by which E-cadherin expression can inhibit EMT consists of the sequestration of β-catenin [23]. Under proper conditions such as Wnt signaling activation or dissolution of adherens junctions, β-catenin can accumulate into the cytoplasm and subsequently travel to the nucleus. There, it can associate with T cell factor/lymphoid enhancing factor (TCF/LEF) proteins to activate transcription of specific target genes [24]. Snail2 [25] and Twist1 [26] are known direct targets of β-catenin. Additionally, both Snail1 and Snail2 have been shown to promote EMT through β-catenin-dependent pathways [27]. In sparse SW480 human colonic adenocarcinoma cultures, β-catenin localized to the nucleus and results in the activation of Snail2 transcription. This maintains reduced E-cadherin expression at the cell membrane. Conversely, cells grown at high density exhibit elevated E-cadherin membrane localization and decreased nuclear β-catenin leading to lower Snail2 expression [28].

In addition to β-catenin release, E-cadherin downregulation can promote EMT through release of additional proteins complexed at cell junctions. The Ajuba protein is normally localized to cell-cell junctions in epithelial cells and directly interacts with the adherens junction protein α-catenin [29]. When not complexed to adhesion proteins, Ajuba can
travel to the nucleus where it cooperates with Snail1 to methylate the E-cadherin promoter and silence its transcription [30], [31].

Aside from loss of cell-cell adhesion, phenotypic and biochemical changes acquired during EMT facilitate cell movement, cell survival and invasion. EMTs result in profound changes in the cytoskeleton [32] allowing for directional motility. Additionally, increased protease secretion [33],[34] permits the degradation of proteins of the extracellular matrix (ECM) to facilitate cell movement beyond the local environment. The mesenchymal cell is also more resistant to cell death. While epithelial cells must maintain cell-cell and cell-ECM adhesion to escape anoikis and apoptosis [35], mesenchymal cells are not subject to such restraints. Activation of anti-apoptotic pathways and suppression of cell death programs [36], [37] and [38] allow mesenchymal cells increased autonomy and motility.

1.1.1 Classification of EMTs

EMTs are categorized into three separate types based on the context in which they occur [2]. Type 1, 2 and 3 EMTs can have different outcomes within a given cellular context, however it is currently not clear what biochemical elements they share and how they differ.
Type 1 EMTs occur during development and are utilized during discrete morphogenic events. For example, the EMT program is activated during gastrulation in a distinct population of epiblast cells. Upregulation of transcription factors such as Snail1 leads to MMP expression facilitating breakdown of the underlying basement membrane and ingestion through the primitive streak [18], [33]. These mesendodermal cells then either undergo reversion of EMT (termed MET) and form the ectoderm or retain their mesenchymal properties and give rise to the mesodermal layer [2]. During neurulation, populations of migratory cells termed neural crest cells are formed at the neuroectoderm-ectoderm junction. Activation of EMT facilitates their delamination and subsequent migration throughout the developing embryo where these cells give rise to various tissues [39].

Type 2 EMTs are associated with inflammation and involve the conversion of epithelial cells to mesenchymal cells in response to physiologic stress and are critical to tissue repair and regeneration. The re-epithelialization stage during wound-healing involves drastic changes in the morphology and function of surrounding keratinocytes. During wound closure, keratinocytes activate a partial and reversible EMT to decrease intercellular adhesion and increase their cell-matrix interaction to migrate over and close a lesion [40]. Type 2 EMT can also be activated in tissues undergoing pathologic stress. For example, liver or kidney damage can
lead to the formation of fibroblasts from existing epithelial cells to promote tissue repair [41], [42]. Although type 2 EMTs normally cease once inflammation has subsided, persistent inflammation can lead to constitutive activation of EMT and tissue or organ damage [43].

Type 3 EMTs are specific to neoplastic epithelial cells that have undergone genetic changes including upregulation of oncogenes and decreased expression of tumor suppressor genes. The subsequent activation of EMT in cancer cells can render them far more invasive and malignant than those cells undergoing type 1 and 2 EMTs. This is possibly due to their ability evade the normal checkpoints and controls of the body. Increasing evidence suggests that the metastatic spread of cancer cells is facilitated through activation of EMT in a subset of tumor cells during cancer progression [11]. The absence of E-cadherin in tumor samples has long been correlated with increased tumor grade and poor prognosis [44],[45], [46] and upregulation of numerous EMT markers is also correlated with decreased patient survival [12],[47], [48] [49]. Additionally, cancer cells at invasive fronts can take on an EMT-like phenotype [50], [51], [52].

Recently, it was discovered that cancer cells that have undergone EMT take on a de-differentiated stem cell-like phenotype [53]. These cancer stem cells (CSCs) are believed to be a discrete population within
a tumor that are characterized by unlimited self-renewal, host-seeding abilities the ability to give rise to more differentiated progenitor cells [54]. CSCs are more resistant to traditional chemo and radiation therapies [55], [56], [57] and their selective re-population after treatment may explain the refractory response to therapy in patients undergoing disease relapse. Consequently, intense research is currently underway to discover their Achilles heel and selective CSC inhibitors such as salinomycin are currently being tested for their effectiveness as cancer therapies [58].

1.1.2 Molecular Control of EMT

Aberrant activation of the EMT program can be hazardous to cellular and tissue homeostasis. For this reason, the transition from epithelial to mesenchymal cell is orchestrated via an intricate and complex set of internal and external signals.

The TGF-β proteins are the well-characterized potent inducers of EMT. After injury, MMPs secreted by stromal cells surrounding a wound can activate TGF-β1 stored in the extracellular matrix and stimulate activation of a temporary and reversible EMT in keratinocytes allowing their migration into the wound site [40], [59]. EMTs occurring during formation of the heart atrio-ventricular (A/V) canal also require TGF-β expression [60]. TGF-β2 and TGF-β3 neutralizing antibodies were both
shown to inhibit EMTs in mouse and chick A/V canal explants, respectively [61].

TGF-β signals can synergize with other major EMT signaling pathways to induce EMT. The Notch signaling pathway has been shown to be a downstream mediator TGF-β-induced EMT. In mammary epithelial cells, TGF-β activation induces the expression of the Notch ligand Jagged1 (Jag1) and EMT progression. siRNA against Jag1 inhibits EMT despite TGF-β expression [62]. Additionally, expression of a dominant-negative form of the Notch ligand Delta1 during avian neural crest formation leads to a decrease in Snail2 expression and disruption of EMT [63].

The Wnt pathway is also involved in EMT activation. Neural crest induction in the chick requires Wnt1-mediated activation of snail2 and expression of a dominant-negative Wnt1 inhibits snail2 transcription and neural crest migration [64]. Mouse mesoderm formation also requires Wnt activation of EMT [65]. Mouse embryonic stem (ES) cells treated with the Wnt inhibitor Dickkopf (Dkk1) lose expression of EMT markers Snail1, N-cadherin and fibronectin [65]. Twist1 is also induced by Wnt1 expression in C57MG and HC11 mouse mammary epithelial cells and its promoter is responsive to β-catenin [26].

Although there are limited studies connecting Hedgehog signaling and EMT, its pharmacologic inhibition in the E3LZ10.7 pancreatic cancer
cell lines restores expression of E-cadherin and decreases Snail1 expression and metastatic potential [66]. Additionally, the Hedgehog effector Gli can induce Snail1 mRNA in RK3E rat kidney cells and results in nuclear translocation of β-catenin and cell transformation [67].

The Src family of tyrosine kinases are found on the plasma membrane of cells and localize with adherens junctions as well as cell-matrix integrin contacts and are required for proper maintenance of epithelial tissue [68]. In addition to maintaining epithelial tissue homeostasis, elevated Src at cell-cell junctions leads to phosphorylation of E-cadherin and β-catenin, E-cadherin endocytosis, adherens junction disassembly and EMT [69]. Src’s effects on cell-cell junction is observed during wound healing where Src is activated to promote cell migration and wound closure. Scratch-wounded A6(1) corneal epithelial cells activate Src along the wound edge [70], addition of a Src inhibitor in these cells leads to a drastic (85%) decrease in wound closure [70]. Src overexpression can also lead to epithelial pathology. Src is overexpressed in many human cancers [71] and transgenic mice expressing a constitutively active human c-src in epidermal basal cells develop hyperplasia and squamous cell carcinomas [72].

Receptor tyrosine kinases (RTKs) are a major group of cell-surface receptors and many of their ligands are inducers of EMT. Epidermal
Growth Factor (EGF) secreted by stromal macrophages can promote EMTs in adjacent tumor cells. These cells, in turn, respond to macrophage-derived EGF signaling by secreting CSF-1, a macrophage attractant and stimulant [59] thereby perpetuating EMT activation. Hepatocyte growth factor (HGF) expression in epithelial cells induces Snail1 expression and cell scattering [73],[74]. Mice deficient in platelet-derived growth factor (PDGF) receptors α and β have defects in epicardial EMT and are unable to form cardiac fibroblasts [75]. Additionally, PDGF-D overexpression in PC3 prostate cancer cells leads to E-cadherin downregulation via mTOR and Bcl-2. Fibroblast growth factor (FGF) [76] and IGF (Insulin-like growth factor) [77] growth factors have also been implicated in mediating EMTs. Ras is a major effector of RTK-mediated EMT [78] and acts through the MAPK pathway to activate EMT transcription factors such as Snail1 [74], Snail2 [79] and Twist1 [80].

1.2 The Snail family of Transcription Factors

The Snail family of transcription factors is a highly conserved group of transcriptional repressors found throughout evolution from worms to humans [81]. They are critical in numerous developmental processes including heart and limb formation, left-right asymmetry, cell movement, cell fate, and cell survival [81]. Among their various functions, Snail family
members have been most extensively studied for their ability to induce EMTs during development of the neural crest, mesoderm and heart [81]. Recently, their role in cancer progression has been the focus of much attention.

Vertebrate Snail family members include Snail1, Snail2 (formally known as Slug) and Snail3 [81]. Snail family proteins are highly conserved within their zinc-finger domains and vary in their overall structure [82]. Outside their zinc-fingers domains, Snail1 proteins are highly variable, showing anywhere from 57-75% similarity [82]. Snail2 proteins are more conserved and share over 90% homology in both their C and N-terminals [83]. They can be identified through a stretch of almost completely conserved 29 amino acids preceding their five tandem zinc fingers [84]. Snail3 proteins also show 70-86% conservation in their zinc-finger domains and can vary considerably in their N-terminal domains [85].

In the mouse, Snail1 is indispensible for normal development. Snail1-null mice are embryonic lethal and display defects in gastrulation and mesoderm formation [84]. Snail2 knockout mice are viable but display severe growth delays at birth as well as anemia, decreased fertility, mild pigmentation defects in the coat, tail and feet and a white forehead blaze [38]. Snail3 mice have not been generated however Snail3 expression is not detected until late in development and is only
observed in the skeletal muscle and thymus in the mouse [86]. In humans, Snail2 deletions have been linked to Waardenburg disease and piebaldism, both characterized by pigmentation defects [87],[88]. Somatic Snail1 and Snai3 mutations or deletions have not yet been identified in congenital human diseases.

Snail1 and Snail2 proteins are highly labile. Both Snail1 and Snail2 are subject to quick ubiquitination in the absence of stabilizing signals. The GSK3-β kinase promotes Snail1 ubiquitination by phosphorylating it at two consensus sequences[89]. The first phosphorylation event leads to Snail1 translocation from the nucleus to the cytoplasm where it is once again phosphorylated by GSK3-β and marked for destruction by the E3 ubiquitin ligase β-TrCP [89]. Activation of Akt, Mitogen-activated protein kinases (MAPK) or Wnt pathways can suppress GSK3-β and lead to stabilization of Snail1 [89]. Snail2 stability is regulated by partner of paired (Ppa), a component of the E3 ligase [90]. During Xenopus neural crest formation, Ppa is expressed at the neural plate border and at early migratory stages, and is excluded from migrating neural crest cells [90]. Ppa directly binds to and promotes Snail2 degradation. The expression of neural crest factors such as Twist1, Sox9 or FoxD3 stabilizes Snail2 expression [90].
1.2.1 Mechanistic Regulation of Gene Expression by the Snail Family

Transcriptional regulation by Snail family members is carried out through both their N and C-terminals. The carboxy-termini of Snail family members contain 4-6 highly conserved C2H2-type zinc-finger DNA binding domains where C and H represent cysteine and histidine residues. These zinc fingers bind the E-box consensus sequence 5’-CAGGTG/5’-CACCTG on target genes to repress their transcription [83] and [81]. In vertebrates, transcriptional repression is also carried out through a non DNA-binding N-terminal Snai/Gfi (SNAG) domain that interacts with histone deacetylases (HDACs) HDAC1, HDAC2 and mSin3A [91]; [81].

As the main focus of my dissertation, Snail2 will be the subject of discussion for the remaining chapter.

1.2.2 Snail2 in Development and the Adult

Snail2 is expressed during numerous morphogenic processes in early embryos. It is first detected in extraembryonic tissue and in the gastrulating mesoderm in chick and zebrafish [92]. In the mouse, Snail2 is expressed in the lateral mesoderm, after mesoderm formation [92]. Snail2 expression is also prominent in the developing limb bud, brachial arches
and optic vesicle [92]. Interestingly, an interchange of expression has been noted between Snail1 and Snail2 in the chick and mouse. In the chick, Snail2 expression in pre-migratory neural crest cells appears to play a predominant role in driving EMT, neural crest induction and migration while in the mouse, Snail1 plays a primary role in these same processes [84]. The same is true for their expression in the primitive streak and mesoderm formation [84].

One of the best-characterized developmental functions of Snail2 is its promotion of neural crest induction and migration. Snail2 is one of the earliest neural crest markers expressed in pre-migratory and migratory neural crest cells of Xenopus [93] and chick [94]. In the chick and zebrafish, Snail2 expression has been shown to be necessary for neural crest migration [84],[95]. Overexpression of Snail2 in the chick neural tube leads to cranial neural crest production [96] and antisense oligonucleotides against Snail2 inhibit crest migration [93],[97],[94]. Neural crest defects appear to be due to failure of Snail2-promoted EMT [94]. Snail2 inhibition does not lead to gross abnormalities in most neural crest derived cell populations in the mouse [98]. Snail2 knockout-mice are viable and homozygous mutants display relatively few pigmentation defects outside of the tail, feet and forehead [98]. This is possibly due to functional compensation and overlapping expression with the related
family member Snail1. Snail1 expression overlaps that of Snail2 in numerous parts of the developing embryo including the migratory neural crest cells, the somites and limb bud [84]. Furthermore, Snail1 has been shown to be functionally redundant with Snail2 in chick neural crest formation [96]. Hence, it is possible that in the absence of Snail2 expression, neural crest cell migration proceeds normally in the mouse through compensation by Snail1. It is interesting to note that there may not be a full compensation within all subpopulations of neural crest cells as evidenced by the appearance of a characteristic white blaze on the forehead of Snail2-null mice as well as areas of depigmentation in the tail and feet. Indeed it has been shown that while Snail1 and Snail2 are expressed within a general area of the developing embryo, their specific expression may also be further segmented into distinct areas [92].

Although Snail2 is not necessary for mesoderm induction or specification in the mouse [92], it is required for emigration of mesoderm precursors from the primitive streak in chick and Xenopus embryos. Snail2 is expressed in the mesendoderm at the start of gastrulation in Xenopus and upregulates expression of dorsal mesoderm genes chordin and cerberus [99]. Expression of a dominant negative form of Snail2 leads to loss of mesoderm gene expression and inhibition of head development.
Chick embryos incubated with antisense oligonucleotides to Snail2 show inhibition of EMT and mesoderm formation [94].

