Exploring the Oncogenic Role of p27 in Regulating Tumor Progression

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EXPLORING THE ONCOGENIC ROLE OF p27 IN REGULATING TUMOR PROGRESSION

By

Dekuang Zhao

A DISSERTATION

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

Coral Gables, Florida

December 2013
EXPLORING THE ONCOGENIC ROLE OF p27 IN REGULATING TUMOR PROGRESSION

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p27 is a cell cycle regulator and an atypical tumor suppressor. However, p27 gene deletions or mutations are rarely observed in human cancers. Instead, p27 is frequently degraded or mislocalized to the cytoplasm in aggressive human cancers. Phosphorylation at T157 or T198 by different PI3K effector kinases leads to p27 cytoplasmic accumulation. However, the functional contribution of phosphorylated and cytoplasmic p27 to cancer progression remains unknown.

By introducing a cell cycle defective (CK-) and double phosphomimetic p27 mutant (T157D/T198D or DD), we found that phosphorylation of p27 at T157 or T198 by PI3K/mTOR directly regulated tumor cell migration and invasion. Targeted inhibition of PI3K/mTOR impaired tumor cell motility and metastasis via modulation of p27. Furthermore, we uncovered a novel oncogenic function of p27 in regulating tumor progression. p27CK-DD induced epithelial-mesenchymal transition (EMT) and transformation of human mammary epithelial cells and enhanced the mesenchymal characteristics and metastatic potential of cancer cell lines. Knockdown of p27 in mesenchymal cell lines with enriched p27T157/T198 reverted EMT and impaired metastatic potential. Mechanistically, p27CK-DD activated STAT3 and facilitated its transactivation of TWIST1 to induce EMT. Pharmacological inhibition of STAT3 or
dominant negative STAT3 (STAT3DN) decreased TWIST1 and reversed p27CK-DD-mediated EMT and tumor progression, while constitutive active STAT3 (STAT3CA) rescued EMT phenotype and metastatic potential in p27 knockdown cells. We also identified a potential signaling feedforward loop containing AKT activation, p27 phosphorylation, STAT3 activation and further AKT activation that might contribute to tumor progression. These findings reveal a novel oncogenic function of p27 to regulate tumor progression through EMT that involves STAT3-mediated TWIST1 regulation. Combined inhibition of both AKT and STAT3 in PI3K/mTOR activated, p27pT157/pT198 enriched human cancers may ultimately have therapeutic potential to limit p27-mediated EMT and cancer metastasis.
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<td>BV</td>
<td>Bevacizumab</td>
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<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
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<tr>
<td>DT</td>
<td>Dissociated tumors</td>
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<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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<td>IVIS</td>
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<td>MET</td>
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<td>TWIST1</td>
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<tr>
<td>TNBC</td>
<td>Triple-negative breast cancer</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Part of this chapter was published in *Clin Cancer Res* 2011;17(1):12-18. As:

p27: A Barometer of Signaling Deregulation and Potential Predictor of Response to Targeted Therapies

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CHAPTER 1: INTRODUCTION

The work described in this thesis expands upon our understanding of how the atypical tumor suppressor, p27, drives human tumor metastasis following its mislocalization to the cytoplasm downstream of oncogenic PI3K/mTOR signaling. The following provides a review of the areas relevant to this work including PI3K/mTOR signaling in cancer metastasis, multifaceted roles that p27 plays in tumor progression, the epithelial-mesenchymal transition and its relevance to the metastatic process and cancer stem cell function.

1.1 PI3K/mTOR SIGNALING IN CANCER

PI3K/mTOR signaling is hyperactivated in many kinds of cancer it plays important role for tumor progression by regulating proliferation, survival and cell migration. My thesis aims to investigate how p27\textsuperscript{KIP1}, when deregulated by PI3K/mTOR signalling, regulates cell migration and metastasis. The following provides a brief overview of this signaling pathway and its contribution to cancer metastasis.

1.1.1 PI3K/mTOR signaling

The PI3K/mTOR pathway is activated in a majority of human cancers through receptor tyrosine kinase activation or amplification, PTEN loss, and activating mutations of PIK3CA or other downstream effectors (Wander et al., 2011a). The mammalian target of rapamycin (mTOR) is the catalytic subunit of two molecular complexes: mTORC1 (mTOR/Raptor) and mTORC2 (mTOR/Rictor). The mTOR signaling links nutrient sensing and mitogenic signals to regulate protein synthesis (Sabatini, 2006). PI3K/mTOR
activation increases cell survival and proliferation (Manning and Cantley, 2007; Ridley et al., 2003; Kolsch et al., 2008).

Receptor tyrosine kinases (RTKs) for IGF-1, HGF, and EGF all signal through PI3K to activate phosphoinositide-dependent protein kinase-1 (PDK1) (Wander et al., 2011a), and many of these RTKs are hyperactivated in cancers. Activated PDK1 further phosphorylates AGC family kinases (homologues of protein kinases A, G, and C), including AKT, serum/glucocorticoid-regulated kinase 1 (SGK1), and ribosomal S6 kinase, polypeptide 1 (RSK1), all of which need secondary phosphorylations to be activated. MAPK1/3 phosphorylates RSK1 to provide secondary stimulation for its activation. mTORC2 could phosphorylate both SGK1 and AKT at the second site to induce their activation (Sarbassov et al., 2005; Hresko and Mueckler, 2005).

One of the major downstream effectors of Akt is mTOR complex 1 (mTORC1), which is activated by AKT-mediated inactivation of the tuberous sclerosis complex (TSC1 and TSC2 proteins). Activated mTORC1 not only upregulates protein biosynthesis required for cell proliferation (Sabatini, 2006), but also phosphorylates the second site of SGK1 to promote its activation (Hong et al., 2008; Garcia-Martinez and Alessi, 2008). Thus, PI3K and mTOR pathways act together to promote cell growth, division, and survival: AKT activates anti-apoptotic mechanisms and the cell cycle; SGK1 regulates insulin and energy metabolism; and RSK1 activates mitogenic transcription factors (Manning and Cantley, 2007; Tessier and Woodgett, 2006; Frodin and Gammeltoft, 1999).
1.1.2 PI3K/mTOR signaling in cancer metastasis

Cancer metastasis is the main cause of death in patients with cancer (Chaffer and Weinberg, 2011). Following transformation and growth of the primary tumor, cells must undergo multistep cell-biological process termed invasion-metastasis cascade to spread local tumor to distal organs (Valastyan and Weinberg, 2011). Cell first lose cellular adhesion and increase invasiveness to invade through surrounding extracellular matrix and intravasate into the blood vessels. These circulating tumor cells need to survive transport through the vasculature and extravasate into the parenchyma of distant organs, where they adapt to the new microenvironment to form distal metastasis (Gupta and Massague, 2006).

Many of these complex metastatic events are orchestrated by critical metastasis drivers and molecular pathways operating in tumor cells. At the same time, the crosstalk between tumor cells and stromal cells in the tumor microenvironment also play important role through this metastasis cascade. Deregulation of these intrinsic and extrinsic signaling pathways allows tumors to generating high-grade and life-threatening malignancies (Valastyan and Weinberg, 2011). A better understanding of these deregulated signaling pathways will provide therapeutic opportunity for patients with metastatic malignancies.

While activation of PI3K/mTOR signaling is critical for tumor cell growth and proliferation, recent data suggest it also drives tumor cell motility, invasion and metastasis (Manning and Cantley, 2007; Ridley et al., 2003; Kolsch et al., 2008). ErbB2 overexpression promotes lung metastasis from orthotopic breast cancers in a SCID mouse model via mTOR/Raptor-dependent p70S6K activation (Klos et al., 2006), and breast
cancer patients whose primary tumors showed p70S6K activation were more likely to develop metastases (Klos et al., 2006). Rictor may also mediate lung metastatic outgrowth of human breast cancer cells, independent of mTOR (Zhang et al., 2010). Both mTORC1 and mTORC2 increase cell motility and metastasis in a colorectal cancer model (Gulhati et al., 2011), and mTORC2-dependent AKT activation promotes lung cancer cell colonization following tail vein injection (Kim et al., 2011).

While increasing data implicate PI3K/mTOR in tumor metastasis, mechanisms underlying this remain poorly defined. The work in this thesis aims to investigate the critical mediators and signaling pathways downstream of PI3K/mTOR activation that might contribute to tumor metastasis. We identify p27 as an important mediator and uncover its oncogenic role in promoting tumor cell migration and metastasis through regulating epithelial-mesenchymal transition.

1.2 EPITHELIAL MESENCHYMAL TRANSITION IN CANCER

The epithelial-mesenchymal transition (EMT) is a biological process in which polarized epithelial cells undergo multiple molecular and cellular changes to acquire mesenchymal phenotype with increased invasive capacity (Kalluri and Weinberg, 2009). During embryonic development, EMT facilitates the redistribution of cells to generate distinct tissue types (Thiery et al., 2009). It plays a significant physiological role during embryogenesis, wound healing and tissue development (Kalluri, 2009). In addition, the EMT program is also activated during organ fibrosis, cancer cell invasion and metastasis (Kalluri and Weinberg, 2009; Thiery, 2002; Micalizzi et al., 2010). Moreover, malignant cells undergoing EMT may also acquire stem cells properties with increased self-renewal
and tumor initiation potential (Mani et al., 2008). Therefore, identification of the critical signaling pathways that regulate EMT during cancer invasion may provide novel therapeutic applications for more effective cancer treatment.

1.2.1 EMT in normal development and tissue injury

The concept of EMT was first proposed by Elizabeth Hay, who used primitive streak cells from chick embryos as a model to prove that epithelial cells undergo dramatic phenotypic changes with transformation to mesenchymal cells (Hay, 1995). This process of EMT is essential for germ layer formation and cell migration in early vertebrate development. Based on the biological context in which they occur, three subtypes of EMT have been proposed: type 1 regulates embryogenesis and organ development; type 2 regulates tissue regeneration and organ fibrosis; and finally type 3 regulates cancer progression and metastasis (Kalluri, 2009). Type 1 EMT generates cells with a mesenchymal phenotype to create new tissues with diverse functions. For example, during embryonic development, epithelial cells of the neuroectoderm give rise to migratory neural crest cells via an EMT program (Bhatt et al., 2013). During development, EMT plays important role in generating mesenchyme that subsequently gives rise to secondary epithelia via MET.

Type 2 EMT starts as a repair event that generates fibroblasts to reconstruct tissues following inflammatory injury or trauma 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 states affecting the liver, lung, kidney and intestine.
(Kalluri and Weinberg, 2009). In addition, ongoing inflammatory signals can stimulate type 2 EMT and eventually lead to organ destruction.

1.2.2 EMT in cancer metastasis

Metastasis is the leading cause of cancer-related death for most tumor types. Despite its devastating consequences, cancer metastasis is an inefficient process that requires cancer cells to overcome a series of challenges to spread from primary to secondary organs (Sethi and Kang, 2011). Cancer cells must undergo multiple steps including local invasion, intravasation, extravasation, and organ colonization to form distant metastasis (Fidler, 2003).

During early tumor invasion, epithelial cancer cells lose apicobasal polarity and cell-cell junctions, and gain mesenchymal phenotypes with increased invasive capabilities to invade through the basement membrane. This early step of metastasis is believed to be initiated by the reactivation of type 3 EMT (Micalizzi et al., 2010). Since epithelial cancer cells are tightly associated with neighboring cells via E-cadherin-containing junctions, cancer cells must break these intracellular junctions through loss of the epithelial phenotype (loss of epithelial E-Cadherin, which mediates epithelial cell-cell adhesion) and acquire mesenchymal characteristics (via induction of mesenchymal markers such as vimentin and N-Cadherin) to gain motility, transgress the basement membrane and invade stromal tissues. These cells typically are seen at the invasive front of primary tumors and considered to be the cells that eventually enter into subsequent steps of metastasis.
Although EMT is now considered the first step in the metastatic cascade, the extent and timing of its contribution to cancer progression has not been well-studied due to the lack of advanced technology to dynamically monitor EMT in vivo. Powerful imaging techniques have recently allowed observation of migration 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 important role during the formation of metastasis (Thiery et al., 2009; Tsai et al., 2012).

1.2.3 EMT markers and transcription regulators

A hallmark of EMT is loss of expression of E-Cadherin, a crucial “gate keeper” of epithelial characteristics. E-Cadherin regulates cell adhesion and its expression is decreased during EMT in embryonic development, in tissue fibrosis, and in cancer (Hay, 1995). Moreover, a cadherin switch, with loss of E-Cadherin and the appearance of N-Cadherin, which is normally expressed in mesenchymal cells, is a commonly used marker of EMT during embryonic development and cancer progression. In addition, appearance of the cytoskeletal marker Vimentin is also commonly used to identify cells undergoing type 3 EMT in cancers, as its expression is positively correlated with increased tumor invasion and metastasis (Satelli and Li, 2011).

The EMT program is initiated by several EMT-inducing transcription factors including Snail, Slug, zinc finger E-box binding homeobox 1 (Zeb1), and Twist1 (Batlle et al., 2000; Cano et al., 2000; Nieto et al., 1994; Aigner et al., 2007; Yang et al., 2004).
These transcription factors can either work individually or together to repress expression of E-cadherin (Stockinger et al., 2001; Onder et al., 2008) and/or upregulate expression of a variety of mesenchymal markers including N-cadherin and Vimentin (Kalluri and Weinberg, 2009). Snail is widely recognized as a repressor of the epithelial marker, E-cadherin, and it also induces expression of mesenchymal markers. Twist1 is a basic helix-loop-helix protein that is transcriptionally active during osteoblast lineage determination and cell differentiation (Bialek et al., 2004). Twist1 can repress E-Cadherin independently of Snail and to upregulate N-Cadherin. As a master regulator of embryonic morphogenesis, Twist1 has been shown to promote cancer metastasis by regulating EMT (Yang et al., 2004). In human breast cancers, high levels of Twist1 expression are observed in invasive lobular carcinoma, a highly infiltrating tumor type associated with loss of E-cadherin expression (Yang et al., 2004).

1.2.4 Signal pathways governing EMT

Several extracellular signal pathways including TGF-β, Wnt/β-catenin, Notch have been implicated in regulating EMT during the course of embryonic development, wound healing and cancer progression (Chen et al., 2010; Asiedu et al., 2011; Yan et al., 2012; Thiery et al., 2009). Of these, TGF-β family cytokines are the most well-studied EMT inducers. During tumor progression, TGF-β signaling induces an EMT program through Smad dependent induction of EMT regulators, including Snail, Slug and Twist1 (Xu et al., 2009). Moreover, TGF-β can also collaborate with other signaling pathways such as Notch, Wnt/β-catenin, to fully induce EMT and maintain the mesenchymal phenotype with invasive and metastatic cancer cells (Zavadil et al., 2004; Zhou et al.,
Wnt signaling can lead to EMT through inhibition of GSK-3β-mediated phosphorylation and degradation of β-catenin. Activated β-catenin, in turn further induces expression of EMT transcription factor. The Notch signaling pathway not only maintains a balance between cell proliferation and differentiation, but also plays important roles in determining cell fate and maintaining progenitor cell populations in several epithelial cell types (Miele, 2006). Notch activation is required for TGF-β-induced EMT and Notch signaling was shown to stabilize Snail expression, resulting in upregulation of EMT and increase of cell migration and invasion (Zavadil et al., 2004; Micalizzi et al., 2010).

STAT3 (Signal transducer and activator of transcription 3) is hyperactivated in many types of cancer and its activation promotes oncogenesis and metastasis (Devarajan and Huang, 2009). Recent findings also link STAT3 activation to the gain of invasive mesenchymal phenotype in human cancers (Xiong et al., 2012; Yadav et al., 2011). The work of this thesis has uncovered a less appreciated signaling pathway via STAT3 as a key driver of EMT to increase tumor cell motility, invasion and metastasis downstream of PI3K-mediated p27 phosphorylation.

1.2.5 The link between EMT and cancer stem cell properties

Increasing data suggest that malignancies arise from stem-like cells that self-renew and generate heterogeneous, more differentiated progeny with lower replicative capacity (Dalerba et al., 2007). Initially characterized in leukemia, cells with stem-like features that initiate tumors with high frequency have been demonstrated in many human malignancies and cancer stem cells (CSCs) appear to play a critical role in tumor
invasion, metastasis, drug resistance, and disease recurrence (Takebe et al., 2011). CSCs can be enriched from bulk cancer cells by selection for expression of specific surface markers. In primary breast cancer, populations expressing surface CD44^+CD24^{low/-} and/or aldehyde dehydrogenase (ALDH1) activity (Al Hajj et al., 2003; Ginestier et al., 2007) are enriched for cells with stem cell like properties in vitro and the ability to initiate xenograft tumors.

Recent experimental data connect EMT and CSCs, and suggest that EMT may facilitate the generation of cancer cells with mesenchymal phenotype for dissemination and also augment the CSC self-renewal properties required for secondary tumor initiation (Mani et al., 2008). Two independent groups recently demonstrated that, in human mammary epithelial cells, ectopic expression of EMT-inducing transcription factors \textit{SNAI-1} or \textit{TWIST1}, or exposure to TGF-\beta resulted in the acquisition of mesenchymal traits, and increased the proportion of CD44^{high}CD24^{low} stem cells and mammosphere formation (Mani et al., 2008; Morel et al., 2008), suggesting a direct link between EMT and the gain of CSC-like properties. Furthermore, both EMT-inducing transcription factors (\textit{TWIST1}, \textit{SNAIL}, \textit{ZEB2}) and the EMT associated genes (encoding Vimentin and Fibronectin) were expressed at higher levels in CD44^+CD24^- stem like cells isolated from human primary breast tissues than in more differentiated epithelial CD44^-CD24^+ cells (Bhat-Nakshatri et al., 2010).

