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Novel Signaling Mechanisms Implicated in Epithelial Mesenchymal Transition and Metastasis of Human Cancers

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NOVEL SIGNALING MECHANISMS IMPLICATED IN EPITHELIAL MESENCHYMAL TRANSITION AND METASTASIS OF HUMAN CANCERS

By
Minsoon Kim

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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NOVEL SIGNALING MECHANISMS IMPLICATED IN EPITHELIAL
MESENCHYMAL TRANSITION AND METASTASIS OF HUMAN CANCERS

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Distant metastases are the leading cause of breast cancer related death. It is important to understand how the metastatic process is regulated in order to develop new therapies that would oppose metastasis and improve cancer outcome. Initiation of metastasis requires cell invasion and escape from the primary tumor into the vasculature followed by colonization of secondary sites. Tumor invasion and intravasation are enabled by the epithelial mesenchymal transition (EMT), a process in which epithelial cells lose polarity and intracellular adhesion and acquire motility and invasiveness. This thesis work has revealed novel mechanisms whereby cancer metastasis is regulated via induction of EMT.

First, this thesis work provides a novel mechanism whereby VEGFA promotes metastasis through activation of EMT. VEGFA is best known as an angiogenic agent, but it also promotes cancer invasion and metastasis through mechanisms that are not fully understood. Our prior work showed VEGFA mediates cancer stem cell (CSC) expansion via induction of SOX2, a key stem cell driver in breast cancer models. Here, we showed VEGFA rapidly upregulates SOX2, leading to SNAI2 induction and EMT. Sox2 downregulates miR-452, a novel metastasis suppressor, that we show directly targets the SNAI2 3’ UTR. VEGFA induction of SOX2 leads to loss of miR452 and upregulation of Slug, driving EMT and metastasis in breast cancer models.
Second, we also uncovered a new mechanism whereby T157 and T198-phosphorylated, deregulated p27 contributes to cancer metastasis. In normal cells, p27 regulates cell cycle and functions as an atypical tumor suppressor. While p27 is rarely completely lost, it is frequently deregulated through either excess degradation or through key C-terminal phosphorylations in human cancers. We and others have previously identified an oncogenic role for p27 in motility, invasion and metastasis resulting from these C-terminal phosphorylations. Here we showed that p27CK-DD induced EMT and enhanced metastatic potential of breast cancer cell lines. Knockdown of p27 in highly metastatic EMT-transformed cell lines with high levels of p27pT157pT198 (p27pTpT) reverted EMT and impaired metastatic potential. Mechanistically, we provides evidence for p27 as a transcriptional co-regulator of cJun. We showed that C-terminally phosphorylated p27 binds and activates cJun, and forms a complex with cJun at an enhancer region upstream of the TGF-β2 gene to induce TGF-β2 and EMT.

My PhD data identify novel pathways in which Sox2, upregulated by VEGFA, contributes to activation of EMT and metastasis through Slug. Furthermore, this thesis work reveals an oncogenic function of p27 to promote tumor progression through EMT via cJun-mediated TGF-β2 induction. Since treatment of metastasis is the final therapeutic frontier, it is hoped that mechanistic insights into acquisition of metastatic potential through EMT may ultimately generate new strategies for opposing metastasis and improving outcome of cancer.
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ABBREVIATIONS

CSC    Cancer stem cell
DT     Dissociated tumors
EMT    Epithelial to mesenchymal transition
ER     Estrogen receptor
IVIS   In vivo imaging systems
JNK    cJun N-terminal kinase
MET    Mesenchymal to epithelial transition
p27KIP1 Cyclin-dependent kinase inhibitor 1B
PR     Progesterone receptor
SOX2   Sex determining region Y-box2
STAT3  Signal transducer and activator of transcription 3
TGF-β1  Transforming growth factor-β 1
TGF-β2  Transforming growth factor-β 2
TGF-βR  Transforming growth factor-β receptor
VEGFA  Vascular endothelial growth factor A
Zeb1   Zinc finger E-box binding homeobox 1
Zeb2   Zinc finger E-box binding homeobox 2
CHAPTER 1: INTRODUCTION
1.1 EPITHELIAL MESENCHYMAL TRANSITION IN CANCER

Initiation of metastasis requires cell invasion and escape from the primary tumor into the vasculature followed by colonization of secondary sites. Tumor invasion and intravasation are enabled by the EMT, a biological process in which polarized epithelial cells undergo multiple molecular and cellular changes to acquire a mesenchymal phenotype with increased invasive capacity (Kalluri and Weinberg, 2009). The work described in this thesis expands upon our understanding of how metastasis is promoted via regulation of epithelial mesenchymal transition (EMT) in human cancer. The following briefly reviews EMT in normal development, tissue injury and cancer metastasis, and reviews pathways and regulators governing EMT.

1.1.1 EMT in normal development and tissue injury

The concept of EMT was first described by Elizabeth Hay, who used chick primitive streak cells as a model to prove that epithelial cells undergo dramatic phenotypic changes when transform into mesenchymal cells (Hay, 1995). This process is essential for germ layer formation and cell migration during early vertebrate development. Based on the biological context in which they occur, three different subtypes of EMT have been proposed: type 1 regulates embryogenesis and organ development; type 2 modulates tissue regeneration and organ fibrosis; and finally type 3 is related with cancer progression and metastasis (Kalluri, 2009).

Type 1 EMT generates cells with a mesenchymal phenotype to form new tissues with diverse functions. For example, during embryonic development, epithelial cells of the neuroectoderm generate migratory neural crest cells via an EMT program (Bhatt et al.,
This type of EMT also plays a significant role in generating mesenchyme that subsequently gives rise to secondary epithelia via MET during development.

Type 2 EMT is represented as a repair event following inflammatory injury or trauma to reconstruct tissues and it is associated with wound healing, tissue regeneration, and organ fibrosis. This type of EMT is closely related to the fibrosis occurring in chronic inflammatory conditions affecting the liver, lung, kidney and intestine (Kalluri and Weinberg, 2009). Moreover, continuous inflammatory signals can induce type 2 EMT and eventually lead to destruction of affected organs.

Type 3 EMT is the focus of my thesis work, and aberrantly appears in neoplastic cells via genetic and epigenetic changes to favor clonal outgrowth and localized invasion from their primary site. This type of EMT primes tumor cells to develop distant metastases and will be discussed in detail in the following section.

1.1.2 EMT in cancer metastasis

Metastasis is the leading cause of cancer-related death for most tumor types. In spite of its devastating outcomes, cancer metastasis is an inefficient process requiring cancer cells to overcome a series of challenges to spread from primary site to secondary organs (Sethi and Kang, 2011). Cancer cells must undergo multiple steps including local invasion, intravasation, extravasation from the vasculature, and colonization to develop distant metastasis in distant organs. (Fidler, 2003).

During early tumor invasion, epithelial cancer cells lose polarity and intra cellular adhesion, and acquire mesenchymal phenotypes with increased motility and invasiveness to break through the underlying basement membrane. This early step towards metastasis is
believed to be initiated by the reactivation of type 3 EMT (Micalizzi et al., 2010). Epithelial cancer cells are tightly associated with neighboring cells via E-cadherin-containing junctions. Progression to a mesenchymal phenotype begins when cancer cells lose epithelial E-Cadherin and acquire the induction of mesenchymal markers such as N-Cadherin and vimentin. These cells, having acquired the capability to invade through the basement membrane, typically are seen at the invasive front of primary tumors and are considered to be the cells that eventually go into subsequent steps of metastasis.

Despite the fact that EMT is presently considered as the initial step in the metastatic process, the extent and timing of its contribution to cancer progression has not been well-investigated due to the lack of advanced technology to dynamically monitor EMT in vivo. Recent imaging techniques have enabled us to observe the movement of individual cells from primary tumors and several studies now provide direct evidence that EMT occurs during tumor progression (Trimboli et al., 2008). It is interesting to note that once disseminated cancer cells reach their destination, the reversal of EMT, which is termed MET (mesenchymal to epithelial transition), also plays an important role during the metastatic process (Thiery et al., 2009; Tsai et al., 2012).

1.1.3 EMT markers and transcriptional regulators

The functional loss of E-Cadherin is considered to be a critical event in acquiring EMT phenotype. E-Cadherin modulates cell adhesion and its expression is decreased during EMT in embryonic development, in tissue fibrosis, and in cancer (Hay, 1995). Moreover, a cadherin switch from E-Cadherin to N-Cadherin is a commonly used as a marker of EMT progression during embryonic development and cancer progression.
Emergence of the cytoskeletal marker Vimentin is also commonly used to recognize cells undergoing EMT in cancers, as its expression is positively correlated with increased tumor invasion and metastasis (Satelli and Li, 2011).

The EMT is regulated by diverse EMT transcription factors that repress CDH1, the gene encoding E-cadherin, directly or indirectly. Snail (Batlle et al., 2000; Cano et al., 2000), Slug (Bolos et al., 2003), zinc finger E-box binding homeobox 1 (Zeb1) (Eger et al., 2005) and zinc finger E-box binding homeobox 2 (Zeb2) (Comijn et al., 2001) can bind the CDH1 promoter and repress its transcription, whereas other factors such as Twist (Yang et al., 2004), Goosecoid (Hartwell et al., 2006) and fork-head box protein C2 (FOXC2) (Mani et al., 2007) repress CDH1 indirectly. Snail is a zinc finger transcription factor active during gastrulation in the developing embryo (Nieto, 2002; Boulay et al., 1987). Slug is a member of the Snail family with a unique conserved motif near the zinc fingers, which is absent in other members and its expression correlates strongly with loss of E-cadherin (Hajra et al., 2002). As a master regulator of EMT, Slug expression has been shown to be associated with cancer metastasis. Slug is overexpressed in various primary tumors including lung, breast, ovarian, prostate, colorectal, and pancreatic cancers and glioma (Alves et al., 2009). Increased Slug expression is associated with high histologic grade, metastasis and poor outcome with recurrence and shorter patient survival in various cancers (Alves et al., 2009; Shioiri et al., 2006; Shih et al., 2005; Liu et al., 2013b; Atmaca et al., 2015; Martin et al., 2005).
1.1.4 Signal pathways governing EMT

The EMT is regulated by diverse molecular networks including TGF-β and Notch, Wnt, Hedgehog, and NF-κB signaling pathways, all of which play central roles in cancer invasion and metastasis (Huber et al., 2005). Wnt signaling can lead to EMT through inhibition of GSK-3β-mediated phosphorylation, ubiquiylation and degradation of β-catenin. Activated β-catenin, thus induces expression of EMT transcription factors. Notch signaling pathway also have been demonstrated as regulator of EMT induction. Notch signaling activation is required for TGF-β mediated EMT and Notch signaling was shown to directly upregulate Snail and Slug, resulting in activation of EMT and increase of cell migration and invasion (Zavadil et al., 2004; Micalizzi et al., 2010; Sahlgren et al., 2008; Niessen et al., 2008).

Of all of the signaling mechanism that activate EMT, the TGF-β family comprises the best-studied EMT inducers. TGF-β family members are secreted peptide growth factors that inhibit normal epithelial growth and whose function is altered in cancer to promote cancer progression (Massague, 2008). During tumor progression, TGF-β signaling induces EMT through activation of Smad-family transcription factors that, in turn, induce expression of EMT regulator genes, including SNAI1, SNAI2 and TWIST1 (Xu et al., 2009). TGF-β can also collaborate with other signaling pathways such as Notch and Wnt, to fully induce EMT and maintain the mesenchymal phenotype in invasive and metastatic cancer cells (Zavadil et al., 2004; Zhou et al., 2012).
1.2 TGF-β SIGNALING IN CANCER

Since my thesis work showed TGF-β2 is a key mediator of EMT by deregulated p27, the TGF-β pathway is reviewed here. This family of secreted peptide growth factors plays roles in diverse cellular processes including growth inhibition, cell migration, cell invasion, extra cellular matrix remodeling and EMT induction (Padua and Massague, 2009). TGF-β was initially discovered as oncogenic cytokine in tumors of mesenchymal origin, but it also has a growth inhibitory function to induce quiescence in normal epithelial cells (Roberts and Wakefield, 2003). p27 was initially identified as a mediator of TGF-β induced cell cycle arrest in normal epithelial cells (Polyak et al., 1994b; Slingerland et al., 1994). In contrast, during malignant tumor progression, TGF-β1 and TGF-β2 are frequently overexpressed and contribute to tumor progression by mediating cell growth, migration and inducing EMT. Given its important role in tumor progression, TGF-β signaling has been extensively studied to elucidate the mechanisms behind its oncogenic action and how downstream mediators play a role in this process. The following briefly reviews TGF-β signaling in normal and transformed cells.

1.2.1 Basics of TGF-β signaling

The TGF-β signaling pathway comprises a transmembrane hetero-tetromeric serine/threonine kinase complex of two TGF-β receptor I (TGF-βRI) and two TGF-β receptor II (TGF-βRII) molecules that form a complex and bind extracellular TGF-β ligands. TGF-β ligand binding induces TGF-βRI and TGF-βRII dimerization, and constitutively active TGF-βRII phosphorylates TGF-βRI, leading to activation of the intracellular SMAD proteins (Jakowlew, 2006; Moustakas et al., 2002). SMAD proteins
are activated by TGF-βRI mediated phosphorylation, oligomerize and translocate to the nucleus to regulate their target gene expression with other co transcriptional factors (Massague, 2012).

The TGF-β signaling pathway is highly conserved in multicellular organisms and it modulates the expression of numerous gene involved in a variety of cellular processes. Specificity for target genes depends on the context in which the signal is being delivered. Coordination of activated SMAD proteins with transcription factors, chromatin remodelers and histone modifiers determine what genes will be targeted and whether target gene expressions will be positively or negatively regulated. Since SMAD proteins are not strong DNA binder, they usually need other co-regulators as binding partners for an efficient binding to DNA and their interaction directs gene expression in different contexts (Massague, 2012).

TGF-β signaling pathway can be trigged by extracellular binding of TGF-β ligands, TGF-β1, TGF-β2, or TGF-β3. Although these three ligands have extensive sequence homology in active domains, they exhibit distinct binding affinities for TGF-β RII. While TGF-β1 and TGF-β3 can directly binds to TGF-β RI and TGF-β RII with high affinity, TGF-β2 can only associate with TGF-β RII with very low affinity. Presence of TGF-β RIII seems to be required to present the TGF-β2 ligand to TGF-β RII, substantially increasing its affinity for binding this particular ligand (Lopez-Casillas et al., 1993). The role of TGF-β1 in metastasis and tumorigenesis has been well studied (Katsuno et al., 2013), but the roles of TGF-β2 and TGF-β3 in these processes remains unclear. It is recently suggested that TGF-β2 signaling is involved in development of melanoma (Reed et al., 1994; Van et al., 1996; Zhang et al., 2009a), glioma (Bodmer et al., 1989; Kieran et al., 2012), pancreatic
cancer (Friess et al., 1993; Von et al., 2001), and breast cancer (Buck and Knabbe, 2006; Ouhtit et al., 2013), and work in my thesis investigated how deregulated p27 contributes to metastasis by activating TGF-β2 expression.

1.2.2 TGF-β signaling in normal cells

TGF-β controls tissue growth and morphogenesis in embryonic development, and activates cytostatic and apoptotic processes to maintain tissue homeostasis. TGF-β signaling restrains cell proliferation in normal skin, lung and mammary epithelial cells by activating cytostatic gene responses including CDK inhibitors. To halt proliferation, p27 (Polyak et al., 1994a; Slingerland et al., 1994), p21 (Datto et al., 1995) and p15 (Hannon and Beach, 1994) are upregulated, and anti-proliferative function of p27 for binding and inhibiting cyclin-CDK is activated (Reynisdottir et al., 1995). In normal cells, the cytostatic function is well regulated, but in cancer cells TGF-β signaling becomes oncogenic with losing their ability to control homeostasis.

1.2.3 Deregulated TGF-β signaling in cancer

Transformed cells can either inactivate core components of pathway, such as TGF-β receptors, or disable downstream effectors leading to disruption the cytostatic effect of TGF-β signaling (Massague, 2008). Many of these mechanisms to elude the cytostatic effects of TGF-β signaling exist in human cancers. TGF-βRII is mutated in colon, gastric, biliary, pulmonary, ovarian and esophageal cancers (Levy and Hill, 2006). TGF-βRI is mutated in ovarian, esophageal, head and neck cancers, and it is epigenetically silenced in gastric cancer (Levy and Hill, 2006). Failed presentation of ligands to receptors are also described in certain cancers. Overexpression of glycoprotein, such as follistatin, appears to
act as ligand trap leading to sequestration of TGF-β in extracellular matrix, and it is associated with bone metastasis in breast cancer (Kang et al., 2003). Smad7, a TGF-βRI antagonist, is overexpressed in endometrial carcinomas and thyroid follicular tumors (Cerutti et al., 2003; Dowdy et al., 2005), and it is associated with chronic inflammation in immune cells within the colonic mucosa (Broderick et al., 2007). SMAD4, downstream effector of pathway, mutations present in a high number of esophageal tumors (Sjoblom et al., 2006) and in pancreatic carcinomas, and SMAD4 inactivation was significantly associated with shorter overall survival of pancreatic cancer patients (Jaffee et al., 2002; Blackford et al., 2009)

Transformed cells can also utilize TGF-β signaling to prime the tumor microenvironment, while leaving cell proliferation unhalted. Through mediating local angiogenic cytokine networks, TGF-β can confer a pro-angiogenic tumor environment. For example, TGF-β targets connective tissue growth factor (CTGF) to stimulate vessel formation (Kang et al., 2003). TGF-β also induces myofibroblasts differentiation into mesenchymal precursors which produce proinvasive matrix metalloproteases (MMPs), IL-8, VEGF, and CXCL12 (De and Mareel, 2003). Secretion of these cytokines and chemokines promotes cell proliferation, invasion, and angiogenesis in cancer (Allinen et al., 2004).