In addition to its functions in development, Snail2 plays an important role in adult epidermal homeostasis and wound repair [100],[101]. Its expression is prominent in many tissues of the adult mouse and human including the colon, heart, kidney glomeruli, liver, ovary, pancreas, placenta, prostate, intestine, spleen, testis and thymus [102]. Although the exact function of Snail2 expression in many of these tissues has not been determined, Snail2 expression is upregulated at wound margins during healing and is important for keratinocyte emigration from wound margins [101]. Snail2-null mouse skin explants show defects in keratinocyte outgrowth and ectopic Snail2 expression accelerates re-epithelialization [101].

Snail2 also suppresses apoptosis in response to DNA damage. The Bcl-2 pro-apoptotic protein Noxa is expressed in response to DNA damage or growth factor deprivation [103]. It interacts with other members of the Bcl-2 family to induce release of mitochondrial cytochrome C and promote apoptosis [103]. Skin from Snail2 knockout mice show increased expression Noxa as well as the anti-apoptotic protein Slurp1 [100]. Snail2 can also directly bind to the promoter of the Puma gene [37]. Puma is another member of the Bcl-2 family and a
downstream target of p53 [104]. Irradiation of mouse hematopoietic cells induces expression of Snail2 leading to direct repression of PUMA. Snail2 knockout mice exposed to normally sub-lethal γ-irradiation die due to bone marrow failure [37]. This defect is rescued in mice deficient in both Snail2 and Puma [37] indicating that Snail2 is a key mediator of apoptosis and cell survival in response to cell damage.

Snail2 expression can also be induced in response to ultraviolet radiation (UVR) [105] and modulates keratinocyte acute inflammatory response to UVR [100]. Snail2 knockout mice are more resistant to sunburn as compared to wild-type mice and show notable decreases in COX2 expression and neutrophil infiltration, possibly due to decreased expression of the chemokines IL1b and Cxcl2 [100].

1.2.3 Pathways regulating Snail2

Snail2 expression can be induced through diverse pathways including TGF-β, Notch1, Wnt, FGF, EGF, and SCF. TGF-β1 induces Snail2 in expression in immortalized keratinocytes [106]. In the embryonic heart of the mouse and chick, TGF-β2 also induces Snail2 expression and EMT. This allows migration of endothelial cells and formation of antrio-ventricular (A/V) mesenchyme [107]. A/V canal explants treated with TGF-β2
antibodies results in decreased Snail2 expression and inhibition of EMT [108].

Notch1, another key regulator of cardiac EMT induces Snail2 expression in human aortic endothelial cells (HAECs). HAECs treated with constitutively active Notch1 increase Snail2 expression [109], as does expression of Notch ligands Jagged1 and Delta like ligand 4 (Dll4) in human mammary epithelial cells (HMECs) [109]. In the avian neural crest, expression of a dominant-negative Notch ligand, Delta-1, inhibits Snail2 expression, EMT and neural crest induction [63].

Snail2 constitutes a downstream target of the Wnt pathway during neural crest formation [64]. A dominant negative form of Wnt1 injected into the neural plate or closing neural tube of chick embryos lead to a marked decrease of Snail2 at the sites of injection [64]. Induction of Snail2 by Wnt signaling appears to be direct. Inspection of the Xenopus Snail2 promoter revealed a consensus binding site for the lymphocyte enhancer factor 1 (LEF1) [25], an important downstream effector of Wnt signaling [110].

Outgrowth of the chick limb bud requires maintenance Snail2 expression through the actions of fibroblast growth factor 4 (FGF-4) [111]. Limb buds incubated with the FGF-4 antagonist, retinoic acid (RA) show loss of Snail2 and arrested limb growth [111]. Snail2, induction by FGF-1
overexpression in NBTII rat bladder carcinoma cells leads to desmosome dissociation and cell spreading [112]. Furthermore, expression of a Snail2 antisense construct inhibits FGF-1 induced EMT [112].

The epidermal growth factor (EGF) and its receptor (EGFR) are potent activators of epithelial outgrowth [113]. In mouse skin explants, both EGF and EGFR can stimulate Snail2 upregulation [113]. Furthermore, EGF promotes robust outgrowth of mouse skin explants derived from wild-type mice [113]. In contrast, mouse skin explants from Snail2 knockout mice are unresponsive to EGF treatment [113]. In U251 human glioblastoma cells, EGF treatment results in a marked increase in Snail2 mRNA [114].

Snail2 was shown to be a target of the stem cell factor (SCF) cytokine. Snail2 knockout mice exhibit many abnormalities common to SCF and c-kit (the SCF receptor) knockout mice [98],[115]. The c-kit/SCF pathway is important in cell homing and survival during hematopoiesis, spermatogenesis, and melanogenesis [98]. Both Snail2 null mice, c-kit and SCF mutant mice display growth retardation, defects in erythroid development, hair and skin pigmentation defects and lower fertility rates. Deletions in Snail2 and deletions or mutations in KIT have been detected in human piebaldism, an autosomal dominant disorder characterized by patches of skin and hair that contain no pigment [88]. SCF treatment in
the human chronic myeloid leukemia cell line, LAMA84, rapidly induces Snail2 expression [98]. Snail2 was shown to mediate the radio-protective functions of the c-kit/SCF pathway. Both Snail2 and c-kit deficient mice are highly susceptible to death due after exposure to γ-irradiation [38]. Additionally, Snail2 expression can rescue irradiation sensitivity in c-kit deficient mice. Interestingly, SCF treatment of Snail2–null mice does not rescue lethality due to γ-irradiation, indicating a central and unique role of Snail2 downstream of c-kit/SCF signaling [38].

1.2.4 Transcriptional Control of Snail2

Snail2 gene expression is modulated through a variety of transcription factors. The Xenopus Snail2 promoter contains a Lef-1 consensus site to which the Lef-1/β-catenin protein complexes bind to activate Snail2 expression [25]. This is in accordance with the role of Snail2 as a downstream target of Wnt in neural crest development [116]. Interestingly, although the murine Snail2 promoter is also responsive to β-catenin [28], it does not contain a Lef-1 consensus sequence [117]. The Snail2 promoter can also be activated by the myogenic transcription factor MyoD and is expressed during myoblast differentiation in muscle regeneration [118]. Snail2 has a well-established role in regulating EMTs during heart cushion formation [119], [108]. Direct activation of the Snail2
promoter by the Notch nuclear effector, CSL (CBF-1, Su-H, Lag-1) is required for vascular-endothelial (VE)-cadherin suppression and EMT induction [109]. The microphthalmia-associated transcription factor (MITF) was shown to target Snail2. The MITF transcription factor controls melanocyte pigment and function [120]. MITF activates Snail2 transcription in vitro by binding to an E-box in its promoter region [87]. Mutation of the Snail2 E-box inhibits its response to MITF [87]. Mutations in both SNAI2 and MITF lead to pigmentation defects and hearing impairment in patients with WS-2 type Waardenburg syndrome [87]. Snail2 can also be activated by the E2A-HLF oncoprotein [121]. Additionally, the p53 tumor suppressor can bind two responsive elements in the murine Snail2 [37] gene promoter to induce its expression.

1.2.5 Transcriptional Targets of Snail2

Snail2 plays a prominent role in suppression of apoptosis. Expression of Snail2 in MCF7 breast cancer cells decreases expression of pro-apoptotic genes p53 [122], BID [122] and DFF40 [122]. ChIP analysis demonstrated direct interaction of Snail2 with their promoters. As previously discussed, Snail2 can also directly suppress PUMA, a key mediator of p53-induced apoptosis [37].
Snail2 controls temporal expression of the tumor suppressor and DNA damage repair protein BRCA2 [123]. BRCA2 is expressed in dividing cells and in response to DNA damage [124]. In the non-dividing cell, BRCA2 is silenced by Snail2 via its recruitment of the transcriptional repressors C-terminal-binding protein-1 (CtBP-1) and histone deacetylase-1 (HDAC-1) to the BRCA2 silencer E-box [123].

Snail2 plays a prominent role in the initiation of EMT through its direct transcriptional regulation of cell-cell adhesion proteins. Loss of tight junctions is an early event in EMT [125]. Tight junctions are located at the apical lateral regions in between epithelial cells [126]. They function to keep cell integrity and help maintain cell polarity by limiting the lateral diffusion of proteins within a cell. Snail2 directly suppresses expression of the integral tight-junction protein claudin-1 [127]. The Claudin-1 promoter contains two E-box motifs that are responsive to Snail2 (Martinez-Estrada, 2006). Mutation of both E-box consensus sequences abrogates Claudin-1 response to Snail2 suppression [128]. The tight junction protein Occludin is also a direct target of Snail2 [122]. In addition to tight junctions, Snail2 also promotes dissociation of desmosomes, one of the strongest cell-cell adhesion complexes; this repression has not been shown to be direct however [112]. Snail2 can directly induce expression of the E-cadherin repressor ZEB1 [129]. Interestingly, silencing of ZEB1 via siRNA leads to an
increase in E-cadherin despite overexpression of Snail2 in the WM164 melanoma cell line, hence, ZEB1 appears to be an important Snail2 target necessary for proper E-cadherin suppression [129]. Integrins are important cell-matrix attachment proteins that regulate cell motility, survival and cell cycle [130]. Snail2 is expressed in basal keratinocytes of the epidermis where it transcriptionally represses the α3 integrin via an E-box in its promoter [131]. Although α3 is the only verified direct Snail2 target, other integrins such as α2 and α6, which are also expressed in keratinocytes contain E-boxes in their promoter region and could be direct Snail2 targets [131].

Given its importance in maintaining epithelial phenotype, E-cadherin remains one of Snail2’s best-characterized targets. Snail2 directly inhibits E-cadherin transcription by binding two E-boxes within the E-pal transcriptional repressor element of the E-cadherin promoter [132]. Snail2 expression inversely correlates with E-cadherin expression in many breast cancer cell lines [9]. And expression of Snail2 in the canine kidney cell line MDCK is sufficient to drive EMT [132]. Snail2 expression also leads to loss of cell-adhesion and subsequent activation of EMT in mesoderm, neural crest and limb bud development [81].

Interestingly, Snail2 expression does not always inversely correlate with E-cadherin expression. The human ovarian cancer cell line SKOV3
expresses both E-cadherin and Snail2 [133] and several mouse epidermal cell lines also express high levels of Snail2 and E-cadherin [134]. Hence, it appears that Snail2 expression is not always sufficient to drive suppression of E-cadherin and promote EMT. It has been suggested that because Snail1 has a stronger affinity for the E-cadherin promoter, in some contexts endogenous Snail2 may function as an immediate but temporary activator of EMT during times of stress. SKOV3 cells express E-cadherin and low levels of Snail2 and quickly upregulates transient expression of Snail2 in hypoxic environments [133]. This Snail2 upregulation leads to a transient decrease in E-cadherin until Snail2 levels are again normalized. E-cadherin levels begin to decrease once again as Snail1 is later upregulated. Hence, Snail2 may be expressed at low endogenous levels in some contexts to serve as an immediate and temporary inducer of EMT while stronger EMT-inducing factors such as Snail1 are activated and act as more potent activators of EMT.

1.2.6 Snail2 in Cancer

Growing evidence has implicated EMT in promoting invasive characteristics of cancer cells through activation of numerous pro-invasive pathways. Given the prominent role of Snail2 in promoting EMT, it is not surprising that Snail2 has been implicated in cancer initiation and
progression. Gene expression profiling of MDCK cells overexpressing Snail2 identified Snail2 as a regulator of numerous pathways promoting tumor progression and invasion such as cell proliferation, angiogenesis and apoptosis [135]. Evidence corroborating these findings is abundant. Transgenic mice overexpressing Snail2 have been shown to develop acute leukemias and sarcomas [136]. In a study of over 100 breast tumor samples, Snail2 expression significantly correlated with invasive ductal carcinomas which were positive for lymph node infiltration [137]. Additionally, Snail2 expression in patients with squamous cell carcinomas has been significantly associated with deeper invasion of cancer cells into surrounding tissue as well as increased lymph node positivity and higher levels of venous invasion [138]. Snail2 knockdown via siRNA has been shown to decrease invasiveness of PC3-16 colon carcinoma cells [139].

Snail2 can promote invasiveness by the modulation of numerous properties in a cell that convert it from a benign stationary cell to one with malignant properties. To invade local tissue, cells must digest through their surrounding extracellular matrix (ECM). This can be done by secretion of numerous enzymes that degrade components of ECM. Snail2 can induce expression of MMP-9, a metalloproteinase that degrades type IV collagen, a major component of the ECM [140]. Snail2 expression in human pancreatic carcinoma (PANC-1) cells upregulates MMP-9 and increases
gelatin degradation and cell invasion [141]. Snail2 also facilitates cell motility through cytoskeletal remodeling and increased pseudopodia formation [140].

Chemoresistance and radioresistance to cancer therapies has been linked to EMT and Snail2 expression [142], [141], [143]. Inhibition of Snail2 via siRNA increases cholangiocarcinoma cell sensitivity to the chemotherapy drug, cisplatin due to increased levels of the pro-apoptotic protein PUMA [141]. A4 ovarian cancer cells resistant to paclitaxel have higher levels of Snail2 as compared to their parental cell line [144]. This increased expression of Snail1 and Snail2 leads to suppression of pro-apoptotic proteins in the p53 pathway [144].

1.3 The Twist1 Transcription Factor

The twist transcription factor was first identified in Drosophila as a zygotic gene involved in dorsal-ventral patterning, gastrulation and mesoderm formation [145] and is found conserved throughout evolution in organisms ranging from the worm C. elegans [146] to the mouse [147] and humans [148]. Twist proteins are essential during numerous developmental processes including morphogenesis, cell fate specification, lineage commitment and EMT [149].
Twist1 is a member of the Twist family of bHLH transcription factors that in vertebrates includes Twist1 and Twist2 [150]. Twist performs its regulatory functions through its highly conserved basic-helix-loop helix (bHLH) motif. The fundamental structure of the bHLH motif consists of a short stretch of basic amino acids and two amphipathic alpha helices separated by a loom domain [151]. Twist1 proteins are highly conserved in the basic residues of their DNA binding domain which are 100% identical between Drosophila, Xenopus, mouse and human proteins [152]. The HLH moiety of Twist1 is 68% conserved between Drosophila, Xenopus, mouse and human Twist [152].

Drosophila twist mutant embryos die early in development due to gastrulation defects and exhibit a twisted torso [153]. Twist1-null mice are embryonic lethal [147] (Wolf, 1991). However, unlike Drosophila mutants, Twist1 mutant mice are able to initiate and proceed through gastrulation. The most prominent phenotype in Twist1-null mice is failure of cranial neural tube closure. Twist1 mutants also display defects in head mesenchyme, brachial arches and limb bud development [154]. Mice heterozygous for Twist1 exhibit craniosynostosis (premature fusion by ossification) of the coronal suture [155], polydactyly (excessive digits) [156] and in rare cases, pre-axial syndactyly (fused digits) [157].
In humans, haploinsufficiency of Twist1 leads to Saethre-Chotzen syndrome, which is characterized with craniosynostosis and syndactyly [158].

Twist1 is subject to proteasome-mediated degradation through polyubiquitination [159]. Its stability is drastically enhanced through phosphorylation of serine 68 (S68) through the MAPK proteins JNK, Erk1/2 and p38 [159].

