COOPERATIVITY BETWEEN EMT AND NON-EMT CELLS IN BREAST CANCERS

PROMOTES METASTASIS VIA

PARACRINE GLI TRANSCRIPTION FACTOR ACTIVATION

by

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Cooperativity between EMT and Non-EMT Cells in Breast Cancers Promotes Metastasis via Paracrine Gli Transcription Factor Activation

Thesis directed by Professor Heide L. Ford

**ABSTRACT**

In recent years, the advent of high throughput sequencing has shown that primary tumors are heterogeneous and consist of diverse cell populations that have different mutation profiles, gene expression patterns and even metastatic potential. Given that a small percentage of cells have the ability to undergo EMT and/or metastasize within the heterogeneous tumors, the overall high incidence of metastasis indicates that EMT and non-EMT cells may interact to affect metastatic outcome. Recently, fate mapping of carcinoma cells that have undergone EMT demonstrated that EMT cells themselves did not colonize secondary sites, and thus the conclusion drawn was that EMT is not required for metastasis, without considering potential crosstalk between EMT and non-EMT cells in a heterogeneous tumor. Herein, we demonstrate that potent EMT-inducing transcription factors Snail1, Twist1 and Six1 function in a non-cell autonomous manner to increase the aggressiveness of (non-EMT) cells that do not express these factors, and that Six1 is key downstream of Snail1 and Twist1 to mediate their non-cell autonomous functions. We show that all three EMT-factors non-cell autonomously activate Hedgehog/GLI signaling in non-EMT cells, and that such signaling is required to increase the aggressive properties of the non-EMT cells. We further demonstrate that EMT cells induce increased metastasis of normally weakly-metastatic non-EMT cells, and that treatment with GANT-61, a Gli1/2 inhibitor, can inhibit this effect. Furthermore, we show that Hh/GLI signaling is non-cell autonomously activated in non-EMT cells via canonical and non-canonical mechanisms, and that only use of downstream inhibitor of the Hh/GLI pathway, GANT-61, is effective in inhibiting the non-cell autonomous phenotypes downstream of all three EMT-factors, while upstream pathway inhibitors such as
cyclopamine, a Smoothened inhibitor, does not work in all contexts. Moreover, expression of each of these EMT-factors consistently positively correlates with Gli1 expression and inconsistently with ligands of the pathway in human breast cancer datasets. Finally, we show that breast cancer patient derived xenografts which express high levels of Twist1, Snail1 and Six1, exhibit activated Hh/GLI signaling and only treatment with GANT-61 (the downstream pathway inhibitor) results in decreased tumor growth, whereas treatment with IPI-926 (a cyclopamine derivative) and hence upstream inhibitor of the Hedgehog pathway, is not efficacious. Our findings establish EMT-factor induced-Gli activation in non-EMT cells, as a key mediator of metastasis, and have important implications for the use of Hh/GLI inhibitors in heterogeneous tumors where EMT is observed in a subset of cells.

The form and content of this abstract are approved. I recommend its publication.

Approved: Heide L. Ford
I dedicate this work to my parents.

Thank you for EVERYTHING. I love you.
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CHAPTER I

INTRODUCTION

Increasing evidence suggests that intratumoral heterogeneity is an important aspect of the tumor landscape. Until recently, lineal evolution was widely accepted, a theory that posits that successive mutations in a single cell could result in a homogenous tumor with one dominant clone. Evidence supporting this theory arose from genetic and molecular profiling of small areas of tumors at static time-points, which was not representative of the genetic complexity of the entire tumor, and thus only a dominant clone was often detected. However, new technologies such as single cell sequencing, next generation sequencing (NGS) as well as high resolution sequencing have allowed a much more refined look at the landscape of human tumors, revealing their heterogeneous nature, as multiple clones with varied genetic mutations have been found within the tumor. While intratumoral heterogeneity was first described many years ago, these recent data lend credence to earlier observations, and have underscored the importance of understanding both genotypic and phenotypic intratumor diversity.

Causes of Intratumoral Heterogeneity

Intratumoral heterogeneity can be divided into two main categories: genetic and non-genetic. As these types of intratumoral heterogeneity have been reviewed in detail elsewhere, they are only briefly described below.

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Parts of this chapter were reprinted with permission from Neelakantan, D., Drasin, D.J. & Ford, H.L. Intratumoral heterogeneity: Clonal cooperation in epithelial-to-mesenchymal transition and metastasis. Cell adhesion & migration 9, 265-276 (2015).
Genetic heterogeneity

High resolution sequencing in tumors has revealed important driver and passenger mutations within cells in the primary tumor \(^7,9\) and has also led to the finding that different cells within the same primary tumor can harbor different genetic mutations. These findings can be reconciled with the theory of clonal evolution \(^2,3\), in which a tumor of monoclonal origin (arising from a single clone that accumulates advantageous “tumorigenic” mutations over time) may become heterogeneous due to the inherent genetic instability of the cells within the tumor (a hallmark of cancer cells \(^14\)). Such instability results in divergent cell populations within the primary tumor that can compete or cooperate as the tumor progresses. It has also been proposed that two or more non-transformed cells within a tissue may accumulate independent mutations and evolve to form a malignant tumor only when they cooperate with one another. In this case, the tumor would be polyclonal in origin and the cells comprising the tumor would consist of different mutations depending both on the clone from which they arose, and on the changes in their genome over time \(^4\).

Regardless of whether tumors arise in a mono- or polyclonal manner, they can evolve to be relatively homogenous, with one dominant clone, or heterogeneous, containing multiple clones. In the latter case, sub-clones may eventually out-compete one another, depending on whether they contain more “advantageous” mutations, or can evolve in parallel, a mechanism known as “branched” evolution. Indeed, ultra-deep sequencing and multiregional genetic analysis of human tumors has confirmed that branched evolution occurs in various tumor types including chronic lymphoblastic leukemia (CLL) \(^15,16\), melanoma \(^17\), renal cell carcinoma \(^9\), and breast cancer \(^18\). Branched evolution may also explain how different clones arising from the same primary tumor display wide mutation spectrums and phenotypic diversity, differing not only in cellular morphology \(^19\), gene expression \(^20\), and response to therapies \(^21,22\) but also in metastatic abilities \(^23-26\). It is important to note, however, that not all genetic mutations within a tumor cell or within
populations of cells have functional or advantageous phenotypic consequences. The concepts of clonal and branched evolution have been thoroughly reviewed elsewhere.\textsuperscript{16,27-29}

**Non-genetic heterogeneity**

Not only are tumor cells inherently unstable, they are also constantly under selective pressure from the tumor microenvironment due to their interaction with the extracellular matrix (ECM) and with cells in the microenvironment such as immune cells, endothelial cells, and fibroblasts. The cells in the primary tumor also face alterations in oxygen and nutrient availability and thus experience distinct selective pressures based on their location within the primary tumor (whether centrally located in the tumor, or located closer to blood vessels).\textsuperscript{30,31} This combination of internal and external pressures allows for outgrowth of subpopulations of cells that may have genetic, epigenetic, and/or phenotypic differences, leading to intratumoral heterogeneity.

An important non-genetic source of heterogeneity within the tumor is thought to arise from differentiation hierarchies that exist within tumor cell populations.\textsuperscript{13} For example, tumor heterogeneity may result from cells within the primary tumor existing in different states of “stem-ness”, a hierarchy that can be regulated and maintained in part by the microenvironment.\textsuperscript{34-36} In this scenario, cancer stem cells (CSCs), which have the ability to self-renew as they asymmetrically divide, can propagate the tumor, whereas non-CSCs within the same tumor would not have this ability. These two different populations of cells (CSC and non-CSC) would then further give rise to diverse sets of cells as they accumulate mutations or epigenetic changes, and undergo selection separately, leading to increased heterogeneity within the tumor. In the CSC theory, a small population of cells within the primary tumor drives tumor progression and metastasis,\textsuperscript{37} and thus the most effective therapies should target this population as well as the bulk of the tumor. Importantly, studies have shown that CSCs within a tumor are resistant to many commonly used therapies,\textsuperscript{11,38,39} and thus when left behind, contribute to metastasis.\textsuperscript{40-42} Recently, the potential of a
bidirectional relationship between CSCs and non-CSCs has been recognized in breast cancer and B cell-lymphoma amongst others, where cells can shift between CSC and non-CSC states throughout tumor evolution\textsuperscript{43-48}. This shift between stem cell-like and non-stem cell-like states can be spontaneous, and has been shown to be under the influence of oncogenes and the tumor microenvironment\textsuperscript{11,43-48}.

The two concepts of clonal evolution and CSCs attempt to explain intratumoral heterogeneity and may be mutually exclusive when defined in the strictest senses. Though there are differences between these theories, specifically in terms of the existence of a differentiation hierarchy and the tumorigenic potential of only a few cells within the tumor (amongst others,\textsuperscript{49}), they do overlap. Clonal evolution and both genetic and non-genetic alterations can give rise to CSCs that have the ability to both self-renew and differentiate to further generate distinct clones within the tumor. Indeed, studies have shown that both CSCs and non-CSCs have different patterns of mutations, indicating that they have evolved from distinct clones in the primary tumor\textsuperscript{50,51} (Figure 1.1).

Much discussion has been devoted to the role of CSCs in tumors, as critical contributing factors to intratumoral heterogeneity\textsuperscript{11-13,27}. However, additional altered differentiation states may contribute to this heterogeneity as well. For example, in epithelial cancers, the trans-differentiation of epithelial tumor cells to more mesenchymal-like cells through an epithelial-to-mesenchymal transition (EMT) may be another critical component of the heterogeneous tumor landscape. This phenomenon can at times be linked to CSCs, as EMT has been shown to increase CSC-like characteristics\textsuperscript{40,44,52}. However, CSC and EMT do not always go hand-in-hand\textsuperscript{53}. Nonetheless, an EMT is highly associated with a gain in metastatic abilities, even when only a small proportion of the tumor has undergone this transition\textsuperscript{54-57}. Thus, EMT may be considered a largely (although not exclusively) non-genetic contributor to intratumoral heterogeneity.
Figure 1.1 Heterogeneity can arise from both genetic and non-genetic mechanisms. Intratumoral heterogeneity can arise through a variety of mechanisms. (A) A strictly genetic mechanism can involve genetic mutations (lightning bolt), some of which may give a clone a fitness advantage over nearby tumor cells (light blue and orange genotype is more fit than green genotype). This model can involve genetic fitness being selected for by the local microenvironment. An increased oxygen and nutrient supply may select for one type of clone (light blue), while the opposite could be true for another clone (orange). This pattern of mutation can repeat over time, yielding a tumor with numerous clones. Such mutations can be stochastic in nature, can be influenced by genomic instability and can be influenced by chemo- and radiation therapy, in addition to other sources. (B) An alternative model involves cancer stem cells (CSCs), which arise due to differentiation hierarchies that may not involve genetic alterations, but may act in combination with mutations. A self-renewing tumor cell, the CSC, can undergo asymmetric division, increasing the overall number of CSC and non-CSC tumor cells. Genetic mutation of a CSC can lead to the outgrowth of a more fit genetic clone (dark blue), that may or may not be affected by the local microenvironment. Additionally, the microenvironment can trigger phenotypic changes, such as what occurs in a hypoxic environment (red cells). Importantly, if the original clone is outcompeted over time, it would not be detectable in patient biopsies.
Can Clonal Cooperation Affect Tumor Initiation and Progression?

A largely unanswered question in the field with respect to intratumoral heterogeneity is whether different subpopulations of clones within the primary tumor interact to affect tumor maintenance and/or progression, or whether cells are actually recruited to the tumor and induced to be tumorigenic. Indeed, recent studies have shown that tumor maintenance may require interaction between clones in a primary tumor, as well as between tumor-initiating cells and progenitor cells in the environment.

Holland and colleagues examined this issue in a study using human platelet-derived growth factor (hPDGF)-induced murine gliomas that are thought to arise from one cell of origin (glial cells) but are heterogeneous in nature. Using two different mouse models of hPDGF-driven gliomas, along with lineage tracing studies, the authors were able to distinguish which cells in the tumor were derived from the cell of origin, versus which cells were not, and were then able to perform tumorigenic and expression analyses on the two different populations. The heterogeneity in these gliomas arises from the ability of the tumor cells to recruit normal neural stem cells as well as progenitor cells (that are not overexpressing hPDGF), but that proliferate to contribute to the tumor mass. Interestingly, when the gliomas were induced by hPDGF in mice with germline deletions of Ink4a, Arf, and/or PTEN, the recruited population (not derived from the cell of origin) was expanded such that it could dominate regions of the tumor. Importantly, transplantation studies revealed that these recruited cells could give rise to gliomas on their own, exhibiting expression profiles and genetic abnormalities that were seen in the glioma cells, but not in the normal progenitor cells. Thus, the authors demonstrated that non-cell-of-origin cells in glioma can actually be transformed non-cell autonomously by the glioma environment, providing a new paradigm for gliomagenesis.
In another such study by Gunther and colleagues, cooperation between distinct clones in a Wnt1-driven murine mammary tumor was shown to be essential for tumor maintenance. In this study, the mouse mammary tumor virus (MMTV)-Wnt1 murine mammary tumor model was employed, where aberrant expression of Wnt1 in the mammary epithelial cells was shown to result in bi-clonal, heterogeneous tumors consisting of basal and luminal epithelial cells. A requirement for Wnt1 in tumor maintenance was shown using an inducible Wnt1 system, since deprivation of Wnt1 led to tumor regression. Interestingly, some cells within this heterogeneous tumor cell population were found to spontaneously acquire Hras mutations. On further analysis of the Hras mutant tumors, the authors found that half of the tumors consisted of basal and luminal cells with identical Hras mutations. On the other hand, the remaining half of the tumors consisted of basal cells that harbored mutant Hras and expressed low Wnt1 levels and luminal cells that contained wild-type Hras and high Wnt1 levels. They also found that the luminal cells within the heterogeneous tumors were the main source of Wnt1 that helped in the maintenance of the tumor mass. When the tumors were deprived of the Wnt1 ligand to imitate targeted therapy, the basal cells recruited other luminal cells to provide the required Wnt1, which led to tumor recurrence. Hence, within the heterogeneous Wnt1-driven mammary tumor, the low Wnt1-expressing, mutant HRas basal cells required Wnt1 from the high-Wnt1 expressing luminal cells to maintain tumor mass, indicating that interclonal cooperation was necessary in this context for tumor maintenance.

Additional studies have provided evidence for clonal cooperativity not only in tumor maintenance, but also in tumor progression. Using a colorectal cancer model, Ellis and colleagues demonstrated that both CSC-like cells and chemoresistant cells within the primary tumor have the ability to confer chemoresistance on surrounding “chemo-naïve” cells. Specifically, colorectal cancer cells were made chemoresistant through chronic
exposure to Oxaliplatin (OxR cells), a common chemotherapeutic agent used in the
treatment of colorectal cancer. Not only did the OxR population of cells have an increased
percentage of CSCs compared to the parental chemo-naïve cells, but the conditioned media
from OxR cells, when placed on chemo-naïve cells, led to increased survival of chemo-naïve
cells in the presence or absence of Oxaliplatin. In addition, subcutaneous injections of
different ratios of OxR and chemo-naïve cells into mice resulted in the largest tumors when
the injections contained equal numbers of both cell types (in a 1:1 ratio), as compared to
injection of either pure population of cells, even though the total number of cells injected into
mice in each case was the same. Since the investigators observed that OxR cells grew at a
slower rate compared to the chemo-naïve cells, the larger mixed in vivo tumors suggest that
both cell types were non-cell autonomously interacting to aid tumor growth. Intriguingly,
effects of the OxR cells was shown to occur over significant distances, as injection of these
cells into one flank of a mouse promoted the growth of chemo-naïve cells that were injected
into the other flank. Thus, these studies again demonstrate that interclonal cooperation is
necessary for tumor maintenance and progression.

These aforementioned studies demonstrate that once a tumor has formed, it can be
composed of phenotypically and/or genotypically distinct clones that interact to the benefit of
one or more clones within the tumor. Thus, while competition between clones may result in
dominant clones with maximum fitness taking over the tumor, clonal cooperation can also
occur, in which co-existence of multiple different clones can impact tumor progression
positively and lead to more aggressive disease. In recent years, interclonal cooperativity has
clearly been demonstrated to impinge on metastatic dissemination.

Metastasis and Intratumoral Heterogeneity

Approximately 90% of cancer related deaths occur due to metastatic dissemination
\cite{57,62}. There is thus an urgent need to develop better therapies to combat metastatic disease
and to improve outcome, and indeed much basic research focuses on gaining a more complete understanding of the molecular mechanisms behind the metastatic cascade. During the process of metastasis, tumor cells need to gain characteristics that enable them to move out of the primary tumor, into and out of the vasculature, and into a secondary site where, perhaps after a period of dormancy, they must regain the capacity to proliferate to colonize at that site 45. Thus, a tumor cell will form clinically detectable metastases only if it is able to successfully navigate the many steps of the metastatic process. Metastasis is thus considered to be an inherently inefficient process 63, with only a small percentage of the cells within the primary tumor having the potential to colonize a secondary site. It would thus seem advantageous to bestow dissemination potential upon as many cells within the primary tumor as possible. This could be achieved through interclonal cooperation and non-cell autonomous mechanisms and would increase the likelihood of metastasis and outgrowth at the secondary site. Thus, while one individual cell may not be able to carry out each of the steps of metastasis, a neighboring tumor cell may allow it to perform a step it normally could not on its own.

There is still some controversy over whether tumor cells innately contain the capability to metastasize, or whether metastatic ability is a phenotype that cells gain from a set of genetic and epigenetic changes that occur throughout tumor progression 26,64-69. It is likely that both these mechanisms could be at play. This question is important to address since current therapies mostly rely on the theory that metastases are similar to the primary tumor from which they arise 70-72. Studies in breast cancer have shown that primary tumor cells left behind post neo-adjuvant treatment have molecular profiles different from matched biopsy samples taken prior to the treatment. Genetic profiling of these residual tumor cells revealed molecular alterations that correlate with poor patient prognosis and might thus be similar to micro-metastases formed in these aggressive cancers 73. On the other hand, there
is ample evidence that both disseminated tumor cells, as well as metastases, are made up of cells with a mutational spectrum that is different from that present in the primary tumor \(^74\).

A multitude of studies have also shown that both tumor initiation and metastatic progression are influenced and often facilitated, by the tumor microenvironment consisting of stromal cells, extracellular matrix and immune cells \(^75\). However, the tumor microenvironment can also serve to negatively regulate tumor progression. For instance, p53 expression in hepatic stellate cells has been shown to non-cell autonomously curb initiation of hepatocellular carcinoma (HCC) by promoting an anti-tumor microenvironment via secretion of senescence promoting factors in the murine liver \(^76\). Fibroblasts in the tumor microenvironment have also been shown to secrete TGF-\(\beta\) ligands, which can suppress tumor progression in the adjacent epithelia \(^77\). But this equation is further complicated by the presence of different subpopulations of cells within the primary tumor itself, which express different genes, have varying metastatic potentials and respond differently to chemotherapy. Given that the primary tumor is populated with distinct cancer cells, it is important to understand whether the cells with metastatic potential within the primary tumor can increase the metastatic potential of the surrounding non-aggressive cells.

**Clonal Cooperation in Metastasis**

One of the first demonstrations of clonal cooperation in metastasis was reported by Fred Miller and colleagues in 1983. Using a set of syngeneic murine mammary carcinoma cell lines derived from a spontaneous tumor arising in a Balb/c mouse \(^24\), the Miller group showed that the tumorigenic, yet non-metastatic 67nr cells metastasized to the lungs only when co-injected intravenously into mice with the highly metastatic 4t1 clones \(^78,79\). The same group also demonstrated that an interaction occurred between syngeneic cell lines with varying metastatic potentials when they were either cultured *in vitro* or orthotopically injected into the mammary fat pads of Balb/c mice. In some cases the more metastatic cells,
when injected into one flank of the mouse, inhibited the in vivo growth rate of the weakly metastatic cells injected into the other flank of the same mouse, a phenomenon that was attributed to the interaction with the immune system of the host mice. In another study, growing the more metastatic and weakly metastatic cells together also resulted in the suppression of growth of the weakly metastatic clones. Around the same time, Fidler and colleagues discovered that clonal interactions between more and less metastatic clones in the B16 murine melanoma model led to stabilization of the metastatic phenotype in the less metastatic clones, again demonstrating the importance of interclonal interactions in metastasis. In 1993, this issue was revisited when David Tarin and colleagues demonstrated that subcutaneous co-injection of intrinsically metastatic and non-metastatic clones of the same murine fibrosarcoma line into mice, although resulting in a heterogeneous primary tumor, produced a significant number of metastases consisting only of the non-metastatic clones. Importantly, subcutaneous injection of only the non-metastatic clones into mice did not result in any metastases. Data from this study yet again suggested that metastatic cells must either impart metastatic characteristics on non-metastatic cells, or that they must somehow aid the non-metastatic cells in reaching the secondary site.

More than a decade later, Xiang and colleagues demonstrated that metastatic BL6-10 murine melanoma cells release exosomes that can increase the metastatic potential of syngeneic non-metastatic F1 melanoma cells. In this study, non-metastatic F1 (parental) melanoma cells metastasized efficiently to the lungs after intravenous injection only when previously cultured with exosomes derived from highly metastatic BL6-10 cells. The exosomes derived from the metastatic cells were profiled and shown to express a previously described metastatic marker Met 72 (a glycoprotein and tumor antigen expressed exclusively by the metastatic BL6-10 cells but not the F1 cells). Interestingly, Met 72 was observed to be expressed by the non-metastatic F1 cells after their treatment with
exosomes released from the metastatic BL6-10 cells, presumably enabling the F1 cells to metastasize (Figure 1.2). This study suggests that had the metastatic and non-metastatic clones co-existed in the primary tumor, the exosomes released by the metastatic clones could stably alter non-metastatic clones, making them more metastatic.

More recently, Berns and colleagues have provided clear evidence that clonal cooperation, under some circumstances, is required for metastasis. These investigators found two populations of cells within a murine model of small cell lung cancer (SCLC): neuroendocrine small cells (NE) and mesenchymal large cells (nonNE), each with distinct gene and protein expression profiles. Importantly, this study showed that crosstalk occurred between the two populations of cells in vitro wherein each cell type had a proliferative advantage when cultured in the presence of the other. Surprisingly, the intrinsically non-metastatic NE cells formed metastases only when co-injected subcutaneously with nonNE cells. Single injections of neither the NE nor nonNE cells formed metastases, suggesting that cooperation between these cells was necessary to facilitate metastasis. Taken together, these studies provide compelling evidence that interclonal cooperativity within the primary tumor can affect metastatic outcome positively by increasing the number of cells with metastatic potential and thus, the overall probability of metastatic occurrence.

**Epithelial-To-Mesenchymal Transition and Metastasis**

Epithelial-to-mesenchymal transition (EMT) is a normal developmental process that is critical for embryogenesis, but has also been observed during wound healing and carcinoma progression.
Non-cell autonomous interactions can increase metastatic propensity: exosomes as an example.
The BL6–10 cell line is a highly metastatic subclone derived from the non-metastatic F1 cell line. Met 72, a previously identified metastasis marker, was found to be expressed on the surface of (A) BL6–10 cells, but not (B) F1 cells. Additionally, Met 72 was expressed on the surface of exosomes derived from BL6–10 cells. (C) Treating F1 cells with exosomes from BL6–10 cells caused F1 cells to become Met 72-positive and significantly increased the metastatic ability of F1 cells.84
Three types of EMT have been defined: 1) EMT that occurs during embryogenesis and is required for processes such as gastrulation, which is referred to as Type I EMT\textsuperscript{86,87}, 2) EMT that occurs during wound healing and tissue regeneration, which is referred to as Type II EMT, and 3) EMT that occurs in cancer cells, which are thought to hijack a process that shares numerous characteristics with developmental EMTs, and is referred to as Type III or “oncogenic” EMT. Thus many of the same signaling pathways and molecules that play important roles during developmental EMTs play similar roles in oncogenic EMT including signaling pathway ligands like Wnt, TGF-\(\beta\) and Hedgehog ligands and transcription factors like Twist1 and Snail1\textsuperscript{56}. In EMT, epithelial cells reduce cell-cell adhesion and apical-basal polarity, enabling them to separate from each other and invade through the basement membrane. This change is accompanied by the formation of filopodia on the cells and a switch from cell-cell adhesion-promoting integrins to those that promote cell-extracellular matrix adhesions\textsuperscript{88}. In addition, remodeling of the cytoskeleton occurs, replacing the static configuration of the epithelium with a more fluid and dynamic state which allows the cells to be more motile\textsuperscript{89}. Morphologically, the more cuboidal shaped epithelial cells often become more elongated through changes to the actin-myosin cytoskeleton,\textsuperscript{89} though this morphological change may not always be observed. This change is often accompanied by downregulation of epithelial proteins such as E-Cadherin and Cytokeratin-18, concordant with the upregulation of mesenchymal proteins such as N-cadherin and Vimentin. Such changes promote migration and invasion, and could thus facilitate metastasis\textsuperscript{86-88}. It should be noted that this conversion in cancer is not always thought to be complete, and in fact recent evidence suggests that carcinoma cells at the leading edge of the tumor as well as in circulation may exist in an intermediate state, expressing both epithelial and mesenchymal markers simultaneously\textsuperscript{57}. These recent findings make it easier to understand how a reversal in EMT may occur at the secondary site (a mesenchymal-to-epithelial transition or MET), to allow for outgrowth of the metastatic lesion. Indeed, the requirement of an
EMT-MET axis in cancer metastasis has been shown in some models. Because EMT during carcinoma progression is thought to be very plastic, the term Epithelial-Mesenchymal Plasticity (EMP) is now frequently used instead of EMT. Importantly, EMP has been shown to occur only within a subpopulation of cells within the primary tumor, thus again demonstrating the heterogeneous nature of tumors.

More recently, some elegant studies have argued against the requirement of EMT for metastasis. In one such study, the authors established triple-transgenic mouse models using Mouse mammary tumor virus-Polyoma middle T-Antigen (MMTV-PyMT) mice crossed to mice described below. MMTV-PyMT mice develop spontaneous breast adenocarcinomas (bearing resemblance to human luminal tumors) that quickly metastasize to the lungs. These mice were crossed to a line in which Cre recombinase expression is driven by the FSP1 (Fibroblast specific protein-1) promoter, a mesenchymal-specific gene that is expressed in the early stages of EMT, as well as to mice that contain a lox-RFP-STOP-lox-GFP constitutively expressed under a β-actin promoter in the Rosa26 locus. This triple cross allowed for lineage tracing during tumor progression, such that when the cells in the primary tumor underwent an EMT, the originally epithelial RFP+ cells would switch to permanently being GFP+, irrespective of whether the metastasized cells underwent the reverse MET process in the lungs. Using this triple transgenic mouse model, the authors showed that cells which metastasized efficiently to the lungs were all RFP+, whereas the surrounding non-tumor tissue (presumably the tumor microenvironment and ECM) was GFP+. From these data, the authors argued that only the epithelial non-EMT cells had metastasized and that undergoing an EMT is dispensable for metastatic progression. Nevertheless, there is little dispute that cells that have undergone an EMT are able to better survive chemotherapy and in some cases later establish metastases. It is also known that in many cases only a small percentage of cells within the primary tumor undergo EMT (or EMP) at any given time, whereas the majority of carcinoma cells appear epithelial. This heterogeneity in the
EMT state of a tumor once again raises the possibility of interactions between the “EMT” and “non-EMT” cells within the primary tumor to affect tumor progression, something that was not considered in the studies described above where EMT was thought not to be required for metastasis.