1.2.4 TGF-β signaling contributing EMT and metastasis

During EMT, epithelial cells undergo multiple molecular and cellular changes to acquire mesenchymal characteristics that enhance their invasive properties (Kalluri and Weinberg, 2009). TGF-β is a key inducer of EMT, both in normal development and in cancers (Derynck and Akhurst, 2007; Thiery, 2003). SMAD mediated induction of
HMGA2 (high mobility group A2) promotes the expression of key EMT transcription factors, Snail, Slug, and Twist (Thuault et al., 2008). TGF-βRII mediated phosphorylation of Par6 can promote dissociation of cell junctions in a SMAD activity independent manner (Ozdamar et al., 2005).

EMT is linked to increased metastatic potential of malignant cells, and growing evidence demonstrates TGF-β’s role in the development of distant metastasis. In breast cancer, TGF-β signaling contributes to breast cancer metastasis to both lung and bone (Chiechi et al., 2013; Drabsch and Ten, 2011). TGF-β/SMAD induced expression of ANGPTL4 (angiopoietin-like 4) enables metastasizing cells to disrupt capillary beds in the lung tissue by dissolution of intercellular junction, and blocking TGF-β signaling with either a dominant negative TGF-βRI or SMAD4 knockdown reduced lung metastasis formation in xenograft mice (Padua et al., 2008). In bone tissue, TGF-β from bone matrix increase permeability to help establishment of secondary niches for metastasizing cancer cells by stimulating a secretion of osteolytic cytokines. Indeed, blocking TGFβ signaling by a dominant-negative TGF-βRII inhibits osteolytic metastasis of human breast cancer (Yin et al., 1999). Moreover, several TGF-β target genes, such as IL11 and CTGF were determined to be important for the establishment of bone metastases (Kang et al., 2005; Kang et al., 2003). These findings suggest that TGF-β can exert a pro-metastatic function to stimulate the establishment of metastasis in cancer.

1.3 VEGF SIGNALING IN CANCER

VEGF was originally identified as an endothelial cell specific cytokine that regulates vascular development during embryogenesis and the formation of new blood
vessel from pre-existing vascular networks (Olsson et al., 2006; Leung et al., 1989; Tischer et al., 1989). It has become apparent that VEGF functions are not restricted to vasculogenesis and angiogenesis (Senger, 2010). VEGF is expressed in a variety of tumors and its overexpression is associated with poor prognosis, cancer progression and metastasis (Goel and Mercurio, 2013; Berns et al., 2003; Manders et al., 2002). The development of new blood vessel is essential for continued tumor growth and metastasis. Without new blood vessel formation, tumor size is restrained due to limited nutrient and oxygen supply. VEGF, secreted by cancer and stromal cells, stimulates endothelial cell invasion and vessel formation (Hicklin and Ellis, 2005). This process plays a central role in both local tumor growth and distant metastasis (Folkman, 1971; Mercurio et al., 2005), but mechanisms underlying this are not fully understood. In the first part of my thesis, I investigated how VEGF promotes metastasis through regulation of EMT. The following provides a brief overview of VEGF signaling and its contribution to cancer metastasis.

1.3.1 VEGF as key regulator of angiogenesis

VEGF family is composed of five secreted glycoproteins including VEGFA, VEGFB, VEGFC, VEGFD and placenta growth factor (PlGF). VEGFA, denoted as VEGF, is first identified as an endothelial cell specific mitogen and additional VEGF forms were identified based on their homology to VEGFA (Kieran et al., 2012). VEGFA is the best characterized and studied isoform among VEGF family members. The VEGFA signaling pathway is initiated by VEGFA binding to receptor tyrosine kinases, including VEGFR1 and VEGFR2. Once VEGFA binds to the extracellular domain of the receptor, the intracellular domains of receptor tyrosine kinases are dimerized. Conformation changes of
receptors induce auto-phosphorylation of tyrosine residues leading to activation of downstream signaling cascade. Of the two tyrosine kinase receptors, VEGFR2 is the main partner of VEGFA action in vasculogenesis and angiogenesis (Olsson et al., 2006).

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is an important physiological process not only in normal development and tissue repair but also in various diseases including cancer. Despite the complex regulation of angiogenesis by numerous proangiogenic and antiangiogenic factors, it is predominantly regulated by single cytokine, VEGFA (Kieran et al., 2012). VEGFA mediates proliferation, sprouting, and migration of endothelial cells and tube formation by these cells (Ferrara, 2002). VEGFA also regulates angiogenic processes such as those involved in wound healing, ovulation, menstruation and pregnancy. VEGFA expression has been associated with critical steps in vasculogenesis and angiogenesis (Shweiki et al., 1993; Jakeman et al., 1993). Deletion of the VEGFA gene is lethal, leading to vascular defects in mice.

1.3.2 VEGF in cancer progression and metastasis

It has become apparent that VEGF functions contribute to cancer progression by inducing angiogenesis and vasculogenesis, which resulting in tumor growth, invasion and metastasis (Senger, 2010). In fact, VEGF is expressed in a variety of tumors and VEGF overexpression has been found to be correlated with tumor progression with poor patient outcome (Goel and Mercurio, 2013; Berns et al., 2003; Manders et al., 2002). VEGF and its receptors are overexpressed in many human hematopoietic tumors including leukemia, lymphoma and multiple myeloma (Bellamy, 2001; Bellamy et al., 2001; Padro et al., 2002). The study of patients with a variety of hematological malignancies has shown that VEGF
expression has been associated with disease progression (Aguayo et al., 2000). VEGF plays an important role not only in hematopoietic malignancies, it is also highly expressed in various solid malignant tumors, and associates with cancer progression.

A solid tumor cannot grow larger than 2-3 mm diameter due to limited nutrient and oxygen supply, the formation of new blood vessel is essential for continued tumor growth. VEGF, secreted by cancer and stromal cells, stimulates endothelial cell invasion and vessel formation (Hicklin and Ellis, 2005). This process plays a central role in both local tumor growth and distant metastasis. In breast cancer, many studies have shown that VEGF plays a pivotal role in disease progression and has a significant impact on patient outcome. VEGF mRNA expressions in invasive breast carcinoma or ductal carcinoma in situ (DCIS) are higher than in normal breast tissue (Yoshiji et al., 1996; Anan et al., 1996; Brown et al., 1995; Lee et al., 1998). Serum VEGFA levels in breast cancer patients are also significantly higher compared to those in patients with benign breast lesions, and both serum and tumor tissue VEGF are positively correlated with tissue VEGF (Ali et al., 2011).

Notably, patients with metastatic breast cancer have higher VEGF levels compare to patients with benign disease or localized disease suggesting VEGF can be used to differentiate the subgroups of breast cancer at higher risk for distant metastasis or recurrence (Adams et al., 2000). Actually, it has been shown that VEGF has strong prognostic value for relapse-free and overall breast cancer survival. Patients with early stage breast cancer, who have increased VEGF expression show a higher probability of recurrence or death than patients with low VEGF levels, even if treated with conventional adjuvant therapy (Eppenberger et al., 1998; Gasparini et al., 1997; Linderholm et al., 1998; Toi et al., 1994). Moreover, tumor VEGF is also associated with bigger tumor size,
negative hormone receptor status, lymph node metastasis and vascular invasion (Ali et al., 2011).

1.3.3 VEGF as mediator of EMT and CSCs

Accumulating data suggest that malignancies originate from stem-like cells which have ability to self-renew and generate heterogeneous, more differentiated progeny with lower replicative capacity (Dalerba et al., 2007). Cells with stem-like features have shown to initiate tumors with high frequency in various human malignancies and those cells appear to contribute to tumor invasion, metastasis, drug resistance, and disease recurrence (Takebe et al., 2011). Cancer stem cells (CSCs) can be distinguished from bulk population of cancer cells by differential expression of specific cell surface markers. In breast cancer, populations expressing surface CD44^+CD24^−low^ and/or aldehyde dehydrogenase 1 (ALDH1) activity are enriched for cells with stem cell like features in vitro and capacity to initiate xenograft tumors in vivo (Al Hajj et al., 2003; Ginestier et al., 2007).

VEGFA was recently shown to increase malignant stem cell abundance in a variety of tumors including glioblastoma (Bao et al., 2006; Hamerlik et al., 2012), skin (Beck et al., 2011) and breast cancers (Zhao et al., 2014b; Goel et al., 2013). Initial studies in skin cancer models elucidated the importance of VEGFA signaling in cancer development and regulating the stemness (Beck et al., 2011; Lichtenberger et al., 2010a). Overexpression of VEGFA promoted cancer stem cell expansion and blocking VEGFR2 decreased cancer stem cell population and self-renewal ability suggesting VEGF signaling through VEGFR2 directly regulates cancer stem cell function and self-renewal. Our study in breast cancer and that of another group also demonstrated that VEGFA contributes to tumor initiation by
increasing cancer stem cell self-renewal via upregulating key stem cell regulators such as Sox2 (Zhao et al., 2014) and Bmi1 (Zhao et al., 2014b; Goel et al., 2013).

CSCs show greater motility and metastatic potential than the bulk tumor cell population and have been postulated to be drivers of tumor metastasis. The EMT program also has been linked to the initiation and/or maintenance of CSCs. Cells undergoing EMT exhibit an increased CSC properties and tumor initiation potential (Fantozzi et al., 2014). In human mammary epithelial cells, enforced expression of EMT transcription factors SNAI1 or TWIST1, or exposure to TGF-β has been shown to increase CSC abundance and ability of self-renewal, suggesting an intimate relationship between CSC and EMT process (Mani et al., 2008; Morel et al., 2008). Moreover, overexpressing various EMT-TFs in mammary cell lines induced transcriptional program to increase CSCs (Tam et al., 2013). Another study showed that the expression of EMT transcription factors and markers were upregulated in stem like cells isolated from human primary breast tissues compared to more differentiated bulk population (Bhat-Nakshatri et al., 2010).

An increased frequency of stem-like cells and expression of EMT related genes has observed in the highly invasive subset of basal-like breast cancers and primary tumor cultures (Azzam et al., 2013a; Ben-Porath et al., 2008). Stem cell and EMT markers are also frequently expressed in circulating tumor cells of metastatic breast cancer patients (Aktas et al., 2009).

VEGFA not only increases the tumor-initiating stem cell population in several different murine and human cancer models (Beck et al., 2011; Zhao et al., 2014b; Goel et al., 2013; Bao et al., 2006; Hamerlik et al., 2012), but is also known to induce EMT and metastasis (Bachelder et al., 2002; Gonzalez-Moreno et al., 2010; Kim et al., 2004). VEGF
can increase breast cancer cell invasiveness by regulating signaling pathways (Bachelder et al., 2002; Yoshiji et al., 1996). VEGF stimulation also induces EMT in normal epithelial cells and in cancer cells by promoting Snail expression and nuclear localization (Mak et al., 2010; Wanami et al., 2008). It was recently shown that VEGF is expressed and secreted more by cancer cells that have undergone an EMT and cancer cell derived VEGF is required for early tumor initiation (Fantozzi et al., 2014) suggesting VEGF may play a role at the interface between EMT and cancer stemness.

1.4  p27: A CDK INHIBITOR WITH DUAL ROLES IN TUMOR BIOLOGY

The second main project of my thesis work investigated how deregulated p27 drives metastatic tumor progression by switching from a tumor-suppressive, anti-proliferative role to an oncogenic, tumor-promoting role. This CDK inhibitor is under complex and coordinated regulation via diverse signaling pathways in both normal and transformed cells. Recent advances have highlighted the significance of p27 phosphorylation events that lead to its deregulation, in directing how this atypical tumor suppressor acquires the ability to stimulate cell migration during tumor progression. The following briefly reviews the function and regulation of p27 in normal cells, and our current understanding of how deregulated p27 may contribute to cancer metastasis.

1.4.1  Normal function as cell cycle regulator

p27 is encoded by CDKN1B and regulates the cell cycle by binding directly to its cyclin partners and the catalytic cleft of its target CDKs, acting as a competitive inhibitor for CDK substrates. Simultaneous binding to Cyclin and CDK is required for efficient CIP/KIP-mediated complex inhibition (Vlach et al., 1997; Chu et al., 2007). Both CDK
and Cyclin binding motifs are located in the highly conserved N-terminal region of p27 (Sherr and Roberts, 1999). C-terminal region of p27 contains nuclear localization sequences, assuring that the newly synthesized protein is translocated to nucleus for binding to its CDK targets (Zeng et al., 2000; Rodriguez-Vilarrubla et al., 2002).

p27 was first identified as an inhibitor of cyclin E-CDK2 and is regulated by contact-inhibition and growth inhibitory cytokines (Koff et al., 1993; Polyak et al., 1994a; Polyak et al., 1994c; Slingerland et al., 1994; Hengst et al., 1994). p27 plays a pivotal role in tissue proliferation during development and in cell cycle control for normal cells. p27 is highly expressed in non-proliferating cells and regulates quiescence and G1 progression. Mice lacking p27 show multi-organ hyperplasia and increased body size, and are susceptible to carcinogen-induced tumors indicating that p27 acts as regulator for cell proliferation and tissue expansion (Vidal and Koff, 2000). While p27 loss causes multi-organ hyperplasia in p27 null mice (Vidal and Koff, 2000), knock in of a mutant form of p27 that is defective for Cyclin-CDK binding causes increased self-renewal of progenitor cells in a variety of tissues including the intestinal and bronchial epithelia (Besson et al., 2007). Cyclin-CDK binding defective p27 knock-in animals show increased self-renewal of lung epithelial progenitor cells and develop lung tumors spontaneously (Besson et al., 2007).

1.4.2 p27 regulation by phosphorylation

p27 is primarily regulated at the post-translational level, however, mechanisms regulating mRNA stability and translation also exist (Hengst and Reed, 1996; Chu et al., 2008). While p27 mRNA levels are constant throughout cell cycle, its translation decreases dramatically as cells exit G0, permitting cyclin E/A-CDK2 complex activation (Chu et al.,
There are key phosphorylation events regulating p27 levels, activity and cellular localization (Connor et al., 2003; Cmielova and Rezacova, 2011). In early G1, p27 phosphorylation at S10 facilitates CRM1-mediated p27 nuclear export (Deng et al., 2004; Connor et al., 2003) (Ishida et al., 2000; Ishida et al., 2002; Rodier et al., 2001) and subsequent degradation. In late G1, the SCFSkp2 (S-phase kinase associated protein 1 (SKP1) / Cullin / F-Box protein: S-phase kinase associated protein 2 (Skp2)) complex-mediated p27 proteolysis is stimulated by cyclin E/A-CDK2-dependent T187 phosphorylation of p27 (Nakayama and Nakayama, 2006). The CDK-inhibitory domain of p27 contains three tyrosines at residues 74, 88 and 89. p27 phosphorylation by Src (Chu et al., 2007) and Abl (Grimmler et al., 2007) at Y74, Y88 and Y89 induces its ejection from the catalytic cleft of CDK2, enabling p27 phosphorylation at T187 to activate SCFSkp2-mediated p27 proteolysis in late G1/S (Chu et al., 2007; Grimmler et al., 2007). Although Y88 is highly conserved among all Cip/Kip family CDK inhibitors, including human p21 and p57, it is not clear whether Src family kinase-mediated phosphorylation regulate stability in an analogous manner.

In addition to the regulatory mechanisms described above, two C-terminal phosphorylations also critically regulate p27 levels and cellular localization. p27 can be phosphorylated by PI3K effector kinases, AKT, SGK, and RSK at T157 and T198 (Wander et al., 2011b). T157 is located in nuclear localization sequences and phosphorylation at this site defers its nuclear import, leading to increased cytoplasmic p27 (Wander et al., 2011b). Phosphorylation at T198 contribute to p27 stabilization in the cytoplasm (reviewed in (Larrea et al., 2009b)). Increasing evidence indicate that the cytoplasmic mislocalization
of p27 is associated with the gain of pro-oncogenic function (Wander et al., 2011b; Besson et al., 2004a; Besson et al., 2008).

1.4.3 CDK independent functions of p27

D-type Cyclin/CDK assembly

Paradoxically, p27 also promotes assembly of Cyclin-CDK complex and induce its nuclear import. Cytoplasmic p27 increases Cyclin D-CDK4/6 complex formation and stabilizes these complexes (LaBaer et al., 1997). p27 phosphorylation by AKT at T157 and T198 facilitates p27 assembly of Cyclin D-Cdk4/6 (Larrea et al., 2008; James et al., 2007). Since neither CDK4/6 nor D-type Cyclin have nucleus localization signal, the associated KIP is required for nuclear import of the complex (Warfel and El-Deiry, 2013) and (Weiss, 2003). Additional phosphorylation may modulate nuclear import of Cyclin D-CDK bound p27.

Stem cell self-renewal

p27 may be involved in self-renewal of stem cells. Notably, mice lacking p27 exhibit multi-organ hyperplasia, increased body size and spontaneous neoplasia (reviewed in (Chu et al., 2008)), suggesting p27 normally limits organ size during embryogenesis. This function of p27 is not related to the CDK inhibitory action of p27, since it is manifest in animals expressing Cyclin-CDK binding defective mutant p27. In p27CK-knock-in mice, a CDK-independent gain of p27 function appears to perturb normal stem cell homeostasis and cause expansion of progenitor/stem cells and spontaneous lung tumor development (Besson et al., 2007). These findings suggest that p27 may have cell
cycle-independent roles to modulate stem cell expansion in various tissues (Besson et al., 2007).

**Cell motility and invasion**

In many cancers, C-terminally phosphorylated p27 appears to gain a cell cycle-independent, oncogenic function to promote cancer cell motility and invasion (Slingerland and Pagano, 2000; Chu et al., 2008). TAT-p27 protein transduction was shown to increase Rac-dependent cell motility (Nagahara et al., 1998). p27 null mouse embryonic fibroblasts (MEFs) exhibit decreased motility compared to wild type MEFs that could be rescued by re-expression of either wild type p27 or mutant p27 that cannot bind Cyclin and CDK (Besson et al., 2004b). Therefore, the motility-promoting effect of p27 appears to be independent of its Cyclin-CDK regulatory functions. Interestingly, overexpression of cytoplasmic p27 promotes invasion and metastasis in various cancer models (Denicourt et al., 2007; Wu et al., 2006) Zhao et al., 2015) and increases glioma cell invasion (See et al., 2010). Thus, while nuclear p27 inhibits Cyclin-CDK complex to restrain cell cycle in normal cells, deregulated cytoplasmic p27 confers pro-oncogenic effects to promote cancer invasion and metastasis in cancer cells. A part of my thesis work aimed to further elucidate how C-terminally phosphorylated p27 contributes to cancer metastasis, and investigated mechanisms under its pro-metastatic actions.