An increased frequency of CD44^+CD24^- stem cell-like cancer cells and expression of EMT-associated genes has observed in the highly invasive subset of basal-like breast cancers and primary tumor cultures (Azzam et al., 2013; Ben-Porath et al., 2008). Immunochemical analysis of invasive breast carcinomas showed that those with higher
expression of mesenchymal markers (N-cadherin, Vimentin) and low expression of epithelial marker E-Cadherin in basal-like breast tumors usually show poor prognosis due to their increased invasive and metastatic ability (Sarrio 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). Recently, EMT was also shown to be associated with maintenance of stemness in human colon cancer (Hwang et al., 2011), suggesting that the generation of CSCs by EMT from cell culture system may also applied to in vivo cancer development.

There is accumulating evidence to support that EMT and acquisition of cancer stem cell features also contribute to drug resistance. For example, in residual breast cancers recovered from patients after chemotherapy, there was a significant enrichment of cells with both CSCs properties and mesenchymal features (Creighton et al., 2009). In lung carcinoma, cells that have undergone EMT showed a decreased sensitivity to EGFR kinase inhibitors (Uramoto et al., 2010).

1.3  p27KIP1: A CDK INHIBITOR WITH DUAL ROLES IN TUMOR BIOLOGY

Since my thesis work investigated how deregulated p27KIP1 (p27) is involved in metastatic tumor progression, the following reviews our current understanding of the roles and regulation of p27KIP1. This CDK inhibitor is subject to complex and coordinated regulation via a variety of signal transduction pathways in both normal and transformed cells. Recent advances have highlighted the importance of both p27 phosphorylation and subcellular localization in dictating how this atypical tumor suppression functions to regulate cell division and cell migration during tumor progression.
1.3.1 p27 regulates cell cycle

Cell cycle progression is driven by cyclins and their associated cyclin-dependent kinases (CDKs). p27 was discovered as an inhibitor of cyclin E-CDK2 (Koff et al., 1993; Polyak et al., 1994; Slingerland et al., 1994), but has since been shown to play dual roles to both promote and inhibit cell cycle progression. In G0, p27 translation and protein stability are maximal as it binds and inactivates nuclear cyclin E-CDK2 to restrain cell cycle progression. The progressive decrease of p27 in G1 permits cyclin E-CDK2 and cyclin A-CDK2 to activate the G1-S transition (Chu et al., 2008). Mice lacking p27 display multi-organ hyperplasia, increased body size and susceptibility to carcinogen-induced tumors, suggesting that p27 acts as a tumor suppressor to control both tissue expansion and cell proliferation (Kiyokawa et al., 1996; Nakayama et al., 1996; Fero et al., 1996). In contrast, early G1, p27 also promotes assembly and nuclear import of D-type cyclin-CDKS to facilitate G1 progression (Larrea et al., 2009b; LaBaer et al., 1997).

1.3.2 p27 regulates cell migration

Cell cycle inhibition, by inhibition of nuclear localized cyclin-CDKs, is a nuclear p27 function. In many cancers, not only is nuclear p27 reduced, but tumors also exhibit different degrees of cytoplasmic p27 mislocalization (Slingerland and Pagano, 2000; Chu et al., 2008). Cytoplasmic p27 appears to acquire a cell cycle-independent oncogenic function to promote cancer cell invasion. TAT-p27 protein transduction was shown to increase Rac-dependent cell motility (Nagahara et al., 1998). p27-/- mouse embryonic fibroblasts (MEFs) show reduced motility compared to wild type MEFs that could be rescued by re-expression of either wild type p27 or mutant p27 that cannot bind cyclins
and CDKs (p27CK-) (Besson et al., 2004). Thus, the pro-motility effects of p27 appear to be independent of its cyclin-CDK regulatory functions. In the cytoplasm, p27 binds RhoA to inhibit RhoA-ROCK mediated actomyosin stabilization (Besson et al., 2004), and this has been implicated in the gain of motility required for tumor invasion and metastasis. In malignant lines, overexpression of cytoplasmic p27 increases tumor invasion and/or metastasis in murine models (Denicourt and Dowdy, 2004; Wu et al., 2006) and promotes glioma cell invasion (See et al., 2010). The potential pro-oncogenic effect of p27 was also supported by the observation that p27CK- knock-in animals show increased lung epithelial progenitor cell self-renewal and lung tumor development (Besson et al., 2007). Thus, while nuclear p27 inhibits CDK2 to restrain cell cycle, excessive cytoplasmic p27 can have pro-oncogenic effects to expand stem cell/progenitor cell populations, and promote cancer invasion and metastasis. Work of my thesis aimed to elucidate further how phosphorylation of p27 by oncogenic signal transduction pathways contributes to its pro-invasion and pro-metastatic actions in cancers.

### 1.3.3 p27 phosphorylation, degradation and subcellular localization

p27 is regulated at transcriptional, translational and post-translational levels (Hengst and Reed, 1996; Chu et al., 2008). p27 mRNA levels usually show little cell cycle periodicity, but may be repressed by PI3K/AKT in a cell type-dependent manner (Medema et al., 2000). In normal cells, p27 protein levels are largely controlled by ubiquitin-dependent proteolysis (Chu et al., 2008). In early G1, mitogens promote p27 phosphorylation at serine 10 (S10) to facilitate nuclear export (Boehm et al., 2002; Connor et al., 2003; Hara et al., 2002); this simultaneously relieves cyclin E-CDK2
inhibition and permits KPC-mediated proteolysis of cytoplasmic p27 (Kamura et al., 2004). Tyrosine phosphorylation of p27 by BCR-ABL (Y74, Y88, Y89) or Src family kinases (Y88 and Y74) impairs the CDK2 inhibitory action of p27. This facilitates subsequent cyclin E-CDK2-dependent T187 phosphorylation of p27, that, in turn, allows it to be targeted by SCFSKP2 for degradation (Figure 1) (Chu et al., 2007; Grimmler et al., 2007). Tyrosine phosphorylation of p27 is also required for the catalytic activation of p27-cyclin D1-CDK4 complexes (Larrea et al., 2008; James et al., 2007). p27 is also subject to regulation by miRNAs 221/222: expression of these miRNAs in prostate cancer and glioblastoma cells has been shown to downregulate p27 levels to drive tumor cell proliferation (Galardi et al., 2007; le Sage et al., 2007).

In addition to the regulatory mechanisms described above, p27 localization appears to be finely tuned during the cell cycle. In early G1, as p27 translation falls, nuclear export of p27 not only relieves CDK2 inhibition, but also promotes p27-D type-cyclin-CDK assembly in the cytoplasm. Transient cytoplasmic retention of newly synthesized p27 is also facilitated in early G1 by PI3K effectors that phosphorylate p27 on T157 and/or T198 which impairs p27 import and promotes cyclin D1-CDK4-p27 complex assembly (Larrea et al., 2009b; Liang et al., 2002; Hong et al., 2008; Larrea et al., 2009a).

1.3.4 Loss of nuclear p27 during tumor progression

Given the central roles of p27 in cellular proliferation and migration, it is no surprise that reduced or mislocalized p27, documented by immunohistochemical analysis in primary tumors, is associated with poor clinical outcome in a diverse variety of human
malignancies (Chu et al., 2008). Progressive loss of nuclear p27 has been observed during progression from benign to in situ and invasive breast cancer (Catzavelos et al., 1997; Han et al., 1999). One study showed that, relative to normal breast duct epithelia where 95% of cells show high nuclear p27, pre-malignant atypical ductal hyperplasia shows modest p27 loss (85%), while ductal carcinoma in situ (40%) and invasive cancer (34%) show greater p27 loss (Han et al., 1999). In ovarian cancer, lesions of low malignant potential showed intermediate p27 levels compared to high levels in normal epithelia, while highly aggressive cancers showed very low nuclear p27 (Sui et al., 2001; Korkolopoulou et al., 2002).

1.3.5 Prognostication of p27 in human cancers

Reduced nuclear p27 is associated with adverse patient outcome in malignancies of epithelial, hematopoietic, and mesenchymal origin (Chu et al., 2008). To date, the application of p27 as a clinical prognostic tool has been encumbered by the lack of uniformity in immunohistochemical (IHC) staining protocols. Most IHC studies of p27 have evaluated only nuclear p27 and there is currently no uniformly accepted guideline for scoring cutoff values for “low” versus “high.” Despite these limitations, multiple studies demonstrate a correlation between reduced nuclear p27 and poor prognosis. This has been observed in lung (Yatabe et al., 1998; Tsukamoto et al., 2001; Hirabayashi et al., 2002) and prostate cancers (Vis et al., 2000; Tsihlias et al., 1998; Yang et al., 1998; Cote et al., 1998; Kuczyk et al., 2001) and more comprehensively in breast cancer, where most analyses show reduced p27 is an independent prognostic indicator of disease relapse.
or death (Catzavelos et al., 1997; Porter et al., 1997; Tan et al., 1997; Han et al., 2003; Porter et al., 2006; Wu et al., 1999; Nohara et al., 2001; Pohl et al., 2003).

While far fewer studies have evaluated the prognostic potential of cytoplasmic p27, this is also correlated with adverse outcome in prostate cancer (Li et al., 2006), gliomas (Piva et al., 1999) and high-grade astrocytomas (Hidaka et al., 2009). In breast cancer, cytoplasmic p27 staining correlated with AKT activation (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002) and predicted poor patient prognosis in univariate analysis (Liang et al., 2002). To date, however, none of these analyses have quantitated both nuclear p27 levels and cytoplasmic p27 mislocalization. An understanding of the potential prognostic and predictive value of p27 (see below) may await this type of study, since reduced nuclear p27 increases proliferation and cytoplasmic p27 drives tumor cell invasion.

1.3.6 Src and MEK/MAPK pathways regulate p27 degradation

In contrast to other tumor suppressors, p27 mutation or deletion is rare in human cancers. Deregulated receptor tyrosine kinases (RTKs) activate Src/BCR-ABL and Ras/MEK/MAPK which contribute to oncogenesis by inducing p27 degradation (Chu et al., 2008; Larrea et al., 2009b).

Many human cancers have increased Src levels or activity (Mayer and Krop, 2010), which would increase tyrosine phosphorylation of p27 to promote SCF^{SKP2}-mediated p27 proteolysis. Indeed, in primary human breast cancers, Src activation is associated with reduced p27 protein (Chu et al., 2007). Similarly, the Src-family kinase, Lyn, and BCR-ABL phosphorylate p27 at Y88 and this is blocked by the ABL kinase
inhibitor drug, imatinib (Grimmler et al., 2007). To further complicate matters, BCR-ABL was recently reported to promote AKT-mediated phosphorylation of p27 at T157 in chronic myeloid leukemia progenitors leading to increased cytoplasmic p27 (Chu et al., 2010). Thus, therapeutic interruption of BCR-ABL may not only increase p27 levels, but also restore its nuclear localization. A potential role for Src family-directed therapy to restore p27 function is in therapeutic development in human cancers.

Oncogenic RTK and Ras trigger MEK/MAPK activation (Sebolt-Leopold, 2008) to mediate either p27 loss or inactivation (Yang et al., 2000; Donovan et al., 2001; Cheng et al., 1998). In epithelial cells, transfected oncogenic Ras mislocalized p27 to the cytoplasm and increased p27-cyclin D-CDK4/6 complexes (Liu et al., 2000). This likely reflects constitutive Ras-mediated PI3K activity. Furthermore, Ras-dependent lung tumorigenesis in murine models is associated with increased cytoplasmic localization of p27 (Besson et al., 2006). Constitutively active N-Ras can also mislocalize p27 via the Ral-GEF pathway (Kfir et al., 2005). In fibroblasts, MEK1 overexpression increased p27 degradation and a MEK1 inhibitor increased p27 stability in one study (Delmas et al., 2001) but led to sequestration of p27 in cyclin D-CDK4 complexes in another (Cheng et al., 1998). In breast cancer cells, p27 proteolysis is activated by HER2 and epidermal growth factor receptor in a MEK/MAPK dependent manner (Yang et al., 2000; Lane et al., 2000).

1.3.7 **PI3K/mTOR signaling regulates p27 cytoplasmic localization**

PI3K/mTOR activation increases cell survival and proliferation, and recent data suggest it also drives tumor cell motility, invasion and metastasis (Manning and Cantley,
2007; Ridley et al., 2003; Kolsch et al., 2008). Oncogenic mTORC1 and mTORC2 activation drives cell proliferation via disruption the CDK inhibitory action of p27. Several PI3K/mTOR effectors including AKT, SGK, and RSK phosphorylate p27 at either T157 or T198, impairing nuclear import of p27 to promote its cytoplasmic mislocalization (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002; Liang and Slingerland, 2003; Hong et al., 2008). PI3K/mTOR driven cytoplasmic mislocalization of p27 not only attenuates its CDK-restraining effect, but also promotes its oncogenic ability to drive cell motility (Wander et al., 2011b). Phosphorylation at T198 also stabilizes p27 (Liang et al., 2007; Kossatz et al., 2006), and mediates p27-RhoA binding and a p27-dependent increase in cell motility (Larrea et al., 2009a). Importantly, PI3K/AKT activation is highly correlated with cytoplasmic p27 localization in breast, renal and thyroid cancers and in certain leukemias (Liang et al., 2002; Kim et al., 2009; Motti et al., 2005; Min et al., 2004; Viglietto et al., 2002; Shin et al., 2002).
20

Figure 1.1: p27 plays multi-faceted roles in the regulation of cell proliferation and cell migration. This schematic representation depicts p27 regulation and function as it relates to the protein’s subcellular localization. While in the nucleus, p27 binds to and inhibits cyclin E-CDK2, preventing G1-S transit. Following SRC/ABL-mediated tyrosine phosphorylation and cyclin E-CDK2-mediated T187 phosphorylation, nuclear p27 is targeted for SCF-SKP2-dependent degradation, allowing cell cycle progression. S10 phosphorylation promotes nuclear export; while in the cytoplasm p27 may play several roles. Oncogenic signal transduction via PI3K/PDK1 or Ras/MAPK results in the hyperactivation of several AGC family kinases (including AKT, SGK, and RSK), that all mediate C-terminal phosphorylation of p27 at T157 and T198. These phosphorylation events cooperate to sequester and stabilize p27.

1.3.8 Targeting critical signal transduction cascades upstream of p27

As reviewed above, diverse oncogenic signaling cascades regulate p27 proteolysis, subcellular localization, and function (see Figure 1.2) (Wander et al., 2011b). The informed use of targeted therapies to inhibit deregulated signaling holds promise for cancer therapy. Several molecular targeting drugs would impact p27 by inhibiting
upstream signaling. In chronic myelogenous leukemia driven by the BCR-ABL kinase, reduced p27 levels result from its constitutive tyrosine phosphorylation and accelerated proteolysis (Grimmler et al., 2007). Imatinib treatment restores p27 levels and inhibits CML cell proliferation (Grimmler et al., 2007). In many other pre-clinical models, pathways driving p27 proteolysis were reversed by targeted therapies: in Her2-overexpressing breast cancer, p27 proteolysis is attenuated by treatment with trastuzumab (Nahta et al., 2004); the same is true of EGFR-overexpressing lung cancers treated with gefitinib (Busse et al., 2000). In a recent study, breast cancer cells treatment with EGFR/ErbB-2 inhibitor, lapatinib, increased p27 and reduced tumor proliferation (D'Alessio et al., 2010). In melanoma models, MEK inhibition increased p27 to induce growth arrest (Kortylewski et al., 2001; Lefevre et al., 2003). Similar results were obtained using a MEK inhibitor in human pancreatic cancer lines driven by activated Ras (Gysin et al., 2005).

Despite its promise from a mechanistic perspective, early clinical trials with rapamycin and its analogs (rapalogs) met with limited success. This may be due, in part, to incomplete blockade of mTORC1 by rapamycin (Choo et al., 2008). Furthermore, inhibition of mTORC1 turns on feedback loops leading to PI3K activation (Harrington et al., 2004; O'Reilly et al., 2006). Feedback PI3K/AKT activation following mTORC1-specific blockade would increase cytoplasmic p27 sequestration and promote p27-dependent tumor cell migration and metastasis. This prompted development of second-generation catalytic site inhibitors that target PI3K, mTOR or both, many of which have shown promise in preclinical studies and early clinical trials (Wander et al., 2011a).
1.4 SUMMARY

As reviewed above, dual PI3K/mTOR inhibitors have potent anti-proliferative and pro-apoptotic effects in several human cancer xenograft models (Serra et al., 2008; Brachmann et al., 2009; Mallon et al., 2010; Cao et al., 2009). While most studies have evaluated drug efficacy against primary xenotumor growth, their ability to inhibit tumor metastasis, the major cause of cancer patient death, has not been systematically evaluated. Work of this thesis in Chapter 3 provides in vivo evidence that PI3K/mTOR activity is critical for the metastatic process in a model of bone metastasis. The highly bone metastatic variant of the MDA-MB-231 breast cancer model, 1833 (Kang et al., 2003), showed PI3K/mTOR activation, high levels of p27pT157 and p27pT198, and p27-dependent motility/invasion in vitro. The novel PI3K/mTOR catalytic site inhibitor, PF-04691502, reduced p27 phosphorylation and cytoplasmic accumulation, and impaired tumor cell invasion. Moreover, this drug effectively impaired outgrowth of bone metastasis in vivo. p27CK-T157D/T198D transfection rendered cells resistant to inhibition of motility/invasion by the PI3K/mTOR inhibitor, suggesting that the activity of PF-04691502 is mediated in part by its action on p27. These data implicate the PI3K/mTOR pathway as a key mediator of tumor metastasis and reveal a novel rationale for application of catalytic-site PI3K/mTOR inhibitors in cancer therapy.