1.3.1 Mechanistic Regulation of Gene Expression by Twist1

Twist1’s HLH moiety is essential for protein-protein interactions while its basic residues interact with target gene DNA. Twist1 can form homodimers and heterodimers with other bHLH proteins thus creating a DNA binding motif through juxtapositioning of the bHLH basic domains to allow specific binding to target gene promoters [160],[149]. Twist regulates gene expression by binding to cis-regulatory elements named E-boxes on a given target gene promoter. E-boxes are binding sites for transcriptional regulation by bHLH factors and are found in many lineage-specific genes [161], [162], [163]. Homo and heterodimerization between Twist and other bHLH proteins in part determines which E-box will be targeted for regulation [164]. Drosophila twist homodimers display an affinity for the CATATG E-box in mobility shift assays whereas
twist/daughterless dimers bind either CACCTG or CATATG [165].

Overexpression of twist homodimers in the *Drosophila* ectoderm or mesoderm induces ectopic muscle development while twist/daughterless (an E12/E47 homolog) dimers inhibit muscle development. The E-box CATCTG is recognized by tissue-specific nuclear factors [166], [167]. Twist1-E12 tethered dimers show much higher trans-activation of CATCTG-Luc reporter constructs than Twist-Twist or Twist-Hand2 tethered dimers.

Twist1 can effect transcription irrespective of DNA binding. This is accomplished through titration of bHLH transcription factors thereby limiting their transactivation activities. E12 and MyoD are key myogenic transcription factors whose dimerization facilitates binding and activation of genes involved in muscle specification. Twist1 can bind both E12 and MyoD to inhibit their dimerization and thereby control and specify correct muscle differentiation and positioning [149]. Twist1 performs similar functions during bone formation in the mouse through interactions with the Runx2 transcription factor, a master regulator of the osteogenic program [149]. Twist1 expression prevents transactivation of Runx2 and controls premature maturation of osteoblast differentiation [168]. It is interesting to note that cells isolated from a Saethre-Chotzen patient show increased levels of the pro-osteoblastic Runx2-regulated protein osteocalcin [168], hence Runx2-Twist interactions may be conserved in
the human. Additionally, Twist1 haploinsufficiency in mice and humans has been linked to craniosynostosis [169] and [155].

The Inhibitor of differentiation (Id) proteins are a dominant-negative forms of HLH transcription factors that form heterodimers with bHLH proteins. As they have no DNA binding activity, they can inhibit DNA binding and thus regulate the transcriptional activities of bHLH transcription factors such as Twist1 [170],[171].

Interactions between Twist and its dimerization partners is tightly regulated through phosphorylation of key residues in the Twist1 bHLH domain. Twist1 and members of the bHLH family such as Hand1 and Hand2 can be phosphorylated at conserved serine and threonine residues within the helix 1 of their HLH domains by PKA and PKC and de-phosphorylated by the B56δ subunit of the PP2A phosphatase [172]. Twist1 mutations leading to disrupted phosphorylation interfere with dimerization and mimic loss of function mutations [173]. Twist1 antagonizes the actions of Hand2 during limb bud development and Twist1:Hand2 dimerization is critical for proper limb bud formation in the mouse [174]. Mutation of the PKA consensus site in Twist1 disrupts its interaction with Hand2 leading to polydactyly [174]. Similar mutations have been linked to Saethre-Chotzen syndrome phenotypes in mice and humans [172]. The phosphorylation status of Twist1 has also been shown to be important for its interaction with
MyoD. The myogenic bHLH protein MyoD is required for muscle cell determination and differentiation [175]. Its expression outside the myotome is antagonized by dimerization with Twist1 thereby preventing ectopic skeletal muscle formation. Mutations in three key arginines disrupts PKA phosphorylation and abolishes interactions between Twist1 and MyoD [175] and [174].

In addition to exerting transcriptional control through its dimerization with bHLH proteins, Twist can carry out transcriptional regulation through histone modification. Twist1 directly interacts with histone acetyltransferase (HAT) domains of the transcriptional co-activators p300/CBP and PCAF via its N-terminal domain, thereby inhibiting their HAT activities [176]. Overexpression of Twist in C2C12 mouse myoblast cells blocked PCAF-enhanced p300 auto-acetylation. Additionally, Twist1 expression in human osteosarcoma (U2OS) cells inhibits the activities of p300-dependent transcription factors MyoD, p53 and VP16 [176]. Twist was also shown to interact with Smad4 and histone deacetylase 1 (HDAC1) to antagonize the BMP signaling pathway [177]. Recent evidence has also shown interaction between Twist and several components of the Mi2/nucleosome remodeling and deacetylase (Mi2/NuRD) complex [178].
1.3.2 Twist1 in Development and the Adult

In the mouse, Twist1 is first expressed in the extraembryonic mesoderm around day 7 and later expressed in the head mesenchyme, somites and the somatic lateral plate and much later in the limb bud and heart [179]. Although data on Twist1 expression in the developing human is limited, human TWIST1 mutations leading to haploinsufficiency share a similar phenotype with Twist1 heterozygous-null mice. Human TWIST1 haploinsufficiency has been linked to Saethre-Chotzen syndrome [158], an autosomal dominant congenital disorder characterized by a premature closure of the cranial sutures and syndactyly [180]. Hence, as in the mouse, Twist1 appears to be necessary for proper development of cranial and limb structures in humans. In the human adult, Twist1 expression is found in mesodermal-derived tissues such as the heart and skeletal muscle [152].

Two of the best-characterized morphogenic events involving Twist-induced EMTs are gastrulation and neural crest induction.

One of the first instances of developmental EMT occurs during gastrulation as the three germ layers are formed [40]. Ventral furrow formation is the first morphogenic event during Drosophila gastrulation.
and is orchestrated by interactions between maternal dorsal as well as zygotic twist and snail genes [181] and [182]. In Drosophila homozygous twist mutants, gastrulation is disrupted and leads to failure of ventral furrow formation and no subsequent mesoderm formation [145]. In wild type embryos, blastoderm cells that form the ventral furrow and eventually give rise to the mesoderm undergo morphologic changes exhibiting typical EMT. They are seen to flatten, constrict, downregulate cell-cell adhesion and invaginate, first forming the ventral furrow then migrating inward to form mesoderm [182]. Presumptive mesoderm cells in twist mutants show defects in EMT. They are seen to initially flatten; however they cannot constrict or invaginate [40]. In the mouse, Twist1 does not appear to be involved in mesoderm formation as Twist1 is only detected after the start of gastrulation [183]. Mouse Twist1 protein is first expressed in the head mesenchyme, somites and the somatic lateral plate and much later in the limb bud and heart [179]. Accordingly, Twist1 mouse mutants do not show gastrulation defects, however they do show defects in cranial neural crest specification and migration [183].

Neural crest cells are a transient population of migratory cells that arise at the border of the neural plate and epidermal ectoderm [40]. After neurulation, neural crest cells undergo EMT and delaminate from the roof of the newly formed neural tube. They then undergo stereotyped
migration throughout the embryo where populations arising at precise rostro-caudal positions eventually give rise to specific cell types including melanocytes, craniofacial cartilage and bone, smooth muscle and cells of the central and peripheral nervous system [39]. Twist is expressed during neural crest formation in many species including *Xenopus* [184], chick [185] and mouse [186] and is important for proper neural crest migration, homing and differentiation [187]. Twist1 knockout mice die at E11.5 and exhibit failure of cranial neural tube closure [183]. Careful inspection of the cranial neural tube region shows defects in neural crest migration. Although craniofacial neural crest induction appears normal, neural crest cells display differentiation and migration defects [186]. The actions of Twist may not be limited to neural crest cells themselves. Wild-type [188] neural crest cells transplanted into Twist1 null mice were unable to correctly travel to their proper location in the brachial arch [188]. This indicates that Twist expression in the mesenchyme may also be important for proper neural crest migration [186].

In the human adult, Twist1 is expressed in white adipose tissue and brown fat and is a positive regulator of fatty acid oxidation [189] and regulator of thermogenesis [190]. Additionally, Twist1 is an essential negative regulator of the inflammatory response through its interaction with key players in the NF-κβ inflammatory pathway [188].
importance of this regulation is demonstrated in Twist1+/−/Twist2+/−
compound heterozygous mice. While Twist1+/− and Twist2+/− mice are
viable and appear relatively normal, Twist1+/−/Twist2+/− mutants exhibit
growth retardation and die soon after birth due to a severe inflammation
syndrome leading to cachexia [188]. Partial regulation of the
inflammatory response by Twist1 occurs through its binding to E-boxes on
the TNFα and IL-1β promoters. TNFα and IL-1β are pro-inflammatory
cytokines and activators of the NF-κβ pathway [191]. Twist1 can bind the
TNFα and IL-1β promoters through homodimerization or heterodimerization
with Twist2 [188]. Additionally, Twist1 can inhibit NF-κβ-dependent
transcriptional activation of TNFα and IL-1β by binding the p65 subunit of
NF-κβ thereby inhibiting p65-mediated transactivation. Twist1 can also
promote B cell survival and differentiation into immunoglobulin-secreting
cells [192]. Twist1 is highly upregulated in chronically inflamed skin and is
induced in T helper lymphocytes to reduce expression of the pro-
inflammatory cytokines IFN-γ, IL-2 and INF-α thus ameliorating the
inflammatory response [193].

1.3.3 Pathways regulating Twist1

Induction of Twist occurs through various pathways including, TGFβ,
NF-κβ, Wnt, FGF and IGF. Twist is present in the mouse and chick palate
during palatal fusion and its expression decreases after palatal fusion [194]. Twist1 expression in the palate is controlled by TGFβ3, a master regulator of palatal fusion [195], [196]. Mouse palates incubated with a TGFβ3 neutralizing antibody decrease Twist1 mRNA expression.

Additionally, exogenous TGFβ3 expression in chicken palate cultures stimulates Twist1 mRNA expression and palates expressing Twist1 siRNA show incomplete palatal fusion [195]. Some redundancy in the system exists however as full palatal fusion was seen in some palates despite the lack of Twist1 inhibition [195]. Twist1 is also highly expressed in immature chondrocytes and is induced by TGF-β to maintain their de-differentiated state [197].

NF-κβ is an important regulator of Twist1 and can in turn be regulated by Twist1. In Drosophila, dorsal, the homolog of NF-κβ induces twist expression in the ventral region of the developing embryo and activates mesoderm formation [198]. In the developing mouse limb bud, NF-κβ expression overlaps that of Twist1 [199]. Inhibition of NF-κβ results in downregulation of Twist1 and perturbs limb bud development [199]. During the inflammatory response, Twist1 is induced by NF-κβ in a negative feedback loop to inhibit uncontrolled amplification of inflammation. NF-κβ- induced expression of Twist1 results in its binding and repression of
various cytokine promoters that would normally perpetuate NF-κβ activation [200].

Among numerous pleotropic functions, Wnt proteins can inhibit cell differentiation. Twist1 is a direct Wnt target and can work in concert with Wnt signaling to maintain a cellular progenitor state. For example, stimulation of HC11 mouse mammary epithelial cells with lactogenic hormones causes their differentiation and induces expression of milk proteins such as β-casein and WDNM1 [26]. Expression of Wnt1 in these cells induces Twist1 expression and is accompanied by a complete absence of β-casein and a decrease WDNM1 expression after hormone stimulation [26]. During chondrogenesis, an early step of bone formation, Twist1 is a target of Wnt signaling and represses chondrocyte gene expression in chondroprogenitor cells [201]. Inhibition of GSK-3β, a negative regulator of Wnt signaling, in ATDC5 chondrogenic cells results in Twist1 expression and suppression of the chondrocyte genes aggrecan and Type II collagen [201]. Conversely, expression of a Twist1 shRNA in GSK-3β-inhibited ATDC5 cells leads to a marked increase in both aggrecan and Type II collagen expression [201].

The fibroblast growth factor receptors (FGFRs) are important regulators of bone formation. Among other locations, FGFRs are expressed in osteogenic fronts of the developing bone including calvarial
sutures and promote osteoblast differentiation [202]. In the mouse, Twist1 expression overlaps that of Fgf2 and Fgf9 in the mid-sutural mesenchyme [203]. Twist1 is induced by Fgf2 and inhibits terminal differentiation of osteoblasts at calvarial sutures [203]. Inactivating TWIST1 mutations and activating FGFR mutations both cause premature fusion of cranial sutures in humans partly due to accelerated osteoblast differentiation [203],[202]. Mutations in FGFR3 and FGFR2 as well as TWIST1 have been detected in individuals with Saethre-Chotzen syndrome [180],[148].

The insulin-like growth factor-1 (IGF-1) controls cell growth and differentiation and can inhibit apoptosis via Twist1 expression. Expression of IGF-1 in NWTb3 fibroblasts leads MAPK-dependent expression of Twist1 and decreases etoposide-induced apoptosis [80]. Introduction of shTwist1 inhibits the anti-apoptotic effects of IGF-1 and leads to increased cell death [80].

1.3.4 Transcriptional Control of Twist1

Current insights into Wnt activation of Twist1 came from an initial report showing that in Drosophila, mutants for the Wnt homolog wingless show decreased expression of twist [204]. A subsequent study demonstrated that the mouse Twist1 promoter was highly responsive to transient transfection of β-catenin in 293 human embryonic kidney (HEK)
cells [26]. Furthermore, expression of Wnt1 in the mouse mammary epithelial cell lines C57MG and HC11 leads to upregulation of Twist1 mRNA and protein [26]. Additionally, mammary tumors derived from Wnt1 transgenic mice express higher levels of Twist1 when compared to their wild-type counterparts [26]. Inhibition of E-cadherin through shRNA and the subsequent nuclear translocation of β-catenin have also been shown to upregulate Twist1 in human mammary epithelial cells [21].

The hypoxia-inducible factor-1α and -2α (HIF-1α and HIF-2α) are transcription factors critical for maintaining oxygen homeostasis. They are translocated to the nucleus in response to low oxygen levels to induce genes involved in hypoxic survival [205], [206]. Cancer cell lines grown in a hypoxic environment induce Twist1 expression in a HIF-dependent manner [206], [207]. Twist1 expression is activated by both HIF-1α and HIF-2α via their binding to intronic HIF-responsive elements (HREs) in the Twist1 promoter [207], and in the first intron, respectively [206]. Hypoxia or HIF-1α expression induces EMT in FaDu hypopharyngeal cancer cells and in MCF-7 breast cancer cells [207]. Inhibition of Twist1 abrogates EMT in response to HIF-1α or hypoxia [207]. Twist1 contains two HREs in its first intron which are both are responsive to HIF-2α binding. Mutation of either one leads to complete inhibition of Twist1 activation [206]. Twist activation by HIF-1α only requires its binding of a single HRE [207].
The Signal transducer and activator of transcription 3 (STAT3) transcription factor regulates expression of genes involved in cell differentiation, proliferation, survival, angiogenesis, and the immune response [208] and is found constitutively activated in numerous cancers [209]. STAT3 is phosphorylated and thereby activated in response to growth factor and cytokine signaling [208]. Its phosphorylation results in homo and heterodimerization with other STAT proteins and their nuclear translocation results in activation of target genes such as Twist1 [208],[210]. The TWIST1 promoter contains five STAT3 consensus binding sites, only one of which is fully responsive to induction by STAT3 [210]. The v-Src kinase induces STAT3-dependent expression of Twist1 in NIH3T3 mouse fibroblasts. Importantly, STAT3 activation and Twist1 overexpression are highly correlated in late stage human breast cancers [210] and Twist1 can rescue STAT3-mediated invasion and migration in the invasive MCF-7 I4 breast cancer cell line expressing an shRNA against STAT3 [210].