**Transcriptional regulation by p27**

Recent studies suggest that p27 may regulate gene expression by binding to various transcription factors. p27 was recently shown to act as a transcriptional co-repressor in a complex with other transcriptional repressors p130, E2F4, HDAC1 and SIN3A (Pippa et al., 2012). p27 directly interacts with p130 and E2F4 by its C-terminal region and p27 is
critical for recruitment of HDAC1 and SIN3A to form complex to repress p27 target gene transcription. Putative p27 target genes were identified by ChIP/ChIP assays and include genes involved in cell proliferation and tissue expansion. (Pippa et al., 2012).

Sox2 is an important transcription factor that maintains self-renewal of normal embryonic stem cells (Marson et al., 2008) and may also drive cancer stem cell expansion (Bass et al., 2009; Leis et al., 2011). It was recently shown that SOX2 is repressed by p27 binding to the main SOX2 regulator site, SOX2-SRR2, located 4KB downstream of the coding exon together with p130, E2F4 and SIN3A (Li et al., 2012). These suggest that p27 may carry out a critical roles as transcriptional regulator. The spectrum of p27 target genes and how they are regulated in normal cells and deregulated in cancer remain to be investigated. A part of this thesis work further expands our knowledge of p27’s role in transcriptional regulation. We show that C-terminally phosphorylated p27 may act as a transcriptional co-activator at key EMT inducing genes to promote PI3K-dependent cancer metastasis. Our findings suggest that p27 may play a previously unappreciated role to govern critical transcriptional programs driving cancer metastasis.

1.4.4 Oncogenic signaling pathways that deregulate p27

Unlike typical tumor suppressors, whose genes are commonly deleted in human cancers, p27 mutations or deletions are rarely observed. This is because p27 can acquire novel actions that drive cancer progression. In cancers, p27 is either reduced due to excess proteolytic degradation or decreased translation, or it is phosphorylated and stabilized in a manner to promote cancer progression (Chu et al., 2008; Larrea et al., 2009b).
p27 is deregulated in cancer by receptor tyrosine kinases that activate Src/BCR-ABL and Ras/MEK/MAPK (Chu et al., 2008; Larrea et al., 2009b). Src levels/activity are increased in many human cancers (Mayer and Krop, 2010). Src phosphorylates p27, decreasing its binding to CDK2, leading to SCF<sup>SKP2</sup> mediated p27 proteolysis. Indeed, in primary human breast cancers, Src activation is associated with reduced p27 levels (Chu et al., 2007). Similarly, the Src-family kinase, BCR-ABL phosphorylate p27 at Y88 (Grimmler et al., 2007; Chu et al., 2010). Src family kinases targeted therapy to restore p27 function is in therapeutic development in human cancers.

PI3K signaling is oncogenically activated in many human cancers (van der Heijden and Bernards, 2010) through deregulated receptor tyrosine kinase activation, PTEN deletion and PIK3CA mutation (Wander et al., 2011a). PI3K signaling plays an important role for tumor progression by modulating proliferation, survival and cell migration. It regulates cell cycle by impairing CDK inhibitory action of p27 by promoting phosphorylations at T157 and T198 within its C-terminal region (Liang and Slingerland, 2003). PI3K effectors, AKT, SGK and RSK, can phosphorylate p27 at T157 and T198 (Liang et al., 2002b; Shin et al., 2002; Viglietto et al., 2002; Liang and Slingerland, 2003), which increases p27-cyclin D-CDK4 assembly (Larrea et al., 2008) and delays p27 import leading to p27 localization in both the nucleus and cytoplasm (Liang et al., 2002a; Viglietto et al., 2002). Phosphorylation at T198 also stabilizes p27 (Liang et al., 2007; Kossatz et al., 2006) and promotes its oncogenic ability to increase cell motility (Larrea et al., 2009a). Indeed, PI3K/AKT activation is highly correlated with cytoplasmic p27 localization in breast, renal, thyroid cancers and leukemias (Liang et al., 2002b; Kim et al., 2009; Motti et al., 2005; Min et al., 2004; Viglietto and Fusco, 2002; Shin et al., 2002).
The diverse oncogenic signaling above may contribute to cancer progression by causing a loss of CDK inhibition by p27 and through a gain of pro-oncogenic function via C-terminal p27 phosphorylation. Thus, therapeutic interruption of these oncogenic pathways may contribute to the efficacy of targeted therapies by restoring of p27 function in cancer.
CHAPTER 2: MATERIALS AND METHODS
This chapter describes the materials and experimental approaches that were used for the work in this thesis. Section 2.1 contains a listing all of the relevant materials: Table 2.1 lists the cell lines used in this work, Table 2.2 lists the various reagents including antibodies and inhibitors. Table 2.3 lists the oligonucleotide sequences used for qPCR and ChIP assays. Section 2.2 contains detailed descriptions of the various experimental methods that were utilized.

2.1 MATERIALS

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<th>Description</th>
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| SUM149PT     | Ham's F12, 5% FBS, 5 µg/ml insulin, 1 µg /ml hydrocortisone, 1mM HEPES | Human breast cancer cell line | S Ethier UColorado |
| DT-22        | IMEM, 10% FBS | Human breast primary dissociated culture, derived from triple negative primary tumor | Dorraya El Ashry UMiami |
| 293T         | DMEM, 10% FBS, 1% Pen/Strep | Lentiviral packaging cell line | Clontech |

Table 2.2: Reagents
Continued Table 2.2: Reagents

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**Table 2.3: Oligonucleotide Sequences**

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**CHIP PRIMERS**

| Human **TGF-β2** | Qiagen GPH1001266(-)19A |
2.2 METHODS

2.2.1 Establishment of p27 phosphomimetic mutant expressing cells

The EGFP-p27CK- vector was provided by Steve Dowdy, UCSD. Constructs of phosphomimetic p27 mutant (EGFP-p27CK-T157D/T198D) were generated by site-directed mutagenesis and transfected into MDA-MB-231, UMUC3, and MCF12A cells with lipofectamine. Stable transfectants expressing similar EGFP-p27 levels were selected for subsequent studies. Different p27 mutants were further subcloned into Lenti-AcGFP vector (Clotech) to generate Lenti-AcGFP-p27CK- and Lenti-AcGFP-p27CK-DD vectors for virus packaging to infect target cells.

2.2.2 Lentivirus production and infection

Lentivirus vectors encoding shSox2, shp27, shcJun and shTGF-β2 were purchased from Open Biosystems or Genecopoeia and 3 different clones of each shRNA were used for lentivirus production and infection. Lentivirus vectors encoding different shRNAs, scramble shRNAs or p27 mutants were co-transfected with Delta VPR and CMV VSVG plasmids (Addgene) into asynchronous 293T with Lipofectamine Plus (Roche). Viral supernatants were collected at 48h and concentrated by ultracentrifugation for 2 h at 22,000 RPM at 4°C. Pellets were resuspended overnight at 4°C. Cells were infected twice in the presence of polybrene (10 μg/ml), and analyzed 3-5 days post infection via both GFP visualization and western blotting.
2.2.3  *Wound-healing migration assay*

Cells were seeded to confluency in 60mm plates and grown for an additional 24h. A linear scratch/wound was made on cell monolayers with a sterile pipette. Photomicrographs were taken of live cells (10x objective) over time and distance migrated measured using ImageJ software (1 μm = 1 pixel). Relative migration was calculated via the following formula: (initial wound distance – final wound distance) / initial wound distance. The values are plotted +/- SEM from three independent scratches.

2.2.4  *Matrigel invasion assay*

Transwell invasion of cells were assayed by seeding $10^5$ cells to the upper chamber of a transwell membrane (Corning) coated with 5 mg/ml of matrigel. After 15 h, cells were fixed in 90% ethanol (10 min), stained with 1% crystal violet (10 min), and washed 3X with PBS. Cells adherent to the underside of the transwell membrane were subsequently visualized at 10x magnification and photographed. Cell numbers were counted and the relative invasion was plotted.

2.2.5  *Automated transwell migration and invasion assays*

Automated transwell migration and invasion assays were carried out using the Real-Time Cell Analysis (RTCA) system from Xcelligence. For transwell migration assays, the upper chambers of CIM plates were seeded with 20,000 cells in serum-free medium, which were subsequently allowed to migrate toward 10% FBS serum in the bottom chambers. For transwell invasion assays, the upper chambers of CIM plates were pre-coated with 5% matrigel and seeded with 20,000 cells, which were subsequently allowed to invade toward
10% FBS serum in the bottom chambers. Automated analysis of cellular density on the underside of the transwell membrane occurred every 30 minutes and was plotted as cell index +/- SEM for at least three wells per group.

### 2.2.6 Quantitative real-time PCR (qPCR)

qPCR analysis were performed as previously described (Lindley and Briegel, 2010; Rieger et al., 2010) using primers for indicated genes listed in table 2.3. *GAPDH* or *18S* were used as an internal control. All samples were performed in triplicates and average C\textsubscript{t} values were normalized to the values of *GAPDH* or *18S*. A comprehensive list of primers used during qPCR is included in Table 2.3.

### 2.2.7 Western blotting

Westerns were as described (Sandhu et al., 1997). A comprehensive list of antibodies used during western blotting (along with the company from which they were purchased) is included in Table 2.1. All western blots were prepared using Millipore PVDF membrane and blocked for one hour in 5% non-fat milk solution. Primary antibody concentration and duration of exposure varied based on individual optimization to each antibody. Secondary incubation at a dilution of 1:5,000 was carried out for one hour. Chemiluminescent imaging was carried out with a variety of ECL reagents, listed in Table 2.2.
2.2.8 Nuclear and cytoplasmic fractionation

Nuclear-cytoplasmic fractionation was performed using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents from Thermo-Scientific. Nuclei were isolated following centrifugation and removal of the cytosolic supernatant. Equal protein concentration in nuclear and cytoplasmic lysates were blotted for indicated proteins. Lamin B2 was used as a nuclear control and β-tubulin was used as a cytoplasmic control.

2.2.9 miRNA screen

MDA-MB-231 cells were treated ±10ng/ml VEGFA for 7 days followed by Ready-to-Use PCR microRNA array, Human panel I+II in 384well plates from Exiqon.

2.2.10 Luciferase assay

293T and MDA-MB-231 were transfected with human SNAI2 3'UTR luciferase reporter plasmid together with miR-452 or control miRNA plasmid. After 48 h, Firefly and Renilla luciferase reporter activity luciferase activity was measured using Luc-Pair™ Duo-Luciferase Assay Kit 2.0 per manufacturer's instructions.

2.2.11 Cell proliferation assay

In vitro cellular proliferation was assessed by plating equal cell number (n=50,000) in a 24well culture plate at time = 0. Triplicate samples were plated in parallel and cells were harvested and counted at day 1, 2, 3 and 4. Average cell counts were calculated and plotted +/- SEM.
2.2.12 **Bioluminescent imaging and analysis**

All animal work was approved and carried out in compliance with the Institutional Animal Care and Use Committee. 5-week old female Balb/c nude mice (Charles River) were used for all xenograft studies. Mice were anaesthetized and injected intraperitoneally with 1.5 mg of d-luciferin (15 mg ml\(^{-1}\) in PBS) and imaged with the Xenogen IVIS system (Xenogen). Bioluminescence (photon flux) was quantitated with time and photon flux values were normalized to the value obtained immediately after xenografting (day 0) so that all mice had an arbitrary starting BLI (Bioluminescence Intensity) signal of 100.

2.2.13 **Experimental lung metastasis assay**

For experimental lung metastasis assays in Chapter 3, MDA-MB-231-luc cells were grown with or without VEGF at 10 ng/ml for 7 days and \(5 \times 10^5\) viable cells in 0.1 ml PBS were injected via tail vein into Balb/C nude mice (Minn et al., 2005a). Successful injection was verified by immediate IVIS imaging following injection. Bioluminescence was monitored by IVIS and quantified as normalized photo flux (Minn et al., 2005a). For lung metastasis BLI plots, a rectangular region of the thorax was utilized for each mouse. Values were normalized to the value obtained immediately after xenografting (day 0) so that all mice had an arbitrary starting BLI signal of 100. Representative individual mice were selected for each experiment and presented along with a standardized scale. Average normalized photon flux for each group is plotted over time +/- SEM. Lungs were photographed and weighted. All animal work was carried out in compliance with the Institutional Animal Care and Use Committee in University of Miami.
2.2.14 Orthotopic xenograft assay

For assays of the effects of p27CK-DD, cJun and TGF-β2 on tumor metastasis, we assayed effects on metastasis from an orthotopic site in Chapter 4. For these orthotopic tumor xenograft assays, $5 \times 10^5$ cells from each of MDA-MB-231 transfected with both EGFP-vector and shRNA control vectors and from MDA-MB231-p27CK-DD transduced with either shcJun, shTGF-β2 or control shRNA were each resuspended ex vivo in a volume of 100 microliters HBSS with matrigel. The different cell lines were then injected into the 4th mammary fat-pad of each of 9 animals per group. Successful injection was verified by IVIS imaging following injection. Tumor growth was monitored by IVIS and measured by twice-weekly calliper assessment and calculated by the following formula: 

\[
\text{area} = \frac{\text{long-side} \times \text{short-side}^2}{2}
\]

For assessment of metastasis, orthotopic primary tumors were completely removed when the size of tumors reached approximately 300 mm³ and mice were monitored every week by IVIS to investigate metastasis from primary region thereafter. In this scenario, total photon flux is presented as an approximate measure of systemic metastasis from the primary site. Representative individual mice were selected for each experiment and presented along with a standardized scale. Average normalized photon flux for each group is plotted over time +/- SEM. All animal work was carried out in compliance with the Institutional Animal Care and Use Committee in University of Miami.

2.2.15 Immunohistochemistry

For analysis of xenograft tumors and metastasized tumors, animal tissues were recovered at necropsy and immediately placed in 10% neutral buffered formalin for 24
hours. Soft tissues were subsequently paraffin embedded, while bone tissues were first decalcified and then paraffin embedded. Tumor sections were cut at 4µM and the lower-most section was stained with Hematoxylin and Eosin (H&E).

### 2.2.16 Immunoprecipitation

UMUC3-LuL2 and MDA-MB-231-1833 cells were treated +/- PF1502 for 48 hours before immunoprecipitation. Cells were lysed and 500-800 µg lysate was incubated with 1 µg of p27 (BD Transduction or Cell Signaling) or Normal mouse/rabbit IgG overnight, then collected on protein A- or G-agarose beads for 2 hours and washed three times with IP buffer. Samples were then analyzed by SDS-PAGE followed by western analysis. Antibody-alone controls were run with all immunoprecipitations.

### 2.2.17 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays for the TGF-β2 promoter were as described (Assou et al., 2007). Briefly, soluble chromatin was prepared from a total of 2 × 10^7 asynchronously growing MDA-MB-231-4175 cells, UMUC3-LUL2 cells, or MDA-MB-231-1833 that were pre-treated with or without PF1502 for 48 hours. The chromatin solution was diluted 10-fold with ChIP dilution buffer (1.1% Triton X-100, 1.2 mm EDTA, 167 mm NaCl, 16.7 mm Tris-HCl, pH 8.1, 0.01% SDS, plus protease and phosphatase inhibitors), and pre-cleared with protein, and blocked with 2 µg of sheared salmon sperm DNA and pre-immune serum. The pre-cleared chromatin solution was divided and utilized in immunoprecipitation assays with either an anti-p27, anti-cJun or an anti-IgG antibody. Following multiple washes, the antibody-protein-DNA complex was eluted from the beads.
by resuspending the pellets in 1% SDS, 0.1 m NaHCO₃ at room temperature for 20 min. After reversal cross-link incubation at 67 °C, protein and RNA were removed by incubation with 10 μg of proteinase K and 10 μg of RNase A at 42 °C for 3 h. Purified DNA was subjected to qPCR with primers specific for the cJun binding sites upstream of the transcriptional start site in the TGF-β2 promoter. The primers for chIP analysis are listed in Table 2.3.

2.2.18 Microarray data acquisition and analysis

RNA was quantified by Nanodrop 8000 Spectrophotometer (Thermo Scientific, Wilmington) and quality verified by RNA 6000 Nano kit (Agilent, Santa Clara, CA) on a Bioanalyzer 2100. Biotinylated cRNA was prepared per Illumina TotalPrep RNA Amplification Kit (Ambion, Inc., Austin, TX) from 400ng RNA. Samples were added to the Beadchip after randomization using a randomized block design to reduce batch effects. Hybridization to the Sentrix Human-HT12 Expression BeadChip (Illumina, Inc., San Diego, CA), washing and scanning were per Illumina BeadStation 500 manual (revision C). Microarray data analysis used Illumina GenomeStudio software.

Preliminary expression data was filtered used the R/Bioconductor package. Expression data from 47,231 probes was log transformed and Quantile normalized. 23,485 Probes having Std. Dev. ≥ 0.1 across all arrays were used for further analysis. Probes significantly differentially expressed (P < 0.0001; Bonferroni-Hochberg corrected) with fold change >1.0 were identified by Student’s T-test. Genes commonly expressed in the parental MDA-MB-231, 1833 scramble and 1833Shp27 were removed from the data set before differences in gene expression between 1833 and 1833shp27 were evaluated by
Student’s T-test. All gene expression analysis followed MAIME standards. Genes differentially expressed after p27 knockdown were compared with both the BMS “Bone Metastatic Signature” of Kang et al. (GSE14244) (7) and the “EMT core signature” of Taube et al. (GSE24202) (27). All genes expressed in the BMS signature were compared and presented in a heatmap. Of over 400 genes significantly altered by p27 knockdown, 40 were present in the EMT signature but altered in the opposite direction, (p<0.005). Genes with significant GSA scores for both analyses are provided in Table 4.1 and Table 4.2 EMT signature genes overlapping with inverse expression following shp27).