As noted earlier, hyperactivation of PI3K/mTOR signaling, through C-terminal phosphorylation of p27 at T157 and T198, promotes cytoplasmic p27 mislocalization, increased invasiveness, and may underlie progression in a variety of cancers (Larrea et al., 2009b). However, the underlying mechanisms of how deregulated p27 downstream of PI3K/mTOR signaling in human cancers contributes to tumor progression are not fully
understood. In Chapter 4, we show that p27, when deregulated by constitutive T157 and T198 phosphorylation functionally contributes to oncogenic progression by regulating cell migration, invasion and metastasis. We further provide novel evidence that this deregulated p27 promotes cancer metastasis through EMT through STAT3-mediated TWIST1 regulation. We also identify a potential signaling feedforward loop containing PI3K/AKT activation, p27 phosphorylation, STAT3 activation and further AKT activation that might contribute to tumor progression. These findings suggest that combined inhibition of both AKT and STAT3 in PI3K/AKT activated, p27pT157/pT198 enriched human cancers may ultimately have therapeutic potential to limit p27-mediated EMT and cancer metastasis.

In the work of Chapter 5, which is not thematically linked to the p27 studies above, I explored a novel role for VEGF in human cancer stem cell self-renewal through VEGFR-2/STAT3 signaling to upregulate MYC and SOX2. In the final chapter of this thesis, future experimental work is proposed to investigate further how C-terminal phosphorylation of p27 may play roles not only to drive EMT, and cancer metastasis, but also promote tumor progression and integrate EMT and metastasis by driving cancer stem cell self-renewal.

In summary, the work in this thesis investigates the oncogenic function of p27 downstream of PI3K/AKT signaling as molecular drivers for cell migration, EMT and cancer metastasis. We identify STAT3 as critical signaling pathway for p27-mediated tumor progression. We also uncover a novel function of VEGF in regulating cancer stem cell self-renewal and metastasis through VEGFR-2/STAT3 signaling. These findings
expand our knowledge of molecular mechanisms underlying cancer metastasis and provide insights for developing novel treatment for inhibiting cancer metastasis.
CHAPTER 2: MATERIALS AND METHODS

This chapter describes the materials and experimental approaches that were used throughout this thesis. Section 2.1 consists of three tables with list of cell lines, reagents and oligonucleotide sequences. Section 2.2 contains details descriptions of different experiment methods that were utilized.

2.1 MATERIALS

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2.2 METHODS

2.2.1 Establishment of stable transfectants expressing p27 phosphomimetic mutants

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 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 vector encoding different shRNAs (Open Biosystems) or p27 mutants was co-transfected with Delta VPR and CMV VSVG plasmids (Addgene) into asynchronous 293T with Lipofectamine Plus. 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 6μg/ml protamine sulfate, and analyzed 3-5 days post infection via both GFP visualization and western blotting.

2.2.3 Wound-healing migration assay

Cells were seeded 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: \((\text{initial wound distance} - \text{final wound distance}) / \text{initial wound distance}\). 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 50% 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 utilizing the Real-Time Cell Analysis (RTCA) system from Xcelligence. For transwell migration assays, CIM plates were seeded in their upper chamber with 20,000 cells in serum-free medium, which were subsequently allowed to migrate toward 10% FBS serum in the bottom chamber. For transwell invasion assays, CIM plates were precoated with 5% matrigel and the upper chamber were seeded with 40,000 cells, which were subsequently allowed to invade toward 10% FBS serum in the bottom chamber. 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 & Briegel, 2010; Rieger et al., 2010) using primers for indicated genes listed in table 2.3 (Lindley and Briegel, 2010), and for GAPDH, which was used as an internal control (Rieger et al., 2010). All samples were performed in triplicates and average C_t values were normalized to the values of GAPDH.

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 Section 2.1. All western blots were prepared utilizing Millipore PVDF membrane and blocked for forty-five minutes 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 above.

2.2.8 Nuclear and cytoplasmic fractionation

Nuclear cytoplasmic fractionation was as described (Liang et al., 2002). Nuclei were isolated following centrifugation and removal of the cytosolic supernatant. Equal cell volumes of nuclear and cytoplasmic lysates were blotted for p27. Nuclear RCC1 was blotted as fractionation control.
2.2.9 Cell cycle analysis

Bromodeoxyuridine (BrdU) pulse labelling and flow cytometry for cell cycle distribution were as described (Sandhu et al., 1997). Triplicate biological replicates were prepared for each sample, and the percent cells in G1, S, or G2 were plotted +/- SEM.

2.2.10 Cell proliferation assay

In vitro cellular proliferation was assessed by plating equal cell counts (n=100,000) in a 100mm culture dish at time = 0. Triplicate samples were plated in parallel and cells were harvested and counted at day 2, 4, and 6. Average cell counts were calculated and plotted +/- SEM.

2.2.11 Bioluminescent imaging and analysis

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). Values were normalized to the value obtained immediately after xenografting (day 0) so that all mice had an arbitrary starting BLI signal of 100. For assessment of lymph node metastasis, orthotopic primary tumors were completely covered with black electrical tape to reduce the large bioluminescent signal. Auto exposures were then acquired and the threshold standards were reduced to allow visualization of nodal tumor burden, should any exist. 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.
2.2.12 Animal studies for drug treatment

Animal work was approved by the Institutional Animal Care and Use Committee. For bone metastasis assays, at least ten animals in each arm were injected with $10^5$ viable cells in 0.1 ml PBS were injected into the left ventricle of 5-week old female Balb/c nude mice (Charles River) +/- prior treatment in culture with 250nM PF1502 or vehicle control over the proceeding 7 days. Mice were injected intraperitoneally with 1.5 mg of d-luciferin (15 mg ml$^{-1}$ in PBS) and imaged by Xenogen IVIS system on day 0 and weekly thereafter as in (Kang et al., 2003). Bioluminescence (BLI) plots of photon flux were calculated for each mouse. Values were normalized to that obtained immediately after xenografting (day 0) such that all mice had a starting BLI signal of 100. Animal were weighed twice-weekly. Leg and spinal tissues were recovered at necropsy at 3 weeks for hemotoxylin and eosin (H & E) staining histopathology unless morbidity required earlier euthanization.

2.2.13 Immunohistochemistry and analysis of human tumor samples

One hundred primary human breast cancer and matched nodal metastasis, and thirty primary node negative breast cancers were obtained from the de-identified tumor bank and database of UMSSCC Tissue Core Facility. p27 evaluated by immunohistochemistry as in (Catzavelos et al., 1997). The % cells showing nuclear and/or cytoplasmic p27 staining was scored independently by two pathologists (C. Milikowski and T. Ince). Means (range) for the node positive group were as follows: age 52 yrs (27-72), tumor size 4.3 cm (1.2-18); # positive nodes 9 (1-53), follow-up 10 yrs (0.8-27.5) . 59% were ER+, 88% grade 2 or 3, with distant relapse in 62%. p27
correlation with node positivity was scored using the Spearman analysis with node number as a continuous variable. Kaplan-Meier curves were generated for node-positive patients after excluding primary tumors that showed no p27 staining (n=3). Statistical significance was assessed by log rank test.

2.2.14 Mammosphere assay

Cells were seeded (2000 or 5000/well) on 6 well ultra-low attachment plates as in (Dontu et al., 2003); spheres >75μM diameter were counted after 10-14 days +/- human recombinant VEGF (R&D Systems) at 10ng/ml renewed every two days (triplicate assays repeated twice). To block the effects of VEGF/VEGFR-2 signaling on mammospheres formation, VEGFR-2 mAb 2C3 (Brekken et al., 2000) (Provided by Dr.Brekken from UT Southwestern Medical Center) was added at 15ug/ml before VEGF treatment. For serial sphere assays, primary spheres were recovered after 7-14 days, gently trypsinized, counted and re-seeded without VEGF for two additional passages to generate secondary and tertiary spheres.

2.2.15 Colony formation assay

Single cell suspensions were mixed with soft agar (6 X10³ cell/ml agar/1 cm well) +/- VEGF added every 2 days. Colonies were counted at 4 weeks.

2.2.16 Fluorescence immunocytochemistry

Fluorescence immunocytochemistry on cultured cells was performed as previously described. Primary antibodies were to E-cadherin (1:1,000), N-cadherin
(1:500), Vimentin (1:500), p-STAT3 (1:1,000), Twist1 (1:500), followed by incubation with Alexa Fluor 594-conjugated secondary antibody (1:200). Nuclei were stained with DAPI in PBS and cells were visualized on a Leitz Axiovert microscope after mounting in Prolong Gold anti-fade reagent (Invitrogen).

2.2.17 Experimental lung metastasis assay

Indicated cancer cell lines were injected via tail vein into 4-6 wk Balb/C nude mice. Bioluminescence was monitored by IVIS and quantified as normalized photo flux (Minn et al., 2005). Lungs were photographed and surface tumors counted.

2.2.18 Orthotopic xenograft assay for VEGF treatment

For orthotopic limiting dilution assays, MDA-MB-231-luc cells were grown with or without VEGF at 10 ng/ml for 7 days as above. One hundred or one thousand cells were injected in 0.05 ml matrigel into one inguinal mammary fat pad of 6 wk old Balb/C nude mice (Charles River) (seven to eight mice per group) as in (Minn et al., 2005) and tumor formation monitored weekly. For analysis of metastasis from primary tumors, 10^6 cells were injected as above. At 1cm diameter, primaries were excised and metastatic bioluminescence was monitored by Xenogen IVIS weekly thereafter. Experiments were conducted per University of Miami Animal Care and Use Committee standards.

2.2.19 CD44 and CD24 staining, ALDEFLUOR assay

The ALDEFLUOR kit (Stem Cell Technologies, Durham, NC, USA) was used to isolate the population with a high ALDH1 enzymatic activity as in (Ginestier et al.,
2007). Cells were suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 mmol/l per 1x10^6 cells) and incubated during 40 min at 37°C. As negative control, for each sample of cells an aliquot was treated with 50 mmol/l diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. After ALDEFLUOR staining, cells were costained with anti-CD24-PE and anti-CD44-APC (BD Pharmigen) for 20 min on ice. ALDEFLUOR was excited at 488 nm, and fluorescence emission was detected using a standard fluorescein isothiocyanate (FITC) 530/30-nm band-pass filter.

2.2.20 Immunoprecipitation

MDA-MB-231 or H23 cells were treated +/- VEGF for 2 hours, lysed and 500 µg lysate was incubated with 1 µg of VEGFR-2 or STAT3 antibodies (Cell Signaling), collected on protein G-agarose beads and washed three times with IP buffer and analyzed by SDS-PAGE followed by western analysis. Antibody-alone controls were run with all immunoprecipitations.

2.2.21 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays for SOX2 promoter or MYC promoters were as described (Assou et al., 2007). Briefly, soluble chromatin was prepared from a total of 2 × 10^7 asynchronously growing MDA-MB-231 cells that were treated +/- VEGF. 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 precleared with protein A beads blocked with 2 µg of sheared salmon sperm DNA and preimmune serum. The precleared
chromatin solution was divided and utilized in immunoprecipitation assays with either an anti-STAT3 antibody 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 PCR with primers specific for the STAT3-binding sites upstream of the transcriptional start site of SOX2 promoter or MYC promoter. The primers for CHIP analysis were listed in Table 2.3.

2.2.22 Statistical analysis

All graphed data presented as mean ± SE from at least 3 experiments used two-tailed Student’s t tests to differences. P values < 0.05 were considered statistically significant. Tests were two-sided unless otherwise specified.

The tumor initializing frequency is calculated by L-Calc™ Limiting Dilution Software (http://www.stemcell.com/en/Products/All-Products/LCalc-Software.aspx) from STEMCELL Technologies Inc.

The statistical differences between growth curves were calculated using "compareGrowthCurves" function of the statmod software package at the following website (http://bioinf.wehi.edu.au/software/compareCurves). This analysis was applied for cellular proliferation assays, orthotopic tumor growth curves, and IVIS metastatic tumor growth curves.

Public gene expression data for 1340 primary breast cancers, compiled from published studies(Kessler et al., 2012) and for 1492 lung cancers(Mishra et al., 2013) was
used to test correlations between \textit{VEGF-A} expression with \textit{STAT3} and \textit{cMYC} using univariate COX analysis or Pearson’s correlation. \textit{VEGF-A} mRNA levels were correlated with patient survival by univariate COX and by Kaplan Meyer analyses. Clinical and pathologic features were not available in these datasets and thus multivariate analysis was not possible.
CHAPTER 3:

TARGETED PI3K/mTOR INHIBITION IMPAIRS TUMOR CELL MOTILITY AND BONE METASTATIC OUTGROWTH VIA MODULATION OF p27

A version of this chapter was published in *Breast Cancer Res Treat* 2013, 138(2):369-381. As:

PI3K/mTOR inhibition can impair tumor invasion and metastasis in vivo despite a lack of antiproliferative action in vitro: implications for targeted therapy

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**Contribution:** Dekuang Zhao was involved in the conception and design of all experimental work for this manuscript and performed experiments and data analysis for Figures 3.2, 3.3, 3.4 and 3.5
3.1 SUMMARY

Oncogenic PI3K/mTOR activation is frequently observed in human cancers and activates cell motility via p27 phosphorylations at T157 and T198. Here we explored the potential for a novel PI3K/mTOR inhibitor to inhibit tumor invasion and metastasis. An MDA-MB-231 breast cancer line variant, MDA-MB-231-1833, with high metastatic bone tropism, was treated with a novel catalytic PI3K/mTOR inhibitor, PF04691502, at nM doses that did not impair proliferation. Effects on tumor cell motility, invasion, p27 phosphorylation, localization, and bone metastatic outgrowth were assayed. MDA-MB-231-1833 showed increased PI3K/mTOR activation, high levels of cytoplasmic p27\textsuperscript{pT157pT198} and increased cell motility and invasion in vitro versus parental. PF04691502 treatment, at a dose that did not affect proliferation, reduced total and cytoplasmic p27, decreased p27\textsuperscript{pT157pT198} and restored cell motility and invasion to levels seen in MDA-MB-231. p27 knockdown in MDA-MB-231-1833 phenocopied PI3K/mTOR inhibition, whilst overexpression of the phosphomimetic mutant p27\textsuperscript{T157DT198D} caused resistance to the anti-invasive effects of PF04691502. Pre-treatment of MDA-MB-231-1833 with PF04691502 significantly impaired metastatic tumor formation in vivo, despite lack of anti-proliferative effects in culture and little effect on primary orthotopic tumor growth. A further link between cytoplasmic p27 and metastasis was provided by a study of primary human breast cancers which showed cytoplasmic p27 is associated with increased lymph nodal metastasis and reduced survival. Novel PI3K/mTOR inhibitors may oppose tumor metastasis independent of their growth inhibitory effects, providing a rationale for clinical investigation of PI3K/mTOR inhibitors in settings to prevent micrometastasis. In primary human breast
cancers, cytoplasmic p27 is associated with worse outcomes and increased nodal metastasis, and may prove useful as a marker of both PI3K/mTOR activation and PI3K/mTOR inhibitor efficacy.
3.2 RESULTS

3.2.1 Metastatic cells show PI3K/mTOR activation and resistance to antiproliferative effects of PI3K/mTOR inhibition at nM PF04691502 doses

To investigate the role of PI3K/mTOR pathway activation in tumor cell invasion and metastasis, we used a well-characterized luciferase tagged MDA-MB-231 (hereafter 231) breast cancer line variant, MDA-MB-231-1833 (hereafter 1833), that consistently shows greater bone metastasis than parental (Kang et al., 2003). The 1833 cells demonstrated enhanced PI3K/mTOR signaling relative to parental 231, with activation of downstream kinases (Figure 3.1A). PF-04691502 (hereafter PF1502) is a potent, catalytic inhibitor of both PI3K and mTOR kinases with anti-proliferative effects at nanomolar doses in vitro, and potent anti-tumor effects on multiple xenografts in vivo (Yuan et al., 2011). Since drug-mediated loss of cell survival or proliferation would abrogate metastasis, drug levels were titrated to determine if effects on motility/invasion could be distinguished from anti-proliferative actions. Treatment of both 231 and 1833 lines at 250 nanomolar concentration inhibited PI3K/mTOR effector kinase activities (Figure 3.1A) but neither 48hr (top) nor 7 day (bottom) continuous drug exposures inhibited subsequent cell proliferation over the following week (Figure 3.1B) nor did this drug dose inhibit 231 or 1833 cell cycle progression (Figure 3.1C). Indeed both 231 and 1833 were resistant to growth arrest by PF1502 at drug doses that arrest other lines (see drug titration in BT-20, 231 and 1833, Figure3.1D). At 250nM, PF1502 did not yield a sub-G1 fraction (Figure 3.1D), nor did it reduce cell viability or induce caspase 3 cleavage (Figure 3.1E).
Figure 3.1: PF1502 inhibits PI3K/mTOR activity in MDA-MB-231 and 1833 without affecting proliferation or caspase activation. (A) Cells were treated 48 h with 250 nM PF1502 or DMSO control prior to Westerns of total and phospho-activated PI3K/mTOR effectors. (B) Cells were pre-treated with 250 nM PF1502 for 48 h (top) or for 7 days (bottom) prior to seeding into cell culture without further drug and subsequent population growth is plotted as cell number over time. Mean cell number from triplicate repeats are plotted/time ± SEM (comparative analysis of growth curves T test‡ p = 0.10, ‡‡ p = 0.20). (C) Flow cytometry of 231 and 1833 cells treated with 250 nM PF1502 or vehicle control for 48 h (Cell cycle distribution after 7 days of therapy was also unchanged, not shown). Drug treatment did not yield any sub-G1 fraction. (D) Flow cytometry for BT20, MDA-MB-231 and 1833 cells treated with increasing nM PF1502 or vehicle control for 48 h. Drug treatment did not yield any sub-G1 fraction. (E) Caspase 3 cleavage assay after 250 nM PF1502 for 48 h (left) or 7 days (right). Positive control in far right lane shows MDA-MB-231 after 72 h of paclitaxel 100 nM.
3.2.2 PI3K/mTOR inhibition impairs tumor cell motility and invasion