Activation of twist via dorsal, an NFK-β related protein, is one of the earliest steps in Drosophila mesoderm differentiation. The Drosophila twist promoter contains cis distal (DE) and proximal (PE) regulatory elements that control its spatial expression in the developing fly [198]. Both the DE and PE each contain two dorsal (dl) binding sites [198]. In the ventral-most regions of the embryo where dl expression is highest, twist expression
is controlled by dorsal binding to the dl PE element [198]. Conversely, dorsal binding in the DE controls twist expression in the lateral region, where dl expression is much lower [198]. In mammals, Twist1 expressed in response to TNFα requires the expression of RelA, a member of the NF-κβ family however RelA has not been shown to directly bind the Twist1 promoter [188].

1.3.5 Transcriptional Targets of Twist1

*snail* was one of the first Twist targets identified. In *Drosophila*, *snail* activation in the mesoderm is induced through binding of twist to the *snail* proximal promoter. Activation of *snail* represses neuroectodermal/mesectodermal fate to ensure proper mesoderm formation [211]. *Snail1* has also been identified as a downstream mediator of Twist1-induced EMT and its expression may facilitate Twist1-induced metastasis [212].

The myocyte enhancer factor 2 (mef2) is a transcription factor that activates genes essential for muscle development in *Drosophila* [213]. *twist* activates *mef2* by binding a conserved E-box within an enhancer element approximately 2kb upstream of its transcriptional start site. Expression of a *twist* mutant during *Drosophila* development leads to loss of *mef2* expression and muscle patterning defects [213].
One of the most prominent sites of Twist expression during *Drosophila* development is the mesoderm. Like twist, tinman is expressed in the fly mesoderm and is necessary for the specification of mesodermal derivatives including heart and muscle tissue [214]. tinman contains three E-boxes within its Element B enhancer region to which twist binds to activate its expression in the trunk mesoderm [214].

Since the identification of Twist1 as an essential mediator of metastasis, many studies have focused on identifying targets of Twist1 that promote cancer cell invasion and metastasis. Twist1 can promote tumor growth and chemoresistance to cisplatin in cancer cells through direct activation of YB-1 [215]. The YB-1 transcription factor is a central regulator of the MEK/ERK pathway [216] and promotes cell survival and tumor growth [215].

Members of the AKT/protein kinase B (PKB) family of serine/threonine kinases are important regulators of cell cycle and cell growth [217] and [218]. AKT2 is overexpressed in many cancers and has been correlated with invasion and metastasis [219]. Two of the four E-boxes in the AKT2 promoter are responsive to Twist1 binding. Activation of AKT2 by Twist1 overexpression leads to increased cell invasion and paclitaxel resistance in MCF7 breast cancer cells [219]. Inhibition of AKT2 through siRNA reduces chemoresistance in MCF7-Twist1 cells [219].
Nuclear translocation of Twist1 triggered by β1 integrin-mediated cell adhesion leads to direct activation of the mesenchymal cell-cell adhesion protein N-cadherin [220]. Blocking of β1 integrin-mediated cell adhesion inhibits nuclear localization of Twist1 and activation of the N-cadherin promoter [220].

Members of the PDGFR family affect gene regulation, cell cycle and are expressed in many cancers [221]. Recently, an elegant study by Eckert et al. demonstrated the platelet-derived growth factor receptor alpha (PDGFRα) tyrosine kinase receptor to be a direct target in Twist1, necessary for Twist1-mediated invadopodia formation [34]. Invadopodia are cellular protrusions present in invasive cancer cells that extend into the extracellular matrix (ECM) releasing proteases that promote ECM degradation, enabling metastatic dissemination [222]. In the same study by Eckert et al., correlative data between Twist1 and PDGFRα was found in human invasive breast tissue arrays indicating that their co-expression may be predict poor patient prognosis [34].

Twist1 can control gene expression through direct activation of BMI1 [223], a component of the polycomb group repressive complex-1 (PRC-1). Polycomb group protein complexes are involved in the silencing and maintenance of gene expression and maintenance of stem cell self-renewal [224]. Twist1 binds and E-box in BMI1’s promoter regulatory region
thereby activating its expression [223]. BMI1 was found to be necessary for EMT induction in Twist1-overexpressing hypopharyngeal tumor FaDu cells. Furthermore, Bmi1 and Twist1 were found to collaborate in repression of the E-cadherin promoter [223].

1.3.6 Twist1 in Cancer

Twist1 was first identified as a potential mediator of cancer cell invasion through gene expression analysis of primary tumors derived from four cancer lines of varying metastatic potential (168FARN, 4TO7, 4T1 and 67NR) which spontaneously arose from a single mammary tumor of a BALB/c mouse [11]. Twist1 was significantly upregulated in the more invasive 168FARN, 4TO7, 4T1 cell lines as compared to the relatively benign 67NR line [11]. siRNA inhibition of Twist1 in the highly metastatic 4T1 cell line drastically attenuated its metastatic potential [11]. Furthermore, microarray gene expression data showed TWIST1 elevated in approximately 70% of the human lobular invasive carcinomas examined [11]. TWIST1 is not significantly expressed in normal breast tissue [11].

Since its identification as an essential mediator of metastatic potential in cancer cells, Twist1 has been the focus of much attention in the cancer field. Its elevated expression has been detected in numerous cancers including diffuse gastric cancer [225], glioblastoma, prostate
cancer [13], melanoma [226] T-cell lymphomas [227] and breast cancer [206]. Importantly, Twist1 expression is highly associated with poor prognosis in many of these cancers. A concerted effort has been made to determine the means by which Twist1 is able to promote metastasis. It has become apparent that Twist1 encourages cancer cell dispersion and colonization through pleiotropic modification of cell behavior.

Twist1 expression in cells results in increased cell invasion, migration and decrease susceptibility to apoptosis [228],[229]. One way by which Twist1 can promote metastasis is by facilitating cell migration and invasion via E-cadherin suppression. As previously discussed, E-cadherin is a key guardian of epithelial phenotype and its downregulation can lead to increased cell motility and invasiveness [10]. Twist1 expression in epithelial cell lines quickly leads to loss of cell-cell adhesion and transcriptional downregulation of E-cadherin [53],[230]. Additionally, E-cadherin is often negatively correlated with Twist1 expression in cancers [231],[4]. Whether or not this is a direct effect is a matter of debate and may depend on cellular context. E-cadherin was demonstrated to be a direct target of Twist1 regulation in MCF7 cell lines overexpressing Twist1 as well as in the Hs578T breast cancer line [4]. This was demonstrated via ChIP and promoter-reporter assays. In contrast, my studies revealed that in the human mammary epithelial cell line (HMEC), E-cadherin downregulation
requires activation of Snail2 through Twist1 [230]. In the absence of Snail2, HMEC cells remain phenotypically epithelial and maintain epithelial marker expression [230] despite overexpression of Twist1. Whatever the verdict will be, it is clear that E-cadherin remains an important downstream target of Twist1.

It has been postulated that tumor maintenance and growth is perpetuated by a discrete population of cells referred to as cancer stem cells (CSCs). Unlike other tumor cells, these CSCs are able to seed new tumors in a host, self-renew and give rise to differentiated, non-cancer stem cell progeny [54]. Induction of EMT in immortalized human mammary epithelial (HMLE) cells through expression of Twist1 (HMLE-Twist), Snail1 (HMLE-Snail) or TGF-β (HMLE-TGF-β) drives cells into a cancer stem cell (CSC) fate, identified by their CD44<sup>high</sup>/CD24<sup>low</sup> signature [53]. When injected into mice, these cells readily form primary tumors whereas HMLE cells expressing a control vector are not able to form primary tumors [53].

One way by which Twist1 may promote cancer cell self-renewal is through its direct activation of BMI1 [223], a component of the polycomb group repressive complex-1 (PRC-1). As a member of PRC-1, Bmi1 promotes chromatin silencing and is involved in maintaining stem cell self-renewal [232]. Inhibition of BMI1 in Twist1-overexpressing hypopharyngeal
tumor FaDu cells results decreased populations expressing the CD44 stem cell marker and drastically impairs tumor formation [223].

In addition to promoting self-renewal, Twist1 can foster tumor viability through imparting chemoresistance. Twist1 expression has been shown to be necessary for chemoresistance in cancer cells [229], [233] in part through its direct interaction with and inhibition of p53 [234].

Insufficient tumor vascularization can result in hypoxia and cell death. Both HIF-1α [207] and HIF-2α [206], important transcription factors regulating oxygen homeostasis, are direct activators of Twist1 in response to hypoxia. This indicates that in a hypoxic tumor environment, Twist1 activation may be implicated in cancer cell survival.

Two previously described Twist1 targets, N-cadherin [220] and PDGFR [34] promote invasion by facilitating degradation of the extracellular matrix (ECM). N-cadherin stabilization of FGFR can lead to expression of the MMP-9 metalloprotease [235] and induction of PDGFR is necessary for invadopodia formation in cancer cells [34]. Interestingly, Twist1 also promotes metalloprotease expression and ECM degradation by suppressing expression of Tissue Inhibitor of Metalloproteinases-1 (TIMP1) [236].

Twist1 can promote oncogene expression and was found to be necessary for Her2/neu induction by Integrin-linked Kinase (ILK) [237].
Expression of Twist1 siRNA leads to a decrease in Her2/Neu protein in the Her2/neu-positive breast cancer cell line SKBR3 [237].

1.4 Summary

Both Twist1 and Snail2 are induced by common signaling pathways and play important roles in many of the same developmental processes such as limb [111], [172], heart [108],[185] and neural crest formation [186], [93]. In Xenopus, Snail2 rescues Twist1 loss of function in neural crest and mesoderm formation [238] and Twist1 expression can stabilize Snail2 protein [90]. Hence, their relationship is clearly an evolutionarily important one. Given the complex interplay between numerous pathways in the induction of both Twist1 and Snail2, it is likely that this relationship is also not a straightforward one. Nonetheless, my studies will attempt to begin the process of dissecting out the roles each play individually and in concert to promote EMT and cancer progression.
CHAPTER II:

SNAIL2 IS AN ESSENTIAL MEDIATOR OF TWIST1-INDUCED

EPITHELIAL-MESENCHYMAL TRANSITION AND

METASTASIS
2.1 Introduction

For carcinoma cells to break away from neighboring cells and invade, they must lose cell-cell adhesion and gain motility [239]. A highly conserved developmental program named Epithelial-Mesenchymal Transition (EMT) has been implicated in promoting dissemination of single carcinoma cells from primary epithelial tumors [240]. The EMT program is activated during many developmental processes – such as mesoderm formation and neural crest development [241]. During EMT, cells lose their epithelial characteristics, including cell adhesion and polarity, and acquire a mesenchymal morphology and the ability to migrate. Biochemically, cells switch off the expression of epithelial markers, such as adherens junction protein E-cadherin and turn on mesenchymal markers, including vimentin and fibronectin [242].

Functional loss of E-cadherin in an epithelial cell has been considered a hallmark of EMT. Forced expression of E-cadherin in certain invasive carcinoma cells can inhibit their ability to invade and metastasize; conversely, blocking E-cadherin function in non-invasive tumor cells activates their invasiveness and metastatic abilities [243, 244]. A partial loss of E-cadherin is also associated with carcinoma progression and poor prognosis in various tumor types [245].
Transcriptional modulation of the E-cadherin gene promoter is a key event to suppress E-cadherin expression during EMT. The human E-cadherin promoter contains E-box elements that are responsible for its transcriptional repression [246, 247]. Several Zn-finger transcription factors, including Snail1 [248, 249], Snail2 [250], ZEB1 [6], and ZEB2 [251], are capable of directly binding to the E-boxes of the E-cadherin promoter to repress its transcription. In addition to these Zn-finger transcription factors, transcription factors belonging to other families have also been shown to be able to regulate EMT in culture and during development. In a search for genes involved in mouse mammary tumor metastasis, the bHLH transcription factor Twist1 is found to be capable of inducing EMT in human mammary epithelial cells. Our previous study also found that the Twist1 transcription factor was essential for the ability of tumor cells to metastasize from the mammary gland to the lung in a mouse breast tumor model [11].

During EMT in metastasis and in embryogenesis, many EMT-inducing transcription factors are often activated simultaneously, such as expression of Twist1, Snail1, Snail2 and ZEB2 in neural crest cells [252, 253]. To understand how these transcription factors coordinate the EMT program, we have used an inducible Twist1 system to address whether
and how Twist1 activates other EMT-inducing transcription factors to suppress E-cadherin and promote EMT and tumor metastasis.
2.2 Materials and Methods

Cell lines

The HMLE and HMLER cell lines are human mammary epithelial cell lines immortalized with hTERT as well as the SV40 large T antigen and the H-Ras oncoprotein (HMLER only) [254]. These lines were obtained from Dr. Robert Weinberg’s lab at the Whitehead Institute for Biomedical Research. HMLE and HMLER were grown in a 1:1 mixture of MEGM Bullet kit (Lonza) and DMEM/F12 (Cellgro) supplemented with Insulin (10µg/mL), human EGF (10ng/mL), L-glutamine (1.2µg/mL) and hydrocortisone (0.5µg/mL). The SUM1315 and MCF7 breast cancer cell line were obtained from the American Type Culture Collection [255]. SUM1315 cells cultured in F12 media (Cellgro) with 5% FBS, human EGF and insulin. MCF7 cells were grown in DMEM supplemented with 10% FBS. All cell lines were grown to a maximum 80% confluency before passaging.

Plasmids and constructs

The Twist1 and Twist1-ER cDNAs in pWZL-Blast (pWB) retroviral vector were previously described [53]. The shTwist1-3 pSP108 lentiviral constructs were previously described [11]. psP108, shGFP-pSP108, pRRLSIN-GFP, H-Ras V12-pBabe, pWzL- pUMCV3 Puro and pCMVΔ8.2R were all purchased through Addgene. shScramble-pLKO.1 was purchased from
Openbiosystems. The pGL4 Luc2 Firefly luciferase reporter vector and Renilla luciferase reporter vector (pGL4[Rluc]) were purchased through Promega. The E47 expression construct was a gift from Dr. Cornelis Murre, University of California, San Diego.

**Generation of Snail2-pGL4 constructs**

BAC clone RP11-845K20 containing the promoter region of human Snail2 DNA was purchased from the Children’s Hospital Oakland Research Institute (CHORI) BACPAC Resources Center. The BAC clone contained 208 kb DNA from chromosome 8 spanning from 49799872 to 50008787. Clones were expanded in DH5-α competent bacteria and DNA extracted and purified. Primers (Table 1) were used to expand the putative Twist1 binding region identified in ChIP-seq results via PCR. PCR products were purified and cloned into the pGL4 luciferase vector (Promega) using Kpn1 and Xhol restriction sites. The mutant Snail2 promoter was generated by mutating the CATCTG E-box to AGTAGG via PCR overlap extension [256]. PCR products were cloned into the pGL4 luciferase vector using Kpn1 and Xhol restriction sites.

**Luciferase reporter assay**

MCF7 cells were seeded in triplicate on a 48-well plate at 8.0x10⁴ cells/well along with 1mL DMEM/well. After 24 hours, 500μL of media was
removed and cells were transfected as follows: 40μL of DMEM was incubated with 3nL/ng DNA of TransIT-LT-1 transfection reagent (Mirus Bio) for 15 minutes at room temperature. After incubation, 10ng of pGL4[Rluc] Renilla plasmid was added to each sample along with 150ng the appropriate reporter pGL4 Firefly plasmid (Snail2prom-Luc or Snail2mut-Luc) and 150ng of Twist1 and E47 as appropriate. The mixture was incubated for 30 minutes at room temperature. After 30 minutes, the mixture was added to dropwise to each well and incubated for 24 hours. The next day, cells were lysed using the Promega Passive lysis buffer. Cell lysates were processed and manually assayed on a luminometer using the Dual-Luciferase Reporter Assay system and protocol (Promega). The firefly luciferase activity was normalized to that of Renilla luciferase.