2.2.19 Expression analysis of VEGFA, SOX2, SNAI2, GABRE genes and miR-452

The METABRIC dataset contains gene expression data for 2136 and microRNA expression for 1448 primary breast cancer samples, with both available in 1302 samples, together with clinical information and disease-specific survival (DSS) outcome data 71,101. METABRIC and the independent Enerly primary breast cancer dataset, containing 101 cases 72, were used to identify a correlation between miR-452 and GABRE expression by Pearson’s correlation. The KM-plotter dataset contains gene expression from primary human breast cancers (n=2553) and was used for analysis of distant metastasis-free survival (DMFS).

For clinical outcome analysis, expression quartiles were employed to test if VEGFA expression alone, or with the other genes, associated with poor DSS (METABRIC) or with poor DMFS (KM plotter) using Kaplan-Meier analysis and the logrank test. Univariate Cox proportional hazards analysis identified hazard ratios with 95% confidence intervals (CI). DSS or DMFS curves were also compared using the logrank
test and the p value from this analysis was displayed in each graph. Data analysis was performed using R statistical software or by using the KM-plotter web tool, as in 102,103.

### 2.2.20 Statistical analysis

All graphed data presented as mean ± standard error of the mean (SEM) from at least 3 experiments. Comparisons of two groups used the two-tailed Student’s t tests for two group. Analysis of variance (ANOVA) was for comparisons of more than two groups. P values < 0.05 were considered statistically significant. Tests were two-sided unless otherwise specified.

Statistical differences between motility or invasion rates from ExCelligence Real Time Cell Analysis of tumor invasion or motility assays and differences between tumor growth curves from in vivo experiments were calculated using "compareGrowthCurves" function of the statmod software package at the following website (http://bioinf.wehi.edu.au/software/compareCurves).
CHAPTER 3:

VEGFA LINKS SELF-RENEWAL AND METASTASIS

BY INDUCING SOX2 TO REPRESS MIR-452, DRIVING SLUG

1 A version of this chapter is accepted and will be published in Oncogene
3.1 SUMMARY

Cancer stem cells appear to have increased metastatic potential, but mechanisms underlying this are poorly defined. Here we show that VEGFA upregulates Sox2 to promote EMT and tumor metastasis. In breast lines and primary breast cancer cultures, VEGFA rapidly upregulates SOX2. Prolonged exposure to VEGFA over seven days subsequently causes SNAI2 induction, EMT, increased invasion and metastasis. We show Sox2 downregulates miR-452, which acts as a novel metastasis suppressor to directly target the SNAI2 3’ UTR. VEGFA stimulates Sox2- and Slug-dependent cell invasion. VEGFA increases lung metastasis in vivo, and this is abrogated by miR-452 overexpression. Furthermore, SNAI2 transduction rescues the suppression of metastasis by miR-452 overexpression. Thus, in addition to its angiogenic action, VEGFA upregulates Sox2 which not only drives stem cell expansion, but also mediates the loss of miR-45, leading to Slug upregulation. These data reveal a novel mechanism whereby cancer stem cells acquire metastatic potential. Prior work showed EMT transcription factor overexpression upregulates CSC. Present work indicates that stemness and metastasis are a two way street: Sox2, a major mediator of CSC self-renewal, also governs the metastatic process.

3.2 INTRODUCTORY REMARKS

VEGFA is a cytokine that regulates vascular development during embryogenesis and the formation of new blood vessel from pre-existing vascular networks (Olsson et al., 2006; Leung et al., 1989; Tischer et al., 1989). VEGFA, secreted by cancer and stromal cells, stimulates endothelial cell invasion and vessel formation (Hicklin and Ellis, 2005). Without new blood vessel formation, tumor size is restrained due to limited nutrient and
oxygen supply. VEGFA is expressed in a variety of tumors and its overexpression is associated with poor prognosis and death from metastasis (Goel and Mercurio, 2013; Berns et al., 2003; Manders et al., 2002). VEGFA functions are not restricted to vasculogenesis and angiogenesis (Senger, 2010). Autocrine VEGFA cooperates with EGFR to drive tumor development (Lichtenberger et al., 2010b) and VEGFA has also been shown to drive tumor metastasis (Hanahan and Folkman, 1996; Hicklin and Ellis, 2005; Mercurio et al., 2005). Indeed, patients with metastatic breast cancer have higher circulating VEGFA levels than those without metastasis (Adams et al., 2000).

Bevacizumab, a humanized monoclonal antibody that targets VEGFA, has been used to treat breast cancer and other malignancies. However, trials in metastatic breast cancer have yielded variable results and the role of this drug is controversial (Sullivan and Brekken, 2010; Shih and Lindley, 2006; Montero et al., 2012). Recent work sheds light on the limited results of bevacizumab in most cancers. Hypoxia caused by inhibition of angiogenesis, upregulates VEGFA expression, contributing to aggressive disease recurrence (Paez-Ribes et al., 2009; Ebos et al., 2009). VEGFA was recently shown to increase tumor initiating stem cell abundance in skin (Beck et al., 2011) and breast cancers (Zhao et al., 2014b; Goel et al., 2013), and in glioblastoma (Bao et al., 2006; Hamerlik et al., 2012). The high local VEGFA induced by hypoxia following bevacizumab treatment would thus also promote expansion of the tumor cell subset with the greatest ability to initiate and disseminate tumors.

Cancer stem cells (CSCs) show greater motility and metastatic potential than the bulk tumor cell population and have been postulated to be drivers of tumor metastasis (Malanchi et al., 2012; Takebe et al., 2011; Picon-Ruiz et al., 2016), but the mechanisms
underlying this are not fully characterized. Metastasis requires cell invasion and escape from the primary tumor into the vasculature followed by colonization of secondary sites. Tumor invasion and intravasation are enabled by the epithelial to mesenchymal transition (EMT), a process in which epithelial cells lose polarity and intracellular adhesion and acquire motility and invasiveness (Thiery, 2002; Kalluri and Weinberg, 2009; Micalizzi et al., 2010; Thiery et al., 2009). The EMT is regulated by diverse molecular networks including TGF-β and Notch, Wnt, Hedgehog, and NF-κB signaling pathways, all of which play central roles in cancer invasion and metastasis (Huber et al., 2005). Downregulated expression of the cell adhesion molecule, E-cadherin, is critical for acquisition of the EMT phenotype and tumor invasion (Onder et al., 2008). Many EMT transcription factors repress CDH1, the gene encoding E-cadherin, directly or indirectly. Snail (Batlle et al., 2000; Cano et al., 2000), Slug (Bolos et al., 2003), Zeb1 (Eger et al., 2005) and Zeb2 (Comijn et al., 2001) can bind the CDH1 promoter and repress its transcription, whereas other factors such as Twist (Yang et al., 2004), Goosecoid (Hartwell et al., 2006) and forkhead box protein C2 (FOXC2) (Mani et al., 2007) repress CDH1 indirectly. Slug, whose expression correlates strongly with loss of E-cadherin, is an important EMT mediator in breast cancer cell models (Hajra et al., 2002).

The EMT program has been linked to the initiation and/or maintenance of CSCs. Enforced expression of EMT transcription factors has been shown to increase cancer stem cell abundance, and stem-like cells exhibit EMT properties such as increased expression of mesenchymal markers and EMT transcription factors, suggesting a link between cancer stem cells and the EMT process (Mani et al., 2008; Morel et al., 2008). However, pathways governing the relationship between cancer stem cells and EMT are not fully defined.
VEGFA not only increases the tumor-initiating stem cell population in several different murine and human cancer models (Beck et al., 2011; Zhao et al., 2014b; Goel et al., 2013; Bao et al., 2006; Hamerlik et al., 2012), but is also known to induce EMT and metastasis (Bachelder et al., 2002; Gonzalez-Moreno et al., 2010; Kim et al., 2004). Our prior work showed VEGFA rapidly activates STAT3 to induce SOX2 and increase the CSC population in breast and lung models (Zhao et al., 2014b). Here, we investigated whether upregulation of Sox2 by VEGFA might play a role not only in CSC expansion but also contribute to the activation of EMT and metastasis.

MicroRNAs (miRNA) are small, non-coding RNAs that regulate transcriptional and post-transcriptional gene expression. Approximately 70% of all genes are regulated by miRNA in eukaryotes (Bartel, 2004; Ceppi and Peter, 2014). miRNAs carry out important functions in development, differentiation, cell cycle progression and apoptosis. Mature miRNAs bind complementary sequences in the 3’ untranslated region of target genes and repress gene expression by inducing mRNA degradation and/or translational inhibition (Chendrimada et al., 2005; Diederichs and Haber, 2007). In cancers, miRNA expression is deregulated by amplification, deletion, mutation, and epigenetic silencing (Lu et al., 2005; Garzon et al., 2009; Iorio and Croce, 2012). Many miRNAs act as oncogenes or tumor suppressors, contributing to malignant transformation and metastatic progression (Iorio and Croce, 2012). miRNAs modulate the metastatic process by targeting metastasis suppressor genes or by repressing metastasis promoting genes (Pencheva and Tavazoie, 2013). Several miRNAs regulate EMT transcription factors including Zeb1, Zeb2 and Snail (Burk et al., 2008; Gregory et al., 2011; Kumarswamy et al., 2012). Indeed, several miRNAs that target EMT transcription factors, such as miR200 that targets Zeb1 (Park et
al., 2008; Korpal et al., 2008; Gregory et al., 2008; Burk et al., 2008) and miR34 that targets Snail (Kim et al., 2011), also repress cancer stem cell self-renewal (Ceppi and Peter, 2014).

Here, we identify a novel pathway in which Sox2, a stem cell driver upregulated by VEGFA, contributes to activation of EMT. VEGFA leads to induction of the stem cell transcription factor gene *SOX2*. Sox2, in turn, mediates repression of miR-452, which is shown to directly target the 3’ UTR of *SNAI2*, leading to EMT induction and breast cancer metastasis.

### 3.3 RESULTS

#### 3.3.1 VEGFA induces EMT and an increase in motility and invasion in breast models

In addition to its angiogenic effects, VEGFA promotes cancer stem cell expansion (Zhao et al., 2014b). VEGFA also drives cancer invasion and metastasis in experimental models (Bachelder et al., 2002; Kim et al., 2004). Cancer stem cell expansion is linked to, indeed potentially driven by upregulation of EMT transcription factors (Mani et al., 2008; Morel et al., 2008), but whether stem cell drivers can also promote EMT has not been fully investigated. To investigate if VEGFA-mediated CSC expansion might also be linked to EMT activation and metastasis, we tested the effect of VEGFA on motility and invasion in aggressive ER negative breast cancer models. Since our earlier work showed a prolonged 7 day exposure to VEGFA caused an irreversibly increase in stem-like cells (Zhao et al., 2014b), all experiments used 7 days of VEGFA (10 ng/ml), unless otherwise indicated. VEGFA-treated MDA-MB-231 showed faster migration on wound healing assays and increased matrigel invasion compared to controls (Figure 3.1A and B). Results were
validated in an ER, PR and Her2 negative primary breast cancer-derived line, SUM149PT (Figure 3.2A and B).

Figure 3.1. VEGFA induces EMT and an increase in motility and invasion. (A) MDA-MB-231 was pre-treated for 24 hours or 7 days ±10ng/ml VEGFA followed by scratch wounding of a confluent monolayer. Photomicrographs were taken at 0 and 12 hrs and mean migration +/-SEM graphed versus controls (C). (B) MDA-MB-231 pre-treated for 24 hours or 7 days ± VEGFA were recovered for real-time matrigel invasion assays. (C-E) EMT marker expression was compared by QPCR after 7 days ±10ng/ml VEGFA (V) in MDA-MB-231 (C), SUM149PT (D) and MCF12A (E). All graphs show mean ± SEM. (C-E) EMT marker expression was compared by QPCR after 7 days ±10ng/ml VEGFA (V) in MDA-MB-231 (C), SUM149PT (D) and MCF12A (E). All graphs show mean ± SEM. *p<0.05
Acquisition of an EMT phenotype is critical for metastasis. Mesenchymal markers (Vimentin, Fibronectin and N-cadherin) were upregulated by VEGFA in MDA-MB-231 and SUM149PT lines and in the immortal but not malignantly transformed human mammary epithelial line, MCF12A. Epithelial markers, including one or both of E-cadherin and Zo-1, were decreased in all three cell lines (Figure 3.1C-E), compatible with a VEGFA-induced EMT.

3.3.2 VEGFA increases motility and invasion by upregulating Slug in breast cancer cells

Expression of major EMT-driving transcription factors (EMT-TFs), Slug, Snail, Zeb1 and Zeb2, was induced over a 7 day VEGFA exposure. The temporal patterns of EMT-TF upregulation during prolonged VEGFA exposure for MDA-MB-231 and MCF-12A are shown in Figure 3.3A-B and Figure 3.4. Of these, SNAI2, which encodes Slug, was the most strongly induced after seven days, and was thus investigated further (Figure 3.2). VEGFA increases motility and invasion in SUM149PT breast cancer cells. (A-B) SUM149PT cells were treated ±10ng/ml VEGFA for 7 days followed by assay of real-time migration (A) and real-time matrigel invasion (B). Statistical differences between motility or invasion rates from ExCelligence Real Time Cell Analysis of tumor invasion or motility assays were calculated using "compareGrowthCurves" function of the statmod software package at the following website (http://bioinf.wehi.edu.au/software/compareCurves).
Figure 3.3. VEGFA increases motility and invasion via slug induction in breast cancer cells. (A-B) SNAI1, SNAI2, ZEB1 and ZEB2 expression levels were assayed by QPCR at indicated times after 10 ng/ml VEGFA in MDA-MB-231 (A) and MCF12A (B). (C-E) MDA-MB-231 cells were transduced with siSNAI2 or control siRNA for 48 hrs. Cells were then treated ±10ng/ml VEGFA for 7 days and then recovered for assays of SNAI2 and Slug expression by QPCR and Western, respectively (C) and assays of migration (D) and matrigel invasion (E). (F) SUM149PT cells were transduced with siSNAI2 or control siRNA for 48 hrs then treated ±10ng/ml VEGFA for 7 days followed by assays of SNAI2 and Slug expression by QPCR and Western, respectively (F) and by real-time matrigel invasion assay (G). All bar graphs show mean ± SEM. *p<0.05
3.3A and B). *Snai2* knockdown prevented VEGFA-mediated increases in cell motility invasion, indicating VEGFA increases migration and invasion via Slug (Figure 3.3C-E). VEGFA also upregulated *Snai2* expression, and *Snai2* knockdown inhibited VEGF-driven invasion in a second model, SUM149PT (Figure 3.3F and G).

**Figure 3.4.** VEGFA upregulates EMT transcription factors motility in breast models. (A-B) Snail, Zeb1 and Zeb2 expression levels were assayed by QPCR at indicated times after 10 ng/ml VEGFA in MDA-MB-231 (A) and MCF12A (B). All graphs show mean ± SEM. *p<0.05
3.3.3 Sox2 is required for VEGFA-driven slug upregulation and increased motility and invasion

Sox2 drives self-renewal in both embryonic stem cells and in several cancer stem cell models (Leis et al., 2011; Xiang et al., 2011) and is a key mediator of VEGFA-driven CSC expansion (Zhao et al., 2014b). CSC are postulated to be major drivers of tumor metastasis and exhibit greater motility and metastatic potential than bulk tumor cells (Malanchi et al., 2012; Takebe et al., 2011; Azzam et al., 2013b). Notably, EMT-TF overexpression leads to expansion of cells with stem-cell characteristics (Mani et al., 2008; Morel et al., 2008). Here we tested if the reverse is also true and if the embryonic stem cell factor, Sox2, might mediate VEGFA-driven EMT. Upregulation of SOX2 expression by VEGFA occurs rapidly, within 1 hr in MDA-MB-231 and SUM149PT (Figure 3.5A) and remains elevated for at least 7 days (Zhao et al., 2014b). SOX2 induction precedes that of SNAI2 by several days. SNAI2/Slug upregulation by VEGFA was prevented by SOX2 knockdown (Figure 3.5B, C and 3.6) and Sox2 was also required for VEGFA-mediated increase in cell motility and invasion (Figure 3.5D) in MDA-MB-231. Findings were validated in the SUM149PT line (Figure 3.5E and F). Moreover, SOX2 overexpression was sufficient to increase Slug expression (Figure 3.5G), invasion and migration in the absence of VEGFA stimulation (Figure 3.5H). Thus, the rapid VEGFA-STAT3-mediated induction of SOX2 (Zhao et al., 2014b) not only precedes, but is required for that of SNAI2 and the increased migration and invasion following VEGFA exposure in both MDA-MB-231 and SUM149PT.
Figure 3.5. Sox2 is required for VEGFA-driven increases in slug, motility and invasion (A) MDA-MB-231 (top) and SUM149PT (bottom) were treated ±10ng/ml VEGFA and SOX2 expression quantitated by QPCR. (B) siSOX2 or control siRNA transfected MDA-MB-231 cells were treated ±10ng/ml VEGFA for 7 days followed by QPCR for SNAI2 expression. (C) MDA-MB-231 transduced with either siSOX2 or control siRNA were treated ± VEGFA for 7 days prior to Western blot for Sox2 and Slug. (D) MDA-MB-231 transduced with either siSOX2 or control siRNA for 48 hrs were treated ± VEGFA for 7 days followed by assays of migration (top) and matrigel invasion (bottom). (E-F) SUM149PT cells transduced with siSOX2 or control siRNA for 48 hrs were treated ± VEGFA for 7 days followed by QPCR for SNAI2 (E) and real-time matrigel invasion assay (F). (G) MDA-MB-231 cells were transected with Sox2 or control vector, and Sox2 and Slug expression were assayed by Western. (H) MDA-MB-231 transduced with Sox2 overexpression or control vector and migration (top) and matrigel invasion (bottom) were assayed using Xcelligence. All data are graphed as mean ± SEM.
3.3.4 miR-452 downregulation is required for VEGFA-mediated increases in slug and invasion

The SNAI2 promoter contains a single, putative Sox2 consensus motif, but Sox2 binding to this motif was not detected after VEGFA treatment. Notably, several studies of global Sox2 DNA binding by ChIP-sequencing also failed to show stable binding of Sox2 to the SNAI2 promoter (Fang et al., 2010; Boyer et al., 2005; Ben-Porath et al., 2008; Engelen et al., 2011). These findings, together with our observation that Sox2 upregulation by VEGFA precedes that of SNAI2 by several days (Figure 3.3A-B and 3.5A) suggested that Sox2-mediated SNAI2 induction is indirect.