Cellular adhesion as measured with the XCelligence Real-Time Cell Analysis system (Figure 3.2A), migration by wound-healing assay (Figure 3.2B), and transwell matrigel invasion (Figure 3.2C) were all decreased by pretreatment of both 231 and 1833 cells with 250nM PF1502 for 48hrs. The highly bone-metastatic 1833 cells showed enhanced migration and markedly enhanced invasion relative to parental 231 (Figure 3.2B&C). Thus, PI3K/mTOR inhibition, at a PF1502 dose that did not affect proliferation or survival, abolished the excess motility and invasion of 1833 over parental 231.
Figure 3.2: PI3K/mTOR inhibition attenuates tumor cell adhesion, motility, and invasion. Cells were pre-treated with PF1502 at 250 nM, unless otherwise indicated, for 2 days prior to assays. (A) Cell adhesion in 1833 cells ± drug (mean ± SEM, T test for final time points vs control, \( p < 0.002 \)) (B) Relative migration 6 h after wounding of a confluent cell monolayer (graphed ± SEM, treated vs untreated T test *\( p = 0.005 \)). (C) Relative transwell matrigel invasion ± SEM (T test of treated vs control *\( p = 0.02 \))

3.2.3 Increased cytoplasmic p27 in 1833 cells is reduced by PF1502 treatment

While nuclear p27 plays an established role to inhibit G1-phase cell cycle progression, recent work has implicated cytoplasmic p27 in the promotion of cell motility (Wander et al., 2011b). PI3K/mTOR effector kinases, including AKT, RSK, and SGK, phosphorylate C-terminal p27 residues T157 and T198 causing its accumulation in the cytoplasm (Wander et al., 2011b). The 1833 cells had higher total p27 levels, p27 phosphorylation at T157/T198 (Figure 3.3A), and cytoplasmic p27 accumulation compared to parental 231 (Figure 3.3B). While most pronounced in 1833, PF1502 (250nM) decreased p27 T157/T198 phosphorylation and reduced cytoplasmic p27 (Figure 3.3A&B) in both lines. Notably, p27 knockdown in 1833 cells (Figure 3.3C) reduced cell adhesion (Figure 3.3D), and impaired motility and matrigel invasion to a similar extent as treatment with PF1502 (see Figure 3.2B&C vs Figure 3.3E&F). Thus, loss of p27 in 1833 cells phenocopied in vitro effects of PI3K/mTOR inhibition on cell adhesion, motility and invasion. In the parental 231 line that lacked high cytoplasmic p27 (Figure 3.3B), p27 knockdown did not significantly affect motility and invasion.
Figure 3.3: PI3K/mTOR inhibition reduces cytoplasmic p27 and p27 knockdown phenocopies PF1502 treatment. Cells were treated 48 h with 250 nM PF1502 or DMSO prior to Westerns of: (A) Total p27, p27pT157 and p27pT198 and (B) p27 in cytosolic (C) and nuclear (N) fractions. Cells were infected with shRNA p27 (+) or control (−) 5 days before the following assays. (C) Relative p27 levels with and without shRNA-mediated knockdown. (D) Cell adhesion. (E) Mean relative migration 24 h after wounding of cell monolayer (mean ± SEM T test *p = 0.01 for 1833 – vs +shp27). (F) Relative transwell matrigel invasion over 24 h (mean ± SEM T test *p < 0.05 for 1833 – vs +).
3.2.4  \( p^{2}{7}^{CK-T157DT198D} \) confers partial PF1502 resistance

If p27 critically mediates PI3K/mTOR effects on cell invasion, a p27 phosphomimetic mutant should oppose the PF1502-dependent reduction in motility and invasion. To test this, a phosphomimetic p27T157DT198D mutant defective for cyclin-CDK interaction (p27^{CK-T157DT198D}, abbreviated p27CK-DD) was introduced into both 231 and 1833 cells (Figure 3.4A). p27CK-DD increased 231 cell transwell motility, and 231p27CK-DD was resistant to inhibition of motility by PF1502 (Figure 3.4B). Similarly, matrigel invasion by 231p27CK-DD was greater than that of parental 231 cells and was not significantly reduced by PF1502 (Figure 3.4C). Introduction of p27CK-DD into 1833 cells—that already expressed high levels of cytoplasmic p27pT157pT198—did not significantly increase matrigel invasion. Notably, while PF1502 impaired invasion in 1833 (Figure 3.2C), 1833p27CK-DD cells were resistant to this effect (Figure 3.4D). Thus, cytoplasmic p27pT157pT198 plays a key role in mediating PI3K/mTOR-dependent motility and invasion.
Figure 3.4: Expression of a phosphomimetic, cell-cycle inert p27 mutant conveys resistance to PF1502. (A) Western analysis shows GFP-p27CK-T157DT198D (p27CK-DD) expression in 231 and 1833 (control represents empty-vector transfection). (B) Migration in 231 ± PF1502 with or without p27CK-T157DT198D, mean ± SEM (T test of 231p27CK-DD [top] and 231 [bottom] with or without drug *p = 0.01, † p = 0.11; 231CK-DD versus. 231 control p < 0.0001). (C-D), Matrigel invasion of 231 (C) and 1833 cells (D) transduced with p27CK-DD, with and without 48 h PF1502 250 nM and graphed ± SEM. (T-tests as indicated: *p = 0.003, † p = 0.068 (left); *p = 0.0002, † p = 0.064 (right)). Representative photomicrographs demonstrate differential cell invasion with and without p27CK-DD expression in 231 and 1833.
3.2.5 PI3K/mTOR inhibition reduces metastasis in vivo

Given the marked effect of PF1502 to inhibit cell motility and invasion, we next assayed its effects on tumor metastasis in vivo. To test if prolonged PI3K/mTOR inhibition in vitro would affect the subsequent ability to establish bone metastasis following intracardiac injection, cells were treated or not with PF1502 250nM for 7d followed by intracardiac injection into nude mice (n=10/group). Animals received no further drug thereafter. These assays did not aim to test anti-tumor effects per se but whether PI3K/mTOR activity at the time of injection was required for manifestation of the metastatic phenotype. Viable tumor burden was quantitated by in vivo imaging system (IVIS) of tumor bioluminescence and representative IVIS images, graphs of photon flux over time and at the end of the experiment, and bone metastasis histopathology are shown in Figure 3.5A-D. Untreated 1833 gave rise to more bone metastases than did 231 over 3 weeks, as expected (Kang et al., 2003). While seven days of 250 nM PF1502 drug pre-treatment did not reduce subsequent cell proliferation in vitro (Fig 3.1), it significantly reduced subsequent bone tumor formation by 1833 (Figure 3.5A-C). Notably, an appreciable reduction in tumor bioluminescence was evident as early as 3 days post-injection, suggesting that PF1502 pre-treatment may modulate early steps in this metastatic model (arterial extravasation and initial establishment of viable microcolonies). PF1502 pre-treatment did not significantly alter bone metastasis by parental 231 (Figure 3.5A-C). The presence of metastatic tumor in bone was verified histopathologically at necropsy (Figure 3.5D), but the decalcification required precluded tumor IHC assays for p27 and Ki67. Thus, at a dose that fails to affect cell survival or
proliferation in vitro, PF1502 reduced the excess tumor cell motility, invasion, and metastasis of highly PI3K/mTOR-activated 1833 to levels seen with the parental 231 line.

It is noteworthy that while it significantly reduced bone metastasis, a one-week PF1502 pre-treatment prior to injection into each of two mammary fat pads did not significantly decrease orthotopic growth of either 231 or 1833 tumors, consistent with its lack of antiproliferative effect in cell culture (Figure 3.5E). Of note, lymph nodal metastasis from primary orthotopic tumors appeared to be reduced by PI3K/mTOR inhibition of injected cells: 4/5 animals injected with untreated cells developed metastases while only 2/5 mice injected with drug pre-treated cells yielded tumors metastatic to nodes.
Figure 3.5: PI3K/mTOR inhibition abrogates bone metastatic outgrowth in vivo. 231 and 1833 were cultured ± 250 nM PF1502 for 7 days prior to intracardiac injection. No further drug was given thereafter. (A) Representative bioluminescence at 0, 7 and 21 days post-injection. (B) Mean normalized photon flux (P/s, log scale) for 1833 (top) and 231 (bottom) ± SEM versus time (comparative analysis of growth curves, *p values as indicated). (C) Mean normalized photon flux (P/s) for 1833 and 231 at day 21 ± SEM. (D) Representative bone metastasis histopathology (tumor outlined in yellow). (E) Growth curves of orthotopic tumors arising from 231 and 1833 cultured in vitro ± 250 nM PF1502 for 7 days prior to mammary fat pad injection († p > 0.50). Animals received no drug therapy.
3.2.6 Cytoplasmic p27 is linked to nodal metastasis and reduced breast cancer patient survival

To further address the link between cytoplasmic p27 and metastasis, p27 localization was assessed by immunohistochemistry in 100 primary breast cancers with matched lymph nodal metastases (see Figure 3.6A). Nuclear p27 staining was greater in primary tumors than in nodal metastases, with the mean % tumor nuclei positive for p27 stain 57.5% in primary tumors and only 40% in nodal metastasis (p< 0.0004). Cytoplasmic p27 staining showed a trend toward increase between primary and nodal tumors. The proportion of tumor cells showing cytoplasmic p27 staining in the primary cancers was positively associated with the number of lymph nodes affected at diagnosis (Spearman’s r=0.22, one-sided p=0.007), an early indicator of distant metastatic potential (Figure 3.6B). Moreover, cytoplasmic p27 was associated with reduced disease-free (two-sided p=0.04, Log-rank test) and overall survival (p=0.05) for these node positive cancers, when compared with cancers showing only nuclear p27 staining at diagnosis (Figure 3.6C&D). These in vivo data further support the link between cytoplasmic p27 and metastatic tumor progression in human breast cancer patients.
Figure 3.6: Cytoplasmic p27 correlates with lymph node invasion at diagnosis and breast cancer outcome. (A) IHC demonstrating predominantly nuclear (left) or strong nuclear + cytoplasmic p27 staining (right). (B) Primary tumor cytoplasmic p27 as it relates to number of nodes positive at diagnosis. (C) Kaplan–Meier analysis of nuclear only versus cytoplasmic positive tumors (N = 21 and N = 73, respectively) and disease-free survival. (D) Kaplan–Meier analysis of nuclear only versus cytoplasmic positive tumors and overall survival.
3.3 DISCUSSION

PI3K/mTOR signaling promotes tumor progression by activating cell proliferation, growth and survival (Sabatini, 2006) and has been implicated in tumor metastasis. Here we provide evidence that a novel catalytic PI3K/mTOR inhibitor may exert anti-tumor effects by opposing metastasis. Inhibition of effector kinases by the PI3K/mTOR inhibitor, PF1502, decreased C-terminal phosphorylation of p27, reduced its cytoplasmic localization, and attenuated tumor cell motility, invasion and metastasis. That these effects occur at a drug dose that failed to inhibit cell proliferation suggests that processes governing tumor metastasis downstream of PI3K/mTOR may be independent of those driving proliferation. Thus, PI3K/mTOR inhibition, in lines that are resistant to the compound’s anti-proliferative action and at a drug dose that fails to prevent primary tumor growth, may have potential to prevent or attenuate the establishment of micrometastatic foci that initiate systemic tumor spread.

While there is evidence that both PI3K and mTOR pathways may contribute to metastasis, the mechanisms mediating this are not well understood. In human breast cancers, pAKT and p4EBP1 were greater in matched distant metastases compared to primary tumors (Akcananat et al., 2008). mTORC2-dependent AKT activation increased ovarian cancer motility in vitro and metastasis in vivo (Kim et al., 2011), and PI3K inhibition prevented metastasis in a murine thyroid cancer model (Furuya et al., 2007). mTORC1 increased migration in gastric cancer cells (Hashimoto et al., 2008), mTORC2 drove migration and invasion in a glioma model (Masri et al., 2007) and both Rictor and Raptor expression are required for metastasis of a colorectal cancer xenograft (Gulhati et al., 2011). While none of these reports distinguished between effects on tumor growth
versus direct effects on metastasis, they raise the possibility that PI3K/mTOR inhibition may not only impair biosynthetic processes driving tumor growth, but also modulate the metastatic process.

The CDK inhibitor, p27, plays dual roles to regulate both cell proliferation and motility. While p27 restrains normal cell proliferation through cyclin-Cdk2 inhibition (Chu et al., 2008), it can also modulate cell motility through mechanisms involving its C-terminal domain (McAllister et al., 2003; Nagahara et al., 1998). p27 can bind RhoA to inactivate RhoA/ROCK and drive cell motility (Besson et al., 2004). Motility effects of p27 may exist in normal cells, since p27-dependent migration is essential for normal cortical neuron development in vivo in murine embryos (Kawauchi et al., 2006; Nguyen et al., 2006). In cancers, oncogenic PI3K/mTOR activation increases p27 phosphorylation at T157 and/or T198 and promotes cytoplasmic p27 mislocalization (Hong et al., 2008; Larrea et al., 2009a; Liang et al., 2002; Viglietto et al., 2002; Shin et al., 2002). Indeed, PI3K-mediated p27 phosphorylation at T198 enhances its binding to and inhibition of RhoA (Larrea et al., 2009a).

The balance between growth inhibitory and acquired pro-motility/metastatic functions may determine effects of p27 in different cancers. For example, p27 knockdown increased proliferation and enhanced primary tumor formation in a Ras-driven mouse tumor model, but impaired tumor invasion (Kelly-Spratt et al., 2009). In many cancers, p27 protein levels are reduced due to miRNA-mediated loss of synthesis or accelerated proteolysis (Chu et al., 2008). In contrast, tumors with activated AKT exhibit abundant cytoplasmic p27 (Chu et al., 2008). Cytoplasmic p27 was implicated in local invasion in an AKT-driven human glioma xenograft (Wu et al., 2006) and
overexpression of cytoplasmic $p27^{CK-}$ enhanced murine melanoma metastasis (Denicourt et al., 2007).

Here we investigated further the link between PI3K/mTOR activation, p27 and cancer metastasis. The well-characterized highly bone-metastatic 1833 model showed activation of PI3K and mTOR effector kinases and a p27-dependent increase in cell motility and invasion in vitro. The dual PI3K/mTOR inhibitor drug, PF1502, inhibited PI3K as evidenced by decreased pPDK1, and effectively impaired activation of both TORC1 and TORC2 substrates. It also reduced C-terminal p27 phosphorylation, cytoplasmic p27 accumulation, and phenocopied effects of p27 knockdown to impair cell adhesion, motility, and invasion in vitro. The T157/T198 phosphomimetic p27CK-DD mutant increased motility and invasion of parental 231 cells. It also promoted resistance to loss of invasion and motility caused by the PI3K/mTOR inhibitor in both 231 and 1833 cells. That the phosphomimetic p27 did not fully reverse PF1502 effects on motility/invasion may reflect that aspartate at T157/T198 fails to fully mimic phosphorylation, or that p27-independent mediators also contribute to PI3K/mTOR driven tumor metastasis.

The present in vivo experimental approach was not intended to mimic patient therapy, but rather to assay the requirement for PI3K/mTOR pathway activity at the time of injection for subsequent primary and metastatic tumor outgrowth. Notably, prior PI3K/mTOR inhibition by PF1502 that failed to restrain cell proliferation during or after one week in culture in vitro, and did not reduce primary orthotopic tumor growth, significantly impaired the subsequent outgrowth of bone metastatic tumors after intracardiac injection in vivo. In addition, nodal metastasis from orthotopic primary
tumors also showed a trend toward reduction with drug pre-treatment prior to injection. Thus, drug effects to impair metastasis in this model appear to occur despite the lack of an anti-proliferative effect, and may modulate tumor cell extravasation and establishment of metastatic foci, as suggested by early IVIS data in the first few days post-injection. Present data extend prior work implicating PI3K and mTOR pathways in tumor growth and metastasis (Gulhati et al., 2011; Thiery et al., 2009), and suggest that drug effects on cell proliferation or growth may be separable from those driving metastasis. Indeed, malignant progression of cells resistant to anti-proliferative effects of a novel PI3K/TOR-KI may still be interdicted at the level of metastasis—an effect that might be missed by traditional phase 1 clinical trials focused on tumor size reduction.

A relationship between cytoplasmic p27 and metastasis is supported by our novel observation that there is a positive correlation between the extent of cytoplasmic p27 staining in primary breast cancers and the number of nodes affected at diagnosis. While loss of nuclear p27 is strongly associated with poor patient outcome (Chu et al., 2008), few studies have addressed the prognostic import of cytoplasmic p27, which is observed in up to 60% of human cancers. Cytoplasmic p27 is correlated with poor outcome in colon and prostate cancers and lymphoma (Chu et al., 2008). p27 localization to cytoplasm in primary breast cancers was associated with AKT activation (Liang et al., 2002; Viglietto et al., 2002; Shin et al., 2002). The present study supports our earlier report associating cytoplasmic p27 in primary breast cancer with a reduced metastasis free interval (Liang et al., 2002), and provides the first association with reduced overall patient survival.
PI3K and/or mTOR inhibitors have shown significant therapeutic promise in several cancers (Wander et al., 2011a). Dual PI3K/mTOR catalytic-site inhibitors have begun to enter clinical trials (Wander et al., 2011a) and have shown improved anti-tumor effects over allosteric mTORC1 inhibitors in pre-clinical models of breast (Serra et al., 2008; Brachmann et al., 2009; Mallon et al., 2010), pancreatic (Cao et al., 2009), and renal cell cancers (Cho et al., 2010), melanoma (Marone et al., 2009), glioma (Mallon et al., 2010; Liu et al., 2009), multiple myeloma (McMillin et al., 2009), and acute myeloid leukemia (Park et al., 2008). The present work suggests the anti-proliferative effects of PI3K/mTOR inhibitor drugs may be distinct from their anti-metastatic action. The latter may result in part from their effects to abrogate cytoplasmic p27 accumulation. This work has implications for the clinical application of this new class of dual catalytic-site PI3K/mTOR inhibitors: in addition to their effects to check primary tumor growth, PI3K/mTOR inhibitors may also help prevent establishment of metastasis by clinically occult tumor cells, proving a rationale for trials investigating their potential to prevent systemic metastases early in the disease course. Furthermore, the presence of cytoplasmic p27 may identify tumors with PI3K/mTOR activation and provide a potential biomarker of PI3K/mTOR inhibitor therapeutic efficacy.
CHAPTER 4:

CYTOPLASMIC P27 PROMOTES TUMOR METASTASIS THROUGH EPITHELIAL-MESENCHYMAL TRANSITION

A version of this chapter will be submitted to Cell as:

Cytoplasmic p27 promotes epithelial-mesenchymal transition and tumor progression via STAT3-mediated Twist1 upregulation.