**EMT-Inducing Factors in Development and Cancer**

Cancer cells are known to utilize embryonic pathways to promote tumor progression via enhancing properties such as migration, invasion, and neovascularization.\(^{56,97,98}\) Thus, many of the same molecular players that control developmental EMTs also regulate oncogenic EMTs. These include signaling pathways like Wnt and TGF-β, transcription factors like Snail, Twist1, and the Six family members, all of which are pro-EMT factors, and various microRNAs (miRNAs), including the miR-200 family members that regulate epithelial identity.\(^{56,97,99}\) During stages of embryogenesis such as gastrulation and cardiogenesis, ligands of signaling pathways like Wnt1 and TGF-β1, 2 and 3 signal in an autocrine and/or paracrine manner to control EMT.\(^{97,100}\) Different sets of cells in the primitive streak, and later the mesoderm, express the ligands or the respective receptors for each of these pathways (with some cells expressing both), and communication between these cells is essential for migration, proliferation and cell-fate determination. Thus, activation of these signaling pathways in one set of cells can non-cell autonomously influence other cells.

MiRNAs are a fairly recently described class of small non-coding RNA molecules which were first discovered in C. elegans as molecules that regulate the timing of differentiation.\(^{101,102}\) Since then numerous miRNAs have been implicated in the process of embryogenesis,\(^ {99,103}\) and they are known to regulate many developmental genes.\(^ {104-106}\) Importantly, they are known to cause Type II EMTs in various systems including during kidney fibrosis and have been implicated in Type III EMTs during cancer progression\(^ {107-109}\). The specific role of miRNAs in non-cell autonomously causing EMT of cells during
development is still unknown. The roles of other EMT-related factors during development have been reviewed extensively elsewhere\textsuperscript{56,103-112}.

In the context of cancer, many of the above mentioned signaling pathways, transcription factors, and miRNAs have been shown to cell autonomously cause oncogenic EMTs and/or to increase metastasis, and their overexpression in various cancers is often associated with lower overall survival and poor prognosis\textsuperscript{97,113,114}. Since some EMT-inducing factors like Wnt1 and TGF-β ligands are secreted molecules, it is not a surprise that secretion of these ligands from cells in the tumor microenvironment affects the tumor cells expressing the appropriate receptors i.e., that they have non-cell autonomous roles in cancer\textsuperscript{77}. However, the non-cell autonomous functions attributed to EMT-inducing factors may result not only from secretion of these molecules from cells in the microenvironment, but also from tumor cells within the heterogeneous tumor. They may also occur in response to alterations in EMT-inducing transcription factors as well as miRNAs. For example, it was recently discovered that malignant cells could release miRNAs in larger exosomes that expressed different surface markers as compared to the exosomes released from normal non-cancerous cells.\textsuperscript{115} Studies such as the murine melanoma study outlined previously (Figure 1.2), have shown that exosomes released from malignant cells can increase the metastatic properties of other cells\textsuperscript{84,116} and exosomes are known to contain mRNA, proteins and miRNAs. This suggests that secreted miRNAs, as well as any other proteins or mRNA, contained within exosomes can non-cell autonomously alter the properties of cancer cells. Indeed, a study done by Taylor and colleagues\textsuperscript{117} showed that tumor-derived exosomes isolated from sera of ovarian cancer patients contained a variety of miRNAs, both similar to and different from the miRNAs expressed by the tumor mass, including some that have been previously associated with increased metastasis in ovarian cancer\textsuperscript{118}.

Finally, it should be noted that EMT-inducing factors exist in a complex web, which may have consequences in a heterogeneous tumor. For example, TGF-β, which activates
Snail during development, can also activate and/or be activated by Snail in the context of cancer\textsuperscript{119-121}. Thus, cancer cells in which Snail has been upregulated may increase their secretion of TGF-β, leading to EMT in neighboring cells that did not originally have increased levels of Snail (but may ultimately upregulate Snail downstream of TGF-β). However, to date, no formal studies have demonstrated that the EMT-inducing transcription factors play non-cell autonomous roles in mediating tumor progression.

**EMT and Intratumoral Heterogeneity**

Although only few cells in a heterogeneous primary tumor may undergo EMT, particularly those at the leading edge of the tumor\textsuperscript{54-57}, it is possible that these few cells may non-cell autonomously increase the potential of surrounding cells to undergo EMT and/or metastasize. Alternatively, it has been proposed that epithelial tumor cells within a heterogeneous tumor may help to maintain the mesenchymal-like state of tumor cells that have undergone EMT\textsuperscript{4}. To elaborate, it has been hypothesized that when some cells within the primary tumor undergo EMT (induced by factors secreted from within the tumor), the other surrounding tumor cells that secreted those EMT-inducing factors remain “epithelial” to continue providing the factors to maintain the EMT phenotype of the other cells\textsuperscript{4}. Thus interclonal cooperation between distinct clones in the primary tumor that have undergone EMT to become mesenchymal-like (EMT cells) and those that have stayed epithelial (non-EMT cells) may be critical for maintaining the EMT status of the tumor cells. Since studies have shown that cells that have undergone EMT also metastasize more efficiently\textsuperscript{56,122}, interclonal cooperation between EMT and non-EMT cells and its possible effect on metastasis, is important to consider. In recent years, a few studies have probed into this cooperation and shown that it may be critical to facilitate metastasis. These studies will be discussed is brief in the following section.
Clonal Cooperation in EMT and the Effect on Metastasis

As mentioned earlier, EMT-inducing factors have the ability to act non-cell autonomously to cause EMT during development. Many studies have shown that a tumor’s microenvironment can affect the EMT status of tumor cells (reviewed more thoroughly in 75,123,124), and factors like TGF-β1, secreted by the microenvironment, have been shown to non-cell autonomously regulate EMT of cells in the primary tumor 125. Few, if any, studies have asked if a mixed population of cells within the tumor itself, some of which have undergone EMT, can cause the surrounding cells to undergo EMT to affect metastatic progression. Recent studies that have demonstrated that EMT is not a pre-requisite for metastasis have ignored the possibility of interactions between the EMT and non-EMT tumor cells to affect the metastatic outcome, irrespective of which cells reach the secondary site 95,96. However, a number of studies have now demonstrated cooperativity between tumor cells that have undergone EMT and those that have not.

Studies first done by Lyons and colleagues looked at interclonal cooperativity between EMT cells and cells that remained epithelial (non-EMT cells) within a carcinoma cell population 126-128. Using BC1 rat mammary carcinoma cells consisting of a mixed population of epithelial (non-EMT) and metaplastic (EMT) cells, this group demonstrated that non-EMT cells induced production of proteinases by EMT cells, which enabled efficient degradation of extracellular matrix. The non-EMT cells also secreted factors that increased growth and attachment of EMT cells 126,128. The EMT cells reciprocated with the production of secreted proteins that conferred multidrug resistance benefiting both cell types 127, indicating that interclonal interactions in the heterogeneous BC1 cell line benefitted both populations of cells.

An elegant study by Tsuji and colleagues demonstrated that, at least in some models, cooperation between EMT and non-EMT cells is necessary to mediate metastasis
In this study, a downstream effector of TGF-β signaling, p12, was used to induce EMT of hamster cheek pouch carcinoma cells. p12 expression increased the invasive phenotype and dissemination of these cells, but did not enable metastatic colonization, regardless of whether the cells were injected into mice subcutaneously, to first form primary tumors, or intravenously, to bypass early metastatic steps. On the other hand, the non-EMT parental cells were able to metastasize, but only when injected into mice intravenously, bypassing the early steps of metastasis including invasion and intravasation into the vasculature. Importantly, when the non-EMT cells were subcutaneously injected into mice along with the EMT cells, both EMT and non-EMT cells could be found in the bloodstream (differentiated by differentially labeling the cells with GFP or DsRed), whereas only the non-EMT cells could be found in the lungs as metastases. These data strongly suggest cooperativity between EMT and non-EMT cells; wherein EMT cells facilitate invasion through the basement membrane and intravasation into the vasculature, which allows the non-EMT cells to access circulation. Once the non-EMT cells are in circulation, they are themselves able to extravasate and grow at the secondary site. Thus, different clonal populations, as represented by EMT and non-EMT cells, were able to interact to produce a favorable metastatic outcome.

Additional studies performed in prostate and bladder cancer models have recently provided further evidence that EMT and non-EMT cells cooperate to affect metastatic outcome. In one study by Thomson and colleagues, the authors found that in contrast to previous studies where the more mesenchymal cells have increased tumor initiating capacities, the more epithelial (non-EMT) cells exhibited a CSC-like gene signature and were less invasive, but more metastatic when injected into mice, as compared to the more mesenchymal-like (EMT) cells. Co-culturing the non-EMT and EMT cells resulted in increasing the in vitro invasive properties of the non-EMT cells. It was also observed that orthotopic co-injection of the EMT and non-EMT clones into mice diminished the growth rate.
of the primary tumor, compared to injection of either clone only, but accelerated the metastatic rate of the metastatic non-EMT cells. Acceleration of metastasis was seen when the EMT and non-EMT cells were co-injected into mice intramuscularly, as well as intravenously. It should be noted that while the non-EMT metastatic cells appeared more epithelial \textit{in vitro}, the cells were able to undergo partial EMT as demonstrated by upregulation of the mesenchymal marker fibronectin and downregulation of the epithelial marker E-Cadherin in the primary tumor. These data support the idea that cellular plasticity may be the critical factor that enables metastatic dissemination and that this plasticity may be transferred non-cell autonomously to other cells. Together, these studies show that cells that have undergone EMT, while not always metastatic in nature, are able to influence those cells in the tumor that have not undergone EMT (or vice versa).

A multitude of studies have now shown that different sub-populations of cells within a primary tumor can interact to affect the outcome of tumor progression. Yet to our knowledge no studies have been performed to decipher the mechanism of interaction between these different cells. Studies have shown that tumor cells expressing EMT-inducing transcription factors like Snail1 can secrete molecules like TGF-\(\beta\) which can act both cell autonomously on the tumor cells and non-cell autonomously on the tumor microenvironment. Yet, to date, EMT-inducing transcription factors have not been shown to play non-cell autonomous roles in affecting neighboring tumor cells in order to mediate tumor progression and affect metastatic outcome. In addition to Twist1, Snail1 and Six1, there numerous other EMT-inducing factors including TGF-\(\beta\), Wnt and FGF signaling pathways, as well as transcription factors such as Zeb1, that play equally important roles in development, and reprise most of those crucial functions in tumor progression. In the following section, we describe the cell autonomous roles of three key EMT-inducing transcription factors Twist1, Snail1 and Six1, during development and cancer, after which I embark on understanding whether they additionally can play a non-cell autonomous role in breast cancer.
**Twist1: A Basic Helix-Loop-Helix Transcription Factor**

**Structure and function**

The basic helix-loop-helix (bHLH) transcription factors belong to a superfamily of transcription regulators that play important roles during development in organisms as diverse as yeast and humans. bHLH proteins are extremely diverse in function, and regulate processes including sex determination and development of the nervous system. They contain a highly conserved region of around 60 amino acid residues in total, the amino and carboxy terminals of which, have their own distinct functions. The amino terminal end of this domain is highly basic in nature and allows the bHLH factors to bind to DNA at specific consensus sequences known as E-boxes. Each bHLH protein binds to its unique E-box sequence resulting in either transcriptional repression or activation. The transcriptional functions of bHLH proteins are further governed by the carboxy terminal end of the region (the helix-loop-helix domain) using which they interact with other bHLH proteins either as homo- or heterodimers. Classically, the bHLH factors have been categorized based on tissue distribution, specificity to binding various DNA sequences, and dimerization abilities. Twist1 is one of the better studied bHLH factors that belongs to a subset of tissue restricted factors. The transcriptional functions of Twist1 are determined not only by its dimerization partners (which ultimately decide the DNA sequences it binds to), but also by phosphorylation states and spatio-temporal expression. Twist1 is specifically expressed in the mesoderm during embryogenesis, and is considered to be a master regulator of development. Its roles in development will be discussed in brief in the following section.

**Role of Twist1 in development**

*Expression of Twist1 during development*

*Twist1 was first identified in Drosophila through its role in mesodermal specification and dorsal-ventral patterning during early embryogenesis, since absence of this gene*
resulted in “twisted” fly embryos with abnormal gastrulation, no discernible mesoderm formation and death at the end of embryogenesis\textsuperscript{132}. In early \textit{Drosophila} embryogenesis, \textit{twist} expression is detected throughout the mesoderm but as embryogenesis progresses, its expression is diminished and retained only in the adult muscle precursor cells\textsuperscript{133}. In mammals, similar to its role in \textit{Drosophila}, \textit{Twist1} expression is mostly restricted to the mesoderm during early mouse embryogenesis. It is detected as early as embryonic day 7.5 (E7.5) in the mesodermal layer and primitive streak, after which its expression decreases. Its expression can be detected in the dental mesenchyme and heart valves until E18. After birth and in adulthood, \textit{Twist1} expression is restricted to the adult stem cells of the mesenchyme, brown and white adipocytes and certain primary osteoblastic cells among others, all of which are derived from the mesoderm, and aids in maintenance of their undifferentiated states \textsuperscript{134,135}. Since \textit{Twist1} expression is limited to the mesoderm and mesoderm-derived tissues, its expression is not detected in mammary epithelial cells in the mammary glands (which are derived from the ectoderm) but can be found in the surrounding fibroblasts\textsuperscript{136}.

\textbf{Functions of Twist1 during development}

In mammals, Twist1 is recognized as master regulator of mesoderm formation. Twist1 causes an EMT in the mesoderm precursors resulting in the loss of epithelial characteristics and gain of mesenchymal properties, enabling the cells to migrate and disperse to form the mesoderm layer \textsuperscript{137}. Twist1 can also repress osteoblastic and muscle differentiation, by means of interacting with and inhibiting Runx2 and MyoD respectively \textsuperscript{138,139}. A crucial role for Twist1 in mouse embryogenesis is highlighted by the fact that \textit{Twist1} KO mice are embryonic lethal and demonstrate defects in neural tube closure \textsuperscript{140}. This phenotype is not surprising since studies have shown that Twist1 regulates migration and potentially adhesion of neural crest cells during embryogenesis\textsuperscript{140,141}. Additionally, the KO embryos display deformed craniofacial structures, impaired growth and differentiation of the branchial arches, and retarded limb development among other abnormalities. As expected,
inducible knock out studies of *Twist1* indicate that Twist1 is not necessary for normal mammary gland function and development, since these mice exhibit normal mammary gland morphogenesis and lactation patterns 136.

In accordance with the crucial role of Twist1 in mesoderm specification and organogenesis, studies show that mutations in its highly conserved bHLH domain result in the Saethre-Chotzen syndrome (SCS) in humans. This syndrome is characterized by deformities in the craniofacial structures, stunted physique, high foreheads, abnormalities in ear formation, and asymmetrical facial structures among others134. Interestingly, the limb deformities and craniofacial abnormalities are recapitulated in heterozygous *Twist1* null mice making them an effective model for the study of this syndrome 142.

**Interactions with developmental networks**

During development, Twist1 interacts with numerous signaling networks that facilitate its regulation of embryogenesis. Twist1's role in limb development is thought to be caused by its upregulation of FGF (Fibroblast growth factor) signaling, which in turn upregulates various components of the Sonic Hedgehog (SHH) signaling network including *Shh, Gli1, Gli2* and *Pcat*140,143. Furthermore, Twist1 is thought to play an important role in organogenesis via its control not only over the FGF and SHH pathways, but also via interactions with the BMP, TGF-β and TNF-α pathways. Interestingly, TNF-α induces NF-κB signaling, resulting in upregulation of *Twist1* to prevent apoptosis in cells134. Twist1 in turn functions to repress NF-κB-mediated increases in cytokine expression as a means to control inflammatory responses 144. These studies together highlight the importance of Twist1’s interaction with multiple signaling networks to facilitate normal development.

Consistent with its crucial role in regulating normal development, predominantly by causing developmental EMT, Twist1 has been recognized for its important pro-tumorigenic roles in numerous cancers.
Role of Twist1 in cancer

Expression of Twist1 in cancer

Twist1 was first recognized as a potential pro-tumorigenic factor when it was discovered to efficiently bypass both myc induced apoptosis as well as p53 induced growth arrest. Twist1 can overcome p53 induced growth arrest by downregulating ARF and potentially interfering with p53’s ability to induce “protective” downstream targets in response to DNA damage. Since this study, Twist has proven to be a potent pro-tumorigenic factor in various cancers including breast cancer, hepatocellular carcinoma (HCC), gastric cancer, bladder cancer, and esophageal squamous cell carcinoma among many others, by playing a role in tumor initiation, progression and metastasis.

The expression of Twist1 is tightly regulated in cancer (Figure 1.3). As mentioned previously, Twist1 can be induced by NF-kB in response to pro-inflammatory cytokines, to inhibit cellular apoptosis. Studies show that intratumoral hypoxia can result in the induction of Twist1 via HIF1-α which in turn facilitates tumor progression. Twist1 can also be induced by Ras or TGF-β signaling, in response to activation of MAP kinase pathways. Alternatively, Twist1, like some other bHLH factors is stabilized by both partner choice and phosphorylation on specific residues, among other mechanisms.

Role of Twist1 in EMT and CSC phenotypes

Twist1 is thought to predominantly cell autonomously function by directly binding to and transcriptionally downregulating genes such as E-Cadherin, an adherens junction molecule and hence causing an oncogenic EMT.
Figure 1.3 Possible regulatory pathways by which Twist1 functions in cancer progression.
Factors that directly or indirectly upregulate Twist1 are listed on the left side. Targets and cellular functions that are directly or indirectly regulated by Twist1 are listed on the right side.
SRC-1, steroid receptor coactivator-1; STAT3, signaling transducer and activator of transcription 3; MSX2, Msh homeobox 2; HIF-1α, hypoxia-inducible factor 1α; NF-κB, nuclear factor kappa B; LMP1, EBV latent membrane protein 1; ILK, integrin-linked kinase; IFN, type I interferon; Axl, Axl receptor tyrosine kinase; miR-10b, micro-RNA 10b; Wnt, wingless and Int; Tcf4, transcription factor 4; Mi-2/NuRD, nucleosome remodeling and deacetylase protein complex; YB-1, Y-box binding protein-1; TIMP1, tissue inhibitor of metalloproteinase-1; VEGF, vascular endothelial growth factor; DKK-1, dickkopf-related protein 1; RhoC, ras homolog C; MMP2, metalloproteinase 2; CXCR4, chemokine (C-X-C motif) receptor 4; CCR7, chemokine (C-C motif) receptor 7; CD29, integrin β1; CD44, CD44 antigen; CD54, inter-cellular adhesion molecule 1; Bmi1, BMI1 polycomb ring finger oncogene; HER2, human epidermal growth factor receptor 2; TNFα, tumor necrosis factor α. Taken from 134.
Indeed, in various contexts Twist1 has been shown to increase invasion, migration, chemoresistance and cancer stem cell (CSC) properties of cancer cells\textsuperscript{40,134,152}, all of which have been linked to its ability cause EMT. Mani \textit{et al} showed that overexpression of Twist1 in human mammary epithelial cells (HMLE cells) causes them to not only undergo EMT (seen by a decrease in epithelial molecules like E-Cadherin and Cytokeratin-18, and increase in mesenchymal markers like N-cadherin and vimentin) but also become more “stem-like” and more metastatic\textsuperscript{40}. Though the ability of Twist1 to promote both EMT and increase CSCs has always been thought to be mechanistically linked, recent studies suggest that Twist1 regulates these phenotypes through different mechanisms\textsuperscript{153,154}. Beck and colleagues showed in a recent elegant study that in skin tumorigenesis, different levels of Twist1 are responsible for different phenotypes; wherein low levels of Twist1 confer stemness and proliferation on the tumor cells, and higher levels of Twist1 induce EMT and enable cells to metastasize\textsuperscript{153}.

Interestingly, studies show that though Twist1 is required for EMT-induction to allow cells to escape the primary tumor site, it has to be downregulated in order for metastatic colonization\textsuperscript{90} indicating that Twist is a key player in the process of EMP which may not always go hand-in-hand with its ability to increase CSCs. More recently, Dragoi and colleagues demonstrated that TGFRβ1-dependent activation of Twist1 can induce distinct cell states via regulation of Zeb1 (another EMT-inducing transcription factor). They showed that Twist1 has the ability to either increase proliferation of cells and allow them to collectively invade or can cause EMT in cells, decrease their proliferation and switch their migratory mode to single-cell\textsuperscript{155}.

\textit{Additional roles of Twist1 in tumor progression}

In addition to directly downregulating E-Cadherin, Twist1 has been shown to promote tumorigenesis by promoting formation of invadopodia (via upregulation of platelet derived
growth factor receptor PDGFRα), which in turn aid in degradation of the ECM, resulting in increased tumor metastasis\textsuperscript{54}. Additionally, studies show that Twist1 induces expression of various miRNAs including miR-10b and miR-424, which further facilitate tumor progression\textsuperscript{156,157}. Interestingly, Twist1-mediated induction of miR-424 in breast cancer cells affects the mesenchymal arm of the EMT process without affecting epithelial markers in cells, placing them in a plastic state, primed for metastatic spread and colonization at a secondary site\textsuperscript{156}. Multiple studies have also shown that Twist1 can regulate VEGF-A (vascular endothelial growth factor-A) leading to increased angiogenesis, further enhancing the metastatic potential of the tumor\textsuperscript{134,158,159}. More recently, Twist1 has been implicated in inducing chemoresistance in cancer cells. Several studies have shown that Twist1 overexpression can cause resistance to paclitaxel and other taxols presumably via upregulation of the pro-survival AKT pathway and evasion of apoptosis\textsuperscript{160}.

These studies collectively indicate that Twist1 is a potent pro-tumorigenic factor that has the ability to cause tumor cells to become more aggressive by inducing diverse phenotypes in them.

**Snail1: A Zing-Finger Transcription Factor**

**Structure and function**

Snail1 and Slug belong to the Snail family of transcription factors and play essential roles during gastrulation in embryogenesis. The members of this family are characterized by a highly conserved carboxy terminus which can contain between 4-6 C\textsubscript{2}H\textsubscript{2} zinc fingers and a more divergent amino terminal region\textsuperscript{161}. While within species the zinc finger domains are conserved, the percent identity between species like *Drosophila* and vertebrate proteins is generally lower and can range from 50% to 70% similarity.

The zinc finger domains in the carboxy termini of these transcription factors are predominantly utilized in binding to specific DNA sequences, although studies have also
shown that the zinc fingers can be used in protein-protein interactions\textsuperscript{161,162}. The DNA sequences recognized by these factors are almost identical to the E-boxes bound by bHLH factors like Twist1, and hence Snail family members are sometimes thought to compete with bHLH factors for binding DNA\textsuperscript{162,163}. This is further realized in that, Snail1 and Twist1 have very similar functions during both development and cancer, and in fact during development, can be expressed temporally in similar cells and tissues. Indeed, studies show that Snail1 requires upregulation of Twist1 to mediate its functions in certain developmental contexts\textsuperscript{56,164}. One key difference between Snail1 and factors like Twist1, is that while Twist1 can either function as a transcriptional repressor or activator, Snail1 has been shown to function predominantly as a transcriptional repressor\textsuperscript{162}. The repressor functions of Snail1, depending on the species, are further regulated by at least two other domains. Snail1 in vertebrates, contains a highly conserved motif known as the SNAG domain in the amino terminus which is required for its repressive activities, while in Drosophila Snail mediated repression is arbitrated via interactions with CtBP (Carboxy-terminal binding protein). Its crucial repressive functions during development have heralded Snail1 as a key regulator of embryogenesis.

**Role of Snail1 in development**

**Expression of Snail1 during development**

Elegant in situ hybridization studies have shown that Snail1 is expressed in a spatio-temporal manner in mesodermal cells during development and can have distinct functions in different organisms. In Drosophila, snail is expressed in the ventral region of the blastoderm and is later observed in the neuroectoderm as well as precursor cells of the wing disc tissues\textsuperscript{162}. In vertebrates, the expression of Snail1 and its homologues is observed in mesodermal cells, neural crest cells, in the primitive streak, mesenchymal cells of tissues like lungs and limbs, and in cardiac mesodermal cells, among others\textsuperscript{161}. Mammary glands
during development are derived from the ectoderm, and since Snail1 is expressed only in the mesoderm, Snail1 has not been implicated in early mammary gland development. Post-embryogenesis Snail1 expression is downregulated and is seldom found in adult tissues. In fact, mis-expression of Snail1 in the adult kidney is enough to disrupt epithelial homeostasis and can lead to fibrosis via induction of Type II EMT.

**Functions of Snail1 during development**

*Snail* was first discovered in *Drosophila* as being essential for embryonic development. Homozygous loss of *snail* resulted in the formation of twisted embryos attributed to malformation of the mesoderm and retraction of the germ band. The protein responsible for this phenotype was identified as zinc-finger containing transcription factor, Snail, and since then a plethora of its homologues which have different functions, have been identified in various organisms. These functions include mesodermal specification, neural crest cell migration, cell division, left-right symmetry regulation and apoptosis. Snail1 is one of the better studied members of this family for its crucial roles during gastrulation, where it is central to repressing alternative cell fates. For example, in *Drosophila* development *snail* expressed in the ventral region of the blastoderm, functions to repress the expression of lateral genes, in order to allow proper mesoderm specification and invagination to occur.

Studies show that Snail1 functions by directly repressing E-Cadherin during gastrulation in both mice and *Drosophila*. In fact, *in situ* hybridization of 7.5-9.5 days post-coitum mouse embryos show that E-Cadherin and Snail1 are reciprocally expressed in mesodermal cells undergoing EMT. Repression of E-Cadherin causes an EMT not only in mesodermal cells, but also palate cells and heart cushion cells which facilitates increased migration of cells aiding in spatial specification. Studies also show that Snail1, similar to Twist1, can recruit histone deacetylases to modify chromatin and facilitate repression of
target genes. Whatever the mode of repression of E-Cadherin, downregulation of E-Cadherin by Snail1 is essential for proper mesoderm ingression and specification during embryonic development. This crucial function of Snail1 during development is further highlighted in Snai1−/− mice that are embryonic lethal. Furthermore, isolated embryos are much smaller than their wild type littermates and contain a highly epithelial mesodermal layer with morphological defects, indicating a failure to undergo EMT.

Given that the primary role of Snail1 during development is repression of E-Cadherin to cause an EMT, and during oncogenic EMT, cancer cells can hijack this crucial developmental process, it is not surprising that Snail1 when re-expressed in certain cancers, plays a similar role in tumorigenesis.

**Role of Snail1 in cancer**

**Expression of Snail1 in cancer**

Increased expression of Snail1 is observed in various cancers including bladder, breast, ovarian, and hepatocellular carcinoma (HCC), among many others. In most cases Snail1 expression is associated with inducing an EMT and hence increases EMT-associated phenotypes like migration, invasion and metastasis. Increased expression of Snail1 has also been shown to correlate with poor prognosis in a number of cancers including gastric and breast cancer and HCC.