Since Sox2 is known to induce several miRNAs to drive stem cell self-renewal, we investigated if a miRNA-driven mechanism might govern Slug upregulation. A miRNA screen of MDA-MB-231 cells before and after VEGFA treatment was performed. Among over seven hundred miRNAs, 47 miRNAs were significantly downregulated by VEGFA (Figure 3.7). The miRNA target prediction software TargetScan (Version 6.2) was used to identify miRNAs decreased by VEGFA that could potentially target SNAI2. Of four potential candidates, miR-452 had the highest probability score for targeting SNAI2 and was investigated herein. A second miRNA target prediction database (microT-CDS version
5.0) verified miR-452 as a putative regulator of SNAI2 expression. VEGFA downregulated miR-452 in both MDA-MB-231 and SUM149PT. miR452 decreased within 6-12 hours and reduced levels persisted after 7 days of VEGFA treatment (Figure 3.8A, bottom panel).

**Figure 3.7. miRNAs are regulated by VEGFA.** MDA-MB-231 cells were treated ±10ng/ml VEGFA for 7 days followed by microRNA array.
To further primary human breast cancer was tested. This culture has been extensively validated and its gene expression and tumor marker profiles resemble those of the cancer from which it was derived (Drews-Elger et al., 2014). Prolonged exposure of DT22 to

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**Figure 3.8.** VEGFA and Sox2 driven miR-452 down-regulation mediates Slug upregulation and breast cancer cell invasion, and miR-452 directly targets the SNAI2 3' UTR. (A) miR-452 expression (QPCR) was compared ± VEGFA for 7 days in MDA-MB-231, SUM149PT and DT22 primary breast cancer culture (top) miR-452 expression levels were assayed by QPCR at indicated times in MDA-MB-231 (bottom) (B) siSOX2 or control siRNA transduced MDA-MB-231 cells were treated ± VEGFA for 7 days and miR-452 expression assayed. (C) MDA-MB-231 was transduced with Sox2 overexpression or control vector and miR-452 expression was assayed by QPCR (D-F) miRNA control or miR-452 vector transduced cells were treated ± VEGFA for 7 days prior to assays of Slug expression by Western (D) and SNAI2 expression by QPCR (E) and real-time matrigel invasion (F). (G-H) MDA-MB-231 were transfected with miR-452 antagonir (inhibitor) or antagonir control followed by assays of SNAI2 by QPCR (G) and of invasion as above (H). All graphs show mean ± SEM. *p<0.05
VEGFA over 7 days also led to miR-452 loss (Figure 3.8A). SOX2 knockdown abrogated the VEGFA driven loss of miR-452, indicating that Sox2 is required for miR-452 downregulation by VEGFA (Figure 3.8B). SOX2 overexpression also reduced miR-452 (Figure 3.8C). To test if VEGFA driven miR-452 down-regulation is essential for VEGFA induced invasion, miR-452 was transduced into MDA-MB-231 and stable clones derived. miR-452 overexpression abrogated VEGFA driven SNAI2 upregulation (Figure 3.8D) and prevented the VEGFA-driven increase in matrigel invasion (Figure 3.8F). Furthermore, inhibition of miR-452 by transfection of a miR-452 antagomir increased SNAI2 expression (Figure 3.8G) and was sufficient to increase matrigel invasion (Figure 3.8H). Thus, miR-452 is required for Sox2-driven Slug upregulation and is critical for VEGFA-driven cell motility and invasion.

3.3.5 miR-452 directly targets SNAI2 3’ UTR to repress slug

Stable overexpression of miR-452 decreased SNAI2 expression (Figure 3.8E) and miR-452 antagomir transfection increased SNAI2 levels in MDA-MB-231 (Figure 3.8G). miRNAs commonly regulate mRNA expression by binding to the 3’UTR. To investigate if miR-452 directly targets the 3’UTR of SNAI2 to repress Slug expression, a reporter assay was performed using the 3’UTR of SNAI2 to drive luciferase expression. 293T and MDA-MB-231 cells were transfected with a human SNAI2 3’UTR luciferase reporter plasmid together with plasmids encoding either the miR-452 precursor or control miRNA and luciferase activity was measured after 48 h. miR-452 transfection significantly reduced luciferase activity, indicating miR-452 targets the SNAI2 3’UTR to repress Slug expression (Figure 3.9A).
There are three putative miR-452 binding sites within the 3’UTR of SNAI2 (Figure 3.9B). A mutant SNAI2 3’UTR clone was constructed in which all three putative miR-452 binding sites were mutationally disrupted. When this mutated vector was co-transfected into 293T cells with the miR-452 precursor plasmid, luciferase activity was not impaired. Thus one or more of these sites is required for miR-452 to inhibit SNAI2 expression (Figure 3.9C). To test if SNAI2 overexpression could rescue the inhibitory effect of miR-452 on VEGFA induced invasion, miR-452 overexpressing MDA-MB-231 cells were transduced with either control vector or human SNAI2 cDNA lacking the 3’UTR region.

Figure 3.9. miR–452 directly targets SNAI2 3’ UTR to repress slug. (A) 293T and MDA-MB-231 were transfected with SNAI2 3’UTR luciferase reporter together with miR-452 precursor or control miRNA plasmid and luciferase activity assayed after 48 h. (B) Sequence alignment of human miR-452 seed regions with SNAI2 3’UTR. (C) SNAI2 3’UTR luciferase reporter plasmid bearing mutations in all 3 putative miR-452 binding sites show loss of luciferase regulation by transfected miR-452 precursor plasmid after 48 h. (D) miR-452 overexpressing MDA-MB-231 was transduced with either human SNAI2 or control vector, then treated ± VEGFA for 7 days followed by real-time matrigel invasion assay. All graphs show mean ± SEM. All graphs show mean ± SEM. *p<0.05
As noted above, miR-452 overexpression abrogated the increased invasion by VEGFA. Overexpression of the 3’UTR deficient \textit{SNAI2} rescued the inhibitory effect of miR-452 on cell invasion (Figure 3.9D), consistent with the notion that miR-452 targets \textit{SNAI2}. Thus, VEGFA-mediated miR-452 downregulation is critical for induction of \textit{SNAI2} and for Slug action on cell motility and invasion.

\textbf{3.3.6 Repression of miR-452 is required for VEGFA dependent cancer metastasis in vivo}

While VEGFA has been shown to drive cancer metastasis (Hirakawa et al., 2005; Kim et al., 2004), mechanisms thereof are largely unknown. To test if VEGFA drives metastasis \textit{in vivo} through regulation of miR-452 and Slug, MDA-MB-231 cells were pre-treated with VEGFA for one week prior to injection by tail vein into nude mice, without further VEGFA treatment after tumor cell injection. Animals were monitored by in vivo imaging system (IVIS). VEGFA pre-treated cells gave rise to a significant increase in lung tumor establishment over the next 5 weeks compared to mock-treated cells. MiR-452 overexpressing cells failed to respond to VEGFA, and showed no effect of VEGFA pre-treatment on tumor metastasis. Notably, transduction of a \textit{SNAI2} cDNA vector lacking the 3’UTR into the miR-452 overexpressing cells rescued the miR-452 mediated inhibition of VEGFA stimulated metastasis \textit{in vivo} (Figure 3.10A-E). VEGFA treatment does not affect MDA-MB-231 cell cycle progression or population growth(Zhao et al., 2014b). Overexpression of miR-452 and \textit{SNAI2} did not change cell proliferation, thus differences in the metastatic tumor burdens of each group are not due to differences in growth rates.

Animals injected with VEGFA-pre-treated cells showed extensive areas of confluent tumor growth on microscopic analysis, precluding accurate enumeration of
Figure 3.10. miR-452 repression is required for VEGFA-induced cancer metastasis in vivo. (A) MDA-MB-231-luc expressing the indicated vectors were pre-treated ±VEGFA and injected via tail vein into nude mice as described in Methods. Representative bioluminescence images of tumor bearing mice at 0 and 5 weeks are shown. The color scale depicts photon flux (photons/s) from xenografted mice. (B) Mean bioluminescence/time of lung metastasis in xenografted mice, graphed as normalized photon flux/time. (C) Mean bioluminescence at 5 weeks (D) Representative images and photomicrographs of lung tumors from indicated groups. (E) Representative immunofluorescence images (4x) of GFP-positive metastasis observed immediately ex-vivo (F) Mean lung weights. All graphs show mean ± SEM. *p<0.05
tumor nodules. As a second measure of lung tumor burden, in addition to IVIS, lung weights were measured. Lung weights were significantly increased in VEGFA treated animals, while those of the VEGFA treated miR-452 overexpression group were not increased compared to controls. Finally, SNAI2 transduction into the miR-452 overexpression group yielded similar lung weights to those in the VEGFA treated group (Figure 3.10F). Thus, VEGFA induced miR-452 repression is required for VEGFA dependent Slug upregulation and cancer metastasis in vivo.

3.3.7 VEGFA, SOX2, SLUG, miR-452, and GABRE expression and prognosis in primary breast cancers

Our in vitro and in vivo models suggest a mechanism in which VEGFA induces EMT and metastasis by activating Sox2, resulting in derepression of SNAI2 through loss of miR-452 (Figure 3.11A). To validate our findings in vivo, we tested if high VEGFA alone or together with high SOX2, SNAI2 and decreased miR-452 expression might identify prognostic subsets of primary human breast cancers. miR-452 is expressed as an intronic transcript from the GABRE gene locus (Kent et al., 2002). Pearson’s correlation analysis of two independent breast cancer datasets, the METABRIC and Enerly datasets (Curtis et al., 2012; Enerly et al., 2011), respectively showed that miR-452 expression correlated strongly with that of its parent transcript, GABRE (R^2 values of 0.484 and 0.786 in the METABRIC and Enerly datasets, respectively; Figure 3.11B), indicating GABRE can be used as a surrogate for miR-452 expression in datasets, such as KM Plotter, that lack microRNA data.
Figure 3.11. Prognostic value of VEGFA, SOX2, SNAI2, miR-452, and GABRE expression in breast cancer patients. (A) Model of mechanism by which VEGFA increases breast cancer invasion and metastasis through Sox2, miR452, and Slug. (B) Correlation of miR-452 and GABRE expression in the METABRIC (left) and Enerly (right) primary breast cancer datasets; $R^2$ values for Pearson's correlation are indicated. (C-D) Kaplan-Meier plots of distant metastasis free survival (DMFS) of all breast cancer patients (C) and those with ER-negative breast cancer (D), stratified by VEGFA expression alone or with sequential inclusion of downstream pathway genes (SOX2, GABRE, SNAI2); data were analyzed and plots generated using the KM Plotter web tool (http://kmplot.com/analysis/). Patients with available clinical data: DMFS, $n=1609$. (E) Kaplan-Meier survival plot of disease-specific survival (DSS) of breast cancer patients from the METABRIC dataset classified by top quartile VEGFA expression (left) or the top 25% patients with the highest mean expression of both VEGFA and SOX2 were classified as "high" (right). Hazard ratios, HR (CI 95%), were determined using Univariate Cox Proportional Hazards analysis and the $p$ values were from the logrank test.
Since Sox2 regulates $SNAI2$ expression via miR-452, we next tested if high $VEGFA$ expression (top quartile) alone or in combination with high $SOX2$, high $SNAI2$ and the lowest quartile $GABRE$/miR-452 expression was associated with distant metastasis free breast cancer survival (DMFS) in the KM Plotter dataset. Differences between groups are shown by graphed Kaplan-Meier curves and hazard ratios (HR) from Univariate Cox Proportional Hazards analysis. Logrank comparison of outcome curves was done and p values are presented in each graph. Of 1609 primary breast cancers in the KM Plotter dataset, those with high $VEGFA$ expression alone showed significantly poorer DMFS (n=1609, HR [95%CI] = 1.45 [1.17-1.81]; p = 0.00082; Figure 3.11C). We next tested elevated $VEGFA$ and $SOX2$ expression, and then evaluated tumors with high levels of $VEGFA$, $SOX2$ and $SNAI2$, and low $GABRE$ (a surrogate for miR-452) expression. Tumors in the top quartile of both $VEGFA$ and $SOX2$ expression showed significantly worse DMFS (HR for recurrence [95%CI] = 1.9 [1.36-2.65]; p = 0.00014; Figure 3.11C), while those with high expression of $VEGFA$, $SOX2$ and $SNAI2$ and the lowest quartile $GABRE$ expression showed an even greater risk of relapse (HR [95%CI] = 2.03 [1.46-2.84]; p = 2.0e-05; Figure 3.11C). A similar analysis showed $VEGFA$ expression was of greater prognostic importance in breast cancers defined as ER-negative by clinical ER protein immunohistochemistry (n=170 with both VEGFA and ER data). Although the top quartile $VEGFA$ expression associated with shorter DMFS (HR [95%CI] = 2.87 [1.69-4.86]; p=4.2e-05), median cutoffs were used to classify "high" or "low" expressers due to the reduced sample size. Using median cutoffs, high $VEGFA$ associated with shorter DMFS (HR [95%CI] = 1.71 [1.01-2.87]; p=0.042; Figure 3.11D). ER-negative cancers with high $VEGFA$ and $SOX2$ expression showed significantly shorter DMFS (HR= 2.72 [95%CI,
Remarkably, among ER-negative breast cancers, those with high \textit{VEGFA}, \textit{SOX2} and \textit{SNAI2} together with low \textit{GABRE} expression showed a 4.54 fold higher risk of metastasis, with DMFS (HR [95%CI] = 4.54 [1.67-12.35]; p=0.0011, Figure 3.11D). These KM-Plotter data identify a very aggressive population within all cancers and in ER-negative breast cancers, in which the mechanistic pathway identified herein appears to be activated. To validate these findings in an independent patient group, a similar analysis was carried out for disease specific survival (DSS) in the METABRIC dataset (n=1286).

This analysis confirmed the prognostic significance of \textit{VEGFA} and showed that elevation of both \textit{VEGFA} and \textit{SOX2} expression associated with a worse survival than did \textit{VEGFA} elevation alone. High \textit{VEGFA} alone conferred a 1.69 fold higher risk of death (DSS HR [95%CI] =1.69 [1.25-2.3], p=0.000595: Figure 3.11E, left), and METABRIC cancers in the top quartile of both \textit{VEGFA} and \textit{SOX2} had even worse outcome (DSS HR [95%CI] =1.76 [1.3-2.38], p = 0.000199; Figure 3.11E, right).

3.4 DISCUSSION

\textit{VEGFA} is best known as an angiogenic agent (Ferrara and Davis-Smyth, 1997) but it also promotes cancer invasion and metastasis through mechanisms that are not fully understood. \textit{VEGFA} not only creates a vascular niche for expanding stem cells (Bao et al., 2006), it was recently shown to increase the stem-like cell population in certain human malignancies, including breast cancer (Beck et al., 2011; Zhao et al., 2014b; Goel et al., 2013; Hamerlik et al., 2012). Hypoxia, caused by angiogenesis inhibitors, stimulates
VEGFA gene expression, and would thus contribute to CSC expansion (Conley et al., 2012) and disease recurrence and progression (Paez-Ribes et al., 2009; Ebos et al., 2009).

CSC have been implicated as drivers of tumor metastasis, however the molecular pathways linking stemness and induction of metastasis are not fully elucidated. Populations bearing surface CSC markers (Todaro et al., 2014; Sheridan et al., 2006; Abraham et al., 2005) or that are enriched for ALDH1 activity (Charafe-Jauffret et al., 2010) have been shown to have greater motility, invasiveness and metastatic potential than the bulk of the cancer population. Recent work in a pancreatic model showed EMT and dissemination may precede overt tumor invasion (Rhim et al., 2012). Circulating tumor cells could be detected during in situ tumor growth, prior to overt invasion. Circulating tumor cells bearing the CSC marker, CD44+, showed much more aggressive self-renewal and tumor generating potential than CD44+-positive cells from the primary tumor site, indicating that escape of stem-like cells from the primary tumor environment is linked to increased self-renewal potential (Rhim et al., 2012).

Sox2 is an important mediator of self-renewal in embryonic stem cells and is an oncogenic driver of CSC in several cancer models, including breast cancer (Leis et al., 2011; Xiang et al., 2011; Picon-Ruiz et al., 2016; Zhao et al., 2014b). Our prior work showed VEGFA mediates CSC expansion via STAT3 driven SOX2 induction in breast and lung cancer models (Zhao et al., 2014b). Proinflammatory cytokines that are upregulated upon breast cancer cell invasion into fat also induce SOX2 to drive CSC self-renewal (Picon-Ruiz et al., 2016). SOX2 knockdown can decrease both CSC and experimental lung metastasis (Xiang et al., 2011) and high SOX2 expression in colon cancer is associated with increased metastasis (Han et al., 2012). Present work reveals Sox2 is necessary for
VEGFA-driven \textit{SNAI2} induction, EMT, and invasion of breast cancer cells and provides a mechanistic link between VEGFA stimulated CSC expansion via \textit{SOX2} induction (Zhao et al., 2014b), and the upregulation of metastatic potential.