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⁵Performed experimental work for figure 4.11 and 4.12
⁶Performed experimental work for figure 4.11 and 4.12
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4.1 SUMMARY

The cell cycle regulator 27 is frequently degraded or mislocalized to the cytoplasm in human cancers. PI3K dependent phosphorylation at T157 or T198 causes cytoplasmic p27 accumulation where it is known to increase cell motility by actin destabilization. Use of a cell cycle defective (CK-) double phosphomimetic p27 T157D/T198D mutant, p27CK-DD, revealed a novel pro-metastatic function of p27. p27CK-DD overexpression induced epithelial-mesenchymal transition (EMT) of human mammary epithelial cells and increased the mesenchymal phenotype and metastasis of breast and bladder cancer cells in vivo. Conversely p27 knockdown in p27pT157/pT198 enriched highly metastatic cancer cells decreased motility and invasion, reverted EMT in vitro, and impaired metastasis in vivo. p27CK-DD activated STAT3 and facilitated its transactivation of TWIST1 to induce EMT. STAT3 inhibition decreased TWIST1 expression, reversed EMT and decreased p27CK-DD induced metastasis, while constitutively active STAT3 rescued both the loss of EMT and metastasis following p27 knockdown in highly metastatic breast and bladder cancer lines. These data extend our understanding of p27 function in human cancer and suggest that PI3K deregulated p27 may promote tumor progression through STAT3 activation and induction of TWIST1 and other EMT transcription factors. Pharmacological inhibition of STAT3 in p27pT157/pT198 enriched human cancers may effectively inhibit p27-mediated EMT and metastasis.
4.2 RESULTS

4.2.1 Generation of p27 phosphomimetic mutants

To study the direct effects of p27 phosphorylation on cell migration and invasion, different EGFP-tagged p27CK- non-phosphorylatable (T to A) or phosphomimetic (T to D) mutants were generated by site-directed mutagenesis and confirmed by direct sequencing (Figure 4.1). Because they fail to bind and inhibit CDKs, these mutants allow us to study the cell cycle-independent oncogenic function of p27.

![Diagram of p27 mutants](image)

**Figure 4.1: Generation of p27 phosphomimetic mutants.** EGFP-p27CK- vector was used as a template to generate EGFP-p27CK- T157D, EGFP-p27CK- T198D and EGFP-p27CK-T157D/T198D mutants by site-directed mutagenesis. All p27 mutants were confirmed by direct sequencing.
4.2.2 Establishment of stable cell lines expressing p27 phosphomimetic mutants

The mutants above were transfected into MDA-MB-231 cells and stable cell lines expressing similar EGFP-p27 were selected for functional analysis (Figure 4.2A). As predicted, these stable p27CK- mutant cell lines showed similar proliferation and cell cycle profiles (Figure 4.2B).

4.2.3 p27CK-T157D/T198D mutants show increased cytoplasmic localization

Phosphorylation of p27 at T157 impairs nuclear import and phosphorylation of T157 and T198 may be processive in vivo. The nuclear and cytoplasmic localization of p27 mutants were quantified by fluorescence microscopy. As shown in Figure 4.3, p27CK-T157D and/or p27CK-T198D mutants show increased cytoplasmic localization.
4.2.4  p27CK-T157D/T198D increases cell migration and invasion

Cytoplasmic p27 has been shown to increase cell motility. To test if each C-terminal phosphorylation (T157 and T198) contributes to cell motility, the cell lines with stable p27 phosphomimetic mutants were assayed by both wound healing and transwell matrigel invasion analysis. Cell migration and invasion were each significantly increased by transfected p27CK-T157D and/or p27CK-T198D phosphomimetic mutants, and the mutant expressing p27CK-T157DT198D together caused the greatest increase in migration and invasion (Figure 4.4).
4.2.5 p27CK-DD induces EMT-like morphological changes in human mammary epithelial cells

As noted above, PI3K/AKT mediated p27 phosphorylation increases cell motility and invasion in cancer-derived cell lines (Wu et al., 2006; Larrea et al., 2009a; Wander et al., 2013), but this has not been shown before in cells that were not malignantly transformed. To test at what point in malignant change the C-terminal phosphorylation of p27 may contribute to tumor progression, we subcloned p27CK-DD into Plvx-AcGFP lentivirus system and introduced p27CK-DD into immortalized, non-transformed human mammary epithelial cell line MCF12A cells (MCF12A-p27CK-DD). Over several weeks
thereafter, MCF12A-p27CK-DD cells underwent a processive morphological switch from the typical cobble-stone-like epithelial appearance to an elongated, spindle-like mesenchymal shape (Figure 4.5), suggesting a possible EMT event.

4.2.6 p27CK-DD induces EMT in human mammary epithelial cells

Expression of p27CK-DD in MCF12A cells was shown in Figure 4.6A. p27CK-DD induced typical EMT-like morphological changes in MCF12A cells (Figure 4.6B). p27CK-DD decreased expression of epithelial markers, such as E-cadherin and increased mesenchymal markers, N-cadherin and Vimentin (Figure 4.6C-D). MCF12A cells expressing only p27CK- did not manifest cellular and molecular changes of EMT (Figure 4.5). Thus, the loss of cell cycle inhibitory function in p27CK- is not sufficient and p27
phosphorylations at T157 and T198 appear to be required for the initiation of p27-mediated EMT.

Cells that undergo EMT acquire a more invasive phenotype (Micalizzi et al., 2010). In keeping with this, p27CK-DD greatly increased both migration and matrigel invasion of MCF12A cells (Figure 6E&F). Similar findings were observed in the hTert-immortalized normal human mammary epithelial line, HME3 (Ince et al., 2007), which showed significant increase of mesenchymal markers, cell migration and invasion following stable p27CK-DD transduction (Figure S4.6). These data suggest that high level expression of p27 defective for CDK inhibition and bearing phosphomimetic mutations at T157 and T198 induces an invasive EMT phenotype in non-tumorigenic human mammary epithelial cells.
Figure 4.6: Overexpression of p27CK-DD induces EMT in immortal normal mammary epithelial cells. (A) Expression of p27CK-DD in MCF12A cells. (B) Phase-contrast images of MCF12A morphology in vector control (MCF12A-C) or p27CK-DD (MCF12A-p27CK-DD). (C) Western of EMT markers and TWIST1 in MCF12A-C and MCF12A-p27CK-DD cells. (D) Immunofluorescence of EMT markers and TWIST1 in MCF12A-C and MCF12A-p27CK-DD cells. (E) Transwell migration of MCF12A-C and MCF12-p27CK-DD cells. (F) Transwell matrigel invasion analysis of MCF12A-C and MCF12A-p27CK-DD cells. All data graphed represent mean of at least 3 repeats +/- SEM. (*p<0.05).
4.2.7 **Twist1 is critical for p27CK-DD induced EMT**

The EMT program can be initiated by a number of different EMT-inducing transcription factors including Snail, Slug, zinc finger E-box binding homeobox 1 (Zeb1), and Twist (Batlle et al., 2000; Cano et al., 2000; Nieto et al., 1994; Aigner et al., 2007; Yang et al., 2004). To elucidate which transcription factors contribute to p27CK-DD -mediated EMT, their expression was assayed by RT-PCT. p27CK-DD modestly increased \textit{SNAI2} (encoding Slug) and \textit{ZEB2} expression and dramatically increased \textit{TWIST1} expression by 20 fold (Figure 4.7A). p27CK-DD also increased TWIST1 protein as demonstrated by both Western blot and by direct immunofluorescence (Figure S4.6: Overexpression of p27CK-DD induces EMT in HME3 cells. (A) Morphological changes of HME3 expressing vector control (HME3-C) or p27CK-DD (HME3-p27CK-DD). (B) Western of EMT markers in HME3-C and HME3-p27CK-DD cells. (C) Transwell migration of HME3-C and HME3-p27CK-DD cells. (D) Transwell matrigel invasion of HME3-C and HME3-p27CK-DD cells. All data graphed represent mean of at least 3 repeats +/- SEM. (*p<0.05).
4.7B&C), suggesting that TWIST1 may play critical role for p27CK-DD-induced EMT. Indeed, *TWIST1* knockdown in p27CK-DD cells significantly attenuated EMT phenotype reflected by a re-expression of epithelial marker and loss of mesenchymal markers (Figure 4.7D), supporting that TWIST1 is essential for the p27CK-DD-induced EMT phenotype.

**Figure 4.7: TWIST1 is critical for p27CK-DD-induced EMT.** (A) RT-PCR of EMT transcription factors in MCF12A-C and MCF-12A-p27CK-DD. (B) Western of TWIST1 in MCF12A-C and MCF-12A-p27CK-DD cells. (C) Immunofluorescence of TWIST1 in MCF12A-C and MCF12A-p27CK-DD cells. (D) The effects of Twist1 knockdown on EMT markers in MCF12A-p27CK-DD cells. All data graphed represent mean of at least 3 repeats +/- SEM. (*p<0.05 and **p<0.01).

### 4.2.8 p27CK-DD enhances the mesenchymal phenotype in cancer cells

We next investigated the ability of p27CK-DD to enhance EMT in cancer cells. p27CK-DD overexpression in the epithelial breast cancer cell line, MCF7, caused a morphological change compatible with EMT progression, and increased mesenchymal markers (N-cadherin and Vimentin) and *TWIST1* expression (Figure 4.8A-D). p27CK-
DD also increased MCF7 cell migration and invasion (Figure 4.8E-F). Moreover, p27CK-DD significantly enhanced the mesenchymal features of a human bladder cancer cell line UMUC3 (data not shown), suggesting that its effects on EMT are not exclusive to breast cancer. Together these data support a novel oncogenic role of p27 to promote EMT in both human mammary epithelial cells and breast and bladder cancer models.

4.2.9 Loss of p27 decreases mesenchymal phenotype in p27pT157/pT198 enriched metastatic cells

To examine whether endogenous p27pT157pT198 contributes to the maintenance of an EMT phenotype, a line derived from MDA-MB-231 (231) with enhanced ability to form lung metastasis, MDA-MB-231-4175 (4175) was compared to the parental line
(Minn et al., 2005). 4175 cells showed strong PI3K pathway activation: AKT, SGK, and PDK1 activation were all increased relative to parental 231 while total kinase levels were similar (Figure 4.9A). 4175 also showed higher p27 levels (Figure 4.9A). When equal levels of endogenous p27 were titrated, 4175 cells showed a marked increase in both p27pT157 and p27pT198 levels compared to 231 cells (Figure 4.9B). Stable knockdown of p27 in 4175 led to an increase of E-cadherin, and a decrease of Vimentin at both mRNA and protein level (Figure 4.9C&D). Transient knockdown of p27 by siRNA caused a decrease in TWIST1 expression within 4 hrs and this was followed by an increase in E-cadherin by 48 hrs (Figure 4.9E&F). Notably, stable knockdown of p27 in 4175 cells did not change the cell cycle profile, compatible with prior observations that in the context of oncogenic PI3K activation p27 loses its cell cycle inhibitory function (Wander et al., 2013) (Figure 4.9G). Migration and matrigel invasion of 4175 cells were both markedly decreased by p27 knockdown (Figure 4.9H&I). Similarly, in the highly metastatic bladder cancer line UMUC3-LUL2 (UMUC3-L) derived from UMUC3, p27 knockdown also decreased Twist1, Vimentin and N-Cadherin levels (Figure 4.9J-M) and matrigel invasion (data not shown). Thus, in metastatic cancers with high PI3K activation, p27pT157pT198 appears to drive and maintain an EMT program promoting increased cell motility and invasion.
**Figure A**

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**Figure B**

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**Figure D**

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**Figure G**

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**Figure I**

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4.2.10 p27CK-DD drives STAT3 activation to induce EMT

To elucidate the oncogenic signaling pathway involved in p27CK-DD-induced EMT, we assayed a panel of signaling kinases known to contribute to EMT. Of these, both total and phosphorylated STAT3 (Y705) were greatly increased in MCF12A-p27CK-DD cells compared to control MCF12A (Figure 4.10A). p27CK-DD expression activated STAT3 and increased its nuclear translocation (Figure 4.10B). To test if STAT3 activation is required for p27-induced EMT, MCF12A-p27CK-DD was treated with a STAT3 inhibitor (VIII 5, 15, DPP). Drug mediated STAT3 inhibition (Figure 4.10D) reversed the mesenchymal phenotype of MCF12A-p27CK-DD cells, shown by morphological change from spindle-like mesenchymal shape to cobble-stone-like epithelial appearance (Figure 4.10C). Interestingly, p27CK-DD also markedly induced AKT activation that was inhibited by treatment with STAT3 inhibitor (Figure 4.10D). The STAT3 inhibitor also increased epithelial marker and decreased mesenchymal marker levels and attenuated Twist1 expression (Figure 4.10E). Transduction of dominant negative STAT3 mutant (STAT3DN) into MCF12A-p27CK-DD cells phenocopied pharmacological STAT3 inhibition (Figure 4.10F), and markedly attenuated p27CK-DD-induced cell migration and invasion (Figure 4.10G&H). Thus, p27CK-DD-
induced EMT is reversible in immortalized mammary epithelial cells and STAT3 signaling plays critical role for the maintenance of this EMT phenotype.

**Figure 4.10:** p27CK-DD drives STAT3 activation to induce EMT. (A) Western of phosphorylated pSTAT3 (Y705) and total T-STAT3 in MCF12A-C and MCF12A-p27CK-DD cells. (B) Immunofluorescence of p-STAT3 in MCF12A-C and MCF12A-p27CK-DD cells. (C) Phase-contrast images of MCF12A-p27CK-DD cells treated with STAT3 inhibitor (STAT3-I). (D) Effects of STAT3 inhibitor (STAT3-I) on Stat3 and Akt activation in MCF12A-p27CK-DD cells. (E) Effects of STAT3-I on Twist1 and EMT markers expression in MCF12A-p27CK-DD cells. (F) Effects of dominant negative STAT3 (STAT3DN) on Twist1 and EMT markers expression in MCF12A-p27CK-DD cells. (G) Transwell migration of MCF12A cells expressing vector control, p27CK-DD or p27CK-DD-STAT3DN. (H) Transwell invasion of indicated cells. All data graphed represent mean of at least 3 repeats +/- SEM. (*p<0.05).
4.2.11 STAT3 inhibition decreases p27CK-DD-induced cancer metastasis

Since the EMT program is critical for cancer cell invasion and metastasis (Kalluri and Weinberg, 2009; Thiery et al., 2009; Micalizzi et al., 2010), we assayed effects of p27CK-DD with and without STAT3 inhibition on invasion and metastasis. In the low metastatic 231 breast cancer line, p27CK-DD transduction increased Twist1, decreased E-cadherin (Figure 4.11A), and markedly increased cell migration and invasion (Figure 4.11B&C). STAT3DN transduction into 231-p27CK-DD cells significantly reversed the mesenchymal phenotype, causing loss of Twist1 and increasing E-Cadherin and decreasing p27-driven cell migration and invasion (Figure 4.11A-C). Furthermore, upon tail vein injection into nude mice, luciferase tagged 231-p27CK-DD generated significantly greater lung metastasis than parental 231 controls (Figure 4.11D-F). Both the number and size of lung metastasis formed were increased, as was cumulative tumor bioluminescence. This gain of metastatic potential was reversed by STAT3 inhibition: STAT3-DN transduction into 231-p27CK-DD significantly attenuated formation of lung tumors, to levels similar to those observed with control 231 cells (Figure 4.11D-F).

To confirm the pro-metastatic effect of p27CK-DD and that STAT3 was a key mediator of its effects, similar experiments were carried out in the UMUC3 human bladder cancer model. p27CK-DD also induced a STAT3-dependent increase of mesenchymal phenotype in vitro and multi-organ metastasis in vivo (Figure 4.11G-J). These data provide strong evidence that p27CK-DD plays a STAT3-dependent oncogenic role in promoting tumor progression by modulating EMT program to drive metastasis.
4.2.12 Restoration of STAT3 activity rescues Shp27-mediated decrease of metastasis

In both highly lung metastatic cell lines, 4175 and UMUC3-L, loss of p27 reverted the EMT phenotype (Figure 4.9). p27 knockdown also decreased STAT3 activation in these lines (Figure 4.12A). Consistent with \textit{in vitro} findings that knockdown of p27 decreased cell migration and invasion, loss of p27 also markedly decreased experimental lung metastasis formation by both 4175 and UMUC3-L cells (Figure 4.12B-G). Since STAT3 activity was decreased by p27 knockdown, a constitutive active STAT3 mutant (STAT3CA) was introduced into 4175-Shp27 cells to test if restoration of STAT3 would rescue the Shp27-mediated loss of metastasis. STAT3CA partially rescued the metastasis formation by 4175-Shp27 cells \textit{in vivo} (Figure 4.12B-D), suggesting that STAT3 serves as a critical mediator of pro-metastatic effects of p27pT157pT198.
Figure 4.12: Restoration of STAT3 activity rescues the loss of metastatic potential following p27 knockdown. (A) Effects of p27 knockdown on STAT3 activation in high metastatic MDA-MB-231-4175 and UMUC3-LuL2 cells. (B) Bioluminescence/time of experimental lung metastasis from xenograft mice that received intravenous injection of 4175 cells expressing the indicated vectors. (C) Representative bioluminescence imaging from indicated 4175 groups. The color scale depicts the photon flux (photons per second) emitted from xenografted mice. (D) Representative images of lungs from indicated 4175 groups. (E) Bioluminescence/time of experimental lung metastasis from xenograft mice that received intravenous injection of UMUC3-L cells expressing indicate vectors. (F) Representative bioluminescence imaging of mice from UMUC3-L groups. The color scale depicts the photon flux (photons per second) emitted from xenografted mice. (G) Representative images of lungs from indicated UMUC3-L groups. Data are graphed as mean ±SEM. (*p<0.05 and **p<0.01).
4.3 DISCUSSION

The development of distant metastasis is an ominous event and the main cause of death in most cancer patients (Valastyan and Weinberg, 2011). There has been substantial progress in recent years in our understanding of the complex process of human tumor metastasis (Gupta and Massague, 2006). Until recently, the lack of knowledge of molecular mechanisms underlying metastasis has limited development of therapies that specifically target tumor spread (Chiang and Massague, 2008). While prior work showed cytoplasmic p27 plays an oncogenic role via RhoA-Rock inhibition to increase cell migration, present data indicate that the phosphorylation of both T157 and T198, functionally contribute to p27-mediated cell migration and invasion in vitro. Here, we provide evidence that deregulated p27 plays a cell cycle-independent, oncogenic role to drive a metastatic cascade in human breast and bladder cancer cells. Overexpression of phosphomimetic p27CK-DD, which mimics p27 phosphorylated at T157 and T198 by PI3K/AKT signaling in human cancers, promotes, whereas p27 knockdown in highly metastatic cells enriched for p27pT157/pT198 impairs tumor invasion and metastasis. Furthermore, our xenograft experiments revealed that the C-terminal phosphorylation of p27 is a critical driver of tumor metastasis in vivo via activation of STAT3, which mediates induction of TWIST1 and other EMT transcription factors.