As seen in development, Snail1 expression is induced by a multitude of factors in different cancers. TGF-β is one of the main inducers of Snail1 in cancers including breast and pancreatic cancer and can in turn be induced by Snail1 in some contexts. Studies also show that Snail1 is induced by a crosstalk between RTKs–Ras, Hedgehog, Notch and/or Wnt signaling pathways (Figure 1.4). Interestingly, once activated by these pathways, Snail1 expression and stability is tightly regulated by its phosphorylation state (dependent on GSK3β and PAK-1) and cellular localization.
Figure 1.4 Schematic diagram of the signaling pathways associated with Snail-induced EMT.
An integrated and complex signaling network, including RTKs, TGF-β, Notch, Wnt, TNF-α, and BMPs signaling pathways, activate the transcription factor Snail, resulting in the induction of EMT. The expression of Snail causes a metabolic reprogramming, confers tumor cells with stem cell-like traits, resistance to immunosuppression, and promotes tumor recurrence and metastasis. Taken from 176.
Role of Snail1 in EMT and CSC phenotypes

Both Snail1 and Slug have been shown to cause an EMT during embryogenesis by specifically repressing E-Cadherin and hence allowing cells to migrate. Similar to their developmental roles, Cano and colleagues and Battle and colleagues first showed that Snail1 repressed E-Cadherin by directly binding to E-box sequences in the E-Cadherin promoter in various tumor cell lines that didn’t express E-Cadherin $^{170,177}$. Stable transfection of Snail1 in epithelial cell lines resulted in the induction of an EMT, with downregulation of E-Cadherin and other epithelial markers and a concomitant increase in mesenchymal markers. The cells also gained a more migratory and invasive phenotype both in vitro and in vivo, indicating that Snail1 is sufficient to cause an EMT in tumor cells $^{170,177}$. Snail1 has also been shown to regulate E-Cadherin expression by recruiting various chromatin modifiers to its promoter such as Sin3A and HDAC1/2 that aid in suppression of E-Cadherin $^{178-180}$ in different cancers. In addition to repressing E-Cadherin, Snail1 has also been shown to both directly and indirectly repress Occludin, claudins and PTEN and activate mesenchymal genes like vimentin and fibronectin ultimately inducing an EMT and increasing metastasis$^{173}$. Breast cancer studies show that EMT induced by Snail1 is accompanied by an increase in the percentage of cancer stem cells, two phenotypes that are often linked $^{40}$. This link, however, is cancer-type dependent, since an elegant study by Celià-Terrassa and colleagues demonstrated in both prostate and bladder cancer models, that overexpression of Snai1 in the enriched “cancer stem cell-like cell” population led to suppression of their renewal and metastatic phenotypes $^{181}$. Interestingly, Snail1 can also regulate the glycolytic switch in basal-like breast cancers, resulting in increased CSCs and tumorigenesis due to increased reactive oxygen species$^{182}$. 
**Additional roles of Snail1 in tumor progression**

Though Snail1 is predominantly associated with EMT, similar to Twist1, it can also induce chemoresistance in tumor cells, which may or may not be dependent on EMT. Interestingly, overexpression of Snail1 can cause resistance to radiation and paclitaxel in ovarian cancer cell lines by repressing a subset of genes that are not involved in EMT. Instead, Snail1 works by enabling the cells to escape p53-mediated apoptosis and in-turn gain stem-cell like characteristics \(^\text{183}\). Additionally, in numerous cancers including pancreatic cancer, melanoma and lung adenocarcinomas, Snail1 overexpression has been shown to be protective against chemotherapeutics such as 5-fluorouracil, gemcitabine and cisplatin \(^\text{173,184}\). In breast cancer, Snail1 has also been shown to reduce Estrogen receptor (ERα) activity and along with NF-κB, maintain tamoxifen resistance in cells \(^\text{185}\). Evasion of the immune system is an additional way by which tumors are thought to become more aggressive, and recently, Snail1 has been implicated in the modulation and regulation of the immune system by, for example, upregulating certain factors like TGF-β to increase the population of T-regulatory cells, which allow the tumor cells to escape immune surveillance \(^\text{184,186,187}\).

Collectively these studies highlight the crucial role of Snail1 as a potent EMT-inducing factor, whose expression in tumor cells allows them to not only become more invasive and migratory, but also become chemoresistant as well as better evade the immune system, ultimately allowing cells to more efficiently colonize at secondary sites.

**Six1: A Homeobox Containing-Transcription Factor**

**Structure and function**

Six1 is a member of the Six family of homeodomain-containing transcription factors, consisting of six members, Six1-6, all of which contain a conserved Six domain and a homeodomain. The Six family members belong to a larger superfamily of homeobox genes,
each of which contain the conserved 61 amino acid-DNA binding motif which is the homeodomain. This helix-turn-helix domain allows homeoproteins to bind to specific DNA sequences in order to activate and/or repress countless genes depending on the co-factors to which they bind.\textsuperscript{112,188} The Six1-6 transcription factors are grouped into a family based on the presence of a conserved Six domain, which is used by the members of this family to bind to different co-factors, and a conserved homeodomain\textsuperscript{112}. The Six domain is involved in mediating interactions with other co-factors (most notably the Eya family of proteins) and depending on the context, dictates whether the Six family members act as transcriptional activators or repressors.\textsuperscript{112} Less is known about the highly divergent and unstructured N- and C-terminals of the Six proteins, but studies have shown that the C-terminus of Six1, mediates its cell cycle specific degradation.\textsuperscript{189}

Six1 lacks intrinsic activation domains and hence requires co-factors like Eya in order to function as a bipartite transcription factor, predominantly leading to transcriptional activation of genes.\textsuperscript{112} In different contexts, Six can also bind to Groucho/Transducin-like enhancer of split via the Six domain leading to transcriptional repression. Other members of the Six family, such as Six2, contain intrinsic activation domains within their C-terminus and possibly rely less on the interactions with Eya family members. However, more recently, a study showed that interactions between Eya1 and Six2 were necessary for progenitor cell expansion during nephrogenesis\textsuperscript{190}, indicating that further studies need to be performed to elucidate the roles of each domain and the resulting interactions with the co-factors.

Nevertheless, numerous studies have been performed to identify the roles of Six1, the most well studied member of the family, in development and cancer.
Role of Six1 in development

Expression of Six1 during development

Studies show that the expression of Six1 is very tightly controlled during development. High mRNA expression of Six1 is observed in cells of the muscle lineage, various sensory structures, developing kidney, craniofacial structures, pituitary gland and thyroid gland to name a few $^{112}$. In-situ hybridization studies in mouse embryos show that Six1 expression can be detected as early as E8.5, strengthens until E12.5 especially in the muscle, but weans off and is barely detectable by E16. This low expression of Six1 continues into adulthood in most tissues $^{112}$. In the mammary gland, expression of Six1 is high in the developing gland, (highest at E17.5) and consequently drops through the processes of pregnancy, lactation and involution. Interestingly, Six1 is not required for normal mammary gland development $^{191}$ since both loss or overexpression of Six1 does not interfere with the development or function of the mouse mammary gland. This is presumably due to compensation by the other family members (Six2 and Six4), whose expression is increased in mammary glands of animals transplanted with Six1−/− embryonic mammary glands $^{191}$. These decreases in the expression of Six1 from development into adulthood suggest that this is very tightly regulated process. Mutational analyses and the study of conserved non-coding sequences in the tetrapod Six1 loci indicate that Six1 expression is most likely regulated by dynamic inputs from Sox, Pax and basic helix-loop-helix proteins, among others, during embryogenesis $^{192}$. More recently, studies done in Zebrafish demonstrate that miR-30a represses Six1 expression during the course of myogenesis $^{193}$.

Functions of Six1 during development

The Six family of homeobox transcription factors, are often regarded as “master regulators” of development since they control a plethora of cellular processes during embryonic development including cell migration, invasion, survival, apoptosis and EMT, to
name a few. Six1 has the ability to regulate cell growth and tissue specification in a context dependent manner. Its critical roles in embryonic development are most dramatically observed in the Six1 KO mice which exhibit severe malformation in various tissues and organs including the thymus, kidney, skeleton, lung, sensory organs, muscle and the brain. The KO studies indicate that the smaller and often missing organs are a result of decreased proliferation and increased apoptosis, resulting in hypoplasia and/or complete loss of tissue, highlighting the critical role of Six1 in regulating these cellular processes. These phenotypes are also most likely caused by the decrease in Six1’s target genes c-myc and gdnf in tissues such as the kidney, amongst others. Six1 has also been shown to promote expansion of progenitor cell populations in the kidney prior to differentiation. More recently, studies have shown that Six1 and Eya1 play pro-proliferative roles in mesenchymal cell populations during lung development, evidenced by decreased proliferation in Six1−/− and Eya1−/− mice and interestingly, increased differentiation in the lung epithelium compared to the wild type mice. Interestingly, Six1, along with its family member Six4, also plays an important role during myogenesis, where they together regulate EMT and consequently cell migration and invasion as well as cell survival. Furthermore, Six1 has also been shown to be important for both the maintenance of stem cells necessary for muscle growth and regeneration in mice as well as muscle progenitor cell proliferation in zebrafish. Together, these studies highlight the crucial role of Six1 during organogenesis in regulating not only EMT, but also proliferation as well as progenitor cell populations.

In accordance with Six1’s critical role during development, studies show that mutations in Six1 are associated with branchio-oto-renal (BOR) syndrome, an autosomal dominant genetic syndrome in which patients present with sensory nerve damage resulting in hearing loss, cleft palates, paralysis of facial muscles and renal defects in some cases.
Given that the process of oncogenesis closely imitates key developmental processes, it is not surprising that Six1, a core developmental transcription factor, has been recognized for its crucial roles in cancer.

**Role of Six1 in cancer**

*Expression of Six1 in cancer*

Numerous studies have demonstrated that Six1 is re-expressed and/or amplified in many epithelial cancers including breast, ovarian, colon carcinoma, HCC and in sarcomas like Rhabdomyosarcoma (RMS); and in each case it is thought to perform similar roles in tumorigenesis as it does during development\(^\text{204-208}\). Research shows that Six1 is overexpressed in almost 50% of primary breast tumors, but in an even greater 90% of metastatic lesions\(^\text{208}\) and high expression of Six1 in the primary tumor significantly correlates with shortened time to relapse, decreased time to metastasis and decreased breast cancer related survival in breast cancer patients\(^\text{206}\). While overexpression of wild type Six1 is thought to occur in most tumors, a gain-of-function mutation was recently discovered in the homeodomain of Six1 in a subset of Wilm’s tumors with higher proliferative indices\(^\text{209,210}\).

**Role of Six1 in EMT, CSC phenotypes and metastasis**

Similar to Twist1 and Snail1, most of the roles performed by Six1 in the context of cancer are related to its developmental functions. Several studies have shown that overexpression of Six1 results in cell autonomous induction of EMT as well as increased percentage of cancer stem cell-like cells within tumors in xenograft mouse models\(^\text{206,211}\). The induction of EMT by Six1 in xenograft mouse models involves increases in TGF-\(\beta\) signaling via upregulation of the receptor TGF\(\beta R1\) as well as a cluster of miRs that encroach on TGF-\(\beta\) signaling via direct downregulation of SMAD7\(^\text{212}\). Additionally, alteration in the
expression of various genes in the TGF-β response signature associated with metastatic disease as well as cell autonomous increases in metastasis is also observed \(^{206,213}\). Interestingly, these phenotypes are also recapitulated in Six1 transgenic mouse models, where misexpression of Six1 in the mammary gland epithelium of adult mice results in tumors with diverse histological subtypes. More importantly, many of these tumors undergo EMT and display stem-cell like features \(^{214}\), highlighting the potency of Six1’s oncogenic properties. In colorectal cancer, Six1 has been shown to activate Zeb1 and concomitantly represses miR-200c, resulting in the repression of E-Cadherin and induction of EMT \(^{204}\). Thus Six1 can cause EMT in different cancer types by regulating various molecules and pathways. Six1 also been shown to be a key regulator of metastasis in several cancers including HCC and RMS \(^{215,216}\). In breast cancer, in addition to upregulating TGF-β signaling, Six1 also induces lymphangiogenesis, the formation of lymphatic vessels within or surrounding the tumor, via upregulation of vascular endothelial growth factor (VEGF-C) and subsequently aids metastatic progression \(^{217}\). Interestingly, many of the pro-tumorigenic phenotypes of Six1 are dependent on its interaction with its cofactor Eya (Figure 1.5).

**Additional roles of Six1 in tumor progression**

In addition to inducing EMT and increasing metastasis in various cancer types, Six1 has also been shown to cell-autonomously promote proliferation by abrogation of cell cycle check points, as well as by direct upregulation of pro-proliferative genes such as Cyclin A1, D1 and c-myc \(^{208,218-220}\). Similar to Twist1 and Snail1, increased Six1 expression has also been linked to chemoresistance and overexpression of Six1 was recently shown to induce resistance to paclitaxel in breast cancer cells via regulation of Bax and Bcl2 \(^{221}\).
Figure 1.5 Role of Six1/Eya complex in cancer.
This figure illustrates the known hallmarks of cancer \(^\text{14}\) in which the SIX1 and EYA family members carry out their tumor-promoting properties. In total, the SIX1/EYA complex play a role in four out of the six original hallmarks, one of the two enabling characteristics and one of the two emerging hallmarks, highlighting the importance of Six1 in tumorigenesis. Taken from \(^\text{195}\).
Furthermore, genomic instability is one of the major hallmarks of cancer cells\textsuperscript{14}, and Six1, in addition to its various other pro-tumorigenic functions, can also aid the oncogenic process by increasing genomic instability and suppressing p53, a potent tumor suppressor\textsuperscript{222,223}. These studies collectively highlight the critical cell autonomous roles of Six1 in the oncogenic process.

Taken together, these studies highlight the crucial cell autonomous roles of all three EMT-inducing transcription factors, Twist1, Snail1 and Six1 during development and how they function in a similar manner during tumor progression. In recent times, immunohistochemical analyses and multiplex high-throughput single cell sequencing of human tumor cells have shown that tumors are phenotypically and genetically heterogeneous\textsuperscript{224} and various studies indicate that different sub-populations of cells within the tumor can cooperate to affect tumor progression. Given that EMT is thought to occur only in a small percentage of cells within the primary tumor\textsuperscript{1}, it is plausible that EMT-inducing transcription factors such as those discussed above, in addition to acting cell autonomously, can also influence neighboring tumor cells to undergo EMT and/or metastasize, and hence increase the overall metastatic efficiency of the tumor.

This thesis focuses on the as yet unrecognized non-cell autonomous roles of three EMT-inducing transcription factors Twist1, Snail1 and Six1 in mediating increased "metastatic" properties of other tumor cells that do not express these factors, and the effects of such interaction on metastatic outcome in breast cancer (Figure 1.6).
Figure 1.6 Graphical model of hypothesis.
Six1, Twist1 and Snail1 are major pro-metastatic, EMT-inducing transcription factors in breast cancer that are thought to be expressed only in a small percentage of cells within the primary tumor. We hypothesize that Six1, Snail1 and Twist1 can non-cell autonomously increase metastasis of the neighboring tumor cells that do not express any of these factors, and hence increase overall metastasis.
CHAPTER II
MATERIALS AND METHODS

Cell Culture, Plasmids And Conditioned Medium Collection

HMLER derivative cell lines were a generous gift from Dr. Robert Weinberg (Massachusetts Institute of Technology; 2009). HMEC-1 cells were a kind gift from Dr. Sean Colgan (UCDenver; 2015). MCF7-Ctrl and MCF7-Six1 cells were generated as previously described. All cell lines were cultured according to ATCC recommendations. Cell lines were routinely checked for mycoplasma and if found positive were either treated or earlier mycoplasma-negative freeze downs were used for experiments. The lines were profiled via short tandem repeat (STR) profiling to confirm their identity (February 2011, April 2015). Transient knockdowns (KDs) of Six1 and TSP1 were performed using ON-TARGETplus SMARTpool siRNAs (L-020093-00-0020-Six1; L-044041-01-0005-TSP1, Dharmacon). Cyclopamine (C-8700, LC Labs), GANT-61 (G9048, Sigma), 5E1 (Developmental Studies Hybridoma Bank, The University of Iowa) and VEGF-C function blocking antibody (sc-1881, Santa Cruz) were used at 5 or 10µM, 3-5µg/ml and 200ng/ml respectively in each experiment. For the in vivo experiment, GANT-61 purchased from MedChem Express (HY-13901) was resuspended in 4:1 corn oil: 100% Ethanol mixture. rhSHH (1845-SH, R&D systems) and rhVEGF-C (2179-VC-025, R&D systems) were used at 1µg/ml in experiments. Vehicle treatment in experiments – combination or single treatment of 100% Ethanol (for cyclopamine), DMSO (for GANT-61), NS-1 (control supernatant for 5E1), Polyclonal Goat IgG (for VEGF-C AB), PBS+BSA (for rhSHH and rhVEGF-C) depending on experiment. Drug concentrations used were chosen based on the current literature. Cells were tagged with pLenti NS-tRFP, pLNXC2-Zsgreen or SFG-nes-TGL-luciferase plasmids. pcDNA3.1 hygro vectors were used for transient overexpression of Six1 and Eya2 and pLKO.1 puro constructs were used for Six1 KD in 4t1 cells. For conditioned medium (CM), equal numbers
of cells were plated and allowed to grow for 24hrs. Next day, the plates of cells were washed and replaced with fresh medium (serum-free or serum-containing medium). CM was collected from the cells 48hrs later, filtered through .45µm filter and stored at -20°C. Repeated freeze-thaws of CM was avoided. For mass spectrometry analyses, CM was filtered using 30KDa molecular weight cutoff columns (UFC903024, EMD Millipore) according to manufacturer’s protocol prior to sample submission to Mass spectrometry core (UCCC).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted using the RNAeasy RNA isolation kit (Qiagen). cDNA synthesis was performed using iScript (Biorad) from 1µg of mRNA. qRT–PCR assays were performed using ssoFast Evagreen supermix (BioRad) and run and analyzed using the Biorad CFX96. The primer sequences used are listed in Table 2.1. For co-culture experiments in the 4t1 system for EMT markers, 67nr-Zsgreen+ cells were cultured with 4t1-tRFP+ cells in a 1:1 ratio (and individually as controls) for 6 and 12 days. The Zsgreen+ and tRFP+ cells were obtained from each condition by flow cytometry and RNA was extracted.

Immunoblot Analysis

Whole cell lysates (WCL) and nuclear extracts (NE) were generated as previously described 206. In brief, RIPA buffer was used to extract WCLs and NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (78833, ThermoFisher) kit was used for NEs. In both cases equal amounts of lysates (35-50µg) were electrophoresed and transferred to PVDF membranes. The membranes were blocked in 5% milk in TBST for 1hr and incubated with primary antibody at 4°C O/N. The antibodies used are listed in Table 2.2. For co-culture experiments for EMT markers, HMLER-Ctrl-GFP+ cells were cultured with HMLER-Snail1 or Twist1-tRFP+ cells in 10:1 or 1:1 ratio and (individually as controls) for 14-16 days. The GFP+ cells (and tRFP+ cells in the control samples) were obtained from each condition by
flow cytometry and WCLs were extracted from them. For CM samples, Lowry assays were first performed on CM to estimate total protein content in each sample. 75-100µg of protein was then precipitated from each sample using established acetone protein precipitation protocols, immediately re-suspended in sample buffer and electrophoresed as described above.

**Immunocytochemistry**

10,000 MCF7 cells were plated in each well of 8-well chamber slides (154534, Nunc Lab-Tek) with different CM and drug conditions and incubated for 48hrs. The cells were fixed using 4% paraformaldehyde (PFA) and stained with primary antibodies at 4°C O/N. Slides were mounted using ProLong® Gold Antifade Mountant with DAPI (P-36931, ThermoFisher). Images were taken using a fluorescence microscope from field of vision containing cells at about 60-80% confluency, since it was observed that E-Cadherin expression in MCF7 cells was substantially affected by cell confluence. Blinded membranous E-Cadherin quantification was performed by dividing the number of cells with membranous E-Cadherin staining by the total number of DAPI stained cells in that field of vision to obtain a percentage.

**Anoikis Resistance Assay**

Cells were cultured in the different CM for 48 hours, after which they were trypsinized, counted and plated on poly-HEMA (12mg/ml in 95% Ethanol) coated plates for 24 hours in CM. Next day, cells were retrieved and re-plated in 96-well plates in full media for 5-6 hours until they attached. The surviving cells were then analyzed using either MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate) assay (Promega), crystal violet staining, or their numbers were counted, to determine their anoikis resistance.
Cell Migration/Wound Healing Assay

Cell migration was measured using a modified scratch assay with culture inserts that create a uniform 500µm gap (80209, Ibidi). 5-6x10^5 cells/ml were plated in 70µl of different CM in each compartment of the insert and incubated O/N. Cells were treated with drugs or vehicle at the time of CM addition. Inserts were removed after 16-18hrs (time point 0hr) and distance migrated by cells in 5-8hrs was measured using DP2-BSW software (v2.2; Olympus). The migration of cells of either represented as distance migrated (µm) or % gap closure.

Cell Invasion Assay

Cell culture inserts (353097, BD Falcon) were coated with 1:20 diluted matrigel (354230, Corning) in serum-free media. 50,000 cells were plated on the matrigel in different CM and/or drug conditions (added when CM was added to cells) and allowed to invade through the membrane towards medium containing 10% FBS in the bottom chamber. The assay was performed either for 8-10hrs (4t1 system) or 18-20hrs (HMLER system). At the end point, cells and matrigel above the membrane were wiped with cotton swabs and cells below the membrane were fixed in 4% PFA and stained with crystal violet. The numbers of invading cells were analyzed either by measuring their absorbance (after crystal violet staining and extraction of the dye using glacial acetic acid) or by counting the cells using a bright field microscope.

Enzyme Linked Immunosorbent Assay (Elisa)

Cells were cultured in serum-free medium and CM was collected after 48hrs. For MCF7 cells, CM was collected from three clonal isolates of each cell type (MCF7-Ctrl and Six1 cells). Equal volumes of samples were loaded on ELISA plates (SHH-ab100639, Abcam; TSP1-DTSP10, R&D). The concentration of protein in the CM was determined by
normalizing the volume of sample loaded to the concentration of total protein in the sample, which was previously measured using Lowry assay.

**Gli1-Gfp Reporter Assays**

3-5x10^4 cells were plated in 24-well plates in different CM and/or drug conditions and simultaneously transfected in different wells with either 7-Gli1-GFP or m-Gli1-GFP reporter. The cells in each condition were also transfected in a separate well with a constitutively expressing GFP-Ctrl vector on the same vector background as the 7-Gli1 and m-Gli1 reporters. The number of GFP^+ cells in each condition (7-Gli1 or m-Gli1, indicating activated Gli signaling in those cells) was counted using a fluorescence microscope. This was normalized to the number of GFP^+ cells in the well containing the GFP-Ctrl vector for each corresponding condition to account for differences in transfection efficiencies of the cells when cultured in different CM and/or drugs. Results from three or more independent experiments using different sets of CM were grouped and graphed as % of 7-Gli1 or m-Gli1 GFP^+ cells. Further details of the reporters will be described elsewhere.

**Mouse Models**

In mixed tumor experiments, Estrogen pellets (mixture of αCellulose and Estradiol) were implanted subcutaneously into mice a day prior to injection with tumor cells as described previously. All animal studies were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committee at the University of Colorado, AMC.

**Zsgreen/tRFP experiment**

MCF7 cells were injected orthotopically in 100µl of a 1:1 mixture of sterile 1XPBS: growth-factor reduced matrigel (354230, Corning) in the nipple of the 4th mammary fat pad in 8-14 week old NOG/SCID female mice for each condition. The conditions were: 1 million
Zsgreen² MCF7-Ctrl cells, 1 million tRFP⁺ MCF7-Six1 cells, 1:1 ratio of 500,000 cells each of Zsgreen² MCF7-Ctrl cells and tRFP⁺ MCF7-Six1 cells, and 1:1 ratio of a million cells each of Zsgreen² MCF7-Ctrl cells and tRFP⁺ MCF7-Six1 cells. This was done to control for both the total number of cells injected as well as for the total number of each cell type injected into the mice. The tumor size was monitored weekly and the mice were sacrificed when the tumor volume reached 2cm³. The lungs of the mice were harvested at the time of death and viewed under a fluorescent microscope. Images were taken at the same intensity and magnification whenever Zsgreen² and tRFP⁺ cells were seen.

**Luciferase/tRFP experiment**

MCF7 cells were injected as described above in female mice less than 1 year of age. The conditions were: 1:1 ratio of 500,000 MCF7-Ctrl-luc: 500,000 untagged MCF7-Ctrl cells, 1:1 ratio of 500,000 MCF7-Six1-tRFP: 500,000 untagged MCF7-Six1 cells, 1:1 ratio of 500,000 MCF7-Ctrl-luc: 500,000 MCF7-Six1-tRFP cells. This served as a control for both total cell number injected as well as number of “tagged” cells injected in the mice. The tumor volume and metastatic progression was followed weekly by measuring both luminescence and fluorescence signal using IVIS imaging software. Mice that received mixed injections were treated either with GANT-61 or vehicle control (4:1, corn oil: ethanol mixture) when their tumors reached 1cm³. Mice were injected every other day for 18 days with 50mg/kg of GANT-61 (or equal volume of vehicle) subcutaneously in the supra-scapular region. This drug dosage and injection method was chosen based on current literature searches. The tumor size and metastatic spread of MCF7-Ctrl-luc cells in singly injected and mixed tumors was compared and analyzed between mouse groups with similar tumor volumes. The tumor size and metastatic spread of MCF7-Six1-tRFP cells was compared and analyzed at the same time point, which corresponded to similar tumor volumes across different injection groups. Luminescence an fluorescent signal from the primary tumor and distant sites in
vehicle and drug treated mice was compared pre- and post-treatment, and analyzed using Living Image software. Singly injected tumor-containing mice were sacrificed when tumor volume reached 2cm$^3$ and mixed tumor-mice were sacrificed at the end of treatment.

**PDX models**

For the triple negative PDX models, PDX tumor fragments were transplanted into the cleared fat pad of 3- to 4-week-old SCID/Beige mice. Mice receiving treatment, were treated either with vehicle, IPI-926 (40mg/kg, oral gavage) or GANT-61 (50mg/kg, subcutaneous injection) once a day for two weeks, starting from a tumor volume of $\approx 200$ mm$^3$. Tumor volume (mm$^3$) was measured twice weekly and calculated as length $\times$ width $\times$ width $\times$ 0.5.

**Mass Spectrometry (Performed By The Biological Mass Spectrometry Core, Ucd)**

**Sample preparation**

CM was collected from three different sets of cells (MCF7-Ctrl and MCF7-Six1 cells) after 48hrs of culture in serum-free medium. The samples were concentrated using a 30KDa molecular weight cutoff column and the top fraction (CM containing proteins above 30KDa) was analyzed. The samples were digested according to the FASP protocol using a 10KDa molecular weight cutoff filter$^{225}$. In brief, samples were mixed in the filter unit with 8 M urea in 0.1 M ammonium bicarbonate (ABC), pH 8.5 and centrifuged at 14,000g for 15mins. all subsequent steps of centrifugation were performed at 14,000g. The proteins were reduced by addition of 100 μL of 10 mM DTT in 8 M urea in 0.1 M ABC, pH 8.5, incubated for 30mins at RT and the device was then centrifuged. Subsequently, 100 μL of 55 mM iodoacetamide in 8 M urea in 0.1 M ABC, pH 8.5 was added to the samples, after which the samples were incubated for 30mins at RT in the dark, and then centrifuged. Following centrifugation, three washing steps with 100 μL of 8 M urea in 0.1 M ABC, pH 8.5 solution were performed, followed by three washing steps with 100 μL of 0.1 M ABC buffer. Protein digestion was carried out in the presence 0.02 % of ProteaseMax (Promega, Madison, WI) detergent at
37°C O/N. Peptides were recovered by transferring the filter unit to a new collection tube followed by centrifugation for 10mins. To complete peptide recovery, filters were rinsed twice with 50 μL of 0.2% FA and 10 mM ABC and peptides recovered by centrifugation. The peptide mixture was desalted and concentrated on Thermo Scientific Pierce C18 Tip.

**Mass spectrometry**

Samples were analyzed on a Q Exactive quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Easy-nLC 1000 UHPLC (Thermo Fisher Scientific) through a nanoelectrospray ion source. Peptides were separated on a self-made 15 cm C18 analytical column (100 µm x 10 cm) packed with 2.7 µm Phenomenex Cortecs C18 resin. After equilibration with 3μL 5% acetonitrile 0.1% formic acid, the peptides were separated by a 225mins linear gradient from 2% to 32% acetonitrile with 0.1% formic acid at 350nL/min. Liquid Chromatography (LC) mobile phase solvents and sample dilutions used 0.1% formic acid in water (Buffer A) and 0.1% formic acid in acetonitrile (Buffer B) (Optima™ LC/MS, Fisher Scientific, Pittsburgh, PA). Data acquisition was performed using the instrument supplied Xcalibur™ (version 3.0) software. The mass spectrometer was operated in the positive ion mode, in the data–dependent acquisition mode. In one scan cycle, peptide ions were first scanned by full MS at resolution 70,000 (FWHM at m/z 200), and then the top 12 intensive ions (2 m/z isolation window) were sequentially subjected to HCD fragmentation and detected at resolution 17,500. Dynamic exclusion was set to 20s. Spray voltage was set to 2.5 kV, S-lens RF level at 55, and heated capillary at 275 °C.