**Generation of shSnail2-1 and shSnail2-2 pSP108 constructs**

The target sequence for shSnail2-1 was designed using the Whitehead Institute for Biomedical Sciences shRNA design website [257] with custom pattern N6AN6TNNHN5A. The Snail2-2 shRNA targeting sequence was designed using Invitrogen’s BLOCK-iT RNAi designer software (https://rnaidesigner.invitrogen.com/rnaiexpress/). All target sequences were screened for unique targeting to Snail2 using NCBI’s Basic Local Alignment Search Tool (BLAST). Oligonucleotides were designed by
incorporating target sequences in addition to flanking BamH1 and BstB1 restriction enzyme sites. All primers are listed in Table 2.

Lentiviral constructs containing shRNA sequences were generated by first cloning each shRNA into pSP-81 vector, then subcloning into the pSP-108 lentiviral vector as follows: Complimentary oligos were re-suspended at 1μg/μL and annealed together by adding 5μL of each sense and antisense oligo to 35μL of double-distilled water along with 5μL of 10X NEB buffer 2 (New England Biolabs). The mixture was incubated for 4 minutes at 95°C, then 10 minutes at 70°C in a 500 mL beaker. The samples were left in the beaker as the water was allowed to cool to room temperature. pSp81 vector was digested using BstB1 and BamH1 purchased through NEB (New England Biolabs). Resulting DNA was purified. Oligos were annealed to pSP-81 for 2 hours at room temperature. Ligated DNA was transformed into DH5-α competent cells and bacteria plated on LB-ampicillin (LB-Amp) Agar plates. Colonies were selected, grown, DNA purified and digested with BamH1 and Sal1. The resulting digested DNA was run on a 1% Agarose gel. Bands at 300bp were excised and purified. The pSP-108 lentiviral vector was linearized with BamH1 and Sal1, run on a 1% Agarose gel and purified. The purified shRNA inserts were ligated into the pSP-108 vector for two hours at room temperature. The resulting ligated samples were transformed into DH5-α
competent cells and plated onto LB-Amp agar plates. Colonies were selected and grown in LB-amp, purified and digested with BamH1 and Sal1 to screen for correct clones. Selected clones were transformed into DH5-α competent cells and plated on LB-Amp agar plates. Colonies were selected and grown in LB-Amp and DNA was purified.

**Viral production and Infection**

Stable cell lines were created via infection of HMLE cells using either lentiviruses, pBabe-puro or pWZL-blast retroviruses. Viruses were generated as follows: 293T cells were plated at 1x10^6 cells per 6cm dish in DMEM (Cellgro) /10%FBS one day before transfection. After 18 hours, cells were transfected as follows: 6µL TransIT-LT1 (Mirus Bio) was added to 150 µL DMEM and incubated for 20 minutes at room temperature. 1µg of viral vector along with 0.9µg of the appropriate gag/pol expression vector (pUMCV3 for pBabe or pWZL or pCMVΔ8.2R for pSP108 vectors) and 0.1µg VSVG expression vector were added to the DMEM/LT-1 mixture. The mixture was incubated for 30 minutes at room temperature. After incubation, the mixture was added to the 293T cells and incubated overnight at 37°C. The following day, the media was replaced. Viral supernatant was harvested at 48 and 72 hours post transfection, filtered through a 0.45µm filter and added to the appropriate HMLE recipient cell
lines along with 6µg/ml protamine sulfate. Stable cells lines were selected
with either 2µg/ml puromycin or 10 µg/ml blasticidin as required.

**Time course**

During the various time courses, cells were seeded in 10-cm dishes
at 600,000 cells/plate two days before protein and RNA harvesting. 4-
hydroxytamoxifen (4-OHT) was added at 20nM as appropriate upon
seeding. Cells were at 80% confluence at time of RNA and protein
collection.

**Protein collection**

Protein was collected by first rinsing the cells with ice-cold DPBS and
lysing in 600µL of SDS lysis buffer (100mM NaCl, 1%SDS, 100mM Tris-Cl)
previously heated to 95ºC. After lysing, samples were collected and
heated to 95ºC for 5 minutes. Each sample was subsequently sonicated
and protein was quantified.

**RNA extraction and quantitative PCR**

Cells were washed with RNAse-free DPBS just prior to RNA
extraction. Total RNAs were extracted from cells using the RNeasy Mini Kit
coupled with DNase treatment (Qiagen). RNA was reverse transcribed
using the High Capacity cDNA Reverse Transcription Kit (Applied
Biosystems). cDNAs were analyzed in triplicate via quantitative PCR using SYBR-Green Master PCR mix (Applied Biosystems) and relevant primers. Reaction conditions were as follows: 40 cycles, 10 min. 95°C, 30 sec 95°C, 1 min. 50°C, 30 sec 72°C. Relative mRNA concentrations were determined by $2^{-(Ct-Cc)}$ where Ct and Cc are the mean threshold cycle differences after normalizing to GAPDH values. Primers used for qPCR are listed in Table 3.

**Quantification of relative Snail2 protein on western blot**

Relative levels of Snail2 proteins between HMLE-shControl, shSnail2-1 and shSnail2-2 were calculated using ImageJ by normalizing the signal of Snail2 to α-Tubulin at each individual time point. Protein levels were graphed by normalizing each cell line at each individual time point to time zero.

**Immunofluorescence**

8-chamber coverslip culture slides (Nunc Lab-Tek) were coated with 0.1% porcine gelatin for 20 minutes and quickly rinsed with PBS. Induced HMLE-TwistER cell lines were seeded three days before each appropriate time point using 12,000 cells/well in 500µL MEGM/DMEM F12 media. Cells were incubated at 37°C with 4-OHT as appropriate. After 3 days, wells were rinsed briefly with PBS that was supplemented with 0.5mM CaCl₂ and
0.5mM MgCl₂ (PBS+) and fixed for 15 min in 4% paraformaldehyde (PFA)/PBS+. Cells were then rinsed with PBS+ and permeabilized with 0.1% Triton X-100/PBS for 10 min., rinsed again in PBS+ and blocked with 5% goat serum/PBS+ for 1 hour. After blocking, wells were rinsed briefly with PBS+ and samples were incubated with appropriate primary antibodies diluted 1:200 in blocking solution overnight at 4°C. The next day, each well was rinsed 3x with PBS+ and incubated for 2 hours at room temperature in the dark with the appropriate Alexa Fluor 488 (green) or 594 (red) secondary antibodies (Invitrogen) diluted in blocking buffer at 1:1000. Each well was briefly rinsed 6x with PBS+. SlowFade/DAPI (Invitrogen) was added to each chamber and wells were then imaged at 20X using Metamorph software. Antibodies used were E-cadherin (BD Biosciences), β-catenin (BD Biosciences), vimentin V9 (NeoMarkers), and Fibronectin (Sigma).

**Invasion and migration assays**

10µg of Matrigel diluted in DMEM was overlaid on 8mm Transwell permeable supports, dried overnight, and reconstituted for 1 hour with standard MEGM media excluding epidermal growth factor (EGF). 40,000 cells were then plated onto each well in triplicate and incubated for 72 hours at 37°C in 750µL complete MEGM media on the bottom chamber and 250µL MEGM media lacking EGF on the top chamber. After 72 hours, the media was aspirated and cells were fixed for 30 minutes in 4% PFA/PBS
then washed in PBS and subsequently soaked and stained with 0.1% crystal violet for 10 min. Each well was rinsed 3x with MilliQ water. A swab was then used to wipe off cells on the top membrane. The chamber was again washed with PBS and dried. Crystal violet was released using 50µL 10% acetic acid and the absorbency was measured at 540 nm using a spectrophotometer. Identical protocols were used for migration assays using transwells without Matrigel.

**Chromatin immunoprecipitation**

5-15 cm plates of HMLE cells were seeded at 1.8x10^6 cells for both 4-OHT and untreated conditions. After 4 days of incubation, HMLE Twist1-ER cells at 85% confluence were cross-linked with 1% PFA for 10 minutes. Cells were then incubated for 5 minutes with 0.4 M glycine to quench the PFA. Plates were placed on ice, rinsed twice with cold PBS and cells scraped off and transferred to a chilled conical tube. The conical tubes were spun at 2000xg at 4°C for 10 minutes and supernatant aspirated. Pellets were washed in ice cold PBS, spun at 4°C, 2000xg and supernatant aspirated. The process was repeated with ice-cold buffer 1 (0.25% Triton X-100, 10mM EDTA, 500µM EGTA, 10mM HEPES at pH6.5) and 2 (200mM NaCl, 1mM EDTA, 500µM EGTA, 10mM HEPES, pH6.5). Cells were then re-suspended in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-Cl at pH 8.1, protease inhibitor cocktail) and sonicated. The chromatin quality was
checked by heating 20μL of sonicated chromatin to 65°C for 30 minutes, adding RNAse then visualizing on a 1% agarose gel. Sheared chromatin was seen at 0.5-1kb. The main chromatin sample was spun down at 14,000 rpm at 4°C for 10 minutes, supernatant was harvested and diluted 1:10 in dilution buffer (1% Triton X-100, 2mM EDTA, 150mM NaCl, 20mM Tris-Cl at pH 8.1, protease inhibitor cocktail). For immunoprecipitation, 50μL of Protein G Dynabeads (Invitrogen) were washed in PBS-0.1%BSA (PBS-BSA) at pH 8.1 and collected via magnet. The process was repeated two times. 5μg of anti-Twist1 or anti-estrogen receptor antibody was added to the beads and incubated 2 hours at 4°C beads were then collected via magnet, and washed in PBS-BSA three times. 1mL of diluted lysates was added to the beads and incubated overnight at 4°C. The next day, beads were sequentially washed at 4°C for 10 minutes in 1mL of each TSE1 (0.10% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-Cl, pH8.1, 150mM NaCl), TSE2 (0.10%, 1% Triton X-100, 2mM EDTA, 20mM Tris-Cl, pH8.1, 500mM NaCl), Buffer3 (250mM LiCl, 1% NP-40, 1% Deoxycholate, 1mM EDTA, 10mM Tris-Cl at pH8.1) and washed twice with 1mL Tris-EDTA (TE). Each sample was spun down at 4°C, 14,000 rpm for 5 minutes and aspirated between each wash. Fragments were eluted from beads by rotating beads in 150μL of 1% SDS, 0.1M NaHCO₃ for 10 minutes at room temperature. Beads were then spun down at 14,000 rpm for 5 minutes at room
temperature. The eluate was saved in a separate tube. The process was repeated and both eluates were pooled. Samples were reverse cross-linked by heating the 300μL of eluate at 65°C overnight. 300μL of input samples were also heated at 65°C overnight. DNA was purified by phenol-chloroform and ethanol precipitation. DNA was amplified via PCR using the Roche Faststart kit using the following settings: 95°C for 4 minutes, touchdown from 65°C by 1°C each cycle for 15 cycles and 55°C for 45 cycles for Snail2 and 30 cycles for GAPDH. PCR fragments were analyzed on a 1.5% agarose gel. Primers used for PCR amplification steps are found on Table 4.

**ChIP-sequencing**

For the ChIP-sequencing analysis, an Illumina sequencing library was prepared from a 10-ng sample of Twist1 ChIP DNA from HMLE-Twist1-ER cells incubated 4 days with 4-OHT, using the ChIP-seq Sample Preparation Kit provided by Illumina. DNA fragments were repaired to generate blunt ends, and a single A nucleotide was added to each end. Double-stranded Illumina adaptors were ligated to the fragments. Ligation products were amplified by PCR, and the DNA between 200 and 400 bp was gel purified. The DNA library was sequenced on the Illumina Genome Analyzer. Cluster generation, linearization, blocking, and sequencing primer reagents were provided in the Solexa Cluster Amplification kits. The
resulting 36-nucleotide sequence reads were mapped to the human genome using the Illumina software suite. Data was graphed and represented by inserting the generated data into the UC Santa Barbara Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway?org=Human&db=hg19&hg sid=193234847) using the March 2006 assembly.

**Promoter sequence alignment**

Analysis of the Snail2 promoter E-box sequences across species was done using ConTra online software as described [258].

**Subcutaneous tumor implantation and metastasis assay**

1 million HMLER cells expressing either the Twist1-pWB+ShScramble-pLK0.1+pRRL (GFP) construct or 4 million HMLER cells expressing Twist1-pWB+shSnail2+pRRL (GFP) were trypsinized and re-suspended in 50μL ice cold PBS, mixed 1:1 with matrigel and kept on ice. Half of the cells were then injected subcutaneously into the left and right flanks of Nude mice. Primary tumors were allowed to develop and their sizes were measured weekly using precision calipers. Mice were sacrificed when primary tumors reached approximately 2 cm in diameter. Lungs were harvested and imaged for GFP positive tumor cells as described below.

**Quantification of GFP-labeled lung metastases**
Lungs of injected mice were removed and each lobe was individually examined and imaged under a fluorescence dissection microscope using Image Pro. The total number of GFP-positive lung colonies was quantified using the ImageJ cell counter software.

**Quantification of tumor burden**

Tumors were completely excised upon necropsy and weighed individually. Graphed tumor weights represent total primary tumor burden. Error bars represent Standard Error of the Mean (SEM). Statistical analysis to determine significance between the samples was performed using a paired T-test. After weighing, primary tumors were embedded in paraffin, sectioned at 10μm and stained with E-cadherin antibody (BD biosciences) and hematoxylin.

**Histology and immunohistochemistry**

Tumors were stained for E-cadherin by first washing sections twice in PBS+, fixing in 4% PFA for 30 minutes, washing in PBS+ and permeabilized for 5 minutes with 0.1% Tween-20 in PBS+. Sections were then washed three times in PBS and blocked with 20% goat serum in PBS+/0.1% Tween20 (PBST). After blocking, the tissue was washed three times in PBS incubated for two hours at room temperature in primary antibody diluted in blocking solution. Subsequently, tissues were washed three times with PBS and
stained with biotinylated secondary antibody using the Vectastain ABC kit and protocol. Nuclei were stained via hematoxylin by dehydrating tumors in xylene twice for 5 minutes, 100% ethanol (EtOH) twice for 5 minutes, 95% EtOH twice for 1 minute, 80% EtOH for 1 minute and 70% EtOH for 1 minute. Sections were then rinsed once in PBS and twice deionized water then left deionized water for 5 minutes and subsequently dipped three times in eosin, rinsed twice in deionized water and left in deionized water for 5 minutes. Tissue was subsequently dehydrated in 70% EtOH for 30 seconds, blow-dried and mounted with permount solution.

**Bioinformatics and statistical analysis**

NCBI’s Gene Expression Omnibus (GEO), a public repository of microarray data, was used as the source for three published microarray datasets derived from breast cancer patients to determine the correlation between Snail2 and Twist1 expression in breast cancer as well as rank the expression of 22,283 gene probe sets against Twist1 expression. GEO identifiers and sample size for the three studies were GSE1456 (STOCKHOLM, n=159), GSE2034 (EMC, n=286) and GSE3494 (UPPSALA, n=236).