To initiate metastasis, individual tumor cells must transgress cell-cell junctions and acquire the ability to invade beyond the surrounding basement membrane (Chaffer and Weinberg, 2011). This early step in the metastasis cascade is believed to be initiated by reactivation of the embryonic EMT program (Micalizzi et al., 2010). Our findings demonstrate that expression of p27CK-DD is sufficient for this EMT-like phenotypic
switch in both normal mammary epithelial cells and tumorigenic cancer cells. In MCF12A, an immortal but non-malignant breast epithelial line and the MCF7 breast cancer models, p27CK-DD induced a typical EMT-like morphological switch with decreased epithelial and increased mesenchymal markers, accompanied by an increase in cell migration and invasion. Conversely, in the highly PI3K-activated metastatic 4175 breast cancer cell line with high endogenous p27pT157/pT198, p27 knockdown reverted the EMT phenotype and invasive ability. Similar findings were observed in the UMUC3-LuL2 bladder cancer model. Both highly metastatic 4175 and UMUC3-LuL2 lines showed higher PI3K activation and higher total and pT157/pT198 levels of p27 than their low metastatic counterparts, and p27 knockdown substantially reduced the formation of experimental lung metastasis in both models. Interestingly, p27 knockdown did not change cell cycle and proliferation rates in either the 4175 or UMUC3-LuL2 lines, compatible with prior observations that in the context of high oncogenic PI3K activation, p27 loses its cell cycle inhibitory function (Wander et al., 2013). As has been also been recently observed for the tumor suppressor p73 in which gene truncation drives an EMT process (Steder et al., 2013), malignant tumor progression not only involves loss of tumor suppressor function of p27, but p27CK-DD acquires a similar oncogenic gain of function to promote metastasis by trigging EMT-like changes. Thus, cancer cells seem to retain and deregulate these cell cycle inhibitors to gain oncogenic functions to promote tumor progression. This may explain why the cell cycle inhibitor p27 is rarely completely lost, despite its decreased expression in human cancers.

The EMT program is initiated by several EMT-inducing transcription factors including Twist1 (Ansieau et al., 2010; Yang et al., 2004). Twist1 acts independently of
Snail to repress E-cadherin and to upregulate N-cadherin. As a master regulator of embryonic morphogenesis, Twist1 has been shown to promote cancer metastasis by activating EMT (Yang et al., 2004). Here, we showed that overexpression of p27CK-DD in low metastatic cells markedly increased TWIST1 at both mRNA and protein levels, while p27 knockdown in p27pT157/pT198 enriched highly metastatic cancer lines decreased TWIST1. Notably TWIST1 knockdown in p27CK-DD overexpressing cells decreased p27CK-DD-induced mesenchymal markers, suggesting that TWIST1 plays the essential role in p27CK-DD-induced EMT.

Aberrant expression and activation of STAT3 occurs in many types of cancers including breast cancer (Lieblein et al., 2008). Activated STAT3 plays critical role in malignant transformation and tumor progression (Inghirami et al., 2005). Recent data suggest that constitutive activation of STAT3 also contributes to EMT and breast cancer metastasis (Marotta et al., 2011b; Devarajan and Huang, 2009; Xiong et al., 2012). We found that both total and phosphorylated STAT3 (Y705) were significantly increased by p27CK-DD in both normal epithelial cells and cancer cells. Conversely, knockdown of p27 in p27pT157/pT198 enriched cancer cells decreased total and phosphorylated STAT3. Pharmacological inhibition of STAT3 or expressing of STAT3DN reversed p27CK-DD-mediated EMT, as evidenced by morphological change from spindle-like mesenchymal shape to cobble-stone epithelial appearance and reversion of mesenchymal to epithelial markers, indicating STAT3 was critical for p27 induced EMT. Furthermore, STAT3 inhibition significantly attenuated both the p27CK-DD-mediated increase in cell invasion and cancer metastasis. Thus STAT3 signaling appears to play a critical role in the maintenance of p27-driven EMT and metastasis. Moreover, STAT3 inhibition also
attenuated p27-DD induced TWIST1 expression and TWIST1 luciferase activity, supporting previous observation that TWIST1 expression is transcriptionally regulated by STAT3 (Cheng et al., 2008; Lo et al., 2007). Indeed, both our Twist promoter-luciferase assays and CHIP analysis suggest that p27CK-DD increased, while p27 knockdown greatly decreased STAT3 occupancy and activation of the TWIST1 promoter, providing a mechanistic link in which constitutive C-terminal p27 phosphorylation would activate an EMT program to drive tumor metastasis through STAT3-dependent TWIST1 induction.

Notably, we also made the novel observation that p27CK-DD markedly increased AKT activation downstream of STAT3 signaling and treatment with a STAT3 inhibitor significantly reduced this AKT activation (Figure 4.10D). Since AKT phosphorylates p27 at both T157 and T198 (Liang et al., 2002; Viglietto et al., 2002; Motti et al., 2004), it is very likely that this p27CK-DD-mediated AKT activation would further induce endogenous p27pT157/pT198 to further activates STAT3, forming a positive feedforward loop for STAT3 activation and TWIST1 induction. Therefore, in the context of PI3K/AKT activation in human cancer, initial phosphorylation of p27 at T157 and T198 (p27pT157/pT198) by AKT could promote tumor progression and metastasis through a STAT3/TWIST1-dependent EMT program. In addition, this deregulated p27 would also drive STAT3-dependent AKT activation, to further phosphorylate p27 and amplify STAT3 activation and EMT contributing thereby to the acquisition and maintenance of metastatic potential (Figure 4.13).

p27 has been shown to act in a cell-cycle independent manner as a transcriptional repressor to downregulate oncogenic pathways in tumorigenesis (Li et al., 2012; Pippa et al., 2012). Nuclear p27 directly interacts with two transcriptional factors, p130
(retinoblastoma-like 2) and E2F4 (E2F transcription factor 4) to form a repressive complex at target promoters (Li et al., 2012; Pippa et al., 2012). We showed p27CK-DD not only activated STAT3, but also increased STAT3 expression. While it is not clear at present precisely how C-terminal phosphorylation of p27 induces STAT3 expression, we postulate that the transcriptional function of p27 might be involved. In non-malignant cells, nuclear p27 might form a repressive complex with p130/E2F4 to inhibit STAT3 expression. During malignant progression oncogenic AKT activation may cause disruption of this repressive complex through C-terminal p27 phosphorylation, with release of the tonic repressive effect of p27 on STAT3 expression. In the context of p27CK-DD overexpression, STAT3 derepression would feedforward to activate AKT and phosphorylate cellular endogenous p27 and further increasing STAT3 expression. Further work will be needed to test this hypothesis.

Interestingly, p27 was also recently shown to downregulate TWIST1 expression through binding TWIST1 gene promoter as part of a repressor complex in undifferentiated human embryonic stem cells (Menchon et al., 2011). We observed that cells expressing high levels of T157 and T198 phosphorylated endogenous p27 and those transduced with p27CK-DD showed high TWIST1-luciferase activity and **TWIST1** expression. Whether C-terminal phosphorylation of p27 downstream of PI3K/mTOR activation may disrupt p27’s repressor function and indeed may convert p27 from a co-repressor to a transactivator at the TWIST1 promoter warrants further investigation. The overexpression of p27CK-DD might similarly disrupt this repressor complex through modulation of endogenous p27 phosphorylation which results in TWIST1 overexpression and EMT induction.
In summary, we have uncovered a novel oncogenic function of p27 to regulate tumor metastasis through activation of an EMT program in both breast and bladder cancer models. Cytoplasmic p27 is observed in human cancers including breast, esophageal, colon, and prostate (Chu et al., 2008) and has been correlated with adverse outcome in prostate cancer (Li et al., 2006), glioma (Piva et al., 1999) and high-grade astrocytomas (Hidaka et al., 2009). In breast cancer, cytoplasmic p27 staining correlated with AKT activation (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002) and predicted decreased time to disease relapse (Liang et al., 2002) and was associated with increased lymph nodal metastasis and poor overall survival (Wander et al., 2013). Here we extend the understanding of p27 deregulation in cancer: p27pT157pT198 activates STAT3 to induce TWIST1 expression, in a manner critical for tumor invasion and metastasis. Present data also raise the possibility that p27 may also shift from a repressor to a STAT3 and TWIST1 activator as a consequence of C-terminal phosphorylation by AKT. Furthermore, we also identified a potential signaling feedforward loop containing AKT activation, p27 phosphorylation, STAT3 activation and further AKT activation that might contribute to tumor progression. Combined inhibition of both AKT and STAT3 in PI3K/mTOR activated, p27pT157/pT198 enriched human cancers may ultimately have therapeutic potential to limit p27-mediated EMT and cancer metastasis.
CHAPTER 5: VEGF DRIVES CANCER INITIATION STEM CELLS THROUGH VEGFR-2/STAT3 SIGNALING TO UPREGULATE MYC AND SOX2

A version of this chapter is under review by *Oncogene*, December, 2013 as

VEGF drives cancer initiating stem cells through VEGFR-2/Stat3 signaling to upregulate Myc and Sox2

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Dekuang Zhao conceived of and designed all experimental work for all figures, and carried out experimental work for all figures with assistance from co-authors. J Sun completed experimental work for Figures 5.4 G and 5.5F. C. Creighton carried out tumor analysis for Figure 5.6
5.1 SUMMARY

Vascular endothelial growth factor-A (VEGF), a well-known tumor angiogenic factor, is also implicated in self-renewal in several tissue types. Recent work indicates VEGF drives malignant stem cells but mechanisms thereof and tumor types affected are not fully characterized. Here, we show VEGF drives breast and lung cancer stem cell self-renewal and provide new mechanistic insight into this. VEGF increased tumor spheres and aldehyde dehydrogenase activity \textit{in vitro} in aggressive triple negative breast cancer (TNBC) lines, dissociated primary TNBC cancers, and in lung cancer lines. VEGF exposure prior to injection increased breast cancer-initiating stem cell abundance \textit{in vivo} yielding increased orthotopic tumors, and also increased metastasis from orthotopic primaries and following tail vein injection without further VEGF treatment. VEGF rapidly activated STAT3 to bind \textit{c-MYC} and \textit{SOX2} promoters and induce their expression. Knockdown of these transcription factors impaired VEGF-induced sphere formation, a proxy for stem cell function. VEGF stimulated VEGFR-2 binding and activation of JAK2/STAT3. VEGFR-2 knockdown or inhibition abrogated VEGF-mediated STAT3 activation, \textit{MYC} and \textit{SOX2} induction and sphere formation. Thus, in addition to angiogenic effects, VEGF appears to increase CSC self-renewal through VEGFR-2/STAT3/MYC-dependent signaling. In the largest analysis of VEGF to date in primary breast and lung cancers (>1300 each), high VEGFA was not only prognostic of poor patient outcome but also strongly associated with STAT3 and MYC expression, supporting the link between VEGFA and CSC self-renewal pathways identified. Antiangiogenics are limited by drug induced hypoxia, which induces VEGF secretion. High VEGF-A tumors may be most likely to escape anti-angiogenics by upregulating
VEGF, driving CSC self-renewal to repopulate post-treatment. Our work highlights the need to better define VEGF-driven cancer subsets and supports further investigation of combined targeting of VEGF and VEGFR-2 or JAK/STAT inhibitors to overcome resistance mechanisms.

5.2 INTRODUCTORY REMARKS

Increasing data suggest that malignancies arise from stem-like cells that self-renew and generate heterogeneous, more differentiated progeny with lower replicative capacity (Dalerba et al., 2007). Initially characterized in leukemia, cells with stem-like features that initiate tumors with high frequency have been demonstrated in many human malignancies and cancer stem cells (CSCs) appear to play a critical role in tumor invasion, metastasis, drug resistance, and disease recurrence (Takebe et al., 2011). CSCs can be enriched from bulk cancer cells by selection for expression of specific surface markers. In primary breast cancer, populations expressing surface CD44^+CD24^{low/-} and/or aldehyde dehydrogenase (ALDH1) activity (Al Hajj et al., 2003; Ginestier et al., 2007) are enriched for cells with stem cell like properties in vitro and the ability to initiate xenograft tumors. As for normal stem cells, CSC self-renewal and cell fate determination appear to be regulated by both intrinsic pathways and extrinsic signals from the tumor microenvironment. Extrinsic signals include cytokine networks that regulate CSC self-renewal (Li and Neaves, 2006; Takebe et al., 2011). Since CSCs mediate tumor initiation, drug resistance and metastasis, signals that govern CSCs function are attractive targets for anti-cancer therapy (Malanchi et al., 2012).
Angiogenesis is crucial for progression of solid malignancies and requires pro-angiogenic factors, chief among which is VEGF (Chung et al., 2010). The VEGF receptor (VEGFR) family contains three transmembrane tyrosine kinase receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4), that regulate the formation of blood and lymphatic vessels (Olsson et al., 2006). While abundantly expressed in endothelial cells, VEGFR-2 has also been shown to be expressed by different types of cancers including breast cancer and non-small cell lung cancers (Guo et al., 2010; Tanno et al., 2004). Secreted by cancer and stromal cells, VEGF-A (hereafter VEGF) preferentially binds VEGFR-2 on endothelial cells to stimulate new blood vessel formation needed to support cancer growth and metastasis (Chung et al., 2010). VEGF/VEGFR-2 signaling has been shown to mediate tumor growth, cell migration and chemoresistance (Lee et al., 2003; Yang et al., 2011). In addition to regulating angiogenesis, VEGF can also synergize with EGFR to promote autocrine growth factor-mediated tumor cell proliferation (Lichtenberger et al., 2010).

VEGF is a mitogen for many normal tissues including hemopoietic tissue, vascular endothelium, cardiomyocytes, and adipose tissue (Kane et al., 2011). VEGF also appears to stimulate stem cell self-renewal in several normal tissues including hemopoietic (Gerber et al., 2002), endothelial (Nourse et al., 2010; Kane et al., 2011), neuronal (Calvo et al., 2011) and adipocyte progenitor cells (Gerber et al., 2002; Verseijden et al., 2009). Recent data indicate that VEGF drives cancer stem cell self-renewal in both glioma (Hamerlik et al., 2012) and skin cancer models (Beck et al., 2011), but mechanisms thereof and the spectrum of tumor types affected in this manner are not fully characterized. Here, we investigated whether and how VEGF may stimulate CSCs
independent of mitogenic or angiogenic effects in breast and lung cancers, two highly prevalent and lethal human malignancies. Present data provide evidence that VEGF drives VEGFR-2 to recruit JAK2/STAT3 leading to cMYC and SOX2 promoter occupancy and induction by STAT3 to form an autocrine loop driving cancer stem cell self-renewal.