**Database searching, protein identification**

MS/MS spectra were extracted from raw data files and converted into mgf (Mascot generic format) files using a MassMatrix (Cleveland, OH). These mgf files were then independently searched against human SwissProt database using an in-house Mascot™
server (Version 2.2.06, Matrix Science). Mass tolerances were +/- 10ppm for MS peaks, and +/- 0.1 Da for MS/MS fragment ions. Trypsin specificity was used allowing for 1 missed cleavage. Met oxidation, protein N-terminal acetylation, and peptide N-terminal pyroglutamic acid formation were allowed for variable modifications while carbamidomethyl of Cys was set as a fixed modification.

Scaffold (version 4.4, Proteome Software, Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified unique peptides.

Statistical Analysis

Prism software (v5.0; GraphPad) was used for all statistical analyses. Two-tailed unpaired Student’s T-test was used when only a pair of conditions was compared. One-way Analysis of Variance (ANOVA) non-parametric followed by Tukey post test was utilized when multiple conditions were being compared and analyzed. Specific analyses used for each experiment is described in the figure legends. *p value <0.05; **p value <0.001; ***p value <0.0001
Table 2.1 Primer sequences for qRT-PCR

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Table 2.2 Antibodies for Western blot analyses and Immunocytochemistry

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<td>1:1000 – WB</td>
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CHAPTER III
SNAIL1, TWIST1 AND SIX1 FUNCTION NON-CELL AUTONOMOUSLY IN BREAST CANCER CELLS

Introduction

In recent years, a multitude of studies have shown that primary tumors are heterogeneous masses of cells and are made up of different sub-populations of cells which have diverse driver mutations, gene expression, proliferation rates and even distinct metastatic potential \(^6\). The existence of such diverse populations of cells, influenced by not only the tumor microenvironment but also by nutrient availability within the tumor, raises the possibility that different cells within the tumor may interact with each other. Furthermore, these interactions may affect not only tumor maintenance, but also tumor progression and metastatic outcomes. Indeed, several studies have now shown that such crosstalk does occur between different populations of cells and it affects both the primary tumor and the metastatic incidence \(^1\). One such interaction observed in primary tumors is between cells that have undergone EMT and those that have not (non-EMT cells). As mentioned previously, cells that have undergone oncogenic EMT, (EMT cells) seldom undergo a complete EMT, and hence more often than not, exist in a partial, “mesenchymal-like” state. In order to simplify their nomenclature in this thesis, they will referred to as EMT cells. Several studies have shown that the interaction between EMT and non-EMT cells in the tumor can increase the metastasis of just the EMT cells, in some cases enable only the non-EMT cells to metastasize, and in other cases increase the overall metastatic efficiency of the tumor \(^1\). This scenario is very dangerous, since most cancer patients die due to their metastatic burden rather than primary tumor burden \(^6\).

Indeed, one such type of cancer where metastatic burden is the major cause of mortality is breast cancer. Breast cancer is the second leading cause of cancer-related
deaths in women in the United States, with over 240,000 cases of invasive disease diagnosed in 2015 alone\textsuperscript{226}. Breast cancer patients with localized disease have a 5-year survival rate of around 99\%, but this dramatically drops to around 25\% when metastases are present. Studies show that metastatic outcome in breast cancer is influenced not only by the EMT status of the tumor, but also by the interaction of tumor cells with their microenvironment\textsuperscript{227}. Recent studies also show that breast tumors, like most other cancers, are heterogeneous\textsuperscript{228}, which suggests that breast cancer metastasis can be influenced by the interactions between different sub-populations of cells that exist within the breast tumor.

In 1983 Fred Miller used syngeneic mouse mammary carcinoma cells derived from a spontaneous mammary tumor that arose in a Balb/c mouse to show that crosstalk could indeed occur between different sub-populations of cells\textsuperscript{24}. He discovered that intravenously co-injecting highly metastatic 4t1 mammary carcinoma cells with non-metastatic 67nr mammary carcinoma cells resulted in the presence of both cell types in the lungs\textsuperscript{78}. They saw similar results when using 168FARN cells, which on their own, invade into the surrounding basement membrane but are not metastatic. In brief, subcutaneous injections of 168FARN resulted in formation of metastatic nodules only on co-injection with 4t1 cells\textsuperscript{78}. Fred Miller’s group further demonstrated that syngeneic cell lines with higher metastatic potential could inhibit the \textit{in vivo} growth rate of a weakly metastatic cell line in mice, via interactions with the immune system of the host mice\textsuperscript{80}. These studies suggest that breast cancer cells follow the same paradigm as seen in other tumor types, wherein subclones in the same tumor can interact to affect tumor progression. Though a few more instances of clonal interaction have been observed in breast cancer\textsuperscript{1}, the mechanisms behind this crosstalk remain largely unknown.

Twist1, Snail1 and Six1 are three EMT-inducing pro-metastatic transcription factors whose cell autonomous roles in breast cancer progression have been extensively characterized. As mentioned previously, they function cell autonomously in breast cancer
cells by inducing EMT, increasing cancer stem cells properties, causing chemoresistance, increasing migration and invasion of cells, and ultimately increasing their metastatic potential \(^{171,206}\). Interestingly, these transcription factors have variable expression levels in different breast cancer cell lines and in patients stratified based on breast cancer subtype. Six1 mRNA is overexpressed in 50% of primary breast tumors and 90% of metastatic lesions\(^{229}\). Though Six1 is expressed in all subtypes of breast cancer, its overexpression correlates with poor prognosis specifically in the luminal B subtype \(^{211}\). Twist1 and Snail1 are expressed predominantly in more basal-like cell lines\(^{230}\), but co-expression of Twist1 and Snail1 can predict poor prognosis in estrogen receptor-positive early breast cancers as well as in claudin-low and basal breast tumors. Studies also show that the gene expression signatures (GES) derived by overexpressing both Snail1 and Twist1 are very similar and are associated with more aggressive breast cancers\(^{119,231}\). Moreover, Twist1 levels are elevated in patients with nodal metastases and Snail1 expression can promote mammary tumor recurrence\(^{232,233}\). Together these studies highlight the importance of Six1, Twist1 and Snail1 in breast cancer progression.

Of interest, only a few cells on the leading edge of an invasive tumor are thought to express factors like Twist1 and studies show that EMT occurs only in a small percentage of cells in the primary tumor\(^{56,95}\). Despite the fact that EMT-inducing transcription factors are often not uniformly expressed throughout the tumor, tumors expressing Twist1, Snail1 and Six1 are highly metastatic. Hence, it is plausible that the few cells that express these factors, undergo EMT and have innate metastatic potential can influence the neighboring tumor cells to increase their metastasis non-cell autonomously. We therefore decided to test this hypothesis in different breast cancer cell lines that specifically express these transcription factors to determine if they can function in a non-cell autonomous manner.
Results

Twist1 and Snail1 can function non-cell autonomously in HMLER cells

To determine whether Twist1 and Snail1 can non-cell autonomously increase metastatic properties of cells that do not express either factor, we used the HMLER system. HMLER cells are human mammary epithelial cells (HMECs) that have spontaneously lost p16\textsuperscript{INK4a}, are immortalized using hTERT and transformed using an oncogenic H-Ras\textsuperscript{V12}.\textsuperscript{234} Studies have previously shown that overexpression of Twist1 and Snail1 in HMLER cells causes them to cell autonomously undergo EMT and become more metastatic.\textsuperscript{40} Hence, we asked if these transcription factors would be able to function in a non-cell autonomous manner, using HMLER-Snail1 and HMLER-Twist1 cells tagged with tRFP and corresponding HMLER-GFP cells (HMLER-Ctrl) which did not express either transcription factor.

To mimic what might occur in the primary tumor, where only a small percentage of cells are expected to express either Twist1 or Snail1,\textsuperscript{1,56} we co-cultured GFP\textsuperscript{*}HMLER-Ctrl cells with tRFP\textsuperscript{*}HMLER-Snail1/Twist1 cells in either a 1:1 or 10:1 ratio, and performed migration assays. When co-cultured with either HMLER-Snail1 or Twist1 cells, the migration of HMLER-Ctrl cells was increased, even when only 1 of 10 cells in the mixture expressed the EMT-inducing transcription factor (Figure 3.1 A-D). We and others have previously shown that both Twist1 and Snail1 can take up to 14 days to stably induce an EMT in HMLER cells in which one can observe morphological changes as well as changes in EMT markers such as E-Cadherin and Vimentin.\textsuperscript{40,156} To determine if this can occur non-cell autonomously as well, we co-cultured the GFP\textsuperscript{*}HMLER-Ctrl cells with tRFP\textsuperscript{*}Twist1 or tRFP\textsuperscript{*}Snail1 cells for 14-16 days (at a 1:1 and 10:1 ratio), sorted the cells using flow cytometry, and extracted proteins from the cells to look at the levels of EMT markers.
Figure 3.1 Snail1 and Twist1 non-cell autonomously increase metastatic properties of control cells without causing changes in EMT markers (continued)
Figure 3.1 Snail1 and Twist1 non-cell autonomously increase metastatic properties of control cells without causing changes in EMT markers.

5h migration assay performed on (A) tRFP+ HMLER-Snail1 cells alone, GFP+ HMLER-Ctrl cells alone, or a mixture of the two cell types at 1:1 or 10:1 ratio. (C) Quantitation of GFP+ cell migration in panel A (B) tRFP+ HMLER-Twist1 cells alone, GFP+ HMLER-Ctrl cells alone, or a mixture of the two cell types at 1:1 or 10:1 ratio. (D) Quantitation of GFP+ cell migration in panel C. (E) Representative image of HMLER-Ctrl cells co-cultured with HMLER-Snail1/Twist1 cells (day 11 of 14). (F) Representative Western blot analyses of GFP+ or tRFP+ sorted from co-culture experiments at day 14. (HDAC1– loading control).

Lanes: 1. GFP+HMLER Ctrl cells, 2. tRFP+ HMLER-Snail1 cells, (3,4) GFP+HMLER Ctrl cells from (3) 1:1 and (4) 10:1 co-culture with HMLER-Snail1 cells, 5. tRFP+ HMLER-Twist1 cells, (6,7) GFP+HMLER Ctrl cells from (6) 1:1 and (7) 10:1 co-culture with HMLER-Twist1 cells.

SEM shown, n≥ 3. One way ANOVA with Tukey post test for (C,D). ***p<0.0001
Surprisingly, no morphological changes or alterations in epithelial or mesenchymal markers were observed in the HMLER-Ctrl cells when co-cultured with HMLER-Snail1 or Twist1 cells for 14 days, (Figure 3.1 E-G). This suggests that EMT and phenotypic characteristics like migration are not always linked downstream of Twist1 and Snail1. These data also provide evidence that EMT-inducing transcription factors like Twist1 and Snail1 expressed breast cancer cells can non-cell autonomously influence neighboring cells.

To determine whether the non-cell autonomous effects of Twist1 and Snail1 on migration were due to a secreted factor, we isolated conditioned media (CM) from HMLER-Twist1 and Snail1 cells, and tested whether these media could increase the migratory and invasive properties of HMLER-Ctrl cells. We found that HMLER-Ctrl cells cultured in CM from HMLER-Snail1 and Twist1 cells have both increased migration and invasion compared to when cultured in Ctrl CM (Figure 3.2 A,B). These data indicate that Twist1 and Snail1 can non-cell autonomously increase the aggressive nature of epithelial, non-EMT tumor cells via secretion of factors into the medium.

**Six1 functions downstream of Twist1 and Snail1 to mediate their non-cell autonomous effects in vitro**

Several studies have shown that EMT-inducing molecules and transcription factors often affect the levels of each other in the tumor cells \(^{119,235,236}\). For example, in colorectal cancer Six1 promotes an EMT through activation of Zeb1, another prominent EMT-inducing transcription factor \(^{204}\). Interestingly, we found that Six1 expression is increased on both the mRNA and protein levels in HMLER-Snail1 and HMLER-Twist1 cells compared to HMLER-Ctrl cells (Figure 3.3 A, B). We thus asked whether Six1 plays a role downstream of Twist1 and/or Snail1 to facilitate their non-cell autonomous effects. To this end, we knocked down (KD) Six1 in the HMLER-Twist1 and Snail1 cells using siRNAs [consistently achieving 70-80% Six1 KD on both the mRNA and protein levels (Figure 3.3 A,B)].
Figure 3.2 CM from Snail1 and Twist1 can non-cell autonomously increase metastatic properties of control cells. 
(A) 6h migration and (B) 18-20h invasion assays of HMLER-Ctrl cells cultured in CM from HMLER-Snail1 or Twist1 cells. SEM shown in representative image with n≥ 3. One way ANOVA with Tukey post test. *p value<0.05, **p value<0.001
cultured the HMLER-Ctrl cells in CM from HMLER-Twist/Snail1 with and without Six1 KD, and carried out in vitro migration and invasion assays. Results from this experiment showed that HMLER-Ctrl cells have significantly decreased migration and invasion when cultured in CM from HMLER-Snail1+Six1 KD cells compared to when cultured in CM from HMLER-Snail1 cells in which Six1 is present (Figure 3.3 C,D).

Similar results were observed with Twist1, wherein HMLER-Ctrl cells had decreased migration and invasion when cultured in CM from HMLER-Twist1 cells with reduced levels of Six1 (Figure 3.3 E, F). Of note, the level of Six1 in the recipient HMLER-Ctrl cells remained low and unchanged in all cases when medium was transferred to the cells that contained siSix1 (Figure 3.3 G). This demonstrates that the observed effects were due to loss of Six1 in the donor HMLER-Twist1/Snail1 cells, and not due to any effects on levels of Six1 in the recipient cells. Thus, Six1 is necessary downstream of Twist1 and Snail1 to non-cell autonomously increase the “metastatic” properties of the cells not expressing these factors.

**Six1 is sufficient to non-cell autonomously induce EMT-like phenotypes in vitro in a human system**

Since Six1 is necessary to mediate non-cell autonomous phenotypes downstream of Twist1 and Snail1, we asked whether Six1, independent of Twist1 or Snail1, is also sufficient to mediate non-cell autonomous phenotypes on tumor cells not expressing Six1. To this end, we developed an in vitro system in which we could examine parameters known to be affected by Six1 in a cell autonomous manner, in cells not expressing Six1.

We used MCF7 cells stably overexpressing Six1 (MCF7-Six1) and the corresponding MCF7-Ctrl cells, which express the Chloramphenicol Acetyl Transferase gene (Figure 3.4 A), in which our laboratory has previously shown that Six1 is sufficient to cell autonomously cause an EMT and increase metastasis.
Figure 3.3 Six1 is required downstream of Snail1 and Twist1 to mediate their non-cell autonomous effects (continued)
Figure 3.3 Six1 is required downstream of Snail1 and Twist1 to mediate their non-cell autonomous effects. (A) qRT-PCR analyses and (B) western blot analyses of WCLs from HMLER-Ctrl, Snail1 and Twist1 cells show that Six1 is upregulated downstream of Snail1 and Twist1 and is knocked down in cells transfected with 150nM of an siRNA pool against Six1 (siSix1) compared to a non-targeting siRNA pool (siNT). Gene expression normalized to 18s. Quantitation of 8h migration (C,E) and 18h invasion assays (D,F) of HMLER-Ctrl cells in CM from HMLER-Snail1/Twist1 +/- siSix1 cells (G) HMLER-Ctrl cells cultured in CM from HMLER-Snail1/Twist1 +/- siSix1 cells don’t exhibit changes in Six1 levels. Gene expression normalized to GAPDH. SEM shown in representative images, n≥6. One way ANOVA with Tukey post test in all cases (ns-not significant) *p<0.5, **p<0.001, ***p<0.0001
It should be noted that in these cells, Six1 does not cell autonomously affect migration or invasion, and thus we examined only EMT parameters as a means to determine whether Six1 could non-cell autonomously increase the aggressive characteristics of non-Six1 expressing cells. Importantly, in contrast to the HMLER system, MCF7-Ctrl cells cultured in CM from MCF7-Six1 cells underwent a shift in the EMT protein expression profile, in which cytokeratin-18 (CK-18), an epithelial marker was downregulated, and Fibronectin (FN1), a mesenchymal marker was upregulated (Figure 3.4 B). In addition, a striking downregulation of E-Cadherin in cell membranes was observed in MCF7-Ctrl cells cultured in CM from MCF7-Six1 cells, another classical sign of cells undergoing an EMT (Figure 3.4 C). An important step in the metastatic cascade is the ability of an epithelial tumor cell to survive detached from extracellular matrix, (i.e., become anoikis resistant), both in the bloodstream and initially at the secondary site, a phenotype that is enhanced by EMT \(^{237}\). In line with the relationship between EMT and anoikis resistance, MCF7-Ctrl cells exposed to MCF7-Six1 CM were able to survive significantly more under unattached conditions (Figure 3.4 D). Together, these findings demonstrate that Six1 is able to non-cell autonomously cause EMT-related phenotypes in cells that do not express Six1, and suggest that while the particular molecular events may differ in different backgrounds, Six1 is both necessary and sufficient to impart aggressive characteristics on cells that do not express Six1.

**Six1 can induce non-cell autonomous phenotypes in additional systems**

To further test if Six1 could function in a non-cell autonomous manner in other systems, we made use of the 4t1 system of cell lines. This system of cell lines consists of 5 syngeneic lines (4t1, 66cl4, 4t07, 168FARN and 67nr) with varying metastatic potential, that were derived from a spontaneous mammary tumor that arose in a Balb/C mouse \(^{24}\).
Figure 3.4 Six1 is sufficient to non-cell autonomously cause an EMT in control cells. 
(A) qRT-PCR analyses of 3 clones each of MCF7-Ctrl and MCF7-Six1 cells (combined data shown) 
(B) Western blot analyses performed on WCLs from MCF7-Ctrl and MCF7-Six1 cells in their own CM, and MCF7-Ctrl cells cultured in CM from MCF7-Six1 cells for 48hrs (β-Tub – β-Tubulin, loading control). 
(C) Representative ICC of E-Cadherin (green) in MCF7-Ctrl cells in MCF7-Ctrl or Six1 CM for 48hrs (Dapi, blue). Quantitation of % membranous E-Cadherin shown, n≥100, scale-20µm 
(D) Representative image of anoikis resistance of MCF7-Ctrl cells cultured in CM from indicated cell types for 24hrs represented as absorbance of cells after crystal violet staining. SEM shown in representative images, n≥3. Two tailed unpaired t-test for (A, C) and One way ANOVA with Tukey post test for (D). ***p value <0.0001
Yang and colleagues first showed, using the above described cell line system, that Twist1 was a major oncogenic factor and exhibited the highest level of expression in the most metastatic cell line, 4t1, compared to the tumorigenic but non-metastatic 67nr cells. Importantly, knocking down Twist1 in 4t1 cells led to a decrease in the number of 4t1 cells in the bloodstream as well as robust suppression of metastasis to lungs *in vivo*, indicating that Twist1 was a major player in the cell autonomous metastatic process of 4t1 cells.

Studies show that co-injection of 4t1 and 67nr cells results in metastasis of the usually non-metastatic 67nr cells, suggesting that crosstalk occurs between these cells, which enables the metastasis of 67nr cells. Hence, we first wanted to test if this crosstalk can occur *in vitro* as well to affect EMT properties and metastatic phenotypes of the “control” 67nr cells. It is important to note that 4t1 metastatic cells appear epithelial in culture and express markers like E-Cadherin and CK-18, while the non-metastatic 67nr cells are more mesenchymal-like and express markers like FN1 and N-Cadherin. Though this does not align with the conventional paradigm of increased EMT and metastasis, 4t1 cells are thought to undergo partial EMT *in vivo* and exist in a more plastic state enabling them to better metastasize. First, to determine if the EMT status of 67nr cells was altered in the presence of 4t1 cells, we tagged the 67nr with Zsgreen (67nr-zsgreen) and 4t1 cells with tRFP (4t1-tRFP) and co-cultured the cells for 12 days. We flow sorted the cells at day 6 and day 12 to exclusively obtain the Zsgreen*+* population from the co-culture, and extracted RNA from the samples to determine if there was a shift in gene expression pattern in the 67nr cells towards that of the 4t1 cells. Indeed, we observed that 67nr cells co-cultured with 4t1 cells expressed increased CK-18 and decreased N-cadherin levels, indicating a shift in their gene expression patterns, similar to the 4t1 cells (Figure 3.5 A, B). To determine if the 4t1 cells could induce non-cell autonomous phenotypic changes in the 67nr cells *in vitro*, we collected CM from the metastatic 4t1 cells, cultured the 67nr cells in this CM, and performed migration and invasion assays as phenotypic read outs.
Figure 3.5 4t1 cells non-cell autonomously alter the phenotype of 67nr cells. (A,B) qRT-PCR analysis on Zsgreen* 67nr cells flow sorted after co-culture with 4t1 cells for 6 and 12 days, n=1. (C) 8hr migration and (D) 10hr invasion assays in 67nr cells in different CM. SEM shown, n=3. One way ANOVA with Tukey post test for (C, D). *p value<0.05, ***p value <0.0001
We observed that 67nr cells cultured CM from 4t1 cells had increased migration and invasion (Figure 3.5 C, D) compared to when cultured in their own CM. Interestingly, we observed that in addition to 4t1 cells expressing higher levels of Twist1, they also express higher levels of Six1 compared to 67nr cells (Figure 3.6 A). Whether or not this endogenous upregulation of Six1 is due to increased expression of Twist1 (since EMT-factors often regulate each other’s levels) is not known. We thus asked whether Six1 in this context, would be both necessary and sufficient to mediate non-cell autonomous effects in the 4t1 system.

In order to test for the necessity of Six1 in this system, we established 4t1 cell lines with stable Six1 knock down using three independent shRNA constructs (Figure 3.6 B). We then collected CM from the shNT cells (transduced with shScramble, and still expressing Six1) and two shSix1 cell lines (transduced with two different shSix1 constructs), cultured the 67nr cells in CM and performed migration and invasion assays. 67nr cells cultured in CM from 4t1 cells that had Six1 KD (shSix1-1, 2) had significantly decreased migration and invasion compared to control conditions (Figure 3.6 C, D). We also performed anoikis resistance assays with 67nr cells cultured in either 4t1 shNT or 4t1 shsix1-1 cells and observed increased anchorage independent growth in the 67nr cells when they were cultured in CM from cells that expressed Six1 versus those in which Six1 was knocked down. The 4t1 cells themselves were also more anoikis resistant compared to the 67nr cells (Figure 3.6 E). These data indicated that Six1 in the 4t1 system of cells, is necessary to non-cell autonomously increase their metastatic properties.

In order to test for sufficiency of Six1 in non-cell autonomously mediating phenotypes in this series of cell lines, we overexpressed Six1 in the 67nr cells, which are non-metastatic and express low levels of Six1 (Figure 3.7 A) and performed CM experiments with the 67nr cells. We also used CM from 4t1 as a positive control to compare the exogenous and endogenous non-cell autonomous activities of Six1.
Figure 3.6 Six1 is necessary to mediate non-cell autonomous functions in the 4t1 system.

(A, B) Western blot analyses on nuclear lysates collected from cells, (shNT- non-targeting shRNA, HDAC1- loading control). Compiled (C) 7hr migration and (D) 10hr invasion assays of 67nr cells cultured in different CM; Solid white/black bars – 67nr/4t1 cells in their own CM. (E) Representative images of anoikis resistance assay performed on 67nr cells cultured in indicated CM for 24hrs. SEM shown, experiments n=2. One way ANOVA with Tukey post test for (C, D). *p value<0.05, **p value<0.001, ***p value <0.0001
We observed, that similar to being cultured in CM from 4t1 cells, 67nr cells cultured in CM from 67nr-Six1 cells had increased migration, invasion and anoikis resistance compared to when cultured in their own CM (Figure 3.7 B-D), demonstrating that Six1 is also sufficient to non-cell autonomously increase the metastatic properties of non-Six1 expressing cells in this system.

**Six1 is sufficient to non-cell autonomously increase metastasis of non-Six1 expressing cells in vivo**

All of the above studies indicate that Six1 is a key player downstream of Snail1 and Twist1, and on its own, to mediate non-cell autonomous phenotypes. Since Six1 expression in MCF7 cells was sufficient to non-cell autonomously induce EMT in MCF7-Ctrl cells *in vitro*, we asked whether Six1 could also mediate non-cell autonomous increases in metastasis *in vivo*.

To this end, we either singly orthotopically injected Zsgreen* MCF7-Ctrl cells or tRFP* MCF7-Six1 cells, or co-injected tagged MCF7-Ctrl and Six1 cells into 8-14 week old female NOG/SCID mice. The mice were sacrificed when their tumors reached 2cm³ and examined for green and/or red metastases in the lungs by visualization of the whole lung under a fluorescent microscope. Interestingly, we found an increase in the number of mice that had Zsgreen*MCF7-Ctrl cells in their lungs when co-injected with MCF7-Six1 cells as compared to when singly injected (Figure 3.8 A,C,D). We also observed Zsgreen* MCF7-Ctrl cells in the lymph nodes of (3/13) mice that received the mixed injections, whereas we found no positive lymph nodes (0/5) in mice that were injected only with Zsgreen* MCF7-Ctrl cells (Figure 3.8 F, G). In contrast, there were no changes in the number of mice that had tRFP* lung metastases, since all the mice that were injected with MCF7-Six1 cells had tRFP* cells in their lungs irrespective of the injection condition (Figure 3.8 B, C, D).
Figure 3.7 Six1 is sufficient to mediate non-cell autonomous phenotypes in the 67nr cells.

(A) Western blot analyses on nuclear lysates collected from cells. Compiled (B) 7hr migration and (C) 10hr invasion assays of 67nr cells cultured in different CM; Solid white/black bars – 67nr/4t1 cells in their own CM. (D) Anoikis resistance assay performed on cells cultured in CM for 24hrs (n=2). SEM shown, experiments n≥3. One way ANOVA with Tukey post test for (B-D). *p value<0.05, ***p value <0.0001
The average number of mice that had tRFP⁺ lymph node metastases also remained comparable (Figure 3.8 E, G). These findings provided the first evidence that Six1 can non-cell autonomously increase the metastatic potential of neighboring non-Six1 expressing cells within the tumor in vivo, but were not very quantifiable. In Chapter V we discuss experiments in which these conditions were repeated in a more controlled and quantifiable manner, leading to the same conclusions.

Discussion

A multitude of studies shows that primary tumors are heterogeneous. Intratumoral heterogeneity affects not only tumor growth, but also metastatic outcome and response to chemotherapeutics¹. The heterogeneity observed in breast tumors is in part due to the distinct differentiation states in which the tumor cells exist. De-differentiated breast tumors that are thought to arise from cancer stem-like cells are often considered to be more basal-like, and more aggressive.²³ Eight Interestingly, high expression of EMT-inducing transcription factors like Twist1 and Snail1 is observed in the de-differentiated basal-like tumors and can correlate with poor outcomes in that subtype of breast cancer.⁴¹⁹,²³⁸ Factors like Twist1 are thought to be expressed only in a small percentage of cells within the primary tumor and yet the tumor as a whole is more aggressive. Given this, and the existence of intratumoral heterogeneity in breast cancer, it is possible that Twist1- and Snail1-expressing cells can influence neighboring tumor cells that do not express these factors and induce them to become metastatic, and hence increase the overall metastatic potential of the primary tumor.