First, to achieve the same distribution of signals across samples within a study, quantile-normalization was performed on an integrated
dataset of .CEL files from individual patients [259]. Probe-level measurement values were converted into gene-level aggregate values using the Median-Polish algorithm [260]. To determine the correlation between Twist1 and Snail2 expression in the breast cancer samples, measurements of the two probe sets were plotted with a linear regression model fit and the Spearman correlation was calculated between the two genes within individual data sets. Twist1 (y-axis) microarray measurements were plotted against Snail2 (x-axis) measurements. Each dot represents a pair of probeset-level aggregated values for the corresponding gene. Genes rankings for correlations with Twist1 were based on the Spearman correlation coefficients for each gene against Twist1.
Table 1: *Snail*2 promoter primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>Wild-type Snail2 Fw</td>
<td>ATGCAGGTACCGAGTAGCGCAGCGCCCTC</td>
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<tr>
<td>Wild-type Snail2 Rv</td>
<td>GTAACCTCGAGTTACGGAACCTGAGCCCCTT</td>
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<tr>
<td>Mutant Snail2 Fw</td>
<td>CGCGCTGGCGCTGCACCAAGTGAGGGAGCCAGGCGGG</td>
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<tr>
<td>Mutant Snail2 Rv</td>
<td>CCCTGCCCCGCTGGCTTCCCTACTTGCTGCCAGCCAGGCGCG</td>
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Table 2: shRNA oligonucleotides

<table>
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<th>Name</th>
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<tr>
<td>shSnail2-1 Fw</td>
<td>CGAAATCTGCAGACCACCATTTTCTGATGTTTTCAAGAG</td>
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<tr>
<td>shSnail2-1 Rv</td>
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<td></td>
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<td></td>
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## Table 3: Quantitative PCR primers

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### Table 4: PCR primers used for chromatin immunoprecipitation assay

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2.3 Results

2.3.1 Twist1 indirectly suppresses E-cadherin transcription to promote EMT

To understand how Twist1 suppresses E-cadherin expression during EMT, we have generated a fusion protein between Twist1 and the modified hormone-binding domain of estrogen receptor (Twist1-ER), which can be activated specifically upon binding to 4-hydroxytamoxifen [261]. We expressed Twist1-ER in the immortalized human mammary epithelial (HMLE) cells via retroviral infection. HMLE cells expressing Twist1-ER underwent morphological changes of EMT upon 4-hydroxy-tamoxifen treatment in 10-12 days (Figure 1A). We harvested these cells at day 18 to characterize the EMT markers and found that activation of Twist1 resulted in loss of cell adhesion and gain of mesenchymal differentiation in HMLE cells (Figure 1B), identical to the EMT program induced by Twist1 [11].

We next examined the expression of E-cadherin mRNA and protein following Twist1 activation. Interestingly, both E-cadherin mRNA and protein levels remained unchanged during the first 7 days after Twist1 activation and only started decreasing afterwards. By the end of the 12 day time course, both E-cadherin mRNA and protein decreased drastically (Figure 1C and 1D), which correlated with loss of cell adhesion in cell morphology (Figure 1A). Given that 4-hydroxytamoxifen activates the
Twist1-ER fusion protein immediately [261], the 6-8 days required to reduce E-cadherin mRNA and protein levels suggest that Twist1 does not directly suppress E-cadherin transcription during EMT.
Figure 1: Twist1 indirectly suppresses E-cadherin transcription to promote EMT. A. Bright-field images of HMLE cells expressing Twist1-ER or a control vector before and after 12 days of 20 nM 4-hydroxytamoxifen (4-OHT) treatment. B. Lysates from HMLE cells expressing Twist1-ER or a control vector were collected before and after 18 days of treatment with 4-OHT, analyzed by SDS-PAGE, and probed for Twist1-ER protein, E-cadherin, β-catenin, γ-catenin, fibronectin, vimentin, N-cadherin, and α-Tubulin. C. Real-time PCR analysis of E-cadherin mRNA expression in HMLE cells expressing Twist1-ER or a control vector treated with 4-OHT. D. Lysates from HMLE cells expressing Twist1-ER or a control vector were collected during 12 days of 4-OHT treatment, analyzed by SDS-PAGE, and probed for E-cadherin and α-Tubulin. HMLE cells expressing Twist1 or a control vector are used as the negative or positive control.
2.3.2 Induction of the transcription factor Snail2 is required for Twist1-induced EMT

Since Twist1 may not directly suppress E-cadherin transcription, we hypothesized that Twist1 might activate other Zn-finger transcription factors such as Snail1, Snail2, ZEB1 and ZEB2 to suppress E-cadherin transcription. During Drosophila mesoderm formation, Twist1 directly induces the expression of Snail1 to promote EMT [262]. To test whether Twist1 induces the Snail family transcription factors in human cells, we examined Snail1 and Snail2 expression following Twist1 activation. Interestingly, Snail2 was induced within four hours upon Twist1 activation (Figure 2A), while Snail1 was only significantly induced at Day 12 (Supplementary Figure 1). Induction of Snail2 protein was also observed in HMLE-Twist1-ER cells two days upon Twist1 activation and in HMLE cells expressing Twist1 (Figure 2B). Since we are interested in possible direct target genes that Twist1 regulates to suppress E-cadherin, we hypothesized that induction of Snail2 by Twist1 could play a key role in the transcriptional repression of E-cadherin.

To test whether induction of Snail2 is required for the ability of Twist1 to induce EMT, we designed two shRNA lentiviral constructs specifically against human Snail2 and expressed them in HMLE cells. Both shSnail2-1 and shSnail2-2 could efficiently suppress Snail2 mRNA and protein below
the basal level in HMLE cells (Figure 2A-2C). We expressed both shRNAs against Snail2 in HMLE cells expressing the inducible Twist1-ER construct and treated the cells with 4-hydroxy-tamoxifen. Interestingly, in the absence of Snail2, Twist1 was not able to induce the morphological changes associated with EMT as observed in HMLE cells expressing a control shRNA construct. Instead, these cells with shSnail2 constructs maintained an epithelial morphology with intact cell-cell adhesions and a cobblestone-like morphology even after 18 days (Figure 2D). Also knockdown of Snail2 did not affect Twist1 expression in these cells (Figure 2B). Together, these data indicate that induction of Snail2 is required for the ability of Twist1 to promote EMT in HMLE cells.
**Figure 2: Induction of the transcription factor Snail2 is required for Twist1-induced EMT.**  
A. Real-time PCR analysis of Snail2 mRNA expression in HMLE cells expressing Twist1-ER or a control vector treated with 4-OHT and in HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl during 12 days of 4-OHT treatment. B. Lysates from HMLE cells expressing Twist1 or a control vector, from HMLE cells expressing Twist1-ER, and from HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl during 12 days of 4-OHT treatment were collected, analyzed by SDS-PAGE, and probed for Twist1 or Twist1-ER, Snail2, and α-Tubulin. C. Quantification of the Snail2 protein level shown in the bottom panel of B. The relative Snail2 protein level was calculated by normalizing the signal of Snail2 to α-Tubulin, and the values at individual time points were compared to the value at Day 0 for each cell line. D. Bright-field images of HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl before and after 12 days of 4-OHT treatment.
A. Graph showing relative Snail2 mRNA levels over time for Twist1-ER and control conditions.

B. Western blot analysis showing Snail2, Twist1, and β-Actin protein levels over time for Twist1-ER and control conditions.

C. Bar graph showing relative Snail2 protein levels over time for different conditions.

D. Images showing cell morphology changes over time for different conditions.
Supplementary Figure 1: Induction of Snail1 mRNA is indirect upon Twist1 activation. Real-time PCR analysis of Snail1 mRNA expression in HMLE cells expressing Twist1-ER or a control vector treated with 4-OHT.
2.3.3 Induction of Snail2 is specifically responsible for suppression of E-cadherin in response to Twist1 activation

To determine how suppression of Snail2 affects expression of various EMT markers, we closely followed the entire time course of Twist1 activation in HMLE-Twist1-ER cells expressing shSnail2 or a control shRNA. First, we examined the expression of E-cadherin following the induction time course, since Snail2 is a direct repressor of E-cadherin [250]. Indeed, suppression of Snail2 in HMLE-Twist1-ER cells completely inhibited the progressive reduction of E-cadherin mRNA and protein upon 4-hydroxytamoxifen treatment (Figure 3A and 3B). In HMLE-Twist1-ER cells expressing a control shRNA, E-cadherin expression started decreasing at Day 7 after Twist1 activation; at Day 18, E-cadherin protein was completely lost and b-catenin disappeared from cell membranes, indicating the loss of adherens junctions upon Twist1 activation (Figure 3C). In contrast, in HMLE-Twist1-ER cells expressing shSnail2, both E-cadherin and b-catenin remained at cell-cell junctions even after 18 days of Twist1 activation (Figure 3C). These results indicate that Snail2 is critical for the ability of Twist1 to suppress E-cadherin and induce EMT.
Figure 3: Induction of Snail2 is required for suppression of E-cadherin during Twist1-induced EMT.  A. Real-time PCR analysis of E-cadherin mRNA expression in HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl during 12 days of 4-OHT treatment.  B. Lysates from HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl were collected during 12 days of 4-OHT treatment, analyzed by SDS-PAGE, and probed for E-cadherin and α-Tubulin.  C. Cells from HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl were immunostained for E-cadherin and β-catenin (green) and nuclei (DAPI, blue) at Day 0, 7 and 18 after 4-OHT treatment.
To understand whether suppression of E-cadherin by Snail2 is the only effect of Snail2 on the entire EMT program, we examined the mRNA and protein expression of EMT mesenchymal markers in response to Twist1 activation. Interestingly, we found that unlike E-cadherin, the initial induction of other EMT markers, including fibronectin, vimentin, and N-cadherin occurred normally in the absence of Snail2 in HMLE-Twist1-ER cells upon 4-hydroxytamoxifen treatment (Figure 4A). However, at Day 7 when E-cadherin was not suppressed in HMLE-Twist1-ER cells expressing shSnail2, fibronectin, vimentin, and N-cadherin proteins failed to increase further to reach the high levels of induction observed in HMLE-Twist1-ER cells expressing a control shRNA (Figure 4A and 4B). We also performed immunofluorescence analysis to examine the expression and localization of these EMT markers and observed similar effects (Supplementary Figure 2). In addition, we attempted to overexpress Snail2 in HMLE cells and found that Snail2 alone was not sufficient to induce EMT in HMLE cells (data not shown). These results suggest that, although Snail2 is not directly responsible for the induction of fibronectin, vimentin, and N-cadherin during EMT, loss of Snail2 and E-cadherin suppression completely blocks EMT morphogenesis and attenuates the expression of mesenchymal markers, including fibronectin, vimentin, and N-cadherin.
Figure 4: Suppression of Snail2 attenuates expression of mesenchymal markers during Twist1-induced EMT. A. Real-time PCR analysis of fibronectin, N-cadherin, and vimentin mRNA expression in HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl during 12 days of 4-OHT treatment. The final folds of induction are indicated in each graph. B. Lysates from HMLE-Twist1-ER cells expressing shSnail2-1, shSnail2-2 or shControl were collected during 12 days of 4-OHT treatment, analyzed by SDS-PAGE, and probed for fibronectin, N-cadherin, vimentin, and α-Tubulin.
Supplementary Figure 2: Expression of fibronectin and vimentin are reduced upon knockdown of Snail2. Cells from HMLE-Twist1-ER cells expressing shRNAs against Snail2 or a control shRNA were immunostained for fibronectin and vimentin (red) and nuclei (DAPI, blue) at Day 0, 7, and 18 after 4-OHT treatment.
2.3.4 Snail2 is a conserved direct transcriptional target of Twist1 in amniotes.

Since Snail2 is induced immediately upon Twist1 activation and plays a critical role in Twist1-induced EMT, we tested whether Twist1 directly binds to the Snail2 gene promoter to activate its transcription. Using Chromatin Immunoprecipitation (ChIP) coupled with high-throughput sequencing, we identified a distinct Twist1 binding E-box domain 306bp from the transcription start site of the human Snail2 promoter (Figure 5A). In contrast, there is no significant enrichment for the 10kb promoter regions of Snail1 or E-cadherin (Figure 5A). To demonstrate that Twist1 specifically binds to the Snail2 promoter, we treated HMLE-Twist1-ER cells with 4-hydroxytamoxifen for four days and performed ChIP analysis to pull-down Twist1-binding DNA fragments. Both Twist1 and ER antibodies were able to specifically enrich the E-box region of the human Snail2 promoter, while there was no specific enrichment for the control GAPDH gene (Figure 5B). To test the specificity of the Twist1-binding E-box on the Snail2 promoter, we tested the ability of Twist1 to activate the isolated 606bp human Snail2 promoter by luciferase reporter assay. We found that Twist1 was able to activate the Snail2 promoter-driven luciferase activity and mutation of the E-box sequence on the Snail2 promoter reduced Twist1-reduced activation (Figure 5C). These data
suggest that Twist1 specifically and directly binds to the E-box on the human Snail2 promoter to activate its transcription.

To test whether the direct regulation of Snail2 by Twist1 is conserved throughout evolution, we searched for the consensus Twist1-binding E-box and its surrounding sequence on the human Snail2 promoter across species. Interestingly, we found that this consensus Twist1-binding sequence (ccaCAtcTGgaagcc) on the Snail2 promoter is highly conserved among amniote genomes examined, including chimpanzee, monkey, mice, rabbit, armadillo, elephant, hedgehog, and chick (Figure 5D). However, this sequence could not be found in Xenopus and Zebrafish, indicating that the direct induction of Snail2 by Twist1 is highly conserved in amniotes.
Figure 5: Snail2 is an evolutionally conserved direct transcriptional target of Twist1 in amniotes. A. Left panels are ChIP sequencing result showing the enriched Twist1-binding peaks at the +5kb to -5kb regions of the human Snail2, E-cadherin and Snail1 promoters. The Y-axis represents the amplitude of the ChIP-Seq signals. Right panel is a close-up view of the enriched Twist1-binding E-box sequence at the -306bp from the transcription start site of the human Snail2 promoter. B. HMLE-Twist1-ER cells were treated with 4-OHT for 4 days. Chromatin was immunoprecipitated using corresponding antibodies and PCR was conducted using one primer set for the Twist1-binding region on the Snail2 promoter and one control set for GAPDH. C. The 606-bp Snail2 promoter or the Snail2 promoter with E-box mutation were transfected with or without Twist1 and its dimerization partner E47 and processed for luciferase promoter assay. D. Alignment of the conserved Twist1-binding E-box (bold) in Snail2 promoters. The number at the start of the sequence indicates the distance from transcription start sites.
### A

**Snail2**

Fold enrichment (log2)

-306 tgtcccgcgcgtggctgcaccaCATCTGgaagccaggcgggcagggcaga

**E-cadherin**

Fold enrichment (log2)

-307 tgtcccgcgcgtggctgcaccaCATCTGgaagccaggcgggcagggcaga

### B

**Day**

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**Snail2**

**GAPDH**

### C

**Relative promoter activity**

- Wt + + -
- Mut - - +
- Twist1 - + +

### D

**Species**

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2.3.5 Induction of Snail2 is required for the ability of Twist1 to promote invasion and metastasis

Given that induction of Snail2 is required for activating the EMT program by Twist1, we next tested whether Snail2 plays an essential role in Twist1-mediated tumor cell invasion and metastasis. As in HMLE-Twist1-ER cells, knockdown of Snail2 also prevented constitutive Twist1-induced EMT in HMLE cells (Figure 6A). We tested whether Snail2 is required for Twist1-induced cell migration and invasion. As shown in Figure 6B, suppression of Snail2 resulted in reduced cell migration and invasion. This data indicate that cell migration and invasion induced by Twist1 require the activation of the entire EMT program. Without Snail2, Twist1 is not sufficient to promote cell migration and invasion.