5.3 RESULTS

5.3.1 VEGF stimulates ALDH1 activity and serial mammosphere formation

Sphere assays reflect the potential to exhibit stem cell traits when cells are removed from their microenvironmental niche (Pastrana et al., 2011). Culture with 10 ng/ml VEGF for 7 days increased mammosphere formation in two ER, PR and HER2 triple negative breast cancer (TNBC) cell lines including the MDA-MB-231 (231) and the oncogene transformed human mammary epithelial line, HMLER3(Ince et al., 2007), and in dissociated tumor cells cultured directly from a primary human TNBC (DT-25) (Bayliss et al., 2007) (Figure 5.1A). Primary MDA-MB-231 spheres formed in the presence of VEGF were dissociated and single cells were seeded serially without further VEGF. The initial 7 day VEGF exposure had a sustained effect over the next 30 days to increase the proportion of sphere forming cells over two further serial passages compared to untreated controls, despite no further VEGF exposure (data for 231 in Figure 5.1A). Spheres required 9-14 days to form and did not result from aggregation in these models. One week VEGF pre-treatment in 2D culture also increased subsequent soft agar colony formation by MDA-MB-231 cells (Figure 5.1B).
VEGF exposure also increased the percentage of cells with aldehyde dehydrogenase 1 activity (ALDH1+) cells in both MDA-MB-231 and DT-25 cells (Figure 5.1C). Sphere formation and ALDH1 activity are properties of stem cells in normal mammary epithelial cells (Ginestier et al., 2007) and are used to assay the abundance of cells with stem like properties in malignant populations. VEGF is a not mitogen for these mammary cancer cells, since it did not increase MDA-MB-231 or DT25 cell numbers over short term 2D culture or change asynchronous cell cycle profiles over 48 hours in 2D or after 14 days in 3D sphere cultures (Figure 5.1D&E).
5.3.2 VEGF pre-treatment increases tumor initiating cell abundance

Since VEGF enriched for cells with stem cell properties in vitro, we next tested if VEGF pre-treatment would increase the proportion of tumor initiating cells titrated by
orthotopic transplantation \textit{in vivo}. Luciferase tagged MDA-MB-231 cells were pre-treated with or without VEGF over one week and then limiting dilutions of 1000 or 100 cells were orthotopically injected into the mammary fat pad of Balb/C nude mice. VEGF pre-treated cells showed a higher tumor-initiating cell frequency and generated tumors with shorter latency than control cells (p=0.01, Figure 5.2A&B). Thus, VEGF significantly increased tumor initiating cell abundance, consistent with its action to increase sphere forming cells.
5.3.3 VEGF pre-treatment increases metastasis-forming tumor cells

Figure 5.2: VEGF exposure increases tumor initiating cell abundance and metastatic potential. MDA-MB-231 cells were pre-treated +/− VEGF for 7 days and injected orthotopically as described. (A) Tumor latency from 1000 and 100 cells is shown. (B) The proportion of injections yielding tumors is tabulated. The frequency of tumor initiating cells was calculated by L-Calc™ Limiting Dilution Software (http://www.stemcell.com/en/Products/All-Products/LCalc-Software.aspx) from STEMCELL Technologies Inc. (C) Tumor growth cure of 10⁶ cell injections. (D) Tumors from 10⁶ cell injections were excised at 1 cm diameter and animals followed for metastasis. Mean bioluminescence (photon flux) of metastasis in tumor-bearing mice tumors is graphed from both groups and representative IVIS images shown. (*p<0.05).
It has been postulated that CSC have greater metastatic potential (Takebe et al., 2011). Since prior VEGF exposure had durable effects on subsequent serial sphere and orthotopic tumor formation weeks after its withdrawal, we tested effects on tumor metastasis. MDA-MB-231 cells pre-treated with or without VEGF for one week prior to injection were introduced into the mammary fat pad. Mammary tumors generated from VEGF-pre-treated cells grew more rapidly than controls (Figure 5.2C, left). Orthotopic primary tumors were excised at 1 cm diameter and animals monitored for metastasis by in vivo imaging system (IVIS). VEGF pre-treatment increased the proportion of tumors that metastasized to lymph nodes (5/7 animals), compared to that of controls (3/7 animals). VEGF pre-treatment significantly increased metastatic tumor burden (mean photon flux in metastasis-bearing animals, p<0.05, Figure 2d left, representative IVIS, Figure 5.2D, right). Thus, VEGF pre-treatment not only increased tumor initiating cell abundance, but also increased their metastatic potential.

We further tested effects of prior VEGF exposure on experimental lung metastasis. MDA-MB-231 cells were pre-treated with or without VEGF for seven days, or pre-treated with anti-VEGF antibody bevacizumab before addition of VEGF (BV+VEGF), prior to tail vein injection and lung tumor formation was monitored by IVIS. VEGF-pretreatment increased, while bevacizumab attenuated early post-intravasation bioluminescence one day after intravenous injection, despite equivalent signals immediately after injection (day 0) in all groups (Figure 5.3A), indicating early effects on intravasation and/or survival. Furthermore, VEGF-pretreatment increased lung tumor establishment over the next six weeks (Figure 5.3A, representative images Figure 5.3B). Lung tumor number and lung weights were significantly increased in
recipients of VEGF-pretreated cells (Figure 5.3C). Notably, 4 of 10 mice injected with VEGF-pretreated cells developed distant metastasis to lymph nodes or bone, while none arose in untreated control and BV+VEGF groups (Representative data Figure 5.3D&E).

To further test if VEGF increased tumor progression by promoting stem cell expansion, pooled lung tumors were dissociated and analyzed for stem cell markers and sphere formation. VEGF-pretreated cells yielded tumors with a higher percentage of ALDH1$^+$ and CD44$^+$CD24$^{low}$ cells and of sphere forming cells, while prior bevacizumab exposure blocked both effects (Figure 5.3F&G). Since VEGF-containing media was removed before cell injection into recipient mice and no additional VEGF was administered thereafter, the increase in tumorigenesis and metastasis cannot be attributed to direct angiogenic effects. Thus, VEGF exposure increased the abundance of cancer cells with stem cell-like features (sphere formation and ALDH1$^+$ activity) in vitro, and exerted long-lasting effects to increase the proportion of tumor initiating cells and enhance metastasis in vivo.
Figure 5.3: VEGF exposure increases experimental lung metastasis. (A) MDA-MB-231 cells were pre-treated -/+ VEGF and +/- bevacizumab (BV+VEGF) for 7 days prior to IV injection. Bioluminescence/time is plotted as normalized photo flux. Representative tumor-bearing mice are shown. (B) Representative lung images and photomicrographs (week 6). (C) Mean lung tumor numbers from each group. (D, E) Distant lymph node and bone metastasis in two representative mice injected with VEGF pre-treated cells. (F, G) Pooled dissociated tumor cells were assayed for % ALDH1+/CD44+/CD24<sup>low</sup> and for sphere formation. Data are graphed as mean ±SEM. (*p<0.05 and **p<0.01).
5.3.4 VEGF induces Sox2 via STAT3 and Myc to promote CSC expansion

Pathways that drive normal embryonic stem cell (ES) self-renewal may be deregulated in cancers to induce malignant stem/progenitor expansion and/or maintenance (Liu et al., 2005). Embryonic stem cell transcription factors including Nanog, Oct3/4, Klf4 and Sox2, are essential for self-renewal and maintain the undifferentiated state of ES. They have also been implicated in CSC self-renewal (Jeter et al., 2011; Yu et al., 2011). VEGF exposure for one week increased *NANOG, SOX2, BMI1*, and *KLF4* expression in MDA-MB-231 (Figure 5.4A). Of these embryonic stem cell transcription factors, *SOX2* was most strongly induced. Sox2 is deregulated in human malignancies (Annovazzi et al., 2011) and increases growth and/or metastasis in lung and breast cancer models (Xiang et al., 2011; Leis et al., 2011), thus, the mechanism of VEGF mediated *SOX2* induction and its importance to VEGF-stimulated stem cell effects were studied further. VEGF increased *SOX2* mRNA within 1-2 hours and protein levels rose by 12 hours (Figure 5.4B).

STAT3 is a master regulator of ES self-renewal and has been implicated as a CSC driver (Bromberg and Wang, 2009; Marotta et al., 2011a). Myc is a known transcriptional target of STAT3 in ES and also promotes self-renewal in ES (Kidder et al., 2008) and hematopoietic stem cells (Meyer and Penn, 2008). Here we assayed their roles as mediators of VEGF effects on CSC. VEGF rapidly activated STAT3 and increased c-Myc expression (Figure 5.4C). We next tested if STAT3 activation was required for VEGF-mediated *MYC* and *SOX2* induction and increased sphere formation. VEGF-mediated Sox2 upregulation was attenuated by either *STAT3* or *c-MYC* knockdown (Figure 5.4D&E). Thus, *SOX2* induction is both STAT3 and Myc dependent.
Furthermore, siRNA mediated loss of STAT3, c-MYC or SOX2 expression each attenuated VEGF-stimulated mammosphere formation (Figure 5.4F). Of these, SOX2 knockdown had the most profound effects, suggesting that SOX2 upregulation by STAT3 and c-Myc is critical for VEGF effects on CSC-like behavior. SOX2 siRNA did not affect cell cycle distribution or short term cell proliferation, thus its attenuation of sphere formation was not an anti-mitogenic effect.

In silico analysis revealed that the human SOX2 promoter upstream region contains multiple c-Myc and STAT3 consensus motifs. VEGF rapidly stimulated binding of both STAT3 and c-Myc to the SOX2 promoter in MDA-MB-231 cells, suggesting direct induction of SOX2 by c-Myc and STAT3 (Figure 5.4G, left and middle). Moreover, VEGF increased STAT3 binding to the c-MYC promoter, which would further amplify the signal (Figure 5.4G, far right). These data suggest that VEGF, produced by tumor or supporting cells, would activate STAT3 in cancer cells and drive C-Myc and Sox2 to promote CSC self-renewal through sustained effects on transcription.
Figure 5.4: VEGF activates STAT3- and Myc-mediated SOX2 induction to drive sphere formation in breast cancer cells. (A) VEGF effects on embryonic transcription factor expression in MDA-MB-231 cells. (B) VEGF increases SOX2 gene and protein expression. (C) VEGF effects on STAT3 activation, c-Myc and SOX2 by Western. (D) Western blot shows knockdown of Sox2, STAT3 and Myc 48 hrs after indicated the siRNA transfections. (E) STAT3 or Myc siRNA decreases VEGF-mediated Sox2 induction. (F) Knockdown of Sox2, STAT3 or Myc attenuates VEGF-mediated increase of mammospheres. (G) ChIP assays show VEGF increases STAT3 and MYC binding to SOX2 promoter and STAT3 binding to MYC promoter. (*p<0.05 and **p<0.01).
5.3.5 VEGF upregulates STAT3/Myc/Sox2 to increase stem cell properties in lung cancer models

To test if effects above might generalize to lung cancer, another highly lethal human malignancy, two non-small cell lung carcinoma lines, Hop62 and H23 were also exposed to VEGF and sphere assays performed. VEGF increased tumor sphere formation (Figure 5.5A) and ALDH1+ activity (Figure 5.5B) without affecting cell proliferation (Figure 5.5C). As in the breast models above, the effect of VEGF on Hop62 self renewal was sustained over two serial sphere assays in the absence of further VEGF (Figure 5.5D). VEGF treatment also induced STAT3 activation and Myc and Sox2 upregulation (Figure 5.5E), and increased occupancy of both MYC and SOX2 promoters by STAT3 (Figure 5.5F), suggesting that VEGF effects on tumor initiating cells are not limited to breast cancer and may have broader relevance to lung cancer and potentially other malignancies.
Figure 5.5: VEGF stimulates STAT3 activation and increases Myc and SOX2 expression in lung cancer lines. (A) VEGF effects on sphere formation in H23 and Hop62 cells. (B) VEGF effects on ALDH1+ cell abundance. (C) VEGF does not change lung cancer cell cycle distribution. (D) VEGF increases serial sphere formation of Hop62 cells. (E) VEGF effects on STAT3 activation, Myc and SOX2 expression. (F) ChIP assays show VEGF activated Myc and STAT3 binding to SOX2 promoter. (*p<0.05 and **p<0.01).
5.3.6 VEGF stimulates VEGFR-2/JAK2/STAT3 activation to drive Myc and Sox2 expression

MDA-MB-231 expresses both VEGFR-1 and VEGFR-2 (Figure 5.6A), in keeping with reports that a variety of tumor cells express VEGF receptors (Guo et al., 2010; Tanno et al., 2004; Ryden et al., 2005). Cells were treated with siRNA to either VEGFR-1 or VEGFR-2 and VEGF effects on pSTAT3, Myc and Sox2 were assayed. Loss of VEGFR-2, but not VEGFR-1 greatly attenuated VEGF-mediated STAT3 activation and prevented Myc and Sox2 upregulation (Figure 5.6A). Furthermore, loss of VEGFR-2 or pre-treatment with specific VEGFR-2 blocking antibody mAb 2C3 (Brekken et al., 2000) also significantly attenuated the VEGF-mediated increase of tumor sphere formation, while VEGFR-1 knockdown did not (Figure 5.6B), suggesting that VEGFR-2 but not VEGFR-1 signaling is required for VEGF-mediated cancer stem cell self-renewal.

VEFG stimulated recruitment of Jak2 and STAT3 by VEGFR-2 as observed in immunoprecipitations of either VEGFR-2 or STAT3 (Figure 5.6C). 2C3 pre-treatment also significantly attenuated VEGF-stimulated STAT3 activation (Figure 5.6D), further indicating that VEGFR-2 and STAT3 interaction is essential for VEGF-mediated VEGFR-2/STAT3/SOX2 signaling. VEGF-driven recruitment and activation of JAK2 and STAT3 by VEGFR-2 was also observed in the H23 lung model and inhibition of VEGFR-2 signaling by its monoclonal antibody 2C3 significantly attenuated lung tumor sphere formation (Figure 5.6E&F). Thus, both breast and lung lines assayed support a model in which VEGF stimulates VEGFR-2 to recruit and activate Jak2/STAT3 driving STAT3 and Myc to induce SOX2 (Figure 5.6G).
Figure 5.6: VEGF enhances VEGFR-2/STAT3 complex formation to induce STAT3 activation. (A) VEGFR-2 knockdownd in MDA-MB-231 reverses VEGF-mediated STAT3 activation and Myc and Sox2 upregulation. (B) Effects of VEGFR-2 knockdown or treatment with blocking antibody 2C3 on VEGF-mediated upregulation of mammosphere formation. (C) VEGF effects on VEGFR2-STAT3 binding in MDA-MB-231. (D) Effects of 2C3 on VEGF stimulated STAT3 activation in MDA-MB-231. (E) VEGF effects on VEGFR2-STAT3 binding in H23 cells. (F) VEGFR-2 blockade by 2C3 attenuates VEGF-mediated increase of mammospheres in H23 cells. (G) Model of VEGF action. (p<0.05, *p<0.05 and **p<0.01).
5.3.7 VEGF-A is associated with STAT3 and c-MYC and poor breast and lung cancer prognosis

That VEGF-A mediates STAT3 activation and MYC induction was further supported by analysis of 1340 primary breast cancers from a public gene expression dataset compiled from published studies (Kessler et al., 2012). Comparison of VEGF-A expression in the different intrinsic breast cancer subtypes identified by gene expression profiling revealed VEGF-A expression was higher in basal like breast cancers (Figure 5.7A), and associated with higher STAT3 (p<1E-15, Pearson’s correlation) and MYC (p<0.0001) expression. Elevated VEGF-A was also associated with worse disease free survival (p=0.01, univariate Cox) (Figure 5.7B). A correlation between increased VEGF-A mRNA and poor patient survival was also observed in a human non-small cell lung cancer dataset (N=1492 patients, p=0.003, univariate Cox) (Figure 5.7C), and elevated VEGF-A was also positively correlated with STAT3 and MYC across lung tumors (p<1E-15 and p<0.01, respectively, Pearson’s).
Figure 5.7: VEGF-A expression according to cancer subtype and relationship to patient outcome.  (A) Analysis of public gene expression data from 1340 primary breast cancers shows VEGF-A mRNA expression is higher in basal-like breast tumors than other intrinsic breast cancer subtypes. (B) In these 1340 human breast cancer patients, VEGF-A expression is associated with decreased disease free survival (p=0.01, Cox). (C) In 1492 human non-small cell lung cancers, VEGF-A expression is associated with worse overall survival (p<0.01, Cox).
5.4 DISCUSSION

VEGF is best known for its angiogenic action on endothelial cells, but also has non-angiogenic effects on other cell types, including normal tissue-specific stem cells. It stimulates normal stem cell compartments in adipogenesis (Cao, 2007) and blood vessel development (Nourse et al., 2010), and also promotes muscle stem cell regeneration, normal neuronal stem cell self-renewal, cardiac stem cell mobilization and survival of embryonic and haematopoietic stem cells (Calvo et al., 2011; Tang et al., 2011; Gerber et al., 2002; Brusselmans et al., 2005; Deasy et al., 2009).

While VEGF promotes self-renewal in these various normal tissue stem cell compartments, a role for VEGF in cancer stem cell (CSC) self-renewal has only recently emerged. In brain tumors, stem cell-like glioma cells (GSC) were shown to promote angiogenesis and tumor growth through increased VEGF secretion (Bao et al., 2006; Folkins et al., 2009; Gilbertson and Rich, 2007). Xenograft blood vessel ablation depleted GSCs, suggesting the vascular microenvironment is critical for GSC maintenance (Bao et al., 2006). Nestin+/CD133+ GSCs directly contact tumor capillaries (Calabrese et al., 2007) and secrete hypoxia-inducible factors including VEGF to promote angiogenesis and glioma growth (Li et al., 2009). Autocrine VEGF-VEGFR-2-Neuropilin-1 signaling was shown to promote glioma growth by regulating stem-like cell viability (Hamerlik et al., 2012). Similarly, VEGF was also shown to stimulate squamous cell skin carcinoma CSCs, suggesting that VEGF not only creates a perivascular niche for CSCs, but may also directly promote their expansion (Beck et al., 2011). A CSC self-renewal pathway in which VEGF acts via Neuropilin-2 to drive Gli was also recently reported in triple negative breast models (Goel et al., 2013).
Present data further support a role for VEGF in CSC regulation and suggest that VEGFR-2-STAT3 mediated induction of MYC and SOX2 is critical to these effects. In three different TNBC models, including a primary dissociated tumor culture, prolonged VEGF exposure over several days increased the proportion of TNBC cells that generate spheres and express ALDH1 activity, and increased soft agar colony formation without exerting a global pro-proliferative effect. The generalizability of these observations to other cancers is supported by similar effects of VEGF on lung cancer lines.