Our data demonstrate that indeed, Snail1 and Twist1 can function in a non-cell autonomous manner in vitro to increase the metastatic properties of migration and invasion in cells that do not express either of these factors.
Figure 3.8 Six1 can non-cell autonomously increase metastasis of non-Six1 expressing tumor cells *in vivo* (continued)
**Figure 3.8** Six1 can non-cell autonomously increase metastasis of non-Six1 expressing tumor cells *in vivo.*

Female NOG/SCID mice were orthotopically injected in the 4\(^{th}\) mammary fat pad either singly with MCF7-Ctrl-Zsgreen or MCF7-Six1-tRFP cells or co-injected with MCF7-Ctrl-Zsgreen and MCF7-tRFP cells (see methods section for more details). Mice were sacrificed when tumors reached 2\(\text{cm}^3\). (A-C) Representative images of lungs of mice injected with (A) MCF7-Ctrl-Zsgreen, (B) MCF7-Six1-tRFP and (C) mixed cells, mounted under a fluorescent microscope. (D) Representation of number of mice with Zsgreen\(^+\)/tRFP\(^+\) cells in the lungs. (E,F) Representative images of lymph nodes of mice injected with (E) MCF7-Six1-tRFP and (F) mixed cells, mounted under a fluorescent microscope. (G) Representation of number of mice with Zsgreen\(^+\)/tRFP\(^+\) cells in the lymph nodes. No Zsgreen\(^+\) cells were observed in the lymph nodes of mice singly injected with MCF7-Ctrl-Zsgreen cells.
Six1, another prominent EMT-inducing pro-metastatic transcription factor, is upregulated in the metastatic Snail1- and Twist1-expressing cells and is required downstream of these factors to mediate their non-cell autonomous effects. Interestingly, Six1 KD in the Snail1 and Twist1 cells moderately decreases their cell autonomous migration (Figure 3.9), indicating that they rely on additional mechanisms other than Six1 upregulation to function cell autonomously. This observation is not surprising given that EMT-inducing factors exist in a complex web and are known to affect the levels of each other. Moreover, both Snail1 and Twist1 indirectly and directly regulate massive transcriptional programs in order to induce EMT and increase metastasis. In the HMLER cells, though increases in the metastatic phenotypes were observed, this was not accompanied by corresponding changes in the EMT status of the cells, suggesting cells can exhibit increased migration and invasion without undergoing an EMT. Thus, fate-mapping studies of EMT cells may not always truly reflect the more aggressive cells, since the two can be disassociated.

Our data further demonstrate that Six1 itself is both necessary and sufficient to mediate non-cell autonomous phenotypes in both human and mouse cell lines, both in vitro and in vivo. In the MCF7 cells where Six1 is the primary driver of EMT and metastasis, (since protein levels of Snail1 and Twist1 are undetectable) we see a non-cell autonomous induction of EMT in the non-Six1 expressing cells, as well as increases in anoikis resistance, an EMT-related phenotype. This is accompanied by increased non-cell autonomous induction of metastasis in weakly metastatic non-Six1 expressing cells in vivo. In the mouse cell lines, though alterations in Six1 levels result in changes in the non-cell autonomous phenotypes, Six1 could still be acting downstream of, or in conjunction with Twist1, which is also differentially expressed in those cells. Nevertheless, Six1 plays an important role in both systems to induce non-cell autonomous phenotypes in the non-Six1 expressing cells.
Figure 3.9 Six1 knockdown downstream of Snail1 and Twist1 moderately affects their cell autonomous migration.

8hr migration assays of (A) HMLER-Snail1 and (B) HMLER-Twist1 cells transfected with 150nM of siSix1 or siNT for 48hrs.

SEM shown, experiments n≥3. Two tailed t-test. *p value<0.05, **p value <0.001
Since we observe increased non-cell autonomous phenotypes in co-culturing as well as conditioned medium experiments, our data suggest that a secreted factor released into the medium by the metastatic cells may be responsible for mediating these effects. Not only does the factor(s) have to function downstream of Twist1 and Snail1, it also has to be expressed in a Six1-dependent manner in different contexts. The following chapters will delve into the non-cell autonomous mechanisms of action of these EMT-inducing transcription factors.

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CHAPTER IV

HEDGEHOG SIGNALING IS ACTIVATED NON-CELL AUTONOMOUSLY DOWNSTREAM OF SNAIL1, TWIST1 AND SIX1

Introduction

There are three main modes of communication in cells: 1) autocrine, in which cells release factors to which they themselves respond, 2) paracrine, which involves long distance communication and factors released by cells that function in other cells in close or far proximity and 3) juxtacrine, wherein factors are released from one cell to an adjacent cell, with which it is in contact. Several studies have shown that tumor cells can interact with each other and with the tumor microenvironment using all of the above means. Factors released by tumor cells include and may not be limited to proteins, DNA/RNA fragments, miRNAs and lipids\(^{239-242}\) and furthermore can be contained within exosomes or be freely secreted depending on how they are processed in the cells.

We determined in our experiments that the factor(s) responsible for mediating non-cell autonomous effects were indeed proteins since trypsin treatment of the CM from metastatic cells (which would degrade proteins) abrogated the phenotypes observed (Figure 4.1). Hence, we focused on protein signaling pathways altered downstream of Twist1, Snail1 and Six1 to determine the mechanism by which these factors can function in a non-cell autonomous manner. A number of signaling networks including TGF-β, Wnt, Notch and Hedgehog, are induced by, and act in concert with these EMT-inducing factors during normal development and are later hijacked in the context of cancer\(^{56,243}\).

The Hedgehog (Hh) family of signaling proteins is one such set of prominent regulators of embryonic development which were discovered in \textit{Drosophila} and whose functions are conserved in vertebrates. In canonical hedgehog signaling, Hh ligands act as morphogens and control numerous developmental processes at various times,
Figure 4.1 Trypsin treatment of cells abolishes non-cell autonomous increases in migration.

22 hr migration assay (to allow cells to attach to plate following trypsin treatment) of HMLER-Ctrl cells in Twist1 CM. SEM shown, n=2.
most notably in the brain, eye and limb bud development. The signaling pathway is very tightly controlled and in mammals is canonically activated when Hh ligands (Sonic hedgehog, SHH; Desert Hh, DHH; or Indian Hh, IHH) bind to their receptors Patched-1 (Ptch1) or Patched-2 (Ptch2). Ptch1 and Ptch2 in turn form complexes with co-receptor proteins: Cell adhesion molecule-related/downregulated by oncogenes (CDON) and Brother of Cdo (BOC), which allows Ptch1/2 to relieve the repression on Smoothened (Smo). This results in the activation of a signaling cascade that ultimately leads to the accumulation of transcriptional activator forms of one or more Gli (glioma associated oncogene homolog) transcription factors (Gli1, Gli2, or Gli3) in the nucleus, and activation of Hh target genes. In the absence of Hh ligands, transcriptional repressor forms of Gli3 and Gli2 are produced to inhibit transcription of target genes. Several studies have previously shown that the Hh pathway can be activated in a non-canonical manner, which can either be Hh ligand independent, independent of Smo regulation of Gli, or independent of Smo itself among other mechanisms of activation. Smo has also been shown to function as a G-protein coupled receptor to activate the Hh pathway.

There are three models of activated Hh signaling in cancer: 1) Activating mutations found in the pathway components including heterozygous inactivating mutations in Ptch or constitutively activating Smo mutations. Such mechanisms of activation are observed in cancers like medulloblastoma and basal cell carcinoma (BCC), where dysregulation of Hh signaling specifically in the stem and progenitor cells is thought to initiate tumorigenesis. 2) Ligand-dependent activation of Hh signaling in epithelial tumor cells where the secreted ligand can act in both an autocrine and juxtacrine manner. This mode of activation is usually observed in other solid tumors including breast cancer, prostate cancer and non-small cell lung carcinoma. In this case activated Hh signaling results in increased proliferation,
Figure 4.2 Canonical Hh signaling.
A schematic of Hh pathway signal transduction derived from developmental and cancer models. (A) In the absence of Hh ligand, Ptc1 is located in the cilium and blocks Smo. Gli transcription factors exist in repressor forms that prevent transcription of target genes. (B) The Hh ligands bind Ptc1 at the cell surface and allow it to move out of the primary cilium, causing the de-repression of Smo and allowing it to activate Gli. The Gli factors are processed to activator forms and translocated to the nucleus to induce the transcription of Hh target genes. Antibodies against the Hh ligands (5E1) block pathway activation by preventing the interaction of Hh ligand with Ptc1. Cyclopamine directly binds and inhibits Smo function. GANT is a direct Gli antagonist, and blocks binding of Gli to DNA. Adapted from 247.
increased percentages of cancer stem cells, and increased tumor invasiveness \(^{248}\). 3) Paracrine activation of Hh signaling which involves crosstalk between tumor cells and cells in the tumor microenvironment. In this model, Hh ligands produced by the tumor cells activate the signaling pathway in the surrounding tumor stroma. The stromal cells then feedback on the tumor cells to facilitate tumor progression \(^{249}\).

Hh signaling has been shown to be activated in breast cancer, though the mechanisms of action are still poorly understood. Interestingly, activation of Hh signaling in mammary stem cells intensifies their tumorigenic potential \(^{in vitro}\) and \(^{in vivo}\)\(^{250}\) indicating that the Hh pathway plays an important role in the breast tumorigenesis. Accordingly, a few studies have revealed mutations in the pathway components like \(Ptch1\) loss and \(Gli1\) amplification but this occurs with low frequency \(^{251}\) compared to Hh ligand activation of the pathway. Patient data suggest that increased Hh signaling is associated with poor prognosis specifically in the triple negative breast cancer subtype \(^{252}\). Furthermore, numerous studies have shown that nuclear Gli expression and the SHH ligand are detected at higher levels in the tumor tissue compared to the surrounding stroma as well as normal breast tissue, and that this increased expression correlates with increased metastasis and poor outcomes \(^{251}\). Given the importance of Hh signaling in cancer, multiple inhibitors have been developed against key players in this pathway. The most prominent pathway inhibitors include: 1) 5E1, a function-blocking monoclonal antibody that binds to and sequesters the ligands and hence keeps the canonical Hh pathway turned off \(^{253}\), 2) Cyclopamine, a naturally occurring plant-derived steroidal alkaloid, that binds to Smo and by producing conformational changes similar to Ptch1, keeps it inactive \(^{254}\) and 3) GANT-61, a small molecule inhibitor that directly binds to the more downstream players in the pathway, Gli1/2 and inhibits them from binding DNA to activate transcription of target genes \(^{255}\). Numerous studies have linked Hh signaling to tumor progression in cancers, thus leading to the use of derivatives of cyclopamine in clinical trials for select patients with BCC, medulloblastoma
and other solid tumors with promising efficacy \cite{256,257}. However, these inhibitors have not shown promise against breast cancer \cite{258}, despite evidence for activation of this pathway in breast tumor cells \cite{251}, which suggests that non-canonical mechanisms of Hh pathway activation (independent of Smo) may be in place in breast cancer cells.

Interestingly, studies have shown that in *Drosophila* eye development, *hh* is a direct target of *sine oculis* (the homolog of Six1 in *Drosophila*) \cite{259}. More recently, Hh/GLI signaling was shown to be activated downstream of Six1 in fibroblasts \cite{260} as well as regulated downstream of Six1 in lung development \cite{200}, providing the first mammalian links of Six1 to Hh signaling. Similarly, Twist1 and Hh signaling are closely linked during development. *Twist* KO mice exhibit defects in limb formation, thought to be a consequence of downregulated FGF signaling and repression of Hh pathway components including *Shh* and *Gli1* \cite{140,143}.

Twist has also been shown to regulate fore- and mid-brain patterning through its regulation of Gli3 activity in addition to regulating the Hh signaling cascade during arch formation and tissue patterning \cite{140}. Snail1 on the other hand, is thought to be a target of Hh signaling in various contexts \cite{261,262}. Only recently, both Twist1 and Snail1 have been linked to the Hh pathway in the context cancer stem cells \cite{263,264}.

Since SHH is a secreted ligand that is capable of functioning in both an autocrine and paracrine manner, and since usual suspects such as TGF-β signaling were not found to be key players downstream of Six1 in mediating its non-cell autonomous effects (see results), we examined whether Hh signaling plays a role in mediating the non-cell autonomous effects of these EMT-inducing transcription factors.
Results

TGF-β signaling is not activated non-cell autonomously downstream of Twist1, Snail1 and Six1

Our data demonstrate that Six1 is both necessary downstream of Twist1 and Snail1, and sufficient on its own, to cause non-cell autonomous changes in tumor cells not expressing Six1. Hence we first examined pathways known to be regulated by Six1 as a means to determine the mechanism by which it imparts its non-cell autonomous effects. Our lab has previously shown that Six1 activates TGF-β signaling, and that this increase in signaling is responsible for numerous cell autonomous roles of Six1 including its ability to induce an EMT as well as increase metastasis. Several studies have also shown that crosstalk occurs between Snail1, Twist1 and TGF-β signaling in breast cancer and other cancers. We thus asked whether TGF-β signaling was activated non-cell autonomously in cells receiving CM from the different cells conditions, using the TGF-β/SMAD-responsive 3TP-luciferase reporter. Since we observed non-cell autonomous phenotypes mediated by Six1 in numerous systems, we focused on the mechanism in the two human cell lines (HMLER and MCF7 cells), as opposed to the mouse lines for physiological relevance. We observed moderately increased (but not significant) activation of the 3TP-luciferase reporter when HMLER-Ctrl cells were cultured in CM from Snail1 or Twist1 expressing cells, and this increase was not altered with Six1KD in the cells expressing HMLER-Twist1/Snail1 cells (Figure 4.3 A,B). Since we demonstrated that Six1 expression is critical for the non-cell autonomous alterations in migration and invasion observed in HMLER cells (Figure 3.3), we excluded the non-cell autonomous contribution of TGF-β signaling to our phenotypes. As previously observed, MCF7-Six1 cells had increased 3TP-luciferase reporter activity when compared to MCF7-Ctrl cells. However, the reporter was not activated in the MCF7-Ctrl cells cultured in CM from the Six1-expressing
cells (Figure 4.3 C), consistent with previous findings that Six1 upregulates TGF-β signaling cell autonomously via increasing the level of TGFβR1 in cells, and not by increasing ligand levels. Thus, these data indicate that both in the HMLER model, as well as the MCF7 model, the Six-mediated non-cell autonomous effects are independent of TGF-β signaling.

**Hedgehog ligands are differentially upregulated downstream of Snail1, Twist1 and Six1**

The Hh/GLI signaling pathway is known to act in concert with Twist1 and Snail1 during development and cancer. Studies have shown that Hh/GLI signaling is also regulated downstream of Six1 and interestingly, *hh* the ligand that activates Hedgehog signaling in *Drosophila* is a direct target of *sine oculus* in *Drosophila* eye development. Since our non-cell autonomous phenotypes are Six1-dependent, we examined which, if any, of the three Hh ligands, SHH, DHH or IHH were upregulated in the HMLER and MCF7 systems downstream of our EMT-factors.

Surprisingly, HMLER-Twist1 and Snail1 cells did not express appreciable mRNA levels of any of the Hh ligands (Figure 4.4 A-C), nor could any increase in SHH be detected in the media from HMLER-Twist1 or Snail1 cells, with or without Six1 KD, as compared to that from HMLER-Ctrl cells (Figure 4.4 D). In contrast, SHH was upregulated on the mRNA level in MCF7-Six1 cells when compared to MCF7-Ctrl cells, while no changes were seen in *Ihh* and *Dhh* expression (Figure 4.5 A-C). MCF7-Six1 cells also expressed increased levels of SHH protein and a marked increase was observed in the levels of SHH in the CM as well (Figure 4.5 D, E). Interestingly, SHH mRNA was significantly decreased in two mutant MCF7-Six1 cells lines, MCF7-DelE133 (a DNA binding mutant) and MCF7-V17E (an Eya binding mutant) (which have previously been shown to have decreased metastasis compared to MCF7-Six1 cells) (Figure 4.5 F). This indicates that the Six1/Eya complex transcriptionally either directly or indirectly upregulates *Shh.*
Figure 4.3 TGF-β signaling is not activated non-cell autonomously downstream of the Twist1, Snail1 and Six1.

(A,B) Analysis of the TGF-β-responsive promoter 3TP-luciferase reporter in HMLER-Ctrl cells cultured in CM from (A) HMLER-Snail1+/-siSix1 cells or (B) HMLER-Twist1+/-siSix1 cells for 48hrs. (C) 3TP-luciferase reporter activity in MCF7-Ctrl cells cultured in MCF7-Ctrl and Six1 CM for 48hrs. SEM shown, compiled experiments n=3 (with different sets of CM). One way ANOVA with Tukey post test in all cases (ns – not significant), **p value<0.001
Figure 4.4 Hh ligands are not upregulated downstream of Snail1, Twist1 and Six1 in HMLER cells. 

(A-C) qRT-PCR analyses of HMLER-Ctrl, Snail1 and Twist1 cells and (D) combined ELISA analyses on 3 sets of CM from HMLER-Ctrl, HMLER-Snail1 and HMLER-Twist1 cells transfected with 150nM of siNT or siSix1 for 48hrs. SEM shown n≥3. One way ANOVA with Tukey post test in all cases (ns – not significant).
Figure 4.5 *Shh* is transcriptionally upregulated by Six1 in MCF7 cells. (A-C) qRT-PCR analyses, (D) western blot analyses of WCLs and (E) ELISA analyses on CM from combination of 3 clones each of MCF7-Ctrl and MCF7-Six1 cells. (F) qRT-PCR analysis on MCF7-Ctrl and MCF7-Six1 wildtype and mutant cells. SEM shown n≥3. Two tailed unpaired t-test for (A-C, E) and One way ANOVA with Tukey post test for (F). (ns – not significant), **p<0.001. ***p<0.0001
Six1 regulates Shh levels in additional cell lines as well (Figure 4.6 A,B). Interestingly, we found that overexpression of Six1 in HMLER-Ctrl cells causes Shh induction only in the presence of its required co-factor Eya2 \(^{266}\) (Figure 4.6 B), suggesting that Six1’s ability to induce Hh ligands is dependent on the availability of an Eya cofactor.

**EMT-inducing transcription factors can activate Gli via canonical and non-canonical mechanisms**

Several studies show that Hh/GLI signaling can be activated in a Hh ligand-independent non-canonical manner \(^{245}\). Thus, we examined whether Gli activity was increased in control cells that received CM from cells expressing Snail1 and Twist1, despite seeing no differences in Hh ligand levels in this system. To this end, a Gli1-specific lentiviral reporter containing 7 Gli1 consensus binding sites fused to GFP (7-Gli1), as well as a mutant Gli (m-Gli1)-GFP reporter (which has mutated consensus binding sites) was transfected into the HMLER cells.

Surprisingly, we found that HMLER cells cultured in CM from HMLER-Twist1 and Snail1 cells which express Six1, have significantly increased 7Gli1-GFP reporter activity (as measured by % GFP\(^+\) cells) when compared to HMLER cells cultured in their own CM (Figure 4.7 A,B). The increased Gli activity was abrogated in Six1 KD CM (Figure 4.7 A, B) and no increased activation of the pathway was observed using the m-Gli1 reporter under any condition (Figure 4.7 A,B). This indicated that HMLER-Twist1 and Snail1 cells require Six1 to activate Gli in cells not expressing the transcription factors. Furthermore, we observed that CM from HMLER-Twist1 and Snail1 cells increased expression of Hh pathway target genes in a non-cell autonomous, Six1-dependent manner in HMLER-Ctrl cells (Figure 4.7 C,D).
Figure 4.6 Six1 can regulate Shh levels in other systems
qRT-PCR analyses of (A) A2780 cells transfected with 100nM of siNT, siSix1 and (B) HMLER-Ctrl cells transfected with 1µg of Six1 and/or Eya2. SEM shown n≥3. Two tailed unpaired t-test for (A) and One way ANOVA with Tukey post test for (B). *p<0.05, **p<0.001. ***p<0.0001
Similarly, MCF7-Ctrl cells cultured in CM from MCF7-Six1 cells exhibited significantly increased Gli activity compared to when cultured in MCF7-Ctrl CM, whereas a significant effect of MCF7-Six1 CM was not observed on the m-Gli1 reporter (Figure 4.7 E). Additionally, MCF7-Ctrl cells cultured in MCF7-Six1 CM also had increased levels of Hh pathway target genes including Gli1, Ptc1, Ptc2 amongst others (Figure 4.7 F). As expected, recombinant SHH added to the medium on MCF7-Ctrl cells resulted in significant activation of the pathway, demonstrating the specific activation of the reporter in response to activation of the Hh pathway (Figure 4.7 G). Additionally, MCF7-Six1 cells themselves demonstrated increased cell autonomous activation of the pathway compared to control cells (and m-Gli1 reporter activation) (Figure 4.7 H) suggesting that Hh/GLI signaling is activated both cell and non-cell autonomously downstream of Six1.

Thus, Six1 is sufficient, and necessary downstream of Snail1 and Twist1, to non-cell autonomously activate Hh/GLI signaling in control cells not expressing these factors. These data also suggest that non-cell autonomous Gli activation by EMT regulators can occur via both Hh ligand dependent and independent mechanisms, and that either the context, or the particular combination of EMT regulators, may mediate the mechanism by which this occurs.

The finding that Gli can be non-cell autonomously activated by EMT-transcription factors in different manners suggests that the type of inhibitor used against the pathway will affect response. Thus, we inhibited the Hh pathway using either 5E1, a function-blocking monoclonal antibody targeting SHH, cyclopamine (that targets Smo acting as an upstream inhibitor of the pathway) or GANT-61 (a small molecule inhibitor that targets Gli1/2, and is thus a downstream inhibitor of the pathway\textsuperscript{255}). Because we could not detect the ligands at all in the HMLER system, only cyclopamine and GANT-61 were used to differentiate between the potential involvement of Smo, versus non-canonical activation of the pathway that bypasses Smo.
Figure 4.7 Hh/GLI signaling is activated non-cell autonomously in Six1-dependent manner (continued)
Figure 4.7 Hh/GLI signaling is activated non-cell autonomously in Six1-dependent manner (continued)
Figure 4.7 Hh/GLI signaling is activated non-cell autonomously in Six1-dependent manner.
48hr 7-Gli1-GFP reporter assays performed in (A,B) HMLER cells treated with CM from HMLER-Ctrl cells or HMLER-Snail1 or Twist1 cells ±/−Six1 KD, (C,D) qRT–PCR analyses of Hh pathway target genes in HMLER-Ctrl cells treated with indicated CM. Gene expression is normalized to PP1B. (E) 48hr 7-Gli1-GFP reporter assays in MCF7 cells treated with CM from MCF7-Ctrl or Six1 cells (F) qRT–PCR analyses of Hh pathway target genes in MCF7-Ctrl cells cultured in different CM for 48hrs. Gene expression is normalized to PP1B. (F, G) 48hr 7-Gli1-GFP reporter assays in (F) MCF7-Ctrl cells with addition of 1µg/ml of rhSHH to media for 48hrs and (H) MCF7-Ctrl and Six1 cells. All Gli1-GFP assays are represented as %GFP+ cells. SEM shown, compiled experiments: n≥3. One way ANOVA with Tukey post test for (A–D,G,H) and two tailed unpaired t-test for (E,F). (ns – not significant); *p value<0.05, **p value<0.001, ***p value<0.0001
Only GANT-61, and not cyclopamine, inhibited non-cell autonomous activation of Gli when HMLER cells were cultured in CM from HMLER-Snail1 and Twist1 cells (Figure 4.8 A). In contrast, all three inhibitors significantly and equally decreased non-cell autonomous activation of the Hh/GLI pathway in MCF7-Ctrl cells cultured in MCF7-Six1 CM (Figure 4.8 B). No differential activity was observed using m-Gli1 reporter in either case (Figure 4.8 A, B). Together, these data suggest that the Hh pathway via Gli can be activated non-cell autonomously by Six1, Twist1, and Snail1 in all settings, albeit through distinct mechanisms.

Discussion

In recent years, Hedgehog signaling has been recognized as a prominent player in tumorigenesis. The effects of Hh signaling are observed in cancers such as BCC and medulloblastoma, where constitutive activating mutants of Smo or inactivating mutants of Ptc1 have been discovered, as well as in lung, colorectal and breast cancer, where mutations are not observed at high frequency, but the pathway is mostly hyperactive248,269-273. Interestingly, the Hh pathway can be activated both via mutations and via increased ligands stimulating "canonical" activation of the pathway, and in a non-canonical manner that is Hh ligand and mutation independent260,274. Studies show that Hh signaling is highly active in the tumor microenvironment273 and there is often crosstalk observed between the Hh ligand-expressing cells in the tumor microenvironment and the tumor cells. Alternatively, tumor cells can secrete SHH in a paracrine manner that is taken up by cells in the tumor microenvironment, which activate Gli signaling and release tumor-promoting cytokines273. Such activation of the pathway has been shown to increase proliferation, survival and stem cell self-renewal as well as metastasis of tumor cells258. While breast cancer cells exhibit activated Hh signaling and increased Hh signaling is associated with poor prognosis in triple negative breast cancers, the mechanisms of its actions are not well understood251.
Figure 4.8 Hh/GLI signaling pathway is activated non-cell autonomously by distinct mechanisms in HMLER and MCF7 cells.

48hr 7-Gli1-GFP reporter assays of (A) HMLER cells cultured in CM from HMLER-Ctrl, Snail1 and Twist1 cells (B) and MCF7 cells cultured in CM from MCF7-Ctrl and Six1 cells +/- 10µM cyclopamine, 10µM GANT-61, or 3-5µg/ml of 5E1. SEM shown, n≥3. One way ANOVA with Tukey post test for all cases (ns – not significant). *p value<0.05, **p value<0.001, ***p value<0.0001
We demonstrate that the Hh ligands are differentially regulated by the three EMT-inducing transcription factors, Snail1, Twist1 and Six1. While Twist1 and Snail1 do not upregulate the ligands in HMLER cells, SHH levels are increased on the mRNA and protein levels, and in the CM media, in the MCF7 cells that express Six1. Interestingly, though Six1 expression alone is not sufficient to upregulate Shh in the HMLER cells, in the presence of Eya2 (a required co-factor for Six1), we observed a dramatic increase in Shh levels. Since Eya2 is a key transcriptional cofactor that regulates Six1’s transcriptional activity as well as cell its autonomous metastatic functions, our data suggest that Six1’s ability to upregulate Shh may depend on the availability of an Eya cofactor, and hence Six1 may either directly or indirectly transcriptionally upregulate Shh. This finding is further supported by examination of SHH mRNA expression in two MCF7 cell lines which express mutant forms of Six1: 1) MCF7-V17E where Six1 cannot interact with Eya and 2) MCF7-DelE133, where Six1 cannot bind DNA, effectively inhibiting its transcriptional activity and inhibiting the induction of Shh. Interestingly, the Shh levels in these two cells lines are significantly lower than that in the MCF7-Ctrl and MCF7-Six1 cells, suggestive of dominant negative effect of the mutant Six1. These data indicate that Six1-Eya complex transcriptionally upregulates Shh. Furthermore, HMLER cells also express lower levels of endogenous Eya2 compared to MCF7 cells (Figure 4.9), which is a possible explanation for why, despite high levels of Six1 in the HMLER-Twist1 and Snail1 cells, upregulation of Shh is not seen. However, these data further suggest that Six1 can function in cells outside of its interaction with Eya cofactors and possibly with other co-activators.

Despite the lack of expression of Hh ligands in the HMLER cells, we observed activation of Gli signaling in cells that received CM from cells expressing Snail1, Twist1 and Six1. This non-cell autonomous activation of Hh/GLI signaling was accompanied by increased expression of several target genes of this pathway including Gli1, Ptch1/2 and CyclinD1 in a Six-1 dependent manner.
Figure 4.9 MCF7 cells express higher endogenous levels of Eya2 compared to HMLER cells.
Western blot analysis of WCLs from "normal" mammary epithelial cells MCF12A, HMLER and MCF7 cells. (n=2)
Therefore, our data indicate that Six1 is required downstream of Snail1 and Twist1 and is sufficient to mediate non-cell autonomous phenotypes as well as activate Gli signaling in different contexts and interestingly, Gli can be activated in a Six1-dependent manner via both Hh ligand dependent and independent means.

Hh ligand independent activation of Hh/GLI signaling in the HMLE cells suggests that there are non-canonical activation mechanisms at play. This hypothesis is additionally supported by the Gli1-GFP reporter assays, wherein use of only GANT-61, the Hh pathway downstream inhibitor efficiently inhibited pathway activation, whereas cyclopamine, the upstream inhibitor did not have any effects. In contrast, in the MCF7-Six1 cells, where we observe upregulation of SHH, all three inhibitors including 5E1 robustly abrogated non-cell autonomous pathway activation.