EMT arising from overexpression of various EMT-TFs has been shown to increase tumor initiating cell abundance (Mani et al., 2008; Morel et al., 2008); moreover, stem like cells exhibit EMT properties such as increased mesenchymal markers and EMT transcription factor expression (Gupta et al., 2009) suggesting an intimate relationship between CSCs and EMT. Mammary cell lines overexpressing various EMT-TFs showed PLC\textgamma-mediated PKC activation leading to a cJun/Fra1-induced CSC transcriptional program (Tam et al., 2013). TGF-\beta and TNF\alpha pathways interact to drive both EMT and upregulate breast CSC properties (Polyak and Weinberg, 2009). Elegant \textit{in vitro} and \textit{in vivo} studies in a Trp53-null mouse breast cancer model showed cross talk between transformed mesenchymal cells and tumor initiating subpopulations in which the mesenchymal cells produced stimulatory ligands driving CSC surface receptors to increase tumorigenicity and metastasis via both Wnt/Fzd7 and CXCL12/CXCR4 pathways (Zhang et al., 2015), suggesting that heterogeneous cell populations with differing stem cell self-renewal may interact with each other to drive pathways governing both self-renewal and metastasis.

miRNAs regulate many processes central to oncogenesis (Ceppi and Peter, 2014). Several miRNAs oppose EMT by targeting EMT transcription factors (Tavazoie et al., 2008). miR-200 targets Zeb1 and Zeb2 (Park et al., 2008; Korpal et al., 2008; Gregory et al., 2008; Burk et al., 2008) as does miR-138 (Liu et al., 2011b), and Snail is targeted by miR-30a (Kumarswamy et al., 2012) and miR-34 (Kim et al., 2011). A number of miRNAs
not only regulate EMT but also serve as key CSC regulators. For example, miR-200 not only inhibits EMT by suppressing Zeb1/2, but also downregulates stem-like cells by targeting Bmi1 (Shimono et al., 2009; Wellner et al., 2009) and the Notch pathway (Brabletz et al., 2011). In addition to its action on Snail, miR-34a also decreases CSC by targeting Myc (Yamamura et al., 2012) and downregulates CD44 expression to decrease prostate CSC (Liu et al., 2011a). miR-128-2 targets both the EMT mediator Snail, and CSC drivers Nanog, KLF4 (Qian et al., 2012) and Bmi1 (Godlewski et al., 2008).

Here we identify miR-452 at the interface between VEGFA-activated CSC self-renewal and EMT, providing a novel connection between the multi-functional cytokine VEGFA, induction of the embryonic stem cell transcription factor Sox2, and EMT. Thus, Sox2 not only governs CSC expansion, but also mediates acquisition of EMT and metastatic potential. VEGFA-induced SOX2 expression is required not only for CSC expansion (Zhao et al., 2014b), but also for VEGFA-mediated SNAI2 induction. VEGFA increased SOX2 expression within an hour, but EMT-TF levels, and in particular that of Slug, rose over several days, suggesting that Sox2 affects Slug indirectly, via an intermediary mechanism. Our miRNA screen identified miR-452 as a putative metastasis suppressor, significantly downregulated by VEGFA in MDA-MB-231 cells. miR-452 is down-regulated in breast cancers compared to normal breast tissue (van Schooneveld et al., 2012). We show miR-452 targets SNAI2 directly and miR-452 loss is required for Sox2-driven Slug upregulation and for VEGFA-driven cell motility, invasion and metastasis in vivo in breast cancer models. Notably, miR-452 expression correlates inversely with glioblastoma survival and inhibits glioma stem cells and tumorigenesis by targeting CSC mediators, Bmi1, LEF1 and TCF4 (Liu et al., 2013a). Thus, miR-452 may not only regulate
EMT via Slug, but may also serve a dual role at the interface between EMT and CSC regulation.

The pathway linking VEGFA to Sox2 upregulation, miR452 loss and \( SNAI2 \) induction is supported by our analysis of two major datasets including over 2500 primary human breast cancers. Although intra-tumor VEGFA upregulation detected by immunohistochemistry has been linked to poor breast cancer outcome, most studies have been small and results controversial (Arias-Pulido et al., 2012; Sa-Nguanraksa et al., 2015). Our analysis showed breast cancers in the highest quartile of VEGFA expression fare worse than all others, and the prognostic value of high VEGFA levels is increased by sequential addition of high \( SOX2 \), \( SNAI2 \) and decreased \( GABRE \) (a surrogate for miR-452) expression. This finding is less important for its prognostic significance than it is as a confirmation of the molecular pathway identified herein. Among aggressive breast cancers expressing high VEGFA, those with SOX2 overexpression define an even more aggressive subgroup in the two independent datasets (KM Plotter and Metabric) evaluated.

VEGFA is a critical mediator of tumor progression. It acts to generate a vascular niche for CSC through autocrine and paracrine action on both tumor and microenvironmental components, and links CSC self-renewal to the acquisition of metastatic potential. To date, targeting VEGFA has had limited success in cancer, and this may be due to anti-angiogenics causing tumor hypoxia, leading to upregulation of both VEGFA and CSC. Since treatment of metastasis is the final therapeutic frontier, it is hoped that mechanistic insights linking VEGFA to tumor initiation and the acquisition of metastatic potential will ultimately generate new strategies for VEGF-pathway targeted intervention.
CHAPTER 4:

C-TERMINALLY PHOSPHORYLATED p27 PROMOTES CANCER METASTASIS BY FORMING A TRANSCRIPTIONAL CO-ACTIVATOR COMPLEX WITH CJUN TO DRIVE TGF-β2 AND EMT
4.1 SUMMARY

In normal cells, p27 restrains the cell cycle by inhibiting cyclin-CDKs. In human cancers, p27 is deregulated by PI3K-dependent phosphorylation at T157 or T198 that impede p27 import leading to p27 localization in both the cytoplasm and nucleus. We and others have identified an oncogenic role for p27 in mediating motility, invasion and metastasis resulting from these C-terminal phosphorylations. The present work investigated mechanisms whereby p27 contributes to EMT and metastasis.

p27 knockdown in highly metastatic MDA-MB-231-1833 cells induced re-expression of metastasis-suppressor genes, and reverted an EMT gene signature. p27 knockdown in PI3K-activated, metastatic lines reduced expression of EMT drivers including SNAI1, SNAI2, ZEB2, and TGF-β2, but not TGF-β1. Transduction of a cell cycle defective (CK-), double phosphomimetic p27CK-DD, into less metastatic or non-transformed cell lines increased SNAI1, SNAI2, ZEB2, and TGF-β2 expression. Notably, TGF-β2 knockout reduced invasion, downregulated EMT drivers and reverted EMT in p27CK-DD transduced cells suggesting p27pT157pT198 promotes EMT and metastasis through TGF-β2.

p27CK-DD over-expression in human mammary epithelial cells increased cJun activity and cJun activity was decreased by p27 knockdown in MDA-MB-231-1833. In silico analysis revealed an AP-1 binding site upstream of the TGF-β2 promoter. Immunoprecipitation showed p27 binds JNK/cJun in the cytoplasm but binds only cJun in the nucleus. ChIP analysis showed p27 co-localized with cJun and RNA PolII at an AP-1 motif -15KB upstream of the TGF-β2 transcriptional start site to induce TGF-β2. Furthermore, p27CK-DD transduced cells show greater p27/cJun binding to the upstream
TGF-β2 enhancer site compared to control cells in Quantitative ChIP assays, suggesting that C-terminal p27 phosphorylation may enhance cJun-driven TGF-β2 gene induction by increasing its binding to the upstream of TGF-β2 promoter.

4.2 INTRODUCTORY REMARKS

Breast cancer is the most common cancer and the second-leading cause of cancer related death in women. Distant metastases are the main cause of breast cancer related death (Chaffer and Weinberg, 2011). Cancer metastasis is a complex process in which tumor cells spread to distant tissues and proliferate in an aberrant tissue niche. Initiation of metastasis requires invasion to escape from a primary tumor. It is enabled by EMT which is a process in which cells lose cellular adhesion and gain increased cell motility and invasiveness (Thiery, 2002; Kalluri and Weinberg, 2009; Micalizzi et al., 2010; Thiery et al., 2009; Satelli and Li, 2011). This process is regulated by many signaling pathways including changes in key transcription factors.

p27 is a cell cycle regulator and a tumor suppressor that normally restrains cell growth. p27 is aberrantly regulated by several oncogenic signaling pathways in cancer (Chu et al., 2008). Normal epithelial cells express high nuclear p27, but human cancers often have reduced nuclear p27 due to increased p27 proteolysis (Catzavelos et al., 1997; Porter et al., 1997; Tan et al., 1997; Tsihlias et al., 1998; Chu et al., 2008). While most human cancers exhibit reduced nuclear p27 levels, these frequently also show cytoplasmic mislocalization of p27. p27 is rarely completely lost in cancers, but it is frequently deregulated: nuclear p27 protein is reduced and/or p27 is mislocalized to the cytoplasm in a majority of human cancers (Buse et al., 1999). Cytoplasmic p27 predicts a poor prognosis.
in several human cancers (Liang et al., 2002b; Fukumoto et al., 2004; Rosen et al., 2005; Li et al., 2006). Increased cytoplasmic p27 is seen in cancers where the PI3K/mTOR pathway is activated. For example, in up to 40% of early breast cancers cytoplasmic p27 is seen in association with AKT activation (Liang et al., 2002b; Shin et al., 2002; Viglietto et al., 2002). The phospho-inositol 3’ kinase (PI3K) pathway is a master regulator of cell differentiation, growth, proliferation, survival, and invasion (Coffer et al., 1998; Toker and Newton, 2000; Vanhaesebroeck and Alessi, 2000) that is oncogenically activated in most human cancers (Lawlor and Alessi, 2001; Vivanco and Sawyers, 2002).

We and others showed that phosphorylation of p27 at T157and T198 (p27pTpT) by PI3K-dependent kinases impairs its nuclear import and leads to stabilization and accumulation in the cytoplasm where it has been shown to promote cell motility, invasion and metastasis in several different tumor models (Larrea et al., 2009b; Wu et al., 2006; Wander et al., 2013). Furthermore, we recently showed that p27 promotes JAK2/STAT3 complex formation to enhance STAT3 activation and STAT3-mediated Twist induction to promote EMT and metastasis. The present work was undertaken to investigate additional mechanisms whereby c-terminally phosphorylated p27 contributes to EMT and metastasis.

4.3 RESULTS

4.3.1 p27 downregulates putative metastasis-suppressor genes and induces an EMT program

Our prior work showed p27 mediates PI3K-induced metastasis in breast and bladder models, and that this requires C-terminal phosphorylation of p27 (Wander et al., 2013; Zhao D. et al., 2015). To further elucidate effects of p27 on metastasis, the gene
expression profiles of parental low metastatic MDA-MB-231 (hereafter 231), a line with low metastatic ability, and its bone-tropic metastatic derivative line, MDA-MB-231-1833 (hereafter 1833) were compared. These differentially expressed genes were compared to the “bone metastatic signature” (BMS) gene expression profile generated by Kang et al (Kang et al., 2003). Expression of BMS genes was compared using gene expression microarrays in parental 231, 1833, and in 1833shp27, in which p27 was stably knocked down (Figure 4.1A). Of note, a profile of genes that were reduced during the transition from low metastatic 231 to highly metastatic 1833 were upregulated following p27 knockdown in 1833 (Figure 4.1A). Thus, loss of p27 promotes re-expression, in 1833 cells, of a subset of putative bone metastasis suppressor genes.

Since aberrant EMT activation is implicated in cancer metastasis, we investigated if p27 may orchestrate aspects of an EMT. While 231 shows features of an EMT-like transformation (Taube et al., 2010), we postulated that mesenchymal characteristics might be further enhanced in 1833. To further explore the effects of p27 loss, genes differentially expressed in 1833 versus 1833shp27 were compared to a previously established “EMT core signature” derived by over-expression of master EMT regulators in immortalized human mammary epithelial cells (Taube et al., 2010). p27 knockdown in 1833 significantly altered the expression of forty genes in this EMT core signature “away from” an EMT phenotype (Figure 4.1B and 4.1C top). EMT core upregulated genes and core downregulated genes are shown in Figure 4.1C (bottom). Genes upregulated in the EMT core signature were reduced by p27 knockdown in 1833, and genes downregulated in the EMT core signature were increased in 1833 with p27 knockdown. Thus, p27 knockdown
reverts expression of an EMT profile, supporting the notion that p27 may drive metastasis, in part, through activation of an EMT transcriptional program.

Figure 4.1. p27 shifts an EMT program. (A) Gene expression analysis of parental 231, metastatic 1833, and 1833shp27 identifies a gene pattern in a bone metastatic signature mediated by p27 (left). (Right) Gene set analyses for BMS downregulated genes in 1833 scr vs.1833 shRNA p27. Shown are ordered gene scores for each gene in the line plot and the average fold change in the heatmap (orange indicates high expression in 1833 shRNAp27 and blue is low). Average fold gene expression changes are indicated by bar graphs. (B) Genes changed with p27 knockdown in the BMS revert away from a previously established EMT core signature. (C) Flow chart shows comparison of p27-regulated genes in both signatures (top). Graphs show changes in individual gene scores with p27 knockdown compared to EMT core signature (bottom).
Gene Symbol | 231 | 1833 | 1833shp27
---|---|---|---
CYP1B1 | 11.16 | 8.9 | 10.15
BCL3 | 8.03 | 7.25 | 8.21
HLA-DPA1 | 7.64 | 6.4 | 7.27
GPRC5C | 7.23 | 6.92 | 7.73
DDX10 | 11.06 | 10.98 | 11.78
HLA-DRA | 9.62 | 8.59 | 9.33
ITGB4 | 6.07 | 5.77 | 6.47
IL1 | 9.42 | 8.85 | 9.53
FTH1 | 10.31 | 9.48 | 10.11
IL15 | 7.56 | 7.22 | 7.78
GBP2 | 7.92 | 7.91 | 8.4
DOR | 5.7 | 5.42 | 5.89
TGFBI | 11.56 | 8.78 | 9.23
MAP4K4 | 8.17 | 7.77 | 8.2
MALL | 7.77 | 7.76 | 8.15
SERPIN1A1 | 7.47 | 5.68 | 6.06
CXCR4 | 8.6 | 7.84 | 8.21
RBM47 | 8.8 | 6.67 | 7.02
S100A3 | 9.97 | 9.27 | 9.62
TMC5 | 5.62 | 5.3 | 5.63
SCAMP4 | 7.43 | 6.97 | 7.28
S100A2 | 7.31 | 6.14 | 6.43
CX3CL1 | 6.16 | 5.47 | 5.72
PPL | 6.12 | 5.99 | 6.22
HIST1H2BH | 5.93 | 5.9 | 6.11
ABCC3 | 8.61 | 6.78 | 6.91
SCNN1A | 6.37 | 5.32 | 5.44
TNFAIP2 | 6.9 | 6.18 | 6.27
WIPF1 | 7.72 | 7.24 | 7.33
CALHM2 | 7.18 | 7.12 | 7.2
KRT7 | 9.63 | 6.88 | 6.93
MCAM | 6.42 | 5.54 | 5.59
POMZP3 | 7.07 | 6.58 | 6.62
CHST15 | 10.2 | 9.62 | 9.65
CSGALNACT1 | 5.54 | 5.2 | 5.23
TUSC3 | 9.26 | 8.11 | 8.13
SLC38A6 | 8.43 | 8.23 | 8.24
ARHGEF10 | 9.58 | 8.8 | 8.8
PON2 | 9.27 | 7.59 | 7.48
COL5A1 | 10.49 | 9.05 | 8.75
CAV2 | 10.9 | 9.82 | 9.25
LAMB1 | 9.16 | 8.17 | 7.96
FKBP11 | 7.67 | 6.73 | 6.6
CTSB | 8.7 | 7.91 | 7.57
TUBA4A | 10.68 | 10.01 | 9.97
DLC1 | 7.12 | 6.51 | 6.24
FN1 | 5.31 | 4.88 | 4.76

| Gene Symbol | 231 | 1833 | 1833shp27
---|---|---|---
SORL1 | 6.01 | 5.59 | 5.58
CLU | 5.68 | 5.51 | 5.45
HLA-DPB1 | 5.09 | 5.04 | 4.92
CCDC68 | 5.67 | 5.64 | 5.63
CNPY2 | 10.47 | 10.47 | 10.42
FST | 9.18 | 9.73 | 8.52
KHDRBS3 | 8.49 | 9.79 | 9.37
FHL1 | 7.68 | 8.78 | 8.45
ADAMTS1 | 8.88 | 10.02 | 9.98
NAP1L3 | 7.54 | 7.73 | 6.85
CTGF | 11.58 | 12.22 | 11.98
FGF5 | 6.08 | 6.66 | 6.47
TRMT1 | 9.61 | 10.2 | 10.03
MTF | 7.35 | 8.1 | 8.09
SOC52 | 8.59 | 9 | 8.74
MRPS18B | 11.41 | 12.02 | 11.98
SRGN | 12.6 | 13.14 | 13.13
HFE | 5.28 | 5.69 | 5.58
SLC4A7 | 7.36 | 7.81 | 7.77
PTK7 | 7.16 | 7.58 | 7.52
NEDD4L | 8.58 | 8.82 | 8.6
FARSA | 9.01 | 9.39 | 9.33
SPP1 | 5.78 | 6.02 | 5.85
E2F8 | 6.31 | 6.47 | 6.23
FAM11A | 5.67 | 5.76 | 5.59
EXOG | 6.59 | 6.67 | 6.51
SOX4 | 7.52 | 8.11 | 8.11
SPAST | 7.41 | 7.92 | 7.93
FNY | 6.56 | 6.94 | 6.97
GSE1 | 9.57 | 10.07 | 10.11
TNKS | 5.96 | 6.06 | 6.11
LRR210 | 10.26 | 11.34 | 11.4
AOX1 | 9.56 | 10.49 | 10.57
SEL1L3 | 5.61 | 6.72 | 6.81
RMN5A | 6.66 | 6.7 | 6.82
R3DMD2 | 6.75 | 6.8 | 6.94
SIX2 | 5.96 | 6.01 | 6.16
PFN2 | 9.64 | 10.49 | 10.68
CRADD | 9.19 | 10.73 | 10.98
SAMD4A | 7.21 | 7.46 | 7.73
SOS1 | 6.07 | 6.37 | 6.8
MMP1 | 9.4 | 13.06 | 13.66
ST3GAL6 | 7.72 | 8.72 | 9.33
DUSP1 | 9.78 | 10.82 | 11.54
HIST1H2AC | 9.23 | 10.36 | 11.11
NCF2 | 7.44 | 7.65 | 8.47