In addition to increasing CSC-like features in vitro, VEGF exposure increased tumor initiating cell abundance in MDA-MB-231, decreased tumor latency and increased orthotopic tumor growth. Furthermore, ex vivo VEGF exposure increased metastasis from both primary orthotopic tumors and following tail vein injection in vivo. Thus, VEGF appears to serve at least two key roles in tumor progression. In addition to its known angiogenic action, VEGF also appears to promote tumor initiating cell expansion and enhance the robustness of metastatic progenitors. The present in vivo studies were not carried out to model anti-neoplastic therapy, but rather to test by ex vivo treatment prior to implantation direct VEGF effects on tumor cells independent of subsequent angiogenesis. In our experiments, animals were not treated further with VEGF after tumor cell injection. Rather, prior exposure to this cytokine appears to provoke STAT3 activity and long lasting effects to sustain CSC behavior over subsequent weeks to months, arguing for effects on tumor formation that are initially independent of angiogenesis. VEGF-STAT3 mediate changes in self-renewal programs via Myc and Sox2 and potentially additional drivers appear to increase tumor initiating cells and drive metastasis, despite the lack of further VEGF treatment in vivo.
VEGFR-2 is a tyrosine kinase receptor essential for VEGF-mediated physiological and pathological responses in endothelial cells (Shibuya, 2008). Although initially thought to be exclusively expressed by endothelial cells, VEGFR-2 is also expressed by different cancers and VEGF/VEGFR-2 can act via both autocrine and paracrine mechanisms to drive cancer cell proliferation and survival (Guo et al., 2010). In non-small-cell lung cancers, a tumor cell-autonomous VEGF/VEGFR-2 feed-forward loop amplifies the signal required for full angiogenic tumor establishment (Chatterjee et al., 2013). Our study provides novel evidence that CSC effects of VEGF are mediated by VEGFR-2 activation of STAT3. In both breast and lung cancer models, VEGF/VEGFR-2 recruits JAK2 and STAT3 to activate STAT3-dependent induction of embryonic stem cell transcription factors, Myc and Sox2. c-Myc and STAT3 cooperatively transactivate each other to activate other embryonic stem cell transcription factors in ES and neural precursors (Kidder et al., 2008; Foshay and Gallicano, 2008). STAT3 has been shown to be required for the growth of CD44+CD24− stem cell-like breast cancer cells in human tumors (Marotta et al., 2011a) and STAT3 and Sox2 have been implicated in not only embryonic stem cell self-renewal and but also in that of CSCs (Nair et al., 2013; Leis et al., 2011).

That VEGF mediates STAT3 activation and MYC induction to drive CSC was further supported by analysis of these factors in the largest cohorts (over 1300 cases in each study) of breast and lung cancer patients assayed for VEGF-A to date. High VEGF-A levels not only predicted poor patient outcome, but were also positively correlated with STAT3 and MYC expression in both tumor types.
The anti-VEGF antibody bevacizumab was the first FDA approved anti-angiogenic therapy and is used in first line therapy of colorectal cancers, and in metastatic non-small cell lung, ovarian and renal cancer and glioblastoma multiforme, in all but the latter together with chemotherapy (Heath and Bicknell, 2009; Boere et al., 2010). Oral small molecule-tyrosine kinase inhibitors of VEGFRs are also approved for liver, kidney and gastrointestinal stromal tumors (Ivy et al., 2009). Despite initial enthusiasm, clinical responses have been modest (Heath and Bicknell, 2009; Boere et al., 2010). Bevacizumab, showed improvement in time to disease progression in early trials with paclitaxel for metastatic breast cancer (Miller, 2003) and while this was also significant in subsequent trials, none showed an increase in patient survival (reviewed in (Montero and Vogel, 2012; Montero et al., 2012)). Pre-clinical studies suggest that bevacizumab promotes more aggressive and metastatic behavior in surviving tumor cells (Ebos et al., 2009; Páez-Ribes et al., 2009). VEGF and VEGFR-directed therapies reduce tumor vessel supply, decrease drug penetration, and increase hypoxia to stimulate even greater VEGF production, overcoming drug effects by driving cell autonomous mitogenic and invasive potential. Indeed, blocking angiogenesis by bevacizumab has been shown to promote subsequent tumor growth due to hypoxia driven CSC self-renewal (Sakariassen et al., 2006; Conley et al., 2012). Present work, together with other recent studies (Beck et al., 2011; Hamerlik et al., 2012), suggest that one factor driving hypoxia-stimulated CSCs is VEGF itself. Increasing data indicate that tumor populations surviving chemotherapy are enriched in stem cells (Creighton et al., 2009; Feng, 2010). Bevacizumab, by augmenting hypoxia would thus increase chemotherapy and VEGF-driven CSC self-renewal.
Given the modest clinical efficacy and pre-clinical data that VEGF-blockade can engender its own resistance, why are present findings of therapeutic interest? Our definitive data in lung and breast cancer patient samples and other smaller prior studies (Han et al., 2001; Cao et al., 2012; Ellis and Hicklin, 2008) suggest that tumor cell VEGFA is upregulated de-novo in a subset of cancers. Since the normal stroma may not be the only or most important target of VEGF-directed therapies, it becomes imperative to identify highly VEGF-driven cancers since these may be most likely to escape anti-angiogenics by upregulating VEGF, driving CSC self-renewal to re-populate post-treatment. Surviving cancer cells secrete VEGF, saturate bevacizumab binding capacity, and stimulate autocrine VEGFR-2 to promote VEGFR-2/STAT3 driven cancer initiating cell recruitment. Targeting VEGFR-2 may enhance the therapeutic efficacy of bevacizumab and help overcome the secondary VEGF upregulation resulting from impaired angiogenesis. We showed that VEGFR-2 blocking antibody treatment greatly attenuated VEGF-mediated tumor sphere formation. Moreover, targeting VEGF/VEGFR-2 downstream signaling by STAT3 knockdown also significantly reduced sphere formation. Current data raise the possibility that combination therapy with VEGF neutralizing antibodies and VEGFR-2 or STAT3 inhibitors may have potential to reduce cancer initiating cell self-renewal and support further pre-clinical and clinical investigation of such novel therapeutic strategies.
CHAPTER 6 FUTURE DIRECTIONS:  
TO INVESTIGATE THE POTENTIAL ROLE OF p27 IN  
CANCER STEM CELL SELF-RENEWAL

6.1 INTRODUCTORY REMARKS

An increasing evidence suggest that diverse solid tumors are hierarchically organized and sustained by a distinct subpopulation of tumorigenic cancer stem cells (CSC) with self-renew and tumor initiating ability (Dalerba et al., 2007). CSCs appear to play a critical role in tumor invasion, metastasis, drug resistance, and disease recurrence (Takebe et al., 2011). Specific surface markers have been used to identify putative CSCs in different types of cancer (Kakarala and Wicha, 2007). In breast cancer, populations expressing surface CD44⁺CD24low/- and/or aldehyde dehydrogenase (ALDH1) activity have been identified as CSCs with the ability to initiate xenograft tumors (Al Hajj et al., 2003; Ginestier et al., 2007).

While our data together with that of other groups provide increasing evidence for an oncogenic role for cytoplasmic p27 in tumor metastasis, accumulating data suggest that cytoplasmic p27 may also regulate cancer stem cell expansion and function (Besson et al., 2007). In acute myelogenous leukemia (AML) CD34⁺ stem cells, p27 levels were shown to be elevated and constitutively phosphorylated at T157 and localized in the cytoplasm (Chu et al., 2010). p27CK- knock in mice have been shown to have an expansion of lung epithelial stem cells and a high incidence of spontaneous lung tumor formation with expansion of progenitor/stem cell populations, suggesting that p27 may have a cell cycle-independent role to promote cancer stem self renewal in a number of tissue compartments (Besson et al., 2007).
Recent data connect EMT and CSCs, suggesting that EMT may facilitate the generation of cancer cells with mesenchymal phenotype to promote dissemination as well as the self-renewal properties for metastatic tumor initiation (Mani et al., 2008). CSCs appear to play a critical role in tumor invasion and metastasis (Takebe et al., 2011). The STAT3 signaling pathway, that we found to be required for p27-driven induction of TWIST1, other EMT regulators and tumor metastasis, has also been shown to be required for the self-renewal of CD44+CD24−/low stem cell–like breast cancer cells (Marotta et al., 2011c). Based on the literature and our observation that deregulated p27 (p27CK-DD) induces a STAT3-dependent EMT that contributes to cancer metastasis, we hypothesize that p27CK-DD may also drive CSC self renewal in the cancer models we have studied.

6.2 PRELIMINARY RESULTS

6.2.1 p27CK-DD increases stem cell surface marker and mammosphere formation

Flow cytometry of CSC markers, CD44 and CD24 indicated that p27CK-DD overexpression in the MCF12A cells significantly increased the proportion of cells with surface markers compatible with CSC. The CD44+CD24−/low stem cell population increased from 20% to 94% in MCF12A-p27CK-DD cells (Figure 6.1A), suggesting that p27CK-DD might have potential role in regulating stem cell properties.

CD44 is a cell surface receptor for hyaluronic acid, and regulates cell migration and adhesion when interacting with other ligands including osteopontin (OPN), collagens, and matrix metalloproteinases (MMPs) (Jothy, 2003). CD44 expression in primary tumors has been linked to aggressive behavior and tumor metastasis, supporting the idea that these stem-like, tumor-initiating cells may also be the cells that survive to
form clinically relevant metastases (Yang et al., 2008; Shipitsin et al., 2007). Recently, the Weinberg group demonstrated that CD44 expression is essential for the growth and tumor-initiating ability of highly tumorigenic mammary epithelial cells (Godar et al., 2008). CD44 regulates adhesion, motility, and proliferation, and its expression is associated with spontaneous metastasis from human breast cancer orthotopic xenograft models (Liu et al., 2010). High surface CD44 is a general marker for CSCs in many types of human cancers including lung, colon, liver and pancreas (Wicha, 2008). In MCF12A cells, p27CK-DD not only increased the proportion of stem cells with surface marker of CD44^+CD24^-/low, but also increased total expression CD44 levels (Figure 6.1B), suggesting a switch from a CD44^- non stem cell population to a CD44^+ stem cell population. Furthermore, p27CK-DD also significantly increased the number and size of mammospheres (a proxy for stem cell function) in both MCF12A and MCF7 cells (Figure 6.1C&D). These preliminary data suggest an oncogenic role for deregulated p27 in regulating CSCs.
Figure 6.1: p27CK-DD regulates stem cell properties. (A) Flow cytometry analysis of CD44+CD24−/low stem cell population in MCF12A-C and MCF12A-p27CK-DD cells. (B) Protein expression of stem cell marker CD44 in MCF12A-C and MCF12A-p27CK-DD cells. (C) Mammospheres formed from 2000 seeded MCF12A-C and MCF12A-p27CK-DD cells are graphed; representative images of mammospheres are shown below. (D) Mammospheres formed from 2000 seeded MCF7-C and MCF7-p27CK-DD cells are graphed; representative images of mammospheres are shown below. All data graphed represent mean of at least 3 repeats +/- SEM. (*p<0.05).
6.2.2  *p27CK-DD induces in vitro transformation of human mammary epithelial cells*

As MCF12A cells stably expressing p27CK-DD were grown to confluence formation of multilayer foci-like structures was observed, indicating a possible transformation event (Figure 6.2A). The oncogenic potential of p27CK-DD was also shown by induction anchorage-independent growth in both MCF-12A and HME3 immortal human mammary epithelial cells (Figure 6.2B&C). Notably, MCF12A is a non-transformed human mammary epithelial cell line without soft agar formation ability (Paine et al., 1992). However, overexpression p27CK-DD results in formation of a large number of soft agar colonies with invasive structures (Figure 6.2B). p27CK-DD also significantly increased the number and size of HME3 colonies upon growth in soft agar (Figure 6.2C). Since CSCs play essential role for tumor initiation, these data suggest that the accumulation of highly stable, C-terminally phosphorylated p27pT157pT198 following PI3K and/or mTOR activation may contribute to a very early stage of tumor formation through regulating CSCs function.
6.3 FUTURE DIRECTIONS

To test the hypothesis that deregulated p27 regulates cancer stem cell properties, future research will be directed to test the effects of p27CK-DD on stem cell markers (CD44⁺CD24⁻/low), stem cell transcription factors (SOX2, KLF4, BMI-1, NANOG), mammosphere formation and tumor initiation ability (in vivo tumorigenicity in limiting dilution tumor formation assays) in multiple cell lines. We also aim to test if p27 knockdown in sorted stem cell-enriched populations will abrogate stem cell properties in vitro and reduce tumor initiation and metastasis in vivo.
6.3.1 Test the effect of p27 on tumor initiation in human mammary epithelial cells

Accumulating evidence suggest that CSCs are responsible for tumor initiation. Our previous data in MCF12A and HME3 cells suggest that p27CK-DD induces transformation since it caused formation of transformed foci in 2D cultured and mediated clonogenic growth in soft agar. However, whether C-terminal phosphorylated deregulated p27 contributes to initial tumor formations remains unknown. To test this, MCF12A-p27CK-DD and HME3-p27CK-DD cells will be injected into the mammary fat pad of NOD-SCID IL2-/- severely immune deficient mice to test whether p27CK-DD is sufficient to induce in vivo tumor formation. Further, we will test whether p27CK-DD may cooperate with and enhance the oncogenic activity of other oncogenes (such as HER2, RAS) that are commonly hyperactivated in breast cancer.

6.3.2 Test the effect of p27 on CSC markers

In breast cancer, CD44⁺CD24⁻/low cells were enriched for xenograft formation compared to bulk tumor cells (Al Hajj et al., 2003). Aldehyde dehydrogenase 1 (ALDH1) activity marks breast cancer cells enriched for stem cell properties and those with both ALDH1⁺ and CD44⁺ CD24⁻/low are most tumorigenic (Ginestier et al., 2007). Our preliminary data indicate that p27CK-DD markedly increased the proportion of stem cells with surface marker CD44⁺CD24⁻/low in non-tumorigenic MCF12A cells and that the MDA-MB-231 cells have fewer CD44⁺CD24⁻/low cells compared to the highly PI3K activated 4175 cells with high cytoplasmic p27. We will further test whether p27CK-DD transduction can increase the proportion of CD44⁺CD24⁻/low cells in tumorigenic 231 cell lines. Conversely, we will test whether loss of p27 in p27pT157/pT198 enriched
metastatic lines MDA-MB-231-1833 and MDA-MB-231-4175 will decrease the the proportion of CD44⁺CD24⁻/low cells.

6.3.3 Test the effect of p27 on the expression of stem cell transcription factors

As for normal stem cells, CSCs self-renewal is tightly regulated, in part, by intrinsic transcription factors (TFs) that integrate extrinsic growth factor signals. During tumorigenesis, deregulated transcription factor expression or activation can promote abnormal self-renewal, proliferation, and differentiation of neoplastic cells. Several stem cell transcription factors, such as SOX2, KLF4, MYC and KLF4 have been shown to be critical for the maintenance of CSCs in various cancer models. A human stem cell transcription factor array will be carried out in p27CK-DD overexpression cells to screen potential transcription factors that were significantly increased by p27CK-DD transduction. Furthermore, we will knockdown these TFs to test their functional contribution to p27CK-DD-mediated stem cell regulation.

6.3.4 Test the effect of p27 on stem cell self-renewal in vitro

Mammosphere formation has been widely employed as a model system to study the self-renewal ability of CSCs. This technique is based on the property of individual stem/progenitor cells to survive and proliferate in non-adherent and serum-free culture conditions, while more differentiated cells undergo anoikis and die in these conditions. Our previous data in MCF12A and HME3 cells show that overexpression of p27CK-DD significantly increased their ability to form mammospheres. We will further test whether
loss of p27 in p27pT157/pT198 enriched metastatic cells will decrease their self-renewal ability to form mammospheres.

6.3.5 Test the effect of p27 on cancer stem cell frequency

Serial limiting dilution xenograft assay is a standard method to test CSC frequency and self-renewal ability in vivo (O'Brien et al., 2010). Mammary CSCs are thought to be able to form more tumors with high frequency and initiate tumors from fewer cells injected into immunodeficient mice. The effects of deregulated p27 on breast cancer stem cell frequency will be tested in both p27CK-DD overexpressing cancer cells and following p27 knockdown cells that have enriched endogenous p27pT157/pT198. We speculate that p27CK-DD might increase, while p27 knockdown decrease the abundance of cells with tumor initiating ability in limiting dilution xenograft assay, supporting that deregulated p27 regulates CSC properties.

6.3.6 Test the effect of p27 knockdown in putative cancer stem cells

To complement the effects of p27 knockdown on CSC properties in p27pT157/pT198 enriched metastatic cells, we will further investigate the direct functional contribution of p27 to CSCs self-renewal in populations of putative CSC that have been isolated by flow sorting from the bulk population of tumor cells in either cell lines or tumors based on their CD44⁺CD24⁻/low surface marker or ALDH1⁺ activity. We anticipate that these putative CSCs may express higher level of endogenous p27pT157/pT198 compared to non-CSCs and loss of p27 will result in a decrease of mammosphere formation and tumor initiation ability.
6.4 CONCLUDING REMARKS

The cell cycle regulator 27 is frequently degraded or mislocalized to the cytoplasm in human cancers. Phosphorylation at T157 or T198 by different PI3K effector kinases leads to p27 cytoplasmic accumulation. While it is well appreciated that cytoplasmic p27 has cell-cycle independent role in regulating cell migration, the underlying mechanisms whereby deregulated p27 by PI3K/mTOR modulates the invasive phenotype as well as its contribution to metastasis remain poorly understood. We demonstrate that targeted inhibition of PI3K/mTOR by a dual catalytic-site inhibitor impairs tumor cell motility in vitro and metastatic dissemination in vivo. We identify cytoplasmic p27 as a critical driver of PI3K/mTOR-dependent tumor cell invasion in vitro and metastasis in vivo. Furthermore, we uncover a novel oncogenic function of cytoplasmic p27 in promoting tumor invasion and metastasis by regulating an EMT program that involves STAT3-mediated TWIST1 upregulation. We also identify a potential signaling feedforward loop containing AKT activation, p27 phosphorylation, STAT3 activation and further AKT activation that might contribute to tumor progression. Together, the work outlined in this thesis provides novel insights into how p27 deregulation downstream of PI3K/mTOR activation contributes to tumor progression and suggests that targeted inhibition of STAT3 or combined inhibition of both AKT and STAT3 in PI3K/mTOR activated, p27pT157/pT198 enriched human cancers may ultimately has therapeutic potential to limit p27-mediated EMT and cancer metastasis.

The future work outlined in this chapter proposes to investigate another novel role of p27 as a putative mediator of CSC self-renewal. Future work to identify the oncogenic role of p27 in regulating CSCs and tumor initiation and to determine the critical signaling
pathway for this effect will not only extends our understanding of how deregulated p27 downstream of PI3K/AKT signaling contributes to tumor progression, but may also reveals a novel therapeutic opportunity for cancer treatment.
References


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