Several studies show that the Hh signaling pathway can be activated both, canonically as well as non-canonically in different cancers. Non-canonical activation of this pathway usually involves activation of Gli transcription factor, independent of Hh ligands and Smo. Such activation will be discussed in detail in the following chapters. Nevertheless, since the mechanism of activation of the pathway differs in the different contexts, but converges on to Gli activation, it suggests that the preferential way to target this pathway in heterogeneous tumors may be to target Gli directly. This question will be explored in subsequent chapters.

**Acknowledgements**

I would like to thank Dr. Michael Lewis and Dr. Xiaomei Zhang (Baylor College of Medicine) for providing the 7-Gli1-GFP reporter system. I want to acknowledge Joshua Cabrera for generating the MCF7-Ctrl, Six1, V17E, and DelE133 stable cell lines and Hengbo Zhou for performing the western blot analysis for Eya protein levels.
CHAPTER V
SNAIL1, TWIST1 AND SIX1 CONVERGE ON GLI TO MEDIATE THEIR NON-CELL AUTONOMOUS PHENOTYPES

Introduction

Hh signaling has now proven to be a major pro-tumorigenic pathway in multiple cancers, including medulloblastoma, BCC, and other solid tumors like pancreatic cancer, breast cancer and certain lung cancers, by contributing to increased EMT, invasiveness, cancer stem cell properties, proliferation and metastasis in various cancers\(^248\). Canonical Hh signaling, as mentioned previously, involves the Hh ligands binding to and activating their receptors Patched (Ptch) 1/2, which in turn relieves its repression on Smoothened (Smo), resulting in activated forms of the transcription factor Gli accumulating in the nucleus and turning on transcription of Hh target genes.

More recently, various non-canonical mechanisms of activation of this pathway have come to light. Independent of Hh ligand and Smo activation, the levels of Gli1 and Gli2 (transcriptional activators of this pathway) can be increased by numerous other mechanisms. Gli2 is directly upregulated by a SMAD3-dependent TGF-β signaling mechanism in pancreatic adenocarcinomas, which in turn mediates activation of the Hh targets genes like Gli1\(^275\). Only pharmacological inhibition of TGF-β inhibits growth phenotypes, while Hh signaling inhibition using cyclopamine does not\(^275\). Similar results were seen in breast cancer, using the metastatic MDA-MB-231 bone-tropic cells lines, where Gli2 was found to be required downstream of TGF-β induced osteolysis. While cyclopamine did not inhibit bone destruction, direct repression of Gli2 was able to rescue the phenotypes, indicating Smo-independent regulation of Gli2 in these cells\(^276\). TGF-β along with Ras signaling has also been shown to increase Gli1/2 levels in bladder cancer and signaling molecules like PKC and AKT can increase Gli1/2 levels by preventing their
degradation in absence of Smo activation. More recently, epigenetic mechanisms that include various chromatin remodelers have been implicated in regulating the levels of Gli1 and Gli2. One of the more interesting regulators of Gli1 activity via protein stabilization, is AMP-activated protein kinase (AMPK). In conditions of deficient cellular energy, AMPK directly phosphorylates Gli1 on three residues resulting in the inhibition of its transcriptional activity. This allows the cells to expend less energy by suppression of the pro-proliferative ability of the Hh pathway. This mechanism of Gli regulation is hijacked in medulloblastoma tumor cells, which can harbor mutations in the three phosphorylation sites on Gli1, thus preventing AMPK from suppressing Gli1 activity. These mutations result in increased Gli1 protein stability, transcriptional activity and hence increased oncogenesis, and highlights the importance of post-translational modifications in the non-canonical regulation of Gli1.

Various other non-canonical mechanisms of activation of this pathway have been reviewed elsewhere (Figure 5.1). Collectively, these studies show that Gli activation does not always require Smo and Hh ligands, and also underscore the importance of Gli activation in various tumor types.

As mentioned previously, Hh signaling can be inhibited using various methods. The most upstream inhibitors of the pathway include 5E1, the monoclonal antibody that binds to and sequesters the Hh ligands, and robotnikinin, a small molecule inhibitor that binds to Shh. Interestingly, robotnikinin does not compete with Smo-cyclopamine interactions and is rendered ineffective when Smo agonists are used to activate the pathway. Cyclopamine, another pathway inhibitor binds to Smo and inhibits it from functioning to activate Gli transcription factors. These inhibitors are effective in abrogation of Hh signaling when it is activated by canonical means. Multiple derivatives of cyclopamine (e.g. Vismodegib, GDC-0449 and IPI-926) are thus in clinical trials for metastatic BCC and medulloblastomas and have proven to be efficacious in their treatment.
Figure 5.1 Non-canonical activation of Hh signaling via Gli1/2 centric mechanisms.
There are several ways for regulating Gli transcription factors independent of Smo and Hh ligands, including TGF-β signaling, SK6 phosphorylation, Ras/MEK/ERK signaling and AKT signaling. Gli1/2 can also work with downstream effectors of EGF signaling to regulate transcription of target genes. Taken from 245.
Given that Gli1/2 is activated by both canonical and non-canonical means, drugs that directly and specifically target Gli1/2 will likely be more efficacious in a wide spectrum of cancers that exhibit activated Hh/GLI signaling.

Our data show that Hh/GLI signaling is activated non-cell autonomously in a Six1-dependent manner in cell lines that express EMT-inducing transcription factors Twist1 and Snail1, potentially by both canonical and non-canonical mechanisms. Hence, we first determined whether abrogation of this pathway would revert our non-cell autonomous phenotypes, and whether targeting Gli directly would be more efficacious than targeting upstream elements of the pathway.

**Results**

**Compounds directly targeting Gli inhibit non-cell autonomous phenotypes mediated by Snail1, Twist1 and Six1**

In our previous experiments using the Gli1-GFP reporter we observed that though Hh/GLI signaling was activated non-cell autonomously downstream of Snail1 and Twist1 and was dependent on Six1 levels, there was a discrepancy in the pathway suppression between the upstream and downstream Hh inhibitors. While GANT-61 (the downstream inhibitor) effectively abrogated Gli1 activity, cyclopamine was unable to do so in all contexts, suggesting that Hh signaling is activated non-canonically (in a Smo-independent manner) downstream of Twist1 and Snail1. In line with this finding, treatment of HMLER-Ctrl cells with cyclopamine had no influence on the ability of CM from HMLER-Snail1 or Twist1 cells to increase migration or invasion (Figure 5.2 A, B). We did not use the 5E1 function-blocking antibody with the HMLER cells, since no changes were detected in the ligand levels when comparing HMLER-Ctrl cells to HMLER-Twist1 or Snail1 cells. These data suggested that the non-cell autonomous phenotypes mediated by Snail1 and Twist1 required non-canonical activation of Hh/GLI signaling.
Figure 5.2 Upstream Hh pathway inhibitor cyclopamine does not revert non-cell autonomous phenotypes induced downstream of Snail1 and Twist1 in HMLER cells. Representative (A) 7hr migration assays and (B) 18hr invasion assays of HMLER-Ctrl cells in CM from HMLER-Snail1 cells and HMLER-Twist1 cells in the presence or absence of indicated inhibitors. SEM shown, n≥3, One way ANOVA with Tukey post test in all cases, **p value<0.001, ***p value<0.0001
In contrast, in the MCF7 system, both cyclopamine and 5E1 had a robust effect on most non-cell autonomous properties induced by Six1 in MCF7-Ctrl cells. Treatment with cyclopamine or 5E1 reversed the decrease in expression of CK-18 and the increase in expression of FN1 that occurred when MCF7-Ctrl cells were cultured in CM from MCF7-Six1 cells (Figure 5.3 A). Membranous E-Cadherin was also robustly restored with the use of these inhibitors (Figure 5.3 B-E), although anoikis sensitivity was not restored in either case (Figure 5.3 F). The results with the upstream inhibitors in MCF7 cells aligned with increased SHH levels detected in the MCF7-Six1 cells and CM, and demonstrate that in the MCF7 system, most, but not all, non-cell autonomous effects mediated by Six1 require SHH and activation of Gli.

**Gli is the key mediator through which the Snail1, Twist1 and Six1 non-cell autonomously alter phenotypes of cells not expressing these factors**

Because we observed activation of Gli1 in recipient cells with all three EMT-inducing transcription factors, but did not consistently observe increased levels of Hh ligands downstream of these factors, our data suggest that upstream inhibitors of this pathway may not be the best approach to treating tumors in which Hh/GLI signaling is activated and EMT is observed. Instead, a better approach to treating these tumors may be through the use of a downstream inhibitor targeting Gli directly. Thus, we examined whether treatment with GANT-61 (the downstream inhibitor of the pathway) would be an effective means to more globally inhibit non-cell autonomous effects of EMT-inducing transcription factors. GANT-61 has been shown to specifically bind to zinc-fingers 2 and 3 of GLI1/2 and hence specifically inhibits their binding to DNA sequences which results in the inhibition of Hh/GLI signaling.
Figure 5.3 Upstream Hh pathway inhibitors cyclopamine and 5E1 revert most non-cell autonomous phenotypes downstream of Six1 in MCF7 cells (continued)
Figure 5.3 Upstream Hh pathway inhibitors cyclopamine and 5E1 revert most non-cell autonomous phenotypes downstream of Six1 in MCF7 cells.

(A) Western blot analyses of WCLs collected from MCF7-Ctrl cells cultured in MCF7-Ctrl and Six1 CM for 48hrs in the presence or absence of indicated inhibitors. (B, D) Representative ICC of E-Cadherin (green) in MCF7-Ctrl cells cultured in MCF7-Ctrl and Six1 CM for 48hrs (Dapi, blue) in the presence or absence of indicated inhibitors; scale – 20µm (B), 100µm (D). (C) Quantification of % membranous E-Cadherin in panel B. (E) Quantification of % membranous E-Cadherin in panel D. n≥ 45-60. (F) Anoikis resistance of MCF7-Ctrl cells cultured in indicated CM + treatment with cyclopamine or 5E1 for 24hrs, SEM shown in representative images, n≥3, One way ANOVA with Tukey post test in all cases, (ns – not significant). **p value<0.001, ***p value<0.0001
Indeed, GANT-61 treatment of HMLER-Ctrl cells cultured in CM from either HMLER-Snail1 or Twist1 reversed the ability of the CM to increase migration and invasion (Figure 5.4 A, B). Furthermore, treatment of MCF7-Ctrl cells with GANT-61 resulted in restoration of CK-18 and FN1 proteins back to control levels in the presence of MCF7-Six1 CM (Figure 5.4 C), as well as in the robust rescue of E-Cadherin on the cell membranes (Figure 5.4 D) and interestingly, a rescue in anoikis sensitivity of MCF7-Ctrl cells (Figure 5.4 E).

These data demonstrate that EMT-inducing transcription factors Six1, Snail1 and Twist1, while regulating Gli via different mechanisms, all converge on its activation to mediate their non-cell autonomous effects, which may have important clinical ramifications for the use of different Hh inhibitors in tumors where cells have undergone EMT.

**Inhibition of Hh/GLI signaling using GANT-61 suppresses non-cell autonomous increases in metastasis *in vivo***

Our preliminary *in vivo* data indicated that Six1 was able to non-cell autonomously increase the metastatic potential of weakly metastatic MCF7-Ctrl cells *in vivo* (Figure 3.8). Since we observed an inhibition of the non-cell autonomous induction of EMT in MCF7-Ctrl cells exposed to MCF7-Six1 CM, with inhibition of Hh/GLI signaling *in vitro*, we further tested whether Gli activation was required for the ability of MCF7-Six1 cells to enhance metastasis of MCF7-Ctrl cells. To more effectively quantitate the differences in metastatic burden when MCF7-Six1 and MCF7-Ctrl cells were co-injected, we luciferase-tagged-MCF7-Ctrl cells (MCF7-Ctrl-luc) and tRFP-tagged MCF7-Six1 (MCF7-Six1-tRFP) and performed an *in vivo* metastasis experiment in the presence or absence of GANT-61.
Figure 5.4 Gli activation is central to the induction of non-cell autonomous phenotypes downstream of Snail1, Twist1 and Six1.
Representative (A) 7hr migration assays and (B) 18hr invasion assays of HMLER-Ctrl cells in HMLER-Snail1/Twist1 CM +/- GANT-61. (C) Western blot analyses of WCLs collected from MCF7-Ctrl cells cultured in MCF7-Ctrl and Six1 CM for 48hrs treated with GANT-61 or vehicle. (D) Representative ICC of E-Cadherin (green) in MCF7-Ctrl cells cultured in MCF7-Ctrl and Six1 CM for 48hrs treated with GANT-61 or vehicle and quantification of % membranous E-Cadherin. n≥45-60; scale ~100µm (E) Anoikis resistance of MCF7-Ctrl cells cultured in indicated CM + treatment with GANT-61 or vehicle for 24hrs. SEM shown in representative images, n=3, One way ANOVA with Tukey post test in all cases, (ns – not significant). *p value<0.05, **p value<0.001, ***p value<0.0001
In this experiment, “singly injected” MCF7-Ctrl cells contained a 1:1 mixture of MCF7-Ctrl-luc and untagged MCF7-Ctrl cells, and “singly injected” MCF7-Six1 cells contained a 1:1 mixture of MCF7-Six1-tRFP and untagged MCF7-Six1 cells. This experimental strategy carefully controls for the tagged cell numbers, when comparing to the 1:1 mixture of MCF7-Ctrl-luc and MCF7-Six1-tRFP cells, a condition that will be referred to as “mixed tumors”. Once the mixed tumor volumes reached 1cm$^3$, the mice were randomized and half were treated every other day with 50mg/kg of GANT-61 (and the other half were treated with vehicle control) for 18 days (Figure 5.5 A). As seen in our pilot experiment, we observed a significant increase in distant luminescence signal of the MCF7-Ctrl-luc cells when they were co-injected with MCF7-Six1-tRFP cells compared to the singly injected MCF7-Ctrl-luc cells when comparing mice across groups bearing similar tumor volumes (Figure 5.5 B, C). The increased distant MCF7-Ctrl-luc signal was dramatically decreased in mice with mixed tumors treated with GANT-61, compared to vehicle control (Figure 5.5 B, D, E). We also observed a decrease in luciferase signal (from MCF7-Ctrl-luc cells) in the primary tumors of mice treated with GANT-61 compared to vehicle (Figure 5.5 B, F, G).

Surprisingly, we observed that MCF7-Six1 cells also grew and metastasized more efficiently in the presence of MCF7-Ctrl cells compared to when MCF7-Six1 cells were “singly” injected (Figure 5.6 A, B). Interestingly, GANT-61 treatment modestly but significantly affected the metastatic ability of the EMT (MCF7-Six1) cells themselves (Figure 5.6 A, C, D), suggesting that they also use the Hh/GLI pathway, but do not depend on it to the same degree as the non-EMT (MCF7-Ctrl) cells. However, GANT-61 treatment did not affect the ability of the EMT cells to grow in the primary tumor (Figure 5.6 A, E, F). Together, these data demonstrate that inhibition of Hh signaling using GANT-61 inhibits both cell and non-cell autonomous mediated metastasis, but that non-EMT cells are more dependent on Gli activation for primary tumor growth and metastasis than the EMT cells.
Figure 5.5 GANT-61 suppresses primary tumor growth and non-cell autonomous induction of metastasis in non-EMT cells (continued)
**Figure 5.5 GANT-61 inhibits non-cell autonomous increase in metastasis in vivo.** (A) Diagrammatic representation of the metastasis experiment. Female NOG/SCID mice were orthotopically injected in the 4th mammary fat pad with either MCF7-Ctrl-luc/MCF7-Ctrl-untagged (1:1) or MCF7-Six1-tRFP/MCF7-Six1-untagged (1:1) cells or co-injected with MCF7-Ctrl-luc and MCF7-Six1-tRFP cells (1:1). Mixed tumors were treated with either 50mg/kg of GANT-61 or vehicle every other day for 18 days once tumor volume reached 1cm³. Primary tumor volume and metastases were measured weekly. (B) Representative bioluminescent imaging of mice at similar tumor volumes for MCF7-Ctrl-luc and mixed injection groups with and without GANT-61 treatment (C) Quantification of MCF7-Ctrl-luc signal (in lymph nodes/lungs - yellow boxed region) in various groups represented as p/s, photons per second. (D, F) Quantification of MCF7-Ctrl-luc signal from (D) distant sites and (F) primary tumor in mixed tumors groups treated with vehicle or GANT-61, represented as fold change over pre-treatment signal. (E, G) Normalized luminescent signal from (E) distant sites and (G) primary tumors of individual mice pre- and post-treatment. SEM shown, two tailed unpaired t-test for all cases, *p value<0.05, **p value<0.001
Figure 5.6 GANT-61 modestly suppresses cell autonomous metastasis of EMT cells (continued)
Figure 5.6 GANT-61 modestly suppresses cell autonomous metastasis of EMT cells. 

(A) Representative fluorescent imaging of NOG/SCID mice at same time point (corresponding to similar tumor volumes) for singly injected MCF7-Six1-tRFP tumors and mixed injection tumor groups treated with GANT-61 or vehicle. 

(B) Quantification of red fluorescent signal from MCF7-Six1-tRFP cells in the primary tumor and distant sites (in lymph nodes/lungs - yellow boxed region) in the singly injected MCF7-Six1-tRFP tumors and mixed tumor groups represented as c/s, counts per second. 

(C, E) Quantification of fluorescent signal from (C) distant sites and (E) primary tumor in mixed tumors groups treated with vehicle or GANT-61, represented as fold change over pre-treatment signal. 

(D, F) Normalized fluorescent signal from (D) distant sites and (F) primary tumors of individual mice pre- and post-treatment, SEM shown, two tailed unpaired t-test for all cases, *p value<0.05, **p value<0.001
Expression of EMT-factors strongly correlates with Gli1, but not Hh ligands, in breast cancer patients.

If Gli activation is an important mechanism through which EMT-inducing transcription factors increase the aggressiveness of tumors, an association between Gli1 mRNA levels, (which itself is a target of the pathway), and the EMT-factors, Six1, Snail1 and Twist1, should be observed in human tumors. In contrast, if the activation of the pathway by these factors via the ligands is not as conserved a mechanism (as observed in our in vitro data), we would anticipate that their correlation with Hh ligands may not be as strong in carcinomas. Examination of numerous publicly available mRNA datasets demonstrate that while many breast cancer datasets showed strong positive correlations between Six1, Snail1 or Twist1 and Gli1, a consistent positive correlation between the EMT-factors and Hh ligands was not observed in those same datasets (Figure 5.7 A-D). Increased levels of each of the EMT-inducing transcription factors and Gli1 also correlated with worsened prognosis in breast cancer patients, as patients with high levels of Six1, Snail1, or Twist1 along with high levels of Gli1 were more likely to have decreased overall survival, decreased relapse-free survival, and decreased metastasis-free survival, in different breast cancer subtypes and grades (Figure 5.8 A-D).

Treatment with Gli inhibitors, but not Smo inhibitors inhibits growth in PDX model showing increased expression of EMT-inducing transcription factors

The relevance of Hh/GLI pathway activation downstream of these factors is further demonstrated on examination of patient derived xenografts (PDX) models, generated by Dr. Michael Lewis and colleagues at the Baylor College of Medicine.
Figure 5.7 Positive correlations of Six1, Snail1, and Twist1 mRNA with Gli1 mRNA in human breast cancer datasets. (A-C) Linear regression analyses examining positive correlation between Gli1 and (A) Six1 in Gluck Breast, (B) Snail1 in Hatzis Breast and (C) Twist1 in TCGA breast. (D) Expressions of the EMT-factors do not consistently correlate with Hh ligand levels. Data obtained from Oncomine.
Figure 5.8 Increased expressions of both Gli1 and each of the EMT-factors predicts poor prognosis in breast cancer patients (continued)
Figure 5.8 Increased expressions of both Gli1 and each of the EMT-factors predicts poor prognosis in breast cancer patients.
High expression of both Gli1 and (A,D) Six1, (B,D) Snail1 and (C) Twist1 predict decreased metastasis-free survival (DMFS), relapse-free survival (RFS), overall survival (OS) and DMFS mixed in breast cancer patients, data obtained from GOBO. (LN- lymph node; neg- negative)

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On interrogation of RNAseq expression data obtained from more than 30 breast PDX models, we found that PDX tumor expressing high levels of *Twist1*, *Snai1* and *Six1* exhibited increased activation of Hh/GLI signaling (as shown by increased levels of pathway targets *Ptch1* and *Ptch2*), despite not consistently expressing high levels of Hh ligands (Figure 5.9 A).

Interestingly, Gli transcription factors, although present, were not always high in tumors that showed an overall activation of Hh signaling, suggesting that the presence of Gli may not be the only predictor for response to Hh pathways inhibitors. We thus chose one tumor in which Gli2 was high (MCI) as well as one tumor in which EMT-factors were high (BCM-2147) and Hh signaling appears activated (not necessarily due to high Gli), to test the hypothesis that breast tumors expressing higher levels of EMT-factors would be more susceptible to inhibitors of Gli than to upstream inhibitors targeting Smo. In tumor-bearing mice treated for 14 days with either IPI-926 (cyclopamine derivate that targets Smo) or GANT-61 (vs. vehicle), only GANT-61 was significantly able to cause growth inhibition (Figure 5.9 B), indicating that Smo-independent activation of Hh/GLI signaling resulting in tumorigenesis, is a mechanism at play in these PDX tumors. Together, these data soundly indicate that convergence on activation of Hh/GLI pathway and specifically Gli is a critical means by which these EMT-factors function in human tumors. These findings suggest that targeting Hh signaling through downstream effectors (Gli) will be more efficacious than upstream Smo inhibitors, in breast tumors expressing EMT-inducing transcription factors.

**Discussion**

Several studies show that activated Hh signaling results in increased tumorigenesis of various cancers. Hh ligands, which can act both cell autonomously and non-cell autonomously (on the tumor microenvironment), activate the canonical Hh pathway via Ptch and Smo.
Figure 5.9 High expression of EMT-factors correlates with increased Hh/GLI pathway activation in PDX tumors (continued)
Figure 5.9 High expression of EMT-factors correlates with increased Hh/GLI pathway activation in PDX tumors.

(A) Heat-map generated from RNA-seq analyses performed on PDX tumors (B) Kaplan-Meier growth inhibition curves of MC1 and BCM-2147 PDX models treated with IPI-926 and GANT-61. (Data generated by Dr. Michael Lewis and colleagues at Baylor College of Medicine, Houston)
More recently, evidence for non-canonical activation of this pathway via direct regulation of Gli, and independent of Smo, has garnered increased attention. Several EMT-inducing, pro-metastatic factors including TGF-β have been implicated in directly increasing Gli1/2 levels and hence causing activation of this pathway in a non-canonical manner.245

Our data demonstrate that non-cell autonomous Gli activation in cells induced by pro-EMT- and metastatic transcription factors Snail1, Twist1 and Six1 is responsible for mediating their non-cell autonomous phenotypes. Although all three factors invoke Hh/GLI signaling, they do so via both canonical and non-canonical means. Six1 is sufficient to increase SHH ligand levels, which in turn activates the Hh pathway. Hence, both upstream and downstream inhibitors of the pathway, which target the ligand/Smo and Gli1/2 respectively, equally and effectively abrogate the non-cell autonomous phenotypes in the MCF7 cells. Interestingly, while cyclopamine and 5E1 do not restore anoikis sensitivity to MCF7-Ctrl cells, treatment with GANT-61 can. These data suggest that there are factors in addition to SHH, which are regulated downstream of Six1 and directly activate Gli signaling non-canonically to affect certain phenotypes. Hence only a drug that targets Gli directly such as GANT-61 is able to effectively abrogate all non-cell autonomous phenotypes.

We also show that GANT-61 can inhibit both cell and non-cell autonomous increases in metastasis in vivo when MCF7-Six1 and MCF7-Ctrl cells are co-injected in mice. While GANT-61 is able to significantly inhibit tumor growth of the MCF7-Ctrl cells, it has no effect on the growth of MCF7-Six1 cells in the primary tumor, suggesting that MCF7-Ctrl cells rely more heavily on Hh/GLI signaling than MCF7-Six1 cells. The modest decrease in cell autonomous metastasis of the MCF7-Six1 cells with GANT-61 treatment is likely due to their reliance on additional mechanisms such as TGF-β signaling in order to metastasize.206 Nevertheless, though numerous studies have shown that GANT-61 can effectively suppress tumor growth in many cancers, to our knowledge our work is first to demonstrate suppression of metastasis.
In the HMLER system, we find that in contrast to MCF7 cells, Gli signaling is activated in a Hh ligand and Smo independent manner, evidenced by the reversal of non-cell autonomous phenotypes only on treatment with Gli1/2 inhibitor GANT-61. In addition to non-canonical activation of the pathway by increasing the levels and/or stability of Gli1/2, several studies show that Smo functions as a G-protein coupled receptor (GPCR) which can activate Gli and hence Hh signaling \(^{279}\). This mechanism of activation is considered non-canonical, since Ptch is not involved in the activation of the pathway. Since studies show that cyclopamine and its derivatives can inhibit Smo from acting as a GPCR\(^{279}\), it is unlikely that this function of Smo is at play downstream of Snail1 and Twist1. Nevertheless all three EMT-inducing transcription factors converge on Gli signaling to mediate their non-cell autonomous phenotypes.

The importance of Gli activation downstream of these factors is further underscored in that Gli1 mRNA expression (Gli1 being a direct target of the pathway) positively correlates with that of Snail1, Twist1 and Six1, in numerous human breast cancer datasets and can predict poor prognosis across many different breast cancer subtypes and grades. More importantly, examination of more than 30 breast PDX tumors indicates that tumors expressing high levels of Twist1, Snail1 and Six1, despite having low levels of the Hh ligands, have increased activated Hh signaling. Moreover, the PDX tumor that expresses high levels of all three EMT-factors and has activated Hh signaling, exhibits decreased tumor growth only when treated with an inhibitor against Gli1/2, GANT-61, while the Smo inhibitor, IPI-926 has no effect. Together these data demonstrate that activation of Hh signaling may be more dependent on presence or absence of the EMT-factors than canonical activation by Hh ligands.

This is especially significant since countless derivatives of Smo-inhibitors like IPI-926 and Vismodegib are currently in clinical trials for numerous cancers. They continue to show promising results in cancers like BCC and medulloblastoma, which usually exhibit mutations
in pathway components resulting in activation of Hh signaling (through Ptch/Smo axis)\textsuperscript{258}. These derivatives have also shown efficacy in other solid tumors like metastatic pancreatic cancer where Hh signaling is thought to be activated via ligand-dependent mechanisms\textsuperscript{270}. In breast cancer, the data are still murky. While some studies show hyperactive Hh signaling via Hh ligands and \textit{Ptch} mutations, our data demonstrate that Hh signaling can be robustly activated via non-canonical, Gli centric mechanisms. Hence, Smo- targeting inhibitors are unlikely to have desirable effects in such patients. Together, our data indicate that use of drugs that directly target more downstream pathway players like Gli, irrespective of which EMT-factor is overexpressed in the cancer, may be the more superior way to effectively inhibit this pathway in heterogeneous tumors.

\textbf{Acknowledgements}

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Hedgehog signaling is a very complex signaling network and is quite poorly understood, despite the plethora of studies that have been performed on its functions and activity. Canonical activation of this pathway involves the contribution of Hh ligands, receptors Patched (Ptch) 1 and 2, Smoothened (Smo) and transcription factors Gli1, Gli2 and Gli3, while activation of the pathway without the involvement of any one of these factors is considered non-canonical. In recent years, several examples of non-canonical activation of Hh signaling have been described in the literature. Studies show that SHH can activate ERK1/2 signaling in cell lines that do not express Smo, by binding to Ptch1. The activation of signaling pathways downstream of Ptch1 (that do not always involve Gli activation) is attributed to its C-terminal domain (CTD) that contains motifs encoding for SH3 binding sites. Ptch1 itself can function in a non-canonical manner by regulating different processes via its CTD. In the absence of ligand binding, Ptch1 CTD acts in concert with caspase proteins to induce cell death in variety of cell types, which is not modulated by Smo antagonists. Furthermore, Ptch1 has been shown to interact specifically with phosphorylated CyclinB1 and affect cell cycle progression and subsequently proliferation of cells. Overexpression of Ptch1 in 293T cells was shown to result in the translocation of CyclinB1 from the nucleus to the cytoplasm which resulted in cell cycle arrest and reduced proliferation. In addition to Smo’s GPCR role in activating Gli factors, ligand activation of Smo and its associated G-protein activity can modulate stress fiber formation and tubulogenesis in endothelial cells, independent of Gli. Ligand
activation of Smo also regulates processes like axon guidance, increased migration of fibroblasts and Calcium level fluctuations, all independent of Gli\textsuperscript{280}.