We next tested whether Snail2 is required for the ability of Twist1 to promote tumor metastasis in vivo. To assess the role of Snail2 in tumor metastasis, we transformed GFP-labeled HMLE-Twist1 cells expressing shSnail2 or a control shRNA with oncogenic Ras (HMLER cells) (Figure 6A). When we implanted these cells subcutaneously in nude mice, HMLER-Twist1 cells expressing a control shRNA generated hundreds of tiny GFP+ micrometastatic lesions in the lung (Figure 6C). Interestingly, when Snail2 was knocked down, HMLER-Twist1 cells failed to form GFP+ metastatic lesions in the lung (Figure 6C), even though the primary tumors reached
similar weight as shControl tumors (Figure 6D). It is worth noting that knockdown Snail2 reduced the initial primary tumor seeding rates, probably due to the difficulty for epithelial cells to establish cell-cell adhesion after being injected subcutaneously in suspension. Therefore we allowed the Snail2 knockdown tumors to grow four more weeks in mice to reach the same primary tumor size as the control tumors (Supplementary Figure 3). Even with longer primary tumor growth time and similar final primary tumor burdens, examination of individual lung lobes revealed that suppression of Snail2 caused a 500-fold reduction in the ability of HMLER-Twist1 cells to form micro-metastasis in the lung (Figure 6D). Immunohistochemistry analysis of the resulting tumors showed that E-cadherin expression was completely absent from cell membranes in HMLE-Twist1 tumors, while suppression of Snail2 resulted in epithelial tumors with strong E-cadherin expression at cell-cell junctions (Figure 6C). Together, these results demonstrate that activation of Twist1 is sufficient, at least in some cell types, to promote tumor cells to disseminate from the primary site to distant organs. Furthermore, the data strongly indicate that Snail2 functions downstream of Twist1 to play a critical role in regulating tumor invasion and metastasis.
Figure 6: Induction of Snail2 is required for the ability of Twist1 to promote invasion and metastasis. A. Left panels are bright-field images of HMLE-Twist1 cells expressing shSnail2-1 or shControl. The right panel shows cell lysates analyzed by SDS-PAGE, and probed for Snail2, Twist1, Ras, and β-Actin. B. HMLE-Twist1 cells expressing shSnail2-1 or shControl were plated on Transwell to assay migration and on Transwell with Matrigel to assay invasion. The percentage of cells that migrated through the insert was quantified. Error bars are standard error of the mean (SEM). C. Top two panels are representative images of lungs from mice injected with HMLER-Twist1 cells expressing shSnail2-1 or shControl. The bottom panel is immunostaining for E-cadherin (brown) in primary tumors from HMLER-Twist1 cells expressing shSnail2-1 or shControl. D. The top graph is quantification of total number of GFP positive colonies in individual lungs. The bottom graph is total primary tumor weight in individual mice carrying tumors of HMLER-Twist1 cells expressing shSnail2-1 or shControl. Error bars are SEM. N = 6 mice for the shControl group, and N = 8 mice for the shSnail2-1 group. Error bars are SEM.
A

B

C

D

Migration
P = 0.0125
Invasion
P = 0.0081

Relative migration

Relative invasion

ShControl

shSnail2-1

ShControl

shSnail2-1

0
1,000
2,000
3,000
4,000
5,000

Number of metastatic colonies per lung

P = 0.0005

P = 0.182

Total final tumor weight (grams)
Supplementary Figure 3: Tumor growth curve for HLMER-Twist1 cells expressing either shControl or shSnail2.
2.3.6 Twist1 and Snail2 are frequently co-expressed in human breast tumors

To further demonstrate that a direct link between Twist1 and Snail2 exists in human tumor cells, we expressed shRNAs against Twist1 in the human breast tumor cell line SUM1315 that expresses high levels of endogenous Twist1 and Snail2. Indeed, when we examined the expression of endogenous Snail2 in these cells, we observed a dramatic reduction of Snail2 expression upon suppression of Twist1 (Figure 7A). This result indicates that expression of endogenous Snail2 is dependent on Twist1 in human breast tumor cells.

To more directly probe the association between Twist1 and Snail2 in human breast tumor samples, we analyzed three published human breast tumor gene expression datasets summing up to 681 primary breast cancers [263-265]. In each data set, we ranked all 22282 genes based on how tight their expression levels are correlated with Twist1 in individual samples. As shown in Figure 7B, in all three datasets, Snail2 was consistently ranked as one of the most correlated genes with Twist1. Furthermore, expression of Twist1 and Snail2 were positively correlated (Supplementary Figure 4). The association between Twist1 and Snail2 in human breast tumor samples provides further support for a functional link between Twist1 and Snail2 in breast tumor progression.
Supplementary Figure 4: Correlation between Twist1 and Snail2 expression in three human breast cancer gene expression datasets. Microarray measurements of Twist1 expression in LOG(2) (y-axis) are plotted against the measurements of Snail2 expression in LOG(2) (x-axis). Each dot is a pair of probeset-level aggregated values for the corresponding gene. The regression line between two genes is drawn as a solid line. The Spearman correlation coefficient, p-value, and the number of patient samples in each study are listed for each dataset.
Figure 7: Twist1 and Snail2 are frequently co-expressed in human breast tumors.  A. Lysates from SUM1315 cells expressing shTwist1 or shControl were analyzed by SDS-PAGE and probed for Twist1, Snail2, and β-Actin.  B. All 22,283 gene probe sets are ranked on the basis of their expression correlation coefficients with Twist1 in their human breast tumor datasets STOCKHOLM (n=159), EMC (n=286), and UPPSALA (n=236) and their distributions are shown in individual graphs. The rank of Snail2 is marked as a vertical line in each graph.  C. A model describing how induction of Snail2 by Twist1 suppresses E-cadherin and promotes EMT and tumor metastasis.
2.4 Discussion

Our study has identified Snail2 as the direct transcription target of Twist1 in repressing E-cadherin during the EMT program. We show that induction of Snail2 is required for the ability of Twist1 to promote EMT in culture and distant metastasis in mice. Furthermore, we identified a conserved Twist1-binding site on the proximate Snail2 promoter in amniotes. These results show that Twist1 induces Snail2 to coordinate the EMT program to promote tumor metastasis (Figure 7C).

Our study revealed an evolutionally conserved link between Twist1 and its direct transcription target Snail2 in suppressing E-cadherin transcription, thus promoting the loss of cell adhesion during EMT. In Drosophila, Twist1 directly induces Snail1 to promote EMT during mesoderm formation [262]. Our study shows that in humans, Snail2, a family member, replaces Snail1 to perform this key function downstream of Twist1 during EMT. Elevated expression of both Twist1 and Snail2 have been observed in lymph node-positive breast tumors and associated with poor outcome [266]. Twist1 and Snail2 are also functionally linked during early embryogenesis. In early Xenopus embryo, loss of Twist1 leads to a decrease in the Snail2 mRNA level, and both of them negatively regulate the expression of Cerberus, an inhibitor of Nodal, BMP, and Wnt signaling [267]. During Xenopus embryonic vasculogenesis, knockdown of Twist1 or
Snail2 provoked similar embryonic hemorrhage, and either Twist1 or Snail2 could rescue the hemorrhage defects due to Myc knockdown [268]. Together, these data show that the direct link between Twist1 and Snail2 is highly conserved and functionally important during embryogenesis and tumor metastasis.

Interestingly, induction of Snail2 is not only required for the ability of Twist1 to suppress E-cadherin, but the entire EMT morphogenesis is blocked in the absence of Snail2. Further analyses of additional EMT markers following the time course of Twist1 activation indicate that genes such as N-cadherin are able to initially increase at similar rates in the absence of Snail2. The initial immediate induction of N-cadherin is possibly due to the direct action of Twist1, together with its dimerization partner E12/E47.

Interestingly, N-cadherin failed to further increase to the high induction level after 7 days of Twist1 activation, the exact time when E-cadherin is supposed to be suppressed upon Twist1 activation. This result indicates that although Snail2 is not directly responsible for the induction of N-cadherin, induction of Snail2 and suppression of E-cadherin are essential to send out a permissive signal to allow further expression of mesenchymal markers and the progression of the EMT program.

The permissive signal that allows progression of the EMT program remains unknown. The disappearance of E-cadherin from adherens
juncti ons results in the release of its partner, b-catenin, into the cytosol, which has the potential to enter the nucleus to activate LEF/TCF-mediated transcription. Although the contribution of the adherens junction-associated b-catenin pool to transcription is still a matter of debate, several studies suggest that b-catenin-mediated transcription can further induce the expression of Snail2 [269] and Twist1 [270], thereby contributing to the EMT program. Furthermore, fibronectin and vimentin have been shown to be b-catenin target genes [271-273], which explains why suppression of Snail2 expression blocks their further induction in response to Twist1 activation. Similarly, ZEB2 has also been shown to not only suppress E-cadherin, but also partially regulate vimentin expression [274]. Together, these studies suggest that suppression of E-cadherin not only breaks the physical barrier to allow cell scattering, but also could actively contribute to activation of mesenchymal traits during EMT.

We found that Snail2 was induced four hours after Twist1 activation; however, reduction of E-cadherin mRNA only became significant seven days after Twist1 activation. This observation raises the question of whether Snail2 alone is sufficient to suppress E-cadherin transcription during Twist1-induced EMT. Several observations could help to explain this phenomenon. First, we found that two additional E-cadherin suppressing transcription factors, ZEB1 and ZEB2, were also induced upon Twist1
activation; and suppression of Snail2 drastically inhibited induction of ZEB1 and ZEB2 upon Twist1 activation. Therefore, induction of ZEB1 and ZEB2 could be required to collaborate with Snail2 to achieve drastic suppression of E-cadherin transcription. A double-negative feedback loop controlling ZEB1/ZEB2 and microRNA-200 family expression could further promote the expression of ZEB1 and ZEB2 during EMT [275-278]. Together, these factors strengthen the ability of cells to suppress E-cadherin expression during EMT. Second, recent studies found that Ajuba LIM proteins, also components of adherens junctions, can travel to the nucleus to serve as co-repressors with the Snail family transcription factors to repress E-cadherin [279, 280]. Therefore, suppression of E-cadherin during EMT progresses in a positive feedback loop. At the beginning of EMT, a slight decrease of E-cadherin causes the weakening of cell adhesion and the release of Ajuba LIM proteins; binding to Snail2 or Snail1, Ajuba LIM proteins can then further suppress E-cadherin transcription. These transcription repressors, including Snail1/Snail2, Ajuba LIM proteins, ZEB1/ZEB2, and the microRNA-200 family, function as a powerful E-cadherin suppression machine to drastically suppress E-cadherin transcription and promote EMT progression.
Chapter II is material that appeared in Cancer Research, 2011. January. 1:71(1):245-54. Casas E, Kim J, Bendesky A, Ohno-Machado L, Wolfe CJ, Yang J. The dissertation author was the primary investigator and author of this paper. Figures 7B and Supplementary Figure 4 were contributed by Jihoon Kim. ChIP sequencing data was generated by Andy Chang.
CHAPTER III:

CONCLUSIONS and FUTURE PERSPECTIVES
3.1 Summary

The Epithelial Mesenchymal Transition is powerful and dynamic genetic program that drastically changes a cell's interaction with its environment, making it more invasive and motile. Pathologic EMTs render a cell refractory to the intrinsic controls with which it co-evolved. In the absence of regulation, these dissident cells have the potential to disrupt normal organ function and can consequently be detrimental to the organism as a whole. EMTs occurring in cancer cells are an especially malignant form of EMT. Unlike in other pathologic EMT's such as those leading to localized fibrosis, EMT's in cancer cells allow and even promote the aggressive invasion of cancer cells to distant organs and tissues resulting in their destruction. Indeed, most cancer patients eventually succumb to cancer due to its metastatic spread and not the primary tumor. Given their potential as drivers of metastatic dissemination, EMTs have recently become the focus of intense study. This burgeoning field is providing important clues regarding the genetic, epigenetic, molecular and phenotypic transformations necessary for the survival and success of a cancer cell.

My studies identify Snail2 as an important direct mediator of Twist1-induced EMT and metastasis. Despite its relatively lower potency as a direct E-cadherin repressor as compared to Snail1, it is clear that Snail2 is
nonetheless an indispensable component of the Twist1-EMT pathway. A better understanding of Snail2’s role in regulating cell invasion and metastasis downstream of Twist1 will require further studies.

3.1.1 Snail2 as an indirect suppressor of E-cadherin

One important consequence of Snail2 transcriptional activation by Twist1 is the downregulation of the adherens junction protein E-cadherin. Interestingly, while previous studies have shown Snail2 expression to be sufficient to drive EMT [112] [28], I have observed that overexpression of Snail2 was not sufficient to promote EMT in immortalized human mammary epithelial (HMLE) cells. This is in agreement with previous studies showing that Snail2 does not always inversely correlate with E-cadherin expression [137], [134] and is therefore not always sufficient to promote EMT. It is clear however, that Snail2 expression, although not always sufficient to drive EMT, can be a necessary prerequisite. For example, NBT-II rat bladder carcinoma cells expressing an shRNA against Snail2 are unable to undergo EMT in response to FGF-1 treatment [112]. Thus, epithelial cells appear to have important mechanisms by which Snail2 expression is inhibited from completely repressing E-cadherin and aberrantly inducing EMT. Our HMLE cells, despite overexpressing Twist1 could not progress to
full EMT in the absence of Snail2. Further studies elucidating how Snail2 expression and EMT are controlled would answer important questions regarding epithelial maintenance, homeostasis and mechanisms that promote pathology. It is possible that induction of Snail2 after Twist1 activation primes the cell for EMT however, full and stable EMT induction requires the induction of multiple E-cadherin repressors and further epigenetic changes to permanently silence E-cadherin expression. The human SKOV3 ovarian cancer cells grown in a hypoxic environment for 12 hours undergo a transient spike in Snail2 expression followed by a transient decrease in E-cadherin [133]. Snail2 levels soon normalize again and this is followed by re-expression of E-cadherin. After 60 hours however, Snail1 is expressed and coincides with more permanent E-cadherin repression [133]. Binding assays have shown that Snail1 binds the E-cadherin promoter with a stronger affinity than Snail2 [132]. It may be that a threshold of Snail2 must be reached before E-cadherin suppression can be observed but stable E-cadherin suppression requires the expression of more potent repressors like Snail1.