Table 4.1. Bone metastatic gene signature regulated by p27. BMS signature genes in 231, 1833, and 1833shp27 cells (fold change listed, log2). These values correspond to data presented in Figure 4.1A.
Table 4.2

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>1833 vs. 1833 shp27</th>
<th>Taube EMT Core List</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1B</td>
<td>3.67</td>
<td>-7.23</td>
</tr>
<tr>
<td>PI3</td>
<td>3.66</td>
<td>-7.96</td>
</tr>
<tr>
<td>COL17A1</td>
<td>2.03</td>
<td>-5.04</td>
</tr>
<tr>
<td>F11R</td>
<td>1.53</td>
<td>-3.85</td>
</tr>
<tr>
<td>RHBDF2</td>
<td>1.49</td>
<td>-2.61</td>
</tr>
<tr>
<td>SERPIND2</td>
<td>1.47</td>
<td>-26.88</td>
</tr>
<tr>
<td>C10orf116</td>
<td>1.42</td>
<td>-1.46</td>
</tr>
<tr>
<td>ITGB4</td>
<td>1.41</td>
<td>-4.76</td>
</tr>
<tr>
<td>HBEGF</td>
<td>1.38</td>
<td>-6.44</td>
</tr>
<tr>
<td>C6orf105</td>
<td>1.38</td>
<td>-3.79</td>
</tr>
<tr>
<td>THBD</td>
<td>1.36</td>
<td>-2.96</td>
</tr>
<tr>
<td>DST</td>
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<td>-4.67</td>
</tr>
<tr>
<td>ALDH1A3</td>
<td>1.33</td>
<td>-8.36</td>
</tr>
<tr>
<td>ANXA8</td>
<td>1.33</td>
<td>-3.95</td>
</tr>
<tr>
<td>PERP</td>
<td>1.33</td>
<td>-3.67</td>
</tr>
<tr>
<td>CLDN1</td>
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</tr>
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<td>-5.27</td>
</tr>
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<td>SLP1</td>
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</tr>
<tr>
<td>C10orf10</td>
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</tr>
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<td>1.23</td>
<td>-4.47</td>
</tr>
<tr>
<td>MYO5C</td>
<td>1.20</td>
<td>-5.32</td>
</tr>
<tr>
<td>CST6</td>
<td>1.20</td>
<td>-4.46</td>
</tr>
<tr>
<td>JUP</td>
<td>1.14</td>
<td>-4.08</td>
</tr>
<tr>
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<td>-4.91</td>
</tr>
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<td>KRT15</td>
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</tr>
<tr>
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</tr>
<tr>
<td>NEBL</td>
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<td>10.25</td>
</tr>
<tr>
<td>COL5A2</td>
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<td>24.63</td>
</tr>
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<td>PRR16</td>
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<td>13.10</td>
</tr>
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<td>3.01</td>
</tr>
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<td>FSTL1</td>
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<td>4.79</td>
</tr>
<tr>
<td>NR2F1</td>
<td>-1.55</td>
<td>38.58</td>
</tr>
<tr>
<td>HAS2</td>
<td>-1.67</td>
<td>7.29</td>
</tr>
</tbody>
</table>

Table 4.2. EMT core gene signature changed by p27 knockdown. EMT core genes that demonstrate inverse expression following p27 knockdown in 1833 cells. These values correspond to data presented in Figure 4.1B and C.
4.3.2 EMT markers and transcription factors are regulated in a p27pT198pT158-dependent manner

To confirm that p27 knockdown drives key EMT markers, E-cadherin, N-cadherin, and Vimentin levels were compared in parental 231 and metastatic 1833 cells with and without stable p27 knockdown. Indeed, 1833 expressed lower E-cadherin and higher N-cadherin and Vimentin than 231 (Figure 4.2A). Stable p27 knockdown increased CDH1 expression in 1833 (Figure 4.2B left), and acute siRNA-mediated p27 increased E-cadherin protein at 48-72 hours (Figure 4.2B right). Thus, loss of p27 reverts a cellular phenotype away from EMT, decreases EMT markers, and reverts elements of the EMT phenotype.

The EMT program is orchestrated by the expression of master transcriptional regulators (EMT-TFs) including Snail, Slug, Twist1, and Zeb2. MDA-MB-231-4175 (4175) (Minn et al., 2005b) and MDA-MB-231-1833 (1833) (Kang et al., 2003) are lung and bone-tropic derivatives of the breast line MDA-MB-231, respectively. UMUC3-LuL2 is a metastatic derivative of the bladder carcinoma line UMUC3 (Overdevest et al., 2011). All three of these lines are PI3K-activated and express high endogenous p27pT157pT198 (Zhao D. et al., 2015). p27 knockdown by siRNA acutely downregulated EMT-TF expression within 12-24 hours (Figure 4.2C).

We previously showed that transduction of a phosphomimetic p27 mutant protein defective in cyclin-CDK binding (p27CK-DD) can induce EMT (Zhao D. et al., 2015). To evaluate effects of C-terminally phosphorylated p27 in more epithelial models, non-transformed mammary epithelial line MCF12A and lines with low metastatic potential, 231 and UMUC3, were transduced with p27CK-DD. In all models tested, p27CK-DD upregulated EMT-TFs (Figure 4.2D).
Figure 4.2. Key EMT markers and transcription factors are regulated in a p27pT198pT158-dependent manner (A) Stable p27 knockdown in metastatic 1833 cells reverts the expression of key EMT markers. (B) In 1833 cells, p27 knockdown increases E-cadherin mRNA (top) and protein re-expression within 48 hours. (C) Acute p27 knockdown reduces several EMT transcription factors in PI3K-activated, metastatic lines. (D) p27CK-DD increases several EMT transcription factors in non-invasive MCF12A and low metastatic 231 and UMUC3. All graphs show mean ± SEM. *p<0.05
4.3.3 TGF-β2 is required for p27pT157pT198 induced EMT and increased invasion

The TGF-β pathway is a major driver of EMT in transformed cells. Notably, TGF-β2 expression was significantly downregulated 4-8 hours following acute p27 loss in all three metastatic models, while that of TGF-β1 was unchanged (Figure 4.3A). Indeed, a time course following acute p27 knockdown in 4175 cells showed loss of p27 protein after one hour, and this was followed by a significant reduction in TGF-β2 protein around 4 hours (Figure 4.3B). In all models tested, TGF-β2 expression and secretion were increased by p27 CK-DD transduction, while that of TGF-β1 was unchanged (Figure 4.3C and D). Thus, C-terminally phosphorylated p27 may turn on an EMT program by first inducing upregulation of the ligand, TGF-β2, with subsequent induction of EMT-TF expression to drive a metastatic phenotype.

Since C-terminally phosphorylated p27 enhances TGF-β2 expression, the effect of TGF-β2 effect on EMT was tested. TGF-β2 exposure upregulated expression of EMT TFs including Snail and Slug and also promoted invasion in 231 and UMUC3 (Figure 4.3E and G). In more the immortal non-transformed breast epithelial MCF-12A cells, TGF-β2 caused a morphological switch from a cobblestone-like epithelial appearance to a spindle-like, mesenchymal shape. To investigate whether TGF-β2 regulates p27pT157pT198 induced EMT and increased invasion (Zhao D. et al., 2015), TGF-β2 was knocked down. p27CK-DD induced invasion and EMT, and the increase in the expression of EMT TFs were all inhibited by knockdown of TGF-β2 indicating TGF-β2 is required for p27pT157pT198 induced EMT and increased invasion (Figure 4.3H-J).
Figure 4.3. TGF-β2 is required for p27pT157pT198 induced EMT and increased invasion. (A) Acute p27 knockdown reduces TGF-β2 mRNA, and not TGF-β1 mRNA, in PI3K-activated, metastatic lines. (B) A timecourse following acute p27 knockdown in lung-tropic, metastatic 4175 cells shows TGF-β2 protein reduced around 4 hours following a drop in p27 protein levels one hour after addition of p27-specific siRNA. (C) TGF-β2 mRNA is upregulated in non-invasive MCF12A and low metastatic 231 and UMUC3 cells expressing p27CK-DD, while TGF-β1 is unchanged. (bottom). (D) p27CK-DD increased TGF-β2 secretion in non-invasive MCF12A and low metastatic 231 and UMUC3 cells. (E) Snail and Slug are upregulated by TGF-β2 treatment in 231 and UMUC3 cells. (F) Morphology of MCF12A cells are switched from epithelial appearance to mesenchymal shape. (G) TGF-β2 increased invasion in 231 and UMUC3 cells. (H) p27CK-DD induced invasion is inhibited by TGF-β2 k/d. (I) EMT marker expression are reversed by TGF-β2 k/d. (J) Upregulated expression of EMT TFs in p27CK-DD expressing cells are downregulated by TGF-β2 k/d. All graphs show mean ± SEM. *p<0.05
4.3.4 cJun activation and its binding to p27 are p27pT157pT198-dependent

We next investigated how p27 directs this specificity. Since a phosphoprotein array comparing proteins and kinases differentially activated in MCF12A cells with and without p27CK-DD revealed that activated cJun (cJunpS63) is significantly increased in p27CK-DD-expressing cells (Figure 4.4), cJun activation were compared in multiple cell lines including 231, 231 p27CK-DD, 1833, 1833 shp27, UMUC3, UMUC3 p27CK-DD, UMUC3-LuL2 and UMUC3-LuL2 shp27. p27CK-DD expression in non-metastatic 231 and UMUC3 significantly increased activated cJunpS63, while p27 knockdown in metastatic 1833 and UMUC3-LuL2 cells reduced cJunpS63 (Figure 4.5A). cJun is a transcription factor that forms part of the heterodimeric AP-1 complex. cJun contributes to oncogenic transformation (Hess et al., 2004a). An in silico search revealed that the genomic region upstream of the TGF-β2 coding sequence contains several putative AP-1 binding sites.

Figure 4.4. p27CK-DD induces activation of diverse kinases including cJun. (A) Phospho kinome array shows proteins and kinases differentially activated in MCF12A cells with and without p27CK-DD (B) Activated cJun (cJunpS63) is significantly increased in p27CK-DD-expressing cells.
Figure 4.5. cJun activation and its binding to p27 are p27pT157pT198-dependent. (A) Western blots show increased cJunS63 in p27CK-DD (DD) expressing 231 and UMUC3 cells and in metastatic 1833 and UMUC3-LuL2 cells compared to parental controls. p27 knockdown in 1833 and UMUC-LuL2 reduces cJunS63 compared to controls. (B) Westerns show the indicated input proteins (left) in lysates of UMUC3 control (C) and UMUC3 p27CK-DD (DD) used for immunoprecipitation of p27 (right). p27 immunoprecipitates contain similar JNK but higher levels of associated cJun in DD compared to parental controls (C). (D) Densitometric quantitative analysis of p27-bound JNK or cJun normalized to total precipitated p27 shows increased p27-bound cJun in p27CK-DD expressing cells. (E-F) Nuclear (N)-cytoplasmic (C) fractionation of MDA-MB-231-1833 (E) and UMUC3-LuL2 (F) cell lysates shows cJun is largely nuclear and JNK is largely cytoplasmic (left). p27 immunoprecipitates show p27-associated cJun and JNK present in cytoplasm and p27-bound cJun in the nucleus (right). All graphs show mean ± SEM. *p<0.05
sites, while the TGF-β1 promoter has none. p27 immunoprecipitation revealed cJun binds cellular p27, and this complex was significantly increased in p27CK-DD-transduced cells (Figure 4.5B and C).

Treatment with PI3K/mTOR inhibitor, PF1502 (250nM), inhibits AKT activation and reduces p27 phosphorylation at T198 (Figure 4.5D left) and T157 (Zhao D. et al., 2015). In UMUC3-LuL2 cells, PF1502 treatment for 48 hours decreased p27/cJun complexes (Figure 4.5D right). Thus, p27/cJun binding in PI3K-activated cells appears to require p27 C-terminal phosphorylation.

We recently showed that p27 promotes JAK2/STAT3 complex formation to enhance STAT3 activation and STAT3-mediated Twist induction (Zhao D. et al., 2015). To test if p27 may facilitate cJun activation by cJun N-terminal kinase (JNK), p27 binding to these proteins was assayed in fractionated cell lysates. In UMUC3-LuL2, JNK was largely cytoplasmic, while both total and phosphorylated cJun localize predominantly in the nucleus (Figure 4.5E and F left). Immunoprecipitation of p27 in fractionated lysate allowed detection of a low level tripartite JNK/cJun/p27 complex in the cytoplasm and a more abundant p27/cJun complex in the nucleus (Figure 4.5E and F right). Taken together, these data support a model in which a p27/JNK/cJun complex forms transiently in the cytoplasm, followed by translocation of activated cJunS63 p27pT157pT198 to the nucleus.

4.3.5 cJun and p27 form a transcriptional complex at the TGF-β2 enhancer region

Recent data suggest that p27 may play a role as a co-regulator of other transcription factors (Pippa et al., 2012). To further investigate the mechanism of p27-activated TGF-
β2 expression, we next assayed if the nuclear p27/cJun complex might bind AP-1/cJun binding motifs upstream of the TGF-β2 promoter. We tested two putative cJun binding sites, identified by our in silico search to contain TPA-response element (TRE) motifs at approximately 17kb upstream and 5kb downstream of the TGF-β2 gene transcriptional start site. Chromatin immunoprecipitation assays showed both cJun and p27 bind to the TRE located approximately 17kB upstream of the TGF-β2 transcriptional start site (base pairs 218500872-79) in 1833 cells (Figure 4.6A). Quantitative ChIP analysis in MCF12A, 231 and UMUC3 shows greater cJun binding to the upstream enhancer site in TGF-β2 in p27CK-DD expressing cells compared to parental controls, suggesting that C-terminal p27 phosphorylation may increase its binding to the TRE upstream of TGF-β2. In addition, cJun binding to the upstream enhancer site in TGF-β2 was increased in PI3K activated 1833, 4175 and UMUC3-LuL2 lines compare to parental controls. In 1833, 4175 and UMUC3-LuL2 cells, PF1502 treatment decreased cJun binding to the upstream enhancer site of TGF-β2 (Figure 4.6B-D). p27 knockdown decreased cJun recruitment to the TGF-B2 enhancer site (data not shown). Dissociation of cJun ChIPs followed by re-ChIP with anti-p27 antibody showed the TGF-β2 upstream enhancer site is co-occupied by cJun and p27. Dual p27-cJun binding was increased in p27CK-DD expressing or PI3K activated cells, and was inhibited by PF1502 treatment. These data suggest that p27pT157pT198 may enhance p27/cJun driven TGF-β2 gene induction (Figure 4.6B-D).

4.3.6 cJun and TGF-β2 is required for p27pT157pT198 dependent metastasis in vivo

Given the importance of EMT program induction and maintenance for cancer metastasis, we next tested the effect of PI3K activated, C-terminally phosphorylated p27 and cJun or
**Figure 4.6. cJun and p27 bind the TGF-β2 enhancer region.** (A) Chromatin immunoprecipitation using cJun and p27 antibodies, respectively, show both proteins bind to a site at -17Kb in the TGF-β2 enhancer in 1833 cells. (B-D) ChIP assays (top) show cJun binding to the TGF-β2 enhancer is greater in p27CK-DD expressing MFC12A, 231 and UMUC3 cells than in control cells. 1833, 4175 and UMUC3-LuL2 show greater cJun binding to the TGF-β2 enhancer than in 231 or UMUC3 cells and cJun binding is decreased by PF1502. ChIP-RE-ChIP (bottom) shows p27 is detected at the cJun-bound TGF-β2 enhancer site. All graphs show mean ± SEM. *p<0.05
TGF-β2 knockdown on metastasis in vivo. 231 cells were transduced p27CK-DD with and without either cJun or TGF-β2 knockdown, and cells were injected into the mammary fat pad of mice and tumor growth was monitored. For assessment of metastasis, orthotopic primary tumors were removed when they reached approximately 300 mm³ and mice were monitored by IVIS for formation of metastasis from primary site. p27CK-DD transduced cells gave rise to a significant increase in metastasis compared to control cells. Notably, knockdown of cJun or TGF-β2 in p27CK-DD expressing cells caused a significant decrease in p27CK-DD stimulated metastasis in vivo (Figure 4.7A-C). These provide strong evidence that p27pT57pT198 mediated cJun activation and TGF-β2 induction are required for p27CK-DD enhanced metastasis in breast models.

Figure 4.7. cJun and TGF-β2 is required for p27pT157pT198 dependent metastasis in vivo. (A) cJun and TGF-β2 expression assayed by western blot or QPCR in MDA-MB-231 p27CK-DD transduced with shcJun, shTGF-β2 or control shRNA. (B) MDA-MB-231 expressing control vector or indicated vectors were injected via mammary fat pad injection into NSG mice as described in Methods. Representative bioluminescence images of tumor bearing mice at 3 weeks after removal of primary tumors are shown. The color scale depicts photon flux (photons/s) from tumor bearing mice. (C) Mean bioluminescence/time of metastasis in xenografted mice is graphed as normalized photon flux/time. All graphs show mean ± SEM. Differences between tumor growth curves were calculated using "compareGrowthCurves" function of the statmod software package at the following website (http://bioinf.wehi.edu.au/software/compareCurves).
4.4 DISCUSSION

Most cancer related deaths result from the development of distant metastases. Despite the considerable advance in our understanding of human cancer metastasis process, the lack of knowledge of molecular mechanisms underlying metastasis has restricted our capacity to specifically inhibit metastasis. Oncogenically deregulated p27 plays a critical role in PI3K-mediated metastasis (Zhao D. et al., 2015). p27 phosphorylation at T198 promotes its binding and inhibition of RhoA activity to increase motility (Larrea et al., 2009a). Furthermore, C-terminally phosphorylated p27 drives JAK2 mediated STAT3 activation, leading to STAT3 mediated TWIST1 induction and EMT (Zhao D. et al., 2015). Present data reveal that p27pT157pT198 also induces EMT by stimulating cJun dependent TGF-β2 induction to promote cancer metastasis.

p27 knockdown reverts the EMT program in PI3K-activated, highly metastatic lines, and also reduced TGF-β2 expression and decreased EMT markers. C-terminally phosphorylated p27 and cJun co-occupy an AP1-consensus motif in the TGF-β2 enhancer to activate TGF-β2 gene expression and TGF-β2 secretion. These data suggest that PI3K activated p27 drives EMT and metastasis by promoting TGF-β2 signaling.