As described previously, in tumorigenesis, many non-canonical mechanisms of Hh pathway activation involve direct regulation of Gli1/2. This is achieved by promoting their transcription, increasing their stability, or regulating their cellular compartmentalization, amongst other means\textsuperscript{245}. EMT related signaling pathways like TGF-\(\beta\) and Ras/MEK signaling have been shown to increase Gli1/2 levels\textsuperscript{245}. Though EMT-inducing transcription factors like Twist1, Snail1 and Six1 have been linked to Hh signaling, until recently there was no evidence for direct regulation of Gli as a mechanism for pathway activation by these factors. Segal and colleagues recently demonstrated that Six1 in conjunction with Eya1, can increase Gli signaling via upregulation of Neuropillin1/2 (Nrp1/2), and in fibroblasts knockdown of either Six1 or Eya1 or Nrp1/2 resulted in attenuated Gli signaling. This pathway was shown to be important for hindbrain development and subsequently dysregulated in a subset of SHH driven medulloblastomas\textsuperscript{260}. Importantly, Six1-Eya1-Nrp1/2 dependent activation of Gli signaling was independent of Hh ligands and Smo function.

In addition to Six1’s more recent links to Gli signaling\textsuperscript{260} and its ability to induce cell autonomous EMT and metastasis via increased TGF-\(\beta\) signaling\textsuperscript{206}, Six1 has been shown by our laboratory to transcriptionally upregulate Vascular endothelial growth factor-C (VEGF-C) in breast cancer cells\textsuperscript{217}. This study showed that VEGF-C secreted by Six1-expressing breast cancer cells resulted in increased lymphangiogenesis and hence increased metastasis. Following this study, another group saw similar results in a cervical cancer model, where Six1 was able to upregulate VEGF-C but in a TGF-\(\beta\)/SMAD dependent manner\textsuperscript{284}.

VEGF-C has been extensively studied for its roles as a lymphangiogenic factor. Increased expression of VEGF-C in several mammary carcinoma models has been shown
to cause intratumoral lymphangiogenesis, increased regional lymph node metastasis as well as distant lung metastasis\textsuperscript{285,286}. Meta-analysis of breast cancer patient data shows that increased VEGF-C expression correlates with shorter overall and disease–free survival\textsuperscript{287} highlighting the important role of VEGF-C in tumorigenesis. VEGF-C secreted by either cancer cells or immune cells like tumor-associated-macrophages, signals by binding to its receptor VEGFR3 mostly expressed on endothelial cells but also on cancer cells themselves\textsuperscript{217}. Binding of the ligand to the receptor triggers receptor dimerization, which initiates an intracellular signaling cascade resulting in increased migration, proliferation and cell permeability resulting in increased metastasis\textsuperscript{288}. Interestingly, VEGFR3 has been shown to dimerize with Neuropillin2 and hence serve as co-receptors for VEGF-C, resulting in increased lymphangiogenesis and endothelial cell survival and migration\textsuperscript{288}. Since VEGF-C is a secreted factor which plays important roles in breast cancer progression and is regulated downstream of Six1, and since the neuropilins, through which VEGF-C acts, have been implicated in Gli activation downstream of Six1, we evaluated the role of VEGF-C in the non-cell autonomous phenotypes mediated by Six1.

**Results**

**VEGF-C is expressed in a Six1-dependent manner downstream of Snail1**

Our data in the HMLER cells indicated that Snail1 and Twist1 required Six1 to mediate their non-cell autonomous phenotypes in cells that did not express any of these factors. Moreover, Hh/GLI signaling was activated in a Six1-dependent manner downstream of Snail1 and Twist1. Interestingly, only inhibition of the pathway using the downstream inhibitor GANT-61 (which directly targets Gli1/2), abrogated the non-cell autonomous increases in migration and invasion facilitated by these factors, while the upstream inhibitor cyclopamine (targeting Smo) had no effects. This, coupled with no changes observed in Hh ligand levels in the HMLER cells expressing Twist1 or Snail1 compared to HMLER-Ctrl cells,
suggests that in the HMLER cells, Hh/GLI signaling is activated downstream of Snail1/Twist1 and Six1 in a non-canonical manner that is Hh ligand- and Smo-independent. Since VEGF-C can be secreted by Six1-expressing breast cancer cells \(^{217}\), we first determined the VEGF-C expression pattern in HMLER cells. We found that HMLER-Snail1 cells expressed higher mRNA levels of VEGF-C than HMLER-Ctrl cells, which was reduced with Six1 KD (Figure 6.1 A). However, we did not observe increased levels of VEGF-C in Twist1-expressing cells. As described above, studies have shown that VEGF-C can function by binding to Neuropilin2 (Nrp2) \(^{288}\) and Nrp2 itself can activate Gli signaling non-canonically \(^{260}\). If the HMLER-Ctrl cells expressed Nrp2, it would suggest that VEGF-C in the CM from HMLER-Snail1 cells would be able to bind to Nrp2 expressed by the HMLER-Ctrl cells, and thus potentially activate Gli signaling non-cell autonomously. We found that HMLER-Ctrl cells express Nrp2 on both the mRNA and protein level (Figure 6.1 B, C). While the variation in Nrp2 mRNA and protein expression in HMLER cells may be due to differences in Nrp2 protein stability, other post-transcriptional modifications or due to a negative feedback loop, our data indicate that HMLER-Ctrl cells express Nrp2. Thus, we hypothesized that Gli signaling in HMLER-Ctrl cells was non-cell autonomously activated by VEGF-C acting downstream of Snail1 and Six1 in this model. Since VEGF-C mRNA expression was unchanged between the HMLER-Twist (+/-Six1 KD) and Ctrl cells, we decided to pursue VEGF-C activation of Gli as mechanism downstream of only Snail1 and Six1.

**Inhibition of VEGF-C abrogates non-cell autonomous activation of Gli signaling and phenotypes downstream of Snail1 and Six1**

To test our hypothesis that Snail/Six1 mediated increases in VEGF-C can activate Gli, we used a function blocking antibody against VEGF-C (VEGF-C AB),
Figure 6.1 VEGF-C and Nrp2 are expressed in HMLER cells.

(A, B) qRT-PCR analysis and (C) western blot analysis on WCLs of HMLER-Ctrl, HMLER-Snail1 and Twist1 cells transfected with 150nM siNT or siSix1. Gene expression normalized to PP1B. SEM shown, n≥2.
which has previously been shown to bind to the C-terminus of the secreted VEGF-C pro-peptide and inhibit its processing to a fully functional VEGF-C molecule \(^{289,290}\).

Blockade of VEGF-C activity using this antibody has been shown to inhibit coronary vessel formation in embryonic quail heart tissue embedded in collagen and VEGF-C associated increases in migration in several breast cancer cell lines \textit{in vitro} \(^{289,290}\). We inhibited VEGF-C by addition of VEGF-C AB or Goat IgG (as a control) to the CM from HMLER-Snail1 cells and performed a Gli reporter assay to determine if blocking VEGF-C would have an effect on Gli activation. Indeed, we observed that HMLER cells cultured in HMLER-Snail1 CM had significantly decreased non-cell autonomous activation of Gli signaling in the presence of the VEGF-C AB compared to control conditions (Figure 6.2 A). Furthermore, addition of human recombinant (rh) VEGF-C to the medium also resulted in significant activation of Gli signaling in the HMLER cells (Figure 6.2 B). In both cases, the m-Gli1 reporter showed negligible pathway activation (Figure 6.2 A, B), indicating that VEGF-C can activate Gli signaling and it does so, downstream of Snail1 in the HMLER cells.

Previous studies have shown that MCF7-Six1 cells express and secrete higher levels of VEGF-C compared to MCF7-Ctrl cells \(^{217}\). Hence we tested whether VEGF-C (in addition to SHH as previously seen) would play a role in activating Gli signaling downstream of Six1 in the MCF7 cells. Since we saw robust effects using cyclopamine in the MCF7 cells, we performed the Gli reporter assays using cyclopamine alone, VEGF-C AB alone, and both in combination, to determine if the combination would additively result in further inhibition of pathway activation. Surprisingly, we found that VEGF-C AB alone had similar inhibitory effects as cyclopamine itself on Gli activation, and the combination did not significantly further enhance these effects (Figure 6.2 C). As seen previously, the m-Gli1 reporter did not show changed activity (Figure 6.2 C). This suggests that VEGF-C can also non-cell autonomously activate Gli signaling in MCF7 cells downstream of Six1.
Figure 6.2 VEGF-C can non-cell autonomously activate Gli signaling.
48hr 7-Gli1-GFP reporter assays in (A) HMLER cells cultured in CM from HMLER-Snail1+/−VEGF-C AB (n=2), (B) HMLER cells with addition of 1µg/ml of rhVEGF-C to media, and (C) MCF7 cells cultured in CM from MCF7-Six1 cells treated with indicated drug/antibodies, represented as %GFP+ cells. SEM shown, compiled experiments n≥3 (with different sets of CM). One way ANOVA with Tukey post test in all cases (ns – not significant), **p value<0.001
We next asked whether blocking VEGF-C would have an effect on the non-cell autonomous phenotypes observed in the HMLER and MCF7 cells, downstream of Snail1 and Six1, respectively. To test this with the HMLER cells, we performed migration and invasion assays with the addition of the VEGF-C AB and found that HMLER-Ctrl cells had significantly decreased migration and invasion when cultured in CM from HMLER-Snail1 cells+ VEGF-C AB, compared to control conditions (Figure 6.3 A, B). As expected, no changes in phenotypes were observed in HMLER-Ctrl cultured in CM from HMLER-Twist1 cells+ VEGF-C AB (Figure 6.3 A, B). Interestingly, treatment of MCF7-Ctrl cells with VEGF-C AB rescued both the CK-18 and FN1 levels, and membranous E-Cadherin back to control levels when the cells were cultured in CM from MCF7-Six1 cells (Figure 6.3 C, D). Together these data indicate that VEGF-C functions downstream of Six1 and Snail1 to mediate their non-cell autonomous phenotype.

**VEGF-C functions specifically downstream of Six1 to mediate the non-cell autonomous effects of Snail1 in HMLER cells**

Our data indicate that VEGF-C can function downstream of Six1 in the MCF7 cells and Snail1 in the HMLER cells. We previously showed that Six1 is required downstream of Snail1 not only to activate Gli signaling non-cell autonomously, but to also mediate its non-cell autonomous functions. Hence, we hypothesized that VEGF-C was acting downstream of both Snail1 and Six1 in the HMLER cells and it was the decrease of VEGF-C in CM from HMLER-Snail1+Six1 KD cells that was responsible for both decreases in the non-cell autonomous Gli activation as well as the observed phenotypes. To test this hypothesis, we added rhVEGF-C to the CM from HMLER-Snail1+siSix1 cells and performed Gli reporter assays and migration and invasion assays on the HMLER cells.
Figure 6.3 Inhibition of VEGF-C abrogates non-cell autonomous phenotypes downstream of Snail1 and Six1.

(A) 7hr migration and (B) 18hr invasion assays in HMLER-Ctrl cells cultured in CM from HMLER-Snail1/Twist1 cells +/- VEGF-C AB. (C) Western blot analyses on WCLs and (D) representative ICC of E-Cadherin (green) in MCF7-Ctrl cells cultured in CM from MCF7-Ctrl and Six1 cells +/- VEGF-C AB for 48hrs (Dapi, blue), scale-20µm, n=2. SEM shown, n≥3. One way ANOVA with Tukey post test in all cases (ns – not significant), **p value<0.001, ***p value<0.0001
Indeed, we found that addition of rhVEGF-C to the CM from HMLER-Snail1+siSix1 cells was able to significantly rescue Gli signaling activation non-cell autonomously in the HMLER cells, which was not seen using m-Gli1 reporter (Figure 6.4 A). Additionally, both the migration and invasion phenotypes of the HMLER-Ctrl cells were rescued with rhVEGF-C compared to control levels (Figure 6.4 B, C). These data demonstrate that VEGF-C is acting downstream of Snail1, and specifically Six1, to facilitate their non-cell autonomous effects.

**Discussion**

Several studies show that Hh signaling can be activated non-canonically independent of Hh ligands and receptor Smoothened (Smo)\(^ {245}\). Signaling pathways like TGF-β, ERK and AKT function to directly increase Gli1/2 levels and hence activate the Hh network without the contribution of Smo\(^ {245}\). Our previous results indicated that Hh/GLI signaling was activated non-cell autonomously in HMLER cells and was not inhibited by upstream inhibitors of pathway that target Hh ligands and Smo. Instead, downstream inhibitor GANT-61 that directly targets Gli1/2 was able to abolish both activation of Gli signaling as well as the associated non-cell autonomous phenotypes. These data suggest that in HMLER cells, Gli signaling is activated in a non-canonical manner that does not involve the ligands and bypasses the upstream receptor Smo.

Our data demonstrate that VEGF-C is expressed in the HMLER cells downstream of Snail in a Six1-dependent manner. Since our laboratory has previously shown that upregulation of VEGF-C in Six1 expressing cells requires interaction with Eya2\(^ {217}\) and HMLER cells express negligible levels of Eya2 (Figure 4.9), it suggests that VEGF-C upregulation in HMLER-Snail1 cells may not fully be dependent on transcriptional upregulation by Six1. This is supported by our data that KD of Six1 in HMLER-Snail1 cells only results in partial decrease in VEGF-C levels compared to HMLER-Ctrl cells (Figure 6.1 A).
Figure 6.4 rhVEGF-C rescues non-cell autonomous phenotypes downstream of Six1 KD in HMLER-Snail1 cells.

(A) 7-Gli1-GFP reporter assays in HMLER cells cultured in CM from HMLER-Ctrl or Snail1 +/- siSix1 cells with 1ug/ml rhVEGF-C added to the CM as indicated, represented as %GFP+ cells. (B) 7hr migration and (C) 18hr invasion assays of HMLER-Ctrl cells cultured in CM from HMLER-Ctrl and HMLER-Snail1 +/- siSix1 cells with addition of 1ug/ml rhVEGF-C to the CM as indicated. SEM shown, n≥3, One way ANOVA with Tukey post test in all cases (ns – not significant), *p value<0.05, **p value<0.001, ***p value<0.0001
Alternatively, Six1 could interact with other Eya family members to regulate VEGF-C in the HMLER system, as HMLER cells do express Eya3 on the protein level. Nevertheless, VEGF-C can activate Hh/GLI signaling since addition of rhVEGF-C to the medium was sufficient to increase 7-Gli1 reporter activity in HMLER cells. Furthermore, blocking VEGF-C in the CM effectively abrogates both non-cell autonomous Gli activation as well as migration and invasion in the HMLER-Ctrl cells, while addition of rhVEGF-C rescues these phenotypes downstream of Six1 KD in Snail1-expressing cells. These data indicate that VEGF-C is both necessary and sufficient downstream of Snail1 and Six1 to mediate their non-cell autonomous phenotypes. Interestingly, while studies show that VEGF-C and other members of VEGF family of proteins including VEGF-A are targets of Hh/GLI signaling during coronary vascular development and tumorigenesis, to our knowledge this study is the first to demonstrate that VEGF-C can activate Hh/GLI signaling.

We hypothesize that VEGF-C functions through Nrp2, since VEGF-C can activate signaling via Nrp2, and Nrp2 has been shown to activate Gli signaling. Furthermore, a recent study showed that C-terminal proteolytic maturation of VEGF-C (inhibited by our function blocking VEGF-C AB) is required for VEGF-C to bind Nrp2. If the VEGF-C/Nrp2 axis is indeed responsible for non-canonical Gli activation, this could provide an explanation for how the antibody renders VEGF-C unable to bind to Nrp2 (expressed by the HMLER-Ctrl cells) and hence inhibits the non-cell autonomous activation of Gli. However, the secreted levels of VEGF-C in the CM from the HMLER cells are yet to be determined. Since we observe discrepancies between the mRNA and protein expression of Nrp2 in HMLER-Ctrl cells, flow cytometry analyses can be performed on the HMLER cells to determine if Nrp2 is expressed on the surface of the cells. The specific role of Nrp2 in our cells can be tested by knockdown of Nrp2 in the HMLER-Ctrl cells or addition of function blocking Nrp2 antibody to the CM. Nevertheless, this non-canonical mechanism of Gli activation via VEGF-C would
potentially explain why only GANT-61 and not cyclopamine inhibited the non-cell autonomous phenotypes downstream of Snail1.

The complete rescue of phenotypes using the VEGF-C AB downstream of Six1 in the MCF7 cells, is surprising since while the activity of VEGF-C is blocked by the antibody, SHH is still presumably present and functional in the CM from MCF7-Six1 cells. This suggests that either the VEGF-C AB has off-targets effects or a more enticing proposition is that inhibition of Gli signaling by VEGF-C results in the suppression of SHH via an unknown mechanism (since SHH is not known to be a direct target of the pathway). Nevertheless, our data indicate that VEGF-C is a potent mediator of non-cell autonomous actions downstream of Six1 and that Six1 can both canonically and non-canonically activate Hh/GLI signaling in the same cells, albeit via different mediators.

The significant yet partial rescue of Gli activity and some non-cell autonomous phenotypes using rhVEGF-C downstream of Six1 KD in HMLER-Snail1 cells, suggests that VEGF-C is not the only factor that mediates the non-cell autonomous phenotypes downstream of Snail1. This is not surprising given that Snail1 regulates a massive transcriptional program and previously has been shown to upregulate several cytokines which could impinge on Gli signaling directly via as yet undiscovered mechanisms. Hence, while our data indicate that VEGF-C plays an important role in non-cell autonomously and non-canonically activating Gli signaling, other factors may also be pertinent. This is especially true downstream of Twist1, where similar to Snail1, only the more downstream pathway inhibitor GANT-61, effectively abrogates both Gli signaling activation and the non-cell autonomous phenotypes. Since VEGF-C levels are not changed between the HMLER-Twist1 and Ctrl cells, it suggests that other molecules are regulated downstream of Twist1 to facilitate its functions.

Nevertheless, together these data indicate that Hh/GLI signaling can be activated non-cell autonomously by non-canonical mechanisms (potentially through VEGF-C)
downstream of Six1 and Snail1 and the end result is increased metastatic properties of cells that don’t express these transcription factors.

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CHAPTER VII

DISCUSSION

Intratumoral Heterogeneity in Breast Cancers

In recent years, the advent of advanced molecular techniques like whole genome and exome sequencing and single cell sequencing have confirmed the existence of both inter- and intratumoral heterogeneity. Intratumoral heterogeneity in breast tumors is a consequence of various contributing factors. One contributing factor to heterogeneity is different populations of cells within a breast tumor that exist in various "differentiation" states, with some cells exhibiting more "basal-like" features and hence are more "mesenchymal-like" than others, which can appear to be more epithelial. The phenomenon is thought to be a reflection of the cell-of-origin of the breast tumor and subsequent transforming events that can often translate into subtype specification in breast cancer. Additionally, genetic and epigenetic alterations as well as non-hereditary causes such as nutrient availability and random activation of signaling pathways are also thought to contribute to breast tumor heterogeneity. Heterogeneous primary breast tumors are thus comprised of diverse subpopulations of cells with different driver mutations, gene expression profiles, chemoresistance profiles and innate metastatic potential.

The existence of such diverse populations of cells within the tumor raises the possibility of crosstalk occurring between the cells which subsequently alters initiation and/or progression of the tumor. The interaction between different clones in the tumor has been hypothesized to occur through adjoining matrix connections or through secretion of diffusible

2 Parts of this chapter were reprinted with permission from 1. Neelakantan, D., Drasin, D.J. & Ford, H.L. Intratumoral heterogeneity: Clonal cooperation in epithelial-to-mesenchymal transition and metastasis. Cell adhesion & migration 9, 265-276 (2015).
factors by one or many clones\textsuperscript{295}. Several studies have shown that secretions from tumor cells can affect the tumor microenvironment, which feeds back to modulate tumor progression. Alternatively, these secreted factors can alter the properties of neighboring cells within the primary tumor itself, in which case, only a few cells would need to gain aggressive characteristics in order to increase metastasis of surrounding tumor cells. An important contributing factor to increased metastasis in epithelial tumors is thought to be the ability of tumors cells to undergo an epithelial-to-mesenchymal transition (EMT), which allows cells to become more aggressive. Interestingly, data show that only a small percentage of epithelial tumor cells may undergo an EMT and/or have increased metastatic potential at any one time\textsuperscript{1,56,95}, yet the resulting metastatic incidence is high. In breast cancer, while studies have shown that subpopulations of cells within breast tumors can interact with each other to affect tumor progression, targetable mechanisms behind these interactions remain elusive.

In this thesis we demonstrate that EMT-inducing transcription factors Snail1 and Twist1 that have been extensively characterized for their cell autonomous roles in breast cancer, have the ability to function in a non-cell autonomous manner \textit{in vitro}. We demonstrate that Snail1 and Twist1 can induce increased migration and invasion of cells that don’t express either factor, via both co-culture and conditioned medium experiments. Interestingly, while Snail1 and Twist1 can cell autonomously cause EMT, the increase in metastatic properties of non-Snail1 and non-Twist1 expressing cells is independent of non-cell autonomous EMT induction in those cells.

\textbf{Six1 Regulation by Snail1 And Twist1}

Much like during development, in various cancers, studies show that EMT-inducing factors exist in a complex web and induce their phenotypes in part by modulating each other’s levels (Figure 7.1). For example, TGF-β and Snail1 are known to function closely in
breast cancer and recently, were together shown to increase Zeb1 levels by multiple mechanisms including increasing protein stability of Zeb1 as well as transcription of Twist1, a pathway which drives home how intricate the process of EMT-induction is. Interestingly, another study by Cano and colleagues performed in canine kidney cells shows that while both Snail1 and Zeb1 are required for EMT induction, only Snail1 along with Snail2 is required for EMT maintenance through epigenetic regulation of miR-200c, once again underscoring the importance of the interactions between various EMT-factors.

In our study we find that Snail1 and Twist1 upregulate Six1, another well studied EMT-inducing pro-metastatic transcription factor, and that they require Six1 to function in a non-cell autonomous manner. Though we show upregulation of Six1 by Snail1 and Twist1 only in one cell line, these transcription factors are often co-expressed in human breast tumors (Figure 7.2 A,B), suggesting that they work in conjunction with each other. Since Snail1, Twist1 and Six1 induce pro-tumorigenic phenotypes in similar types of cancers, including ovarian, HCC and lung cancer, it possible that their interactions are not limited to breast cancer alone. To our knowledge this is first study to demonstrate a link between Snail1, Twist1 and Six1 in cancer, though the exact mechanism of Six1’s upregulation is still unclear. Since we see an increase in Six1 mRNA levels downstream of Twist1 in HMLER cells and we find E-box binding sites in the promoter region of Six1, we speculate that Twist1 may directly upregulate Six1. Furthermore, since other bHLH factors can regulate Six1 expression during embryogenesis, it is plausible that Twist1 activates Six1 in oncogenesis, but this will have to be confirmed with electromobility shift assays (EMSA), luciferase reporter and ChIP assays. Regulation of Six1 by Snail1 proves to be more complicated, since Snail1 predominantly functions as a transcriptional repressor. We speculate that one of the mechanisms may be via regulation of miRNAs downstream of Snail1.
Figure 7.1 EMT-inducing factors exist in a complex web. Several studies show that EMT-inducing factors function in cancer by affecting the levels of each other. Six1 was recently added to this web via its interaction with Zeb1 and miR-200c\textsuperscript{204,298}. In this thesis we demonstrate that Six1 functions downstream of Snail1 and Twist1 in breast cancer cells. Adapted from \textsuperscript{119}. * Shown in \textsuperscript{204,298}
Figure 7.2 Expression of Six1 and Twist1/Snail1 positively correlate in breast cancer datasets.
Linear regression analyses examining positive correlation between Six1 and (A) Snai1 in Bonnefoi and Miller Breast and (B) Twist1 in Bittner and TCGA Breast obtained from Oncomine.
Our laboratory has previously shown that a multitude of miRNAs are regulated downstream of Snail1\textsuperscript{156}, any of which may function directly or indirectly to regulate Six1 levels. Since with this study and others, the role of Six1 has been firmly established in the complex interaction web of EMT-factors, it is only a matter of time before Six1’s novel collaborative roles with other EMT-factors is recognized.

**Non-Cell Autonomous Roles of Six1**

In this thesis, we further extensively demonstrate in various human and murine mammary carcinoma cell lines, that Six1 itself is sufficient to non-cell autonomously increase the aggressive properties as well as induce EMT (unlike Snail1 and Twist1) in non-Six1 expressing cells. Because the non-cell autonomous effects in the different systems did not always result in alterations in typical EMT markers, but in all cases enhanced properties associated with EMT and increased aggressiveness, it suggests that undergoing EMT (specifically alterations in EMT markers) can be disassociated from increased aggressiveness. Indeed, our data demonstrate that EMT cells can indeed enhance metastatic properties in cells that may not themselves have fully undergone an EMT. These data suggest that fate mapping of EMT cells may not truly reflect the role of these cells in metastatic dissemination, since in all cases examined, the aggressiveness of the epithelial cells (measured by varying parameters) was increased by the presence of and/or conditioned medium from EMT cells. Furthermore, the requirement of EMT for metastasis to occur has been disputed, and more recent studies using elegant transgenic mouse models have argued that oncogenic EMT is not a requirement for metastasis to occur\textsuperscript{95,96}. However, these studies have not addressed the possibility of interactions that may occur between EMT and non-EMT cells within a heterogeneous primary tumor, resulting in the metastasis of either cell type, and have rather only examined whether the cells that grow out at the secondary site have undergone an EMT.
In this thesis, we demonstrate that Six1-expressing cells that have undergone EMT (EMT cells) are able to increase the metastatic potential of non-Six1 expressing, non-EMT cells in vivo. Interestingly, the EMT cells themselves are able to metastasize better, indicating symbiotic cooperation between EMT and non-EMT cells in the primary tumor that positively affects metastatic outcome.

**Consequences of Clonal Cooperativity**

From the studies performed in this thesis as well as those summarized previously, it is clear that crosstalk occurs between the different cell populations comprising the primary tumor, resulting in beneficial outcomes for one or more clones. It is unlikely given the sequencing studies carried out in various cancers that one type of dominant clone will take over the entire tumor, since these studies are able to detect the presence of multiple clones within the primary tumor. Rather, data suggest that it can be beneficial for both the aggressive and non-aggressive clones to have the other present. Whether the cooperation between different clones results in positive tumor progression and increased metastasis, or whether it results in the stunting of tumor progression, is likely under the regulation of many factors and probably heavily influenced by the tumor microenvironment. Microenvironmental changes and pressures have been shown to be partly responsible for the outgrowth of these different sub-populations of cells at secondary sites, and could possibly play a role in making one direction of interaction between these cells more dominant than the other.

While we observe in our study that the Six1-expressing (EMT cells) increase metastasis of non-Six1-expressing (non-EMT cells), several questions remain to be answered. Though we see non-cell autonomous induction of EMT in non-EMT cells in vitro in our MCF7 model, it is unclear whether this phenomenon occurs in vivo, although clearly the EMT cells promote the metastasis of the “non-EMT” cells. Along similar lines, it is unknown whether the result of this cooperation is permanent. To elaborate, we are unaware
whether the EMT cells in our system only temporarily change the non-EMT cells; just enough to allow them to escape into the bloodstream and colonize at a secondary site. Thus, these cells with “pseudo” metastatic potential might revert to their non-aggressive versions in the metastatic site.