Given the importance of maintaining proper epithelial structure and function, it is likely that full E-cadherin suppression requires the concerted efforts of numerous molecular signals. Snail2 appears to be an important gatekeeper in this process. Snail1 [8], Snail2 [132] and Twist1 [4] have all
been shown to bind the E-cadherin promoter to repress its transcription but Twist1 cannot itself drive EMT in the absence of Snail2 despite induction of Snail1 mRNA in our HMLE cells. Hence, transcriptional repressors of E-cadherin are themselves tightly regulated and the mere expression of one is not sufficient for transcriptional repression of E-cadherin in HMLE cells. How might the expression Snail2 regulate the actions of downstream E-cadherin transcriptional repressors? Snail2 may promote EMT by activating transcription of Axin2 leading to stabilization of Snail1. Both Snail1 and Snail2 are highly unstable proteins [132] and are quickly ubiquitinated in the absence of stabilizing signals [90],[89]. The GSK3-β kinase is a key regulator of Wnt signaling and can promote Snail1 ubiquitination [89]. GSK3-β binds nuclear Snail1 and translocates it from the nucleus to the cytoplasm [89]. In the cytoplasm, Snail1 is phosphorylated by GSK3-β and subsequently ubiquitinated [89]. The Wnt target gene Axin2 inhibits Snail1 ubiquitination by sequestering GSK3-β in the cytoplasm [281]. I have observed Axin2 mRNA induction in as little as 2 days following Twist1-ER activation in HMLE cells. Axin2 mRNA levels continue to increase after 12 days of Twist1-ER activation. Significantly, I have observed that Axin2 is not induced in Twist-ER cells expressing an shRNA against Snail2. Additionally, Snail1 mRNA levels increase regardless of Snail2 expression after 7 days of Twist1-ER activation. Therefore,
induction of Snail1 appears to be a direct result of Twist1 activation. Twist1 activation is concomitantly followed by Snail2 transcription and induction of Axin2. Snail2 expression after Twist1 activation may serve to stabilize Snail1 and lead to transcriptional inhibition of E-cadherin and EMT progression. Hence, although Snail1 is induced after Twist1-ER activation, it cannot be stabilized in the absence of Snail2 and EMT cannot progress.

Induction and stabilization of Snail1 by Snail2 may also promote E-cadherin downregulation by inducing additional E-cadherin repressors such as ZEB1 and ZEB2. Both ZEB1 and ZEB2 mRNA’s are induced approximately 7 days after Twist1-ER activation and I have observed that their induction is largely Snail2-dependent. Both ZEB1 and ZEB2 have been shown to be upregulated in Snail1-induced EMT [282],[283]. Furthermore, Snail1 can directly regulate ZEB2 protein expression by preventing the processing of a large intron located in its 5’ UTR that is necessary for ZEB2 protein expression [283].

Another possible level of E-cadherin regulation after Twist1 expression may be through repression of microRNAs. miR-200 family microRNAs maintain epithelial phenotype by suppressing expression of ZEB1 and ZEB2 [284]. In mesenchymal cells, increased levels of ZEB1 and ZEB2 inhibit promoter expression of miR-200 family microRNAs though binding of their E-boxes [285]. Overexpression of miR-200a or miR-200b is
sufficient to revert Madin Darby canine kidney Pez (MDCK-Pez) cells from mesenchymal to epithelial-like phenotype through repression of ZEB1 and ZEB2 [285]. We have not probed for the expression of microRNAs in Twist1-activated HMLE cells however human Twist1 has been detected on the miR-200 and miR-205 promoters in normal immortalized urothelial cells via ChIP [286]. Twist1 expression may initially suppress transcription of miR-200 family proteins, leading to an increase in ZEB1 and ZEB2 levels and further suppression of miR-200 microRNA thereby maintaining EMT.

3.1.2 Control of an EMT permissive signal by Snail2 and Twist1 synergy

My studies determined that Snail2 is necessary for EMT progression after Twist1-ER activation. Interestingly, Snail2 expression is not sufficient to suppress E-cadherin and promote EMT. Hence both Twist1 and Snail2 expression are required to provide a permissive signal leading to EMT progression. Although the source of this permissive signal is not yet clear, studies indicate that it may originate in the intracellular portion of the E-cadherin protein. Expression of a dominant negative E-cadherin (DN-Ecad) protein retaining only the intracellular domain of E-cadherin does not result in EMT. However, those cells that express an shRNA against E-cadherin (shEcad) lose cell-cell adhesion, upregulate expression of
mesenchymal markers N-cadherin and vimentin and downregulate the epithelial markers cytokeratin-8 and γ-catenin [21]. Furthermore, mice injected with HMEC cells expressing shE-cadherin and not DN-Ecad form metastatic colonies in the lung. This indicates that the intracellular tail of E-cadherin plays an important role in signaling the loss of cell-cell adhesion, EMT progression and invasion. The intracellular tail of E-cadherin is complexed to a number of possible signaling proteins including β-catenin, γ-catenin, α-catenin, p120 [287]. Numerous studies have shown that nuclear localization of β-catenin, a downstream mediator of Wnt signaling, is involved in promoting EMTs [27], [77], [288], [21]. Indeed, Twist1 [201] and Snail2 [25] have been shown to be direct Wnt targets. It has been proposed that β-catenin, once released from E-cadherin can travel to the nucleus to modulate gene activity and thereby EMT. Overexpression of E-cadherin has been shown titrate β-catenin proteins and inhibit target gene activation [289]. Interestingly, overexpression of constitutively active β-catenin is not sufficient to decrease E-cadherin expression or activate expression of EMT markers N-cadherin or vimentin in HMLE cells [21]. This indicates that additional factors are required for β-catenin-mediated EMT. One possible Twist1 target that may promote β-catenin-induced EMT is Snail1. Snail1 was previously shown to physically interact with β-catenin via its zinc-finger
domains to stimulate the transcriptional activity of β-catenin thereby promoting expression of Wnt target genes [290]. Snail1 and β-catenin can be regulated by GSK3-β [89], a key regulator of Wnt signaling. In the absence of Wnt signals, cytoplasmic GSK3-β phosphorylates β-catenin to promote its degradation and inhibit its nuclear translocation [24]. Nuclear GSK3-β can regulate Snail1 expression by promoting Snail1 ubiquitination. The Wnt target gene Axin2 inhibits Snail1 ubiquitination by sequestering GSK3-β in the cytoplasm. As previously mentioned, I have observed the induction of Axin2 mRNA in response to Twist1-ER activation in HMLE cells and this induction is Snail2-dependent. It is possible that the induction of Axin2 downstream of Snail2 allows stabilization of Snail1 thereby promoting its interaction with β-catenin and the expression of Wnt target genes associated with EMT. Ajuba-LIM proteins can also be released upon dissolution of cell-cell junctions and can interact with Snail proteins in the nucleus to act as co-repressors [30]. Ajuba-LIM proteins are involved in cell growth and differentiation and have been shown to regulate the actions of Snail1 and Snail2 [29]. In Xenopus, XSnail1 or XSnail2 mRNA fail to expand the neural crest in embryos depleted of Ajuba LIM proteins XLIMD1 and XWTIP [30].
3.1.3 Snail2 and cancer stem cells

The observation that tumor seeding and metastasis are inefficient processes and that not all cells constituting a tumor are themselves tumorigenic lead researchers to postulate the existence of cancer stem cells (CSC's). CSCs are defined as a population of cells within a tumor that have the ability to seed tumors in an animal hosts, self-renew and give rise to more differentiated progeny [54]. Although distinct cell populations with higher tumorigenic capacities have been isolated in tumor cells, it is unclear whether these populations are absolutely necessary for the propagation of tumor growth and metastasis. Numerous studies have demonstrated that, indeed, there appear to be select and privileged populations of cells within a tumor that have the capacity for self-renewal and tumor seeding [291]. In a search for cancer cells with tumorigenic potential, researchers injected various populations of cancer cells isolated from breast cancer patients into mice and probed for markers that specifically identified cells with high tumor-regenerating capacities. They found that those cells expressing the adhesion cell-surface marker CD44 were especially tumorigenic while those expressing the CD24 adhesion marker were unable to regenerate tumors [291]. These $\text{CD}44^{\text{high}}/\text{CD}24^{\text{low}}$ cells represented 11-35% of the total tumor population [291]. Amazingly, as little as 100 injected cells were able to
reconstitute tumors whereas unsorted populations required $5 \times 10^4$ cells [291]. Additionally, when inoculated back into mice, these CD44\text{high}/CD24\text{low} cells were able to give rise to phenotypically heterogeneous populations of cells. Hence, it appears that tumorigenicity is bestowed upon a select population with a given tumor and that these cells are responsible for cancer propagation.

Recent studies indicate that EMT activation results in the generation of CSCs [53]. Induction of EMT in HMLE cells by either Twist1, Snail1 or TGF-β lead to an increased number of cells with CD44\text{high}/CD24\text{low} signature [53]. Additionally, these cells exhibit stem cell properties including the ability to self-renew and give rise to more differentiated progeny. [53]. Notably, when comparing sorted populations of CD44\text{high}/CD24\text{low} and CD44\text{low}/CD24\text{high} cells, the study found that CD44\text{high}/CD24\text{low} cells were especially enriched in mesenchymal cell markers and expressed low levels of E-cadherin [53]. In agreement with this study, our lab also detected an induction of CD44 mRNA in HMLE cells after 4-OHT activation of Twist1-ER. Evidence suggests that CD44 may be a Wnt target. Tcf4 mutant mice show a complete loss of CD44 [292] while CD44 is overexpressed in Apc mutant mice. Tcf4 is a positive transcriptional regulator of Wnt target genes while APC negatively regulates Wnt signaling output [293]. We have shown that expression of shSnail2 in Twist1-ER activated HMLE cells
inhibits upregulation of Wnt target genes fibronectin [294] and vimentin [295]. Additionally, as previously mentioned induction of another Wnt target gene, Axin2 is also inhibited in Twist1-ER shSnail2 cells. Finally, transient expression of Snail2 in MCF7 cells leads to partial nuclear translocation of β-catenin and can also activate the TOPFLASH-Luciferase reporter (a nuclear β-catenin reporter) [296]. This implies that Snail2 may play a role in facilitating induction of Wnt target genes and inducing CSC fate downstream of Twist1. Current evidence has recently emerged supporting this hypothesis. Snail2 overexpression in MCF7 and MCF-10A breast cancer cells leads to induction of a CD44+/CD24- cell population [297]. Additionally, MCF7 cells exposed to TNFα become more invasive and upregulate CD44 in a Snail2-dependent manner [296]. Also, MCF7 cells stably transduced with the NF-κB subunit p65 increase mammosphere formation (MS) capacity, an indicator of cell stemness whereas MS formation was drastically decreased in the same cells also expressing shSnail2 [296]. These findings indicate that Snail2 has the capacity to impart stemness in mammary epithelial cells leading to increased invasiveness and self-renewal capabilities. Our findings make sense in light of these results. First, my study found Twist1+shSnail2 HMLE cells to be less invasive than those expressing a control vector. Additionally, the metastatic capabilities of Twist1+shSnail2 HMLE cells were
drastically impaired compared to Twist1+shControl cells despite the fact that both cell lines overexpressed Twist1. Moreover, the impaired primary tumor growth rates in Twist1+shSnail2 HMEC cells necessitated inoculation of twice as many cells as Twist1+shControl cells. Even under these conditions, we had to wait longer for Twist1+shSnail2 HMEC tumors to grow to sufficient size to probe for lung metastasis. The decreased self-renewal capabilities in cells expressing shSnail2 are in concordance with a role for Snail2 in generating CSCs downstream of Twist1. The fact that these tumors were able to grow out at all may be due to the ability of HMEC cells to spontaneously generate CD44+/CD24- cell populations [298]. However, the inability for these tumors to seed metastatic colonies indicates that CD44 expression may be necessary for CSC renewal but may not be sufficient to endow a cell with metastatic potential. Of note, neither Twist1-ER+shSnail2 nor Twist1+shSnail2 cells showed reduced growth in vitro as compared to their control counterparts. This may indicate that spontaneous CD44+/CD24- conversion is generally inefficient in the context of the harsh in vivo environment. Future experiments looking at expression levels of CD44 in Twist1 cells vs. Twist1+shSnail2 cells will shed light on the possible role of Snail2 downstream of Twist1 in the generation of CSC and help explain its necessary role in driving tumor growth and the metastatic dissemination of cancer cells.
3.1.4 Activation of a specific arm of the Twist1 pathway by Snail2

Twist1-ER+shSnail2 cells treated with 4-OHT remain phenotypically epithelial despite activation of Twist1. Interestingly, these cells do not remain strictly epithelial in their gene expression. Inspection of mesenchymal markers such as vimentin and N-cadherin shows that mRNA expression is strongly suppressed in the absence of Snail2. Axin2 mRNA was also almost completely inhibited. Conversely, I detected Snail1 mRNA despite in activated Twist1-ER+shSnail2 cells. Hence, there appears to be a distinct group of genes that are differentially affected by the absence of Snail2. Since Twist1 cells expressing Snail2 have decreased migration, invasive and metastatic abilities, this implicates genes specifically induced by Snail2 expression in conferring characteristics essential for tumor growth and colony seeding. Comparisons of gene expression profiles in Twist1-ER vs. Twist1-ER+shSnail2 cells can be exploited to elucidate which genes or pathways are absolutely necessary for invasion and metastatic dissemination.
3.1.5 Targeting EMT in cancer

Despite many advances in cancer therapeutics, there exists a fundamental conundrum. Successful treatment of a primary tumor appears to promote the inadvertent selection of more aggressive, treatment-resistant populations of cells [299], [300], [301]. These cells may stay dormant and after a given time begin to propagate again. Recurrent growths are more refractory to conventional treatment and quickly lead to death. Whereas traditional therapies have targeted primary tumor growth inhibition, it is clear that this is not always sufficient to keep cancer indefinitely at bay. Hence, the cancer field is now focusing increasing efforts to target cancer relapse by determining the mechanisms by which chemo and radioresistance occurs.

Growing evidence indicates that cells having undergone EMT are especially resistant to drug therapies and appear to be selected for during treatment [300]. For example, L3.6pl and AsPC-1 pancreatic cancer cells selected for gemcitabine resistance downregulate E-cadherin and increase nuclear β-catenin and vimentin as compared to their parent cells [302]. Additionally, a study of breast cancer patient biopsies taken before and after treatment with lapatinib, an EGFR/HER2
inhibitor, found an increase in CD44+ cells as well as an increase in the CSCs population as evidenced by mammosphere formation [299]. Given the ability of EMTs to increase CSC populations within a tumor, they may promote chemo and radioresistance through a variety of mechanisms that are not currently entirely clear. One well established mechanism by which EMTs promote cell survival is through evasion of apoptotic mechanisms. As previously mentioned, Snail1 [144], Snail2 [37] and Twist1 [303] have all been demonstrated to selectively target apoptotic proteins and obstruct their functions. It is not surprising then that, inhibition of these transcription factors has been found to increase response to therapy in vitro [304], [233]. Hence, therapies targeting EMT factors may have promise. Indeed, there are currently a number of cancer therapies that target upstream pathways known to induce and maintain EMT. For example, numerous tyrosine kinase inhibitors such as imatinib (PDGFR inhibitor), erlotinib (EGFR inhibitor) and sorafenib (PDGFR/KIT inhibitor) have all had success in suppressing tumor growth in the clinic [305]. The search is on the way for more specific EMT inhibitors however we are still in the infancy of this new and exciting field and only time will tell if indeed targeting of CSCs or EMT will lead to higher long-term success rates in the treatment of cancer.
3.1.6 Closing

Despite the plethora of information that has amassed in the recent years we still do not fully understand how EMTs elicit such a diversity of context-dependent cellular responses and how they contribute to the spread of cancer. Both pathologic and non-pathologic EMT’s are characterized by the loss of E-cadherin and gain of mesenchymal markers, hence it is important to determine the intrinsic properties of a cancer cell that make it especially invasive in the context of EMT. This will require careful inspection of the individual components that drive EMT and contribute to cancer propagation and malignancy. My studies have begun to elucidate the downstream factors that are necessary for Twist1-induced EMT, invasion and cancer metastasis. Snail2 is essential for the suppression of E-cadherin transcription downstream of Twist1, however its induction also appears to have numerous other pleiotropic effects that permit and drive EMT and malignancy. Future studies will be focused on determining what factors and pathways are specifically controlled by Snail2 after Twist1 induction.


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