EMT is a critical process that governs the metastatic process, and key inducers are needed to set this process in motion. TGF-β is a crucial driver of EMT in transformed cells (Heldin et al., 2012). Here, we showed that p27 knockdown in PI3K-activated, metastatic cells with high endogenous p27pT157pT198 decrease expression and secretion of TGF-β2. Moreover, siRNA mediated loss of TGF-β2 decreased SNAI1 and SNAI2 expression. Conversely, p27CK-DD overexpression upregulates both gene expression and secretion of TGF-β2. While considerable date inform the role of TGF-β1 in cancer, a growing body of
evidence has suggested that TGF-β2 may specifically play an important role in tumor progression. TGF-β2 appears to mediate metastasis in a variety of tumors including glioma (Kingsley-Kallesen et al., 2001), melanoma (Zhang et al., 2009b), pancreatic cancer (Schlingensiepen et al., 2011; Cui et al., 2014), and breast cancer (Beisner et al., 2006). In breast cancer models, increased TGF-β2 promoter activity enhances lymph node metastasis (Beisner et al., 2006). An antisense oligonucleotide specific for TGF-β2 has shown therapeutic efficacy in early clinical trials (Jaschinski et al., 2011). The present data also suggest that TGF-β2 is an important mediator of p27CK-DD mediated metastasis in breast and bladder cancer models.

Recent evidence suggested that p27 regulates transcription of pluripotent genes by acting as a transcriptional co-repressor of E2F4 and p130 mediated differentiation in quiescent cells (Pippa et al., 2012; Li et al., 2012; Menchon et al., 2011). cJun is aberrantly activated in many human cancers and has critical roles in malignant transformation and tumor progression (Hess et al., 2004b). While p27 knockdown in high p27pT157pT198 expressing cells decreased cJun activation, p27CK-DD overexpression significantly increased phosphorylated activated cJun in immortal mammary epithelial cells and low metastatic lines. Here, we demonstrate that p27pT157pT198 forms a complex with JNK/cJun and localizes with cJun to the nucleus where it binds as transcriptional co-

![Mechanistic model of p27pTpT mediated induction of TGF-β2 expression.](image)

Figure 4.8. Mechanistic model of p27pTpT mediated induction of TGF-β2 expression.
activator with cJun at an upstream enhancer region of the $TGF-\beta 2$ gene to drive gene expression and TGF-\beta 2 secretion and signaling.

p27 thus serves as a central signaling node to drive EMT and metastasis, integrating both the PI3K and TGF-\beta signaling pathways. Notably, cJun and p27 recruitment to the $TGF-\beta 2$ enhancer, and $TGF-\beta 2$ gene expression was decreased by PI3K inhibition. It is tempting to speculate that p27 action as a transcriptional regulator may depend on the activity of upstream PI3K-driven kinases. As a result of oncogenic PI3K signaling mediated p27 C-terminal phosphorylation, the repressive transcriptional regulatory complex comprised of p27-E2F4, p130, HDAC1 and SIN3A may be disrupted and p27 may bind cJun and other transcriptional factors to activate genes that induce EMT. Further work will be required to define the spectrum of p27 bound genes co-regulated by cJun and to define other p27-associated complexes that may govern EMT and metastasis.
CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS
5.1 SUMMARY

Distant metastases are the leading cause of cancer related death. While the development of new therapies to oppose metastasis may improve patient outcome, it is not fully understood how the metastatic process is regulated. Work of my thesis has revealed novel mechanisms of EMT induction to drive cancer metastasis. Work of this thesis in Chapter 3 showed that VEGFA promotes metastasis via EMT activation. Our prior work showed that VEGFA, while best known as an angiogenic agent, also stimulates CSC expansion via SOX2, major mediator of CSC induction in breast cancer models. VEGFA is also known to promote metastasis but molecular mechanisms linking the pro-metastatic and CSC driving actions of VEGFA were not known. My thesis work showed VEGFA upregulated SOX2 not only drives CSC expansion, but also drives EMT, and increases invasion and metastasis in breast cancer lines through downregulation of miR-452. miR-452 is identified as a novel, direct repressor of SNAI2 by binding to the SNAI2 3’ UTR, and miR-452 loss is required for Sox2 driven Slug upregulation and invasion in vitro and the pro-metastatic effects of VEGFA in vivo. This pathway is supported by analysis of two different breast cancer cohorts comprising nearly 3000 patients, in which combined overexpression of VEGFA, SOX2 and SNAI2 with miR-452 downregulation is shown to contribute to poor patient outcome. These data reveal a new mechanistic link between VEGFA-mediated upregulation of an embryonic stem cell transcription factor, Sox2, EMT induction and metastasis and support a growing body of evidence that cancer stem cell upregulation and EMT are a two way street.

In Chapter 4, we identify a mechanism whereby PI3K-activated, deregulated p27 drives metastasis via EMT induction. Oncogenic activation of PI3K/mTOR signaling,
through C-terminal phosphorylation of p27 at T157 and T198, increases tumor cell invasiveness and drives progression in a variety of cancers (Larrea et al., 2009b). However, the mechanisms whereby C-terminal phosphorylation of p27 by PI3K/mTOR signaling kinases contributes to cancer metastasis are not fully understood. We show that p27, when deregulated by constitutive T157 and T198 phosphorylation, contributes functionally to oncogenic progression by increasing cell migration, invasion and metastasis. We further provided novel evidence that C-terminally phosphorylated p27 binds a JNK/cJun complex, to enhance cJun activation and p27 is co-recruited with cJun to bind a TGF-β2 enhancer and activate transcription. Furthermore, we show that cJun and TGF-β2 are required for p27CK-DD-driven breast tumors metastasis from an orthotopic site. The work in this thesis has revealed a novel pro-oncogenic function of p27 downstream of PI3K/AKT as a molecular driver of EMT and cancer metastasis, in that it binds cJun and serves as a coactivator of cJun-mediated TGF-β2 induction.

5.2 SIGNIFICANCE

Most cancer related deaths are caused by distant metastases. In spite of the extensive advances in our comprehension of tumor metastatic process, the lack of knowledge of molecular mechanisms underlying metastasis has limited our ability to specifically restrain metastasis. The work of my thesis expands upon our understanding of how metastasis is promoted via regulation of EMT in human cancer. Initiation of metastasis requires cell invasion and escape from the primary tumor into the vasculature, which is enabled by EMT. My thesis work has revealed a novel mechanism whereby VEGFA contributes to the metastatic process. VEGFA, best known as an angiogenic agent, is
expressed in various tumors and its overexpression is associated with poor prognosis and
death caused by metastasis (Goel and Mercurio, 2013; Berns et al., 2003; Manders et al.,
2002). VEGFA functions are not restricted to angiogenesis (Senger, 2010) and it has also
been shown to drive tumor metastasis (Hanahan and Folkman, 1996; Hicklin and Ellis,
2005; Mercurio et al., 2005). Indeed, patients with metastatic breast cancer have higher
circulating VEGFA levels than those without metastasis (Adams et al., 2000).

VEGFA targeted therapy using bevacizumab has been investigated for the
treatment of breast cancer and other malignancies. However, trials in metastatic breast
cancer have showed variable results and the efficacy of this drug is controversial (Sullivan
and Brekken, 2010; Shih and Lindley, 2006; Montero et al., 2012). VEGFA blockade, by
inhibition of angiogenesis, creates tumor hypoxia. Hypoxia stimulates HIF-1α dependent
VEGFA induction (Goel and Mercurio, 2013), which would stimulate the most aggressive
hypoxia tolerant, chemo-resistant CSC to reseed local and metastatic niches contributing
to aggressive disease recurrence. Indeed, tumors surviving bevacizumab were shown to
have increased CSC, due to the effects of hypoxia to upregulate VEGFA (Conley et al.,
2012). CSCs show greater metastatic potential than the bulk tumor cell population and have
been postulated to be drivers of tumor metastasis (Malanchi et al., 2012; Takebe et al.,
2011; Picon-Ruiz et al., 2016), but the mechanisms underlying this are not fully
characterized.

Here we identify novel pathway in which Sox2, upregulated by VEGFA,
contributes to EMT and metastasis through loss of miR-452 and Slug upregulation. This
pathway is supported by analysis of two major datasets including over 2500 primary human
breast cancers. Our analysis showed breast cancers in the highest quartile of VEGFA
expression fare worse than all others, and the prognostic value of high VEGFA levels is increased by sequential addition of high SOX2, decreased GABRE (a surrogate for miR-452) and high SNAI2 expression indicating this pathway activation leads to very aggressive population within breast cancer. This work suggests a novel rationale and a potential strategy for targeting VEGFA pathways in cancer. Instead of targeted inhibition of VEGFA alone, combined use of drugs that inhibit VEGFA signaling at multiple levels, such as inhibitors of VEGFR, Slug inhibitor and/or artificial miR-452 treatment might inhibit cancer metastasis with successful effect.

My thesis work also has revealed a novel pro-oncogenic function of p27 downstream of PI3K/AKT as a molecular driver of EMT and cancer metastasis via cJun-mediated TGF-β2 induction. In normal cells, p27 restrains the cell cycle by inhibiting cyclin-CDKs. In human cancers, p27 is deregulated by PI3K-dependent phosphorylation at T157 or T198 that leads to an oncogenic role for p27 in mediating motility, invasion and metastasis. We previously showed p27pTpT drives metastasis by promoting STAT3 activation, STAT3-dependent TWIST1 induction, and EMT (Zhao D. et al., 2015). The present work demonstrates that p27 knockdown in MDA-MB-231-1833 reverts the EMT phenotype and reverse the expression profiles of genes whose mRNA levels are modulated by EMT core transcription factors. These findings suggest that p27 may regulate EMT at the transcriptional level. Indeed, our data demonstrates that p27 binds to and activates cJun and p27-cJun co-activate TGF-β2 gene expression to drive EMT in a T157 and T198 phosphorylation-dependent manner, supporting to the notion that p27 act as transcriptional regulator to induce EMT and promote subsequent metastasis. This thesis work opens a new paradigm in which pro-oncogenic actions of p27 include more than cytoskeleton
disruption. This work sheds light on a novel function of p27 as a phosphorylation-dependent transcriptional regulator with implications for cancer.

This work may also identify new targets for cancer therapy. PI3K pathway is frequently activated in many human cancers (Lawlor and Alessi, 2001; Vivanco and Sawyers, 2002) and preclinical or clinical studies with multiple PI3K inhibitors showed some antitumor activity (Fruman and Rommel, 2014). However, clinical efficacy of these drugs in cancer have shown limited efficacy, due to diverse reasons such as insufficient target inhibition, off target effects, resistance, tolerability and bypass pathway activation (Engelman, 2009; Rodon et al., 2013). Due to high selective pressure and rapid evolution of cells within the tumor population, targeted therapies using a single agent almost invariably fail to prevent tumor progression. Combination treatment strategies with other agents may overcome negative effects of mono-therapy and improve the efficacy of drugs. This might not only allow lower the doses required for each drug, leaving non-tumorigenic cells less affected, but also cancer cells will have fewer signaling pathways activated to evade targeted therapy. Our data provides a novel rationale for use of PI3K/mTOR inhibitors together with TGF-β2 signaling inhibitors in PI3K-activated, p27pT157pT198 enriched human cancers to prevent systemic spread in the course of tumor progression. In addition, C-terminal phosphorylation of p27 might be implicated as a promising therapeutic biomarker that identifies tumor types and/or patients most likely to benefit from combined PI3K/mTOR and/or TGF-β2 inhibitor treatment.

Taken together, the work in this thesis has revealed a novel pro-oncogenic function of p27 downstream of PI3K/AKT as a molecular driver of EMT and cancer metastasis via cJun-mediated TGF-β2 induction. We also identify another novel pathway in which Sox2,
upregulated by VEGFA, contributes to EMT and metastasis through loss of miR-452 and Slug upregulation. These findings expand our knowledge of molecular mechanisms underlying cancer metastasis and provide insights to generate novel strategies for opposing cancer metastasis and improving patient outcome.

5.3 FUTURE DIRECTIONS

It has been shown that deregulated, C-terminally phosphorylated p27 associates with new binding partners to promote oncogenic events independent of its CDK-binding role. Interestingly, it was recently shown that p27 can modulate transcription. Earlier work revealed that p27 binds genomic DNA and acts as a transcriptional co-repressor with p130, E2F4, HDAC1, and SIN3A (Pippa et al., 2012). Notably, the C-terminal region of p27 was required to recruit HDAC1 and SIN3A to DNA-bound p27/E2F4/p130. Another study showed that p27 co-represses the SOX2 gene (Li et al., 2012), which encodes an important embryonic stem cell transcription factor and driver of cancer stem cells self-renewal (Bass et al., 2009; Leis et al., 2011). p27 appears to co-repress SOX2 together with p130, E2F4 and SIN3A at the SOX2-SRR2 regulatory element (Li et al., 2012).

Work in this thesis identified that PI3K-activated p27pTpT forms complex with cJun and co-occupy an enhancer site upstream of the TGF-β2 gene to induce TGF-β2 expression leading to EMT and metastasis suggesting p27 may also play a role as transcriptional co-activator. In the final chapter of this thesis, future experimental work is proposed to identify transcriptional targets of p27 and investigate whether p27 modulates EMT transcription program as co-regulator.
5.3.1 Identify putative p27 target genes implicated in EMT transcriptional program

Since we showed that PI3K-activated, C-terminally phosphorylated p27 binds cJun and is co-recruited to a TGF-β2 gene enhancer motif as a putative co-activator, we will analyze expression of p27 bound genes in 1833, with or without 250 nM PF1502, a PI3K/mTOR inhibitor that potently decreases p27pTpT. We will perform a comprehensive analysis combining ChIP with high throughput sequencing using the Illumina HiSeq platform to identify expression of p27 bound gene. ChIP-Seq analysis will assess p27 gene occupancy with or without PF1502 to test how p27 phosphorylation at C-terminus affects p27 binding on DNA sequence to regulate transcriptional program. Genome-wide p27 binding sites will be mapped in 1833 and PF1502 treated 1833 cells. As a second model, we will compare p27-bound genes in the non-malignant breast epithelial line, MCF12A, and MCF12A p27CK-DD. We will compare p27 binding patterns on DNA with patterns of gene activation or repression. Total RNA will be isolated and rRNA depleted fractions subjected to high throughput sequencing (RNA-Seq) using the Illumina HiSeq platform. These assays will identify which p27-bound genes, which will be detected by ChIP-Seq, are differentially expressed in 1833 with or without PF1502 and in MCF-12A vs MCF12A p27CK-DD. Gene expression differences will be compared to the genome-wide p27 binding assays to gain a comprehensive picture of how p27pTpT binding relates to gene expression implicated in EMT transcriptional program. Loss of p27pTpT with PF1502 treatment (Wander et al., 2013) is expected to decrease both p27 binding and activation of target genes driving EMT. Since we showed by ChIP that p27 and cJun co-occupy an enhancer upstream region of TGF-β2, forming a potential coactivator complex, we will also analyze global cJun-DNA binding by ChIP-Seq in lines indicated above and compare
this to RNASeq gene expression data. This will determine which putative p27 target genes are co-regulated by cJun and if cJun independent DNA binding sites may also exist for p27.

5.3.2 Prioritizing EMT related genes bound by p27 and cJun for further ChIP analysis.

We showed p27 and cJun interact in nuclear extracts and p27pTpT upregulates TGF-β2 expression and p27 binds a cJun-consensus motif within TGF-β2 enhancer region together with cJun. Thus, AP1 sites and cJun consensus motif bound by both cJun and p27 will be a priority for further characterization. We will investigate whether p27 is co-recruited with cJun to DNA enhancer or promoter AP-1 sites. We showed that cJun is strongly activated in PI3K activated high p27pTpT cell lines and binding of p27 and cJun to a cJun consensus TRE motif in the TGF-β2 enhancer was increased in those cell lines in which TGF-β2 is expressed more highly than in parental 231. Notably, cJun binding to this site was decreased by p27 knockdown and by PI3K inhibition with PF1502, which reduces p27pT157pT198. Thus, putative promoter or enhancer sites that are stably bound by both p27 and cJun on ChIP-Seq will be investigated with ChIP assays, and it will be tested whether the binding is p27 C-terminal phosphorylation dependent. In addition, we will compare known AP1 (Zhao et al., 2014a) and cJun (Li et al., 2011) binding sites with cJun/p27 bound genomic region identified herein.

5.4 CONCLUDING REMARKS

Distant metastases are the main cause of cancer related death. While it is important to find new strategies to oppose metastasis, mechanisms governing the metastatic process
remain poorly understood. Work of my thesis has provided novel mechanisms whereby cancer metastasis is regulated via induction of EMT. Here, we identified a novel pathway in which Sox2, upregulated by VEGFA, contributes to activation of EMT. VEGFA leads to induction of the stem cell transcription factor gene SOX2. Sox2, in turn, mediates repression of miR-452, which is shown to directly target the 3’ UTR of SNAI2, leading to EMT induction and cancer metastasis. This thesis work also revealed that p27pT157pT198 as a critical driver of PI3K/mTOR-dependent cancer cell invasion in vitro and metastasis in vivo. Furthermore, we uncovered a novel, oncogenic function of p27 to promote invasion and metastasis by up-regulating an EMT program that involves cJun mediated TGF-β2 induction. Our data suggest that C-terminally phosphorylated p27 may act as transcriptional regulator to promote recruitment and activation of oncogenic transcription factors implicated in the EMT program. Present findings extend the current paradigm of CDK independent p27 actions to reveal a novel, broader p27 function as a phosphorylation-dependent transcriptional regulator with implications for cancer and differentiation.

Together, the work of this thesis provides novel mechanistic insights into how cancer metastasis is promoted via EMT induction. These findings may ultimately lead to new therapeutic strategies to limit p27 and VEGF mediated EMT and cancer metastasis.
References