Furthermore, the kinetics of this process are undefined. It is unclear whether the non-EMT cells in the primary tumor which have been influenced by the EMT cells, potentially play a role in aiding the EMT cells to colonize at the secondary site. It is known that cancer cells can be dormant at the secondary site for long periods of time, and microenvironmental changes are known to be responsible for bringing cancer cells out of dormancy. In light of symbiotic cooperation between tumor cells, it is possible that intrinsically non-aggressive, non-EMT, yet cancerous cells within the primary tumor, metastasize first, to the secondary sites (with the aid of the EMT cells) and secrete factors that form a more habitable niche for the EMT (metastatic) cells that follow. This niche could enable the EMT cells to transition from dormant to active metastases, and perhaps could also alter the characteristics of the EMT cells to appear more epithelial. Alternatively, it is possible that the EMT cells metastasize first, paving way for the non-cell autonomously influenced (non-EMT) cells to follow, with both theories putting a new spin on the original “seed and soil” hypothesis. The third and most likely alternative is that both EMT and “non-EMT” escape the primary tumor together, aiding each other in the metastatic process to colonize at a secondary site together. In our experiments with the MCF7 cells, though the non-EMT cells require the EMT cells and hence metastasize with increased efficiency only in their presence, the EMT cells themselves have increased metastasis in the presence non-EMT cells, indicating that these cells interact with each other to facilitate overall increased metastasis. Most of the biologically important questions raised above, can now be answered given the recent advancements in molecular genetic techniques and real-time microscopy facilities. Nonetheless, since mutually beneficial cooperation can lead to increased tumor
progression and metastasis, it is pertinent to define the molecular mechanisms behind these interactions.

**Gli Signaling is the Central Node Downstream of Snail1, Twist1 and Six1**

The possibility that cells with metastatic potential can increase the metastasis of surrounding tumor cells suggests that in order to curb the overall incidence of metastasis, it will be important to identify factors that facilitate this conversion and inhibit their activity. Indeed, intratumoral heterogeneity can have dire consequences on patient survival, as studies have shown that different populations of cells within the tumor react differently to chemotherapeutic drugs. Many studies have also shown that EMT cells are often more “cancer stem cell-like” and are resistant to chemotherapy. However, if these cells are also capable of non-cell autonomously inducing EMT-like properties in neighboring tumor cells, and rendering neighboring cells resistant to chemotherapy, a larger than anticipated percentage of the tumor may not respond to chemotherapy. Eradication of such heterogeneous tumors would require strategies to both purge the CSC/EMT cells and curb their ability to convert other surrounding cells. Since non-genetic factors can contribute to heterogeneity by generation of CSCs or alterations in EMT-status, and since CSC/EMT cells are often chemoresistant, one could envision a scenario in which we try to shift the population of cells towards a homogenous chemosensitive and/or differentiated state by targeting factors released by the CSCs and/or EMT cells. A homogenous tumor made of similar cells would then be easier to target (Figure 7.3).

In this thesis, we demonstrate that all three EMT-inducing transcription factors Snail1, Twist1 and Six1 converge onto Hedgehog/GLI signaling in order to non-cell autonomously increase the aggressive properties of cells that don’t express these factors. Interestingly, their mode of activation of the Hh pathway differs, despite the fact that Six1 is a key driver of non-cell autonomous activation of Gli in all contexts.
Figure 7.3 Targeting heterogeneous tumors.
The differentiation hierarchy existing within an epithelial tumor, making it heterogeneous in nature, can be due to both the presence of cancer stem cells (CSCs) and non-CSCs as well as cells that have undergone an epithelial-to-mesenchymal transition (EMT; EMT cells) and those that remain epithelial (non-EMT cells). Current evidence suggests that bidirectional conversion between non-CSCs and CSCs is possible, and that CSCs do not always go hand-in-hand with EMT cells. Additionally, intratumoral heterogeneity can arise from both (A) non-cell autonomous interactions between cells in the tumors and (B) signals from the microenvironment. Future therapeutics should focus on identifying and targeting molecules that function non-cell autonomously within the tumor and are secreted from the microenvironment, which will aid in driving the tumor toward homogeneity. Conventional therapy may then be used on the resulting homogenous tumor leading to tumor regression. Taken from 1.
The Hh pathway has been shown to be activated in cancers both canonically (via Hh ligands) as well as non-canonically via Gli (independent of Hh ligands and Smo) through various mechanisms. In our studies we observe that Six1 is able to non-cell autonomously activate Gli by upregulating and secreting high levels of the Hh pathway ligand, SHH. Hence, in a context where Six1 is the main EMT mediator, its non-cell autonomous in vitro effects can be abrogated equally efficiently using both upstream inhibitors (5E1 and cyclopamine) and downstream inhibitors of the Hh pathway (GANT-61 which targets Gli1/2). Interestingly, although the non-cell autonomous functions of Twist1 and Snail1 are dependent on Six1 expression, no increase in SHH levels is observed, and the non-cell autonomous phenotypes are only abrogated with the most downstream inhibitor of the pathway, GANT-61. We hypothesize that the ability of Six1 to upregulate SHH is via transcription and hence dependent on its interaction with required co-factor Eya proteins. Upregulation of SHH on co-transfection with Six1 and Eya2 is further evidence for this speculation. Thus, the inability of Six1 to regulate SHH levels downstream of Snail1 and Twist1 is most likely due to limiting levels of Eya2 expressed by the cells.

Furthermore, we demonstrate that VEGF-C is a potential player downstream of Snail1 and Six1 to mediate their effects, but remains unchanged downstream of Twist1, thus suggesting that yet another mechanism exists downstream of Twist1 and Six1 to activate Gli signaling. Our data also indicate that VEGF-C plays a non-cell autonomous role downstream of Six1 in contexts independent of Snail1, and blocking VEGF-C using a neutralizing antibody works to inhibit the EMT phenotypes as efficiently as blocking Hh signaling, despite the presence of SHH. This suggests that VEGF-C can function to increase EMT (as has been previously observed in a non-cell autonomous manner independent of Hh signaling and its role in increasing lymphangiogenesis. Alternatively, it is possible that crosstalk exists between VEGF-C signaling and SHH, whereby abrogation of VEGF-C activity results in downregulation of SHH activity, though this is yet to be tested.
experimentally. It is also unlikely that activation of Hh/GLI signaling is the only mechanism by which these factors mediate their non-cell autonomous phenotypes. Since tumor cells within the primary tumor communicate not only in a paracrine manner but also via juxtacrine interactions, it is plausible that additional factors and mechanisms play a role in these interactions in vivo. We have identified other factors that are differentially secreted by Six1 and non-Six1 expressing cells, and will discuss this further in Appendix A.

Nonetheless, activation of Gli signaling is a prominent event downstream of Snail1, Twist1 and Six1 and our data suggest that the context in which the EMT-factors are expressed, as well as perhaps the particular combination of the factors, dictate how this pathway is activated. Furthermore, it suggests a unified theme in that Snail1, Twist1 and Six1 converge on non-cell autonomous activation of Gli, and thus has implications for how heterogeneous tumors containing both EMT and non-EMT cells should be targeted.

To examine this latter question, we first tested whether GANT-61 was able to inhibit the ability of Six1 to non-cell autonomously increase the metastasis of non-Six1 expressing cells, and found that it indeed was effective in doing so. GANT-61 also had a modest but significant effect on the metastasis of MCF7-Six1 cells themselves but surprisingly, was unable to inhibit the growth of EMT cells (expressing Six1) in the primary tumor, indicating that the EMT cells do not rely solely on Hh signaling for tumor maintenance. In contrast, as shown both in vitro and in vivo, the main effector of the non-cell autonomous phenotypes in the non-EMT cells, induced by the EMT cells, is Gli. Because Hh signaling is active in the tumor microenvironment, it is likely that the actions of GANT-61 are both through effects on the tumor cells as well as other cells in the tumor environment. While GANT-61 is known to affect tumor growth in numerous contexts, to our knowledge, this work is the first to demonstrate its suppression of metastatic spread.

When making a comparison between Gli activation via canonical and non-canonical means, we found that Gli1, which itself is a pathway target, positively correlates with each of
the EMT-inducing transcription factors, across multiple breast cancer datasets, whereas such a strong and consistent correlation is not observed with the EMT-factors and the Hh ligands. Furthermore, high expression of Gli1 and the EMT-factors predicts worse prognosis across breast cancer subtypes and grades. These data prompted us to use PDX models to directly compare the efficacy of upstream inhibitors targeting Smo (IPI-926) and downstream inhibitors targeting Gli (GANT-61). Firstly, we found that expression of the three EMT-factors correlated strongly with activation of the Hh pathway in these PDX models while the expression of Hh ligands, did not. These data argue that EMT-factors may thus be predictive of response to Gli inhibitors, but less predictive of response to upstream inhibitors of the Hh pathway. Indeed, we show that a PDX tumor with elevated levels of Six1, Snail1 or Twist1 is more sensitive to growth inhibition by GANT-61, while the Smo inhibitor has no effect. Together these data indicate that Gli activation is a key event downstream of these EMT-factors (Figure 7.4), and hence targeting Gli may be more efficacious than using an upstream pathway inhibitor, irrespective of which transcription factor is expressed in breast cancers.

Furthermore, as alluded to previously, since intratumoral heterogeneity can arise as a consequence of both, interactions of tumor cells within the primary tumor as well as between the tumor cells and their microenvironment, the Hh pathway is arguably a near perfect target if tumor homogeneity is the desired. The studies performed in this thesis and by others demonstrate that activated Hh signaling can function both within the tumor and in the microenvironment to facilitate metastasis. Hence using a downstream Hh inhibitor to target the pathway such as GANT-61 (in combination with other therapies, discussed below) will inhibit both these interactions and thus will be efficacious in treating heterogeneous tumors.
Figure 7.4 Graphical representation of thesis results.
In heterogeneous primary tumors, where a small percentage of cells express EMT-inducing transcription factors Twist1 or Snail1 and hence undergo EMT and/or are metastatic, they upregulate Six1 to function in a non-cell autonomous manner. All three EMT-factors converge on activation of Hedgehog/GLI signaling via diverse mechanisms. Six1 is able to non-cell autonomously increase metastasis of neighboring non-Six1 expressing (non-EMT) cells which can be inhibited by GANT-61. (dotted lines – not proved conclusively)
Therapeutic Consequences

Hh signaling in recent years has come to be recognized as a major pro-tumorigenic signaling pathway in multiple cancer types, activated either by (Hh ligand-independent) mutations in pathway components or via increased Hh ligand levels. Activated Hh signaling is observed in many cancers including medulloblastoma, BCC, non-small cell lung carcinoma, breast cancer and pancreatic cancer. This has resulted in the use of derivatives of cyclopamine that target Smo such as vismodegib (GDC-0449) and sonidegib (LDE-225), in clinical trials for patients with BCC and medulloblastoma, where Hh signaling is often the major driver of tumorigenesis. These cancers exhibit activating mutations in Smo and/or inactivating mutations in Ptc1, and while this results in Hh ligand-independent constitutive activation of Hh signaling, the cancer cells still respond to canonical inhibition of pathway using inhibitors target Smo. Unfortunately, cyclopamine derivatives in trials for patients with late stage metastatic melanoma, pancreatic cancer and breast cancer often in combination with other receptor tyrosine kinase inhibitors, have been less successful.\(^\text{244}\)

This is not surprising, given our data that Gli signaling is activated downstream of Snail1, Twist1 and Six1 both canonically via Smo and in a non-canonical manner that is Smo-independent. More impressively, patient derived xenograft tumors expressing high levels of these factors, respond to GANT-61 more effectively than an upstream Smo inhibitor, indicating that Gli is the more targetable pathway player. More recently, studies have shown that cancer patients treated with Smo antagonists often develop resistance to drugs targeting Smo, via mutations that either render Smo incapable of being bound by the inhibitors, or which activate the Hh pathway (in Smo-independent manner) via Gli.\(^\text{303,304}\) Thus, these data, together with my studies in this thesis, make a strong case for the use of more downstream antagonists of Gli, like GANT-61, in order to more effectively inhibit this signaling pathway in different cancers.
Additionally, our *in vivo* studies indicate that while non-EMT cells rely on Hh/GLI signaling for tumor maintenance and metastasis, EMT cells are less sensitive to GANT-61 inhibition. These data suggest that GANT-61 in combination with additional therapies would be a more efficient way to target both EMT and non-EMT cells in primary heterogeneous tumors. Our studies demonstrate that Six1 is a key player downstream of EMT-factors Snail1 and Twist1 and that they require Six1 to mediate their non-cell autonomous functions. Hence it would be prudent to target Six1, except that historically, therapeutically targeting transcription factors has proven to be difficult and ineffective due to various off-target effects. However, we demonstrate in this thesis, that in contexts where Six1 can increase Hh ligand levels, it does so with the help of Eya, its required transcriptional cofactor. Several other studies have also shown that Six1’s pro-EMT and metastatic abilities are dependent on Eya proteins. In our laboratory, we are currently working on developing a small molecule inhibitor that can inhibit the Six1-Eya interactions and testing its ability to render Six1 incapable of carrying out its pro-metastatic functions. Thus we hypothesize that use of inhibitors like GANT-61 in combination with not only standard therapeutics, but also novel inhibitors of EMT-factors like the anti-Six1-Eya drugs, will be the most proficient means to target heterogeneous tumors as a whole.

**Concluding Remarks**

The overarching goal of cancer research is to determine the mechanisms by which a cell becomes cancerous and more importantly, aggressive. Spectacular advancements in this field have ensured that localized tumors are curable, yet metastases, both regional and distant remain untreatable. The biological equation between the primary tumor and arising metastases, is further complicated by intratumoral heterogeneity that exists in tumors, which is rarely taken into account by most current therapies. Since this heterogeneity is now known to be influenced by both genetic and non-genetic factors, and since clones in the
tumor that cooperate with one another, can harbor different advantageous mutations, more attempts at combination therapies may increase our changes of successfully inhibiting tumor progression. The evidence of intratumoral heterogeneity has added an additional layer to an already complex problem of how to target cancers, as we become cognizant that not only is every person’s tumor different (intertumoral heterogeneity); there are also vast differences present within a person’s tumor as well, and in many cases, between the primary tumor and metastases. Our studies add to the body of literature by identifying a mechanism by which different populations of cells within a heterogeneous primary tumor interact with each other to increase overall metastatic incidence. These studies will ultimately aid in outlining and designing more effective therapeutic combinations that can be efficacious clinically and will thus have far-reaching implications for prognosis and therapeutics.
REFERENCES


APPENDIX A

UNBIASED APPROACH TO IDENTIFY NOVEL MOLECULES REGULATED DOWNSTREAM OF EMT-INDUCING TRANSCRIPTION FACTORS

In this thesis we demonstrate that Snail1, Twist1 and Six1 increase metastatic properties of cells not expressing these factors via non-cell autonomous activation of Hedgehog/GLI signaling. Snail1, Twist1 and Six1 are each prominent EMT-inducing transcription factors that regulate massive transcriptional programs and hence the levels of a myriad numbers of genes and proteins. Moreover, tumorigenesis is a highly intricate process that requires the integration of complex signals not only between cells within the primary tumor, but also between the tumor cells and their microenvironment. Hence, it is likely that additional novel secreted molecules and signaling pathways are activated downstream of Snail1, Twist1 and Six1 to mediate their non-cell autonomous phenotypes in a heterogeneous tumor, although our data do suggest that Gli activation is a major mechanism of their non-cell autonomous effects.

In order to approach this problem in an unbiased manner, we performed mass spectrometry analysis on conditioned medium (CM) from tumor cells, which previously has been shown to yield a plethora of useful information. Since in this thesis we demonstrate that Six1 is a key mediator of non-cell autonomous phenotypes downstream of Snail1 and Twist1, we focused on identifying novel proteins secreted into the CM from Six1-expressing cells with the idea of extrapolating that data to Snail1 and Twist1. Since numerous studies show that tumor cells can secrete in excess of 500 proteins into the media, we first separated the molecules in the CM from MCF7-Ctrl and MCF7-Six1 cells by molecular weight using 30KDa molecular weight cutoff filter columns, followed by testing their activity in migration assays. We previously observed that HMLER-Ctrl cells cultured in CM from MCF7-Six1 cells had increased migration compared to when cultured in CM from MCF7-Ctrl
cells (Figure A.1 A), indicating that non-cell autonomous activity can be transferred between cell systems and that factors in the CM from the Six1-expressing cells are responsible for mediating these effects.

Since we observed such robust phenotypic effects, we used this cross-system migration assay as readouts to test the activity of the 30KDa-filtered CM from MCF7-Ctrl and Six1 cells. Surprisingly, we found that the “top” fraction of CM (containing molecules with molecular weight >30KDa) had higher migratory activity when placed on the HMLER-Ctrl cells compared to the “bottom” fraction (containing molecules with molecular weight <30KDa) (Figure A.1 B). In this thesis we demonstrate that high levels of SHH are secreted into the CM from MCF7-Six1 cells which would be present in the bottom fraction since SHH is a 19KDa protein. Since the bottom fractions of the CM from MCF7-Ctrl and MCF7-Six1 induced smaller differences in migration (Figure A.1 B) despite presumably containing SHH, it suggests one of two things. 1) An inhibitor of SHH exists in the bottom fraction (<30KDa) of the CM whose activity intensifies on CM fractionation or 2) an “activator” of SHH exists in the top fraction (>30KDa) which is lost on CM fractionation. In either scenario, SHH is rendered ineffective. Nevertheless, since we detected similar trends using different sets of CM from MCF7-Six1 and MCF7-Ctrl cells, we analyzed the proteins present in the top fraction of CM using mass spectrometry analysis. Surprisingly, despite controlling for cell numbers from which CM was collected as well as detecting no differences in basal cell death levels between the Six1 and non-Six1 expressing cells, far more hits were obtained from MCF7-Ctrl CM than from the MCF7-Six1 CM. Furthermore, in addition to secreted proteins (as expected), numerous nuclear proteins as well as traditionally non-secreted proteins were found in the CM samples, including serum contaminants in spite of using serum-free media. This suggested that the “secretome” obtained from the CM was more a reflection of apoptosis and cellular lysis, than secretion.
Figure A.1 HMLER-Ctrl cells have increased migration in CM from MCF7-Six1 cells. 5-7hr migration assay of HMLER-Ctrl cells in (A) CM from MCF7-Ctrl and Six1 cells and (B) CM from MCF7-Ctrl and Six1 cells filtered through 30KDa Molecular weight cutoff column; representative image. SEM shown, n>5. Two tailed unpaired t-test for (A), ***p value <0.0001
Interestingly, these results are in line with a study performed by Villanueva and colleagues on the secretome of MCF7 cells, where similar levels of conventionally non-secreted proteins were found in the CM. Since they observed substantial cell lysis in MCF7 cells cultured in serum-free media only after 48hrs, they concluded with subsequent rigorous experiments that breast cancer cells can secrete traditionally non-secreted proteins via various mechanisms including exosomes. Nevertheless for our purposes, we generated a list of differentially secreted proteins by MCF7-Ctrl and Six1 cells, with fold change (Six1/Ctrl or Ctrl/Six1) greater than 1.5 and present in at least two out of three sets of CM submitted for mass spectrometry analyses. Interestingly, we found a greater number of proteins that have anti-tumorigenic roles secreted by MCF7-Ctrl cells, than proteins that have pro-tumorigenic roles, secreted by MCF7-Six1 cells, which could be a reflection in part, of the greater number of hits obtained from MCF7-Ctrl CM.

Since Six1 is a transcription factor, and in an attempt to further narrow down candidate proteins, we determined which, if any, of our top hits were transcriptionally regulated by Six1 using qRT-PCR analyses. We found that a number of proteins that were differentially secreted were also changed on the mRNA level downstream of Six1, including thrombospondin-1 (TSP1), and filamin-A (FLNA) (Figure A.2 A-E). Since in this thesis we demonstrate that Six1 functions downstream of Snail1 and Twist1 to mediate their non-cell autonomous phenotypes, we determined if the factors altered on the mRNA level downstream of Six1 in the MCF7 cells, were also changed downstream of Snail1 and Twist1 with or without Six1 KD in HMLER cells. We found that only TSP1 was consistently altered between the HMLER-Snail1/Twist1 cells and HMLER-Ctrl cells, but it remained mostly unchanged with Six1 KD (Figure A.2 F). These data indicate that despite remaining unchanged in the Six1 KD HMLER cells, TSP1 was still transcriptionally downregulated downstream of all three EMT-inducing factors, Six1, Snail1 and Twist1.
Figure A.2 Several proteins from mass spectrometry analysis are transcriptionally regulated downstream of EMT-factors.

qRT-PCR analyses of (A-E) combination of 3 clones each of MCF7-Ctrl and MCF7-Six1 cells and (F) HMLER-Ctrl and HMLER-Snail1/Twist1 cells +/-Six1 KD. SEM shown, n=3. Two tailed unpaired t-test for (A-E) and One way ANOVA with Tukey post test for (F). *p value <0.05, **p value <0.001, ***p value <0.0001
Thrombospondin-1 is a 450KDa homotrimeric glycoprotein, discovered as the most abundant protein secreted by platelet cells and since then has been shown to be secreted by a variety of cell types including endothelial cells and numerous cancer cells\textsuperscript{306}. TSP1’s tumorigenic role is quite controversial, since several groups often in the same cancer types have described TSP1 as having both anti-tumorigenic and pro-tumorigenic functions. The role of TSP1 in any given cancer type often depends on its cellular localization (membrane bound v/s secreted) as well as the presence of other factors\textsuperscript{306} that cause its proteolysis, yielding fragments that can have either pro- or anti-tumorigenic functions.

In breast cancer, TSP1 has predominantly been shown to have anti-tumorigenic roles including inhibition of proliferation, increased cell adhesion, decreased motility and most importantly inhibition of angiogenesis\textsuperscript{306,307}. Analysis of breast cancer cell lines show that metastatic cancer cells express at least 3 fold reduced expression of TSP1 than “normal” breast cell lines\textsuperscript{307}, and examination of patient data reveals that increased TSP1 expression often correlates with favorable prognosis and reduced risk for cancer recurrence\textsuperscript{308} indicating that downregulation of TSP1 possibly facilities increased cancer aggressiveness. To our knowledge, TSP1 has never been directly linked to Six1 in the context of cancer and only recently, was shown to be regulated by Twist1 and Snail1 in a developmental and cancer context, respectively\textsuperscript{184,309}. Since we observed repression of TSP1 mRNA downstream of all three transcription factors, we hypothesized that downregulation of TSP1 is an additional mechanism by which Six1, Snail1 and Twist1 perform their pro-tumorigenic functions in breast cancer.

To test this hypothesis, we first determined whether TSP1 was downregulated on the protein level in the EMT-factor expressing cells. Interestingly, we found that not only did the Six1/Snail1/Twist1-expressing cells have decreased protein levels of TSP1 (Figure A.3 A,B), they also secreted significantly lower levels of TSP1 into the CM compared to the corresponding control cells (Figure A.3 C,D).
Figure A.3 Thrombospondin-1 (TSP1) is downregulated on the protein level and in CM from Six1/Snail1/Twist1-expressing cells.

(A, B) Western blot analyses on WCLs from (A) MCF7-Ctrl and Six1 cells and (B) HMLER-Ctrl and HMLER-Snail1/Twist1 +/- siSix1. (C) Western blot analyses on CM from 3 clones of MCF7-Ctrl and Six1 cells. (D) ELISA analyses on CM from HMLER-Ctrl and HMLER-Snail1/Twist1 +/- siSix1. n≥2, NSB- non-specific band from ponceau S stain.
Since TSP1 has been shown to inhibit migration of endothelial cells and hence curb angiogenesis in vivo, and is differentially expressed between the EMT-factor-expressing and control cells, we next asked whether endothelial cells migrate differentially when cultured in CM from Six1/Snail1/Twist1-expressing cells and control cells. Our preliminary results indicate that indeed, immortalized endothelial (human microvascular endothelial cells, HMEC-1) cells have decreased migration when cultured in CM from control cells, compared to when cultured in CM from EMT-factor-expressing cells (Figure A.4 A, B).

To specifically test whether TSP1 was responsible for the decreased migration of endothelial cells cultured in CM from control cells, we first knocked down TSP1 in MCF7-Ctrl cells using siRNAs and found that it resulted in ~50% less secreted TSP1 in the CM from siTSP1-transfected cells compared to non-targeting siRNAs (Figure A.4 C, D). We then performed migration assays using the CM from TSP1 KD cells and found that KD of TSP1 in MCF7-Ctrl cells was able to rescue the non-cell autonomous decrease in endothelial cell migration to levels comparable to when cultured in CM from MCF7-Six1 cells (Figure A.4 E), indicating that TSP1 is responsible for the decrease in migration of endothelial cells in the control CM. Next, we tested whether the tumor cells themselves would have differential migration with altered levels of TSP1 and found that HMLER-Ctrl cells cultured in CM from MCF7-Ctrl + siTSP1 cells had increased migration, while CM from MCF7-Six1 cells with TSP1 KD did not have effect (Figure A.4 F). These preliminary data suggest that TSP1 has the ability to function in a non-cell autonomous manner and affect both, the cells in the tumor microenvironment (endothelial cells), as well as the tumor cells themselves, albeit to different extents. Furthermore, partial rescue of the migratory phenotypes in the tumor cells indicate the presence of other factors involved in the crosstalk between the aggressive and non-aggressive cells within the tumor.
Figure A.4 TSP1 is required for non-cell autonomous increase of endothelial and tumor cell migration.

(A,B) 7hr migration assay in HMEC-1 cells cultured in CM from (A) HMLER-Ctrl, Snail1 and Twist1 cells and (B) MCF7-Ctrl and Six1 cells. (C) Western blot analyses on CM from MCF7-Ctrl and Six1 cells transfected with 150nM siTSP1 or siNT for 48 hrs and (D) corresponding quantification. (NSB – non-specific band from membrane) (E,F) 7-8hr migration assays of (E) HMEC-1 cells and (F) HMLER-Ctrl cells cultured in CM from MCF7-Ctrl and Six1 cells +/- siTSP1. SEM shown for (B, F), n=1, One way ANOVA with Tukey post test for (B,F). **p value <0.001, ***p value <0.0001
While numerous additional studies have to be performed to validate these results as well as to extend the scope of phenotypes, our preliminary data that TSP1 is negatively regulated downstream of Six1, Snail1 and Twist1 is quite compelling. This then raises the questions of a) how TSP1 is regulated by these EMT-factors and b) does this mechanism work in conjunction with activation of Hh/GLI signaling? Interestingly, while TSP1 has not been directly linked with Six1, multiple molecules regulated downstream of Six1 have been linked with TSP1. Myc, CyclinD1 (targets of Six1\textsuperscript{112}) and miR cluster 17-92 (which is closely related to miR cluster 106b-25 upregulated by Six1\textsuperscript{212}) have all been shown to repress TSP1 function\textsuperscript{310-312}. Furthermore, p53, which was recently shown to be downregulated by Six1\textsuperscript{222}, is a positive activator of TSP1\textsuperscript{313}, indicating that Six1’s regulation of TSP1 may not direct. On the other hand, Twist1 has been shown to regulate TSP1 directly during development depending on its dimerization partner\textsuperscript{309} and hence may directly repress TSP1 during tumorigenesis as well. The mechanism of TSP1 regulation by Snail1 may be via direct transcriptional repression, since Snail1 is a potent transcriptional repressor, but this remains to be experimentally verified.

It is highly probable that Six1, Snail1 and Twist1 function in heterogeneous tumors by upregulating Hh/GLI signaling as well as downregulating factors like TSP1 (Figure A.5), along with the regulation of various other factors that may non-cell autonomously influence tumor progression. Our studies provide preliminary evidence for a novel pro-tumorigenic mechanism of action for three prominent EMT-inducing transcription factors; specifically I show that Six1, Snail1 and Twist1 may be able to repress an anti-angiogenic factor TSP1, resulting in increased migration of endothelial cells, and hence vascularization of the tumor and subsequent increase in metastasis.
EMT-factors like Snail1, Twist1 and Six1 function both cell and non-cell autonomously by modulating signaling pathways like Hedgehog (Hh)/GLI signaling as well as anti-angiogenic factors like thrombospondin-1 (TSP1) to increase overall metastatic incidence.
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REFERENCES FOR APPENDIX A


