p27 Drives PI3K-dependent Cancer Metastasis by Activating EMT Transcription Factors to Induce an EMT Program

Alexandra H. Besser

University of Miami, alexbesser@gmail.com

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p27 DRIVES PI3K-DEPENDENT CANCER METASTASIS BY ACTIVATING EMT TRANSCRIPTION FACTORS TO INDUCE AN EMT PROGRAM

By
Alexandra Besser

A DISSERTATION

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p27 DRIVES PI3K-DEPENDENT CANCER METASTASIS BY ACTIVATING EMT TRANSCRIPTION FACTORS TO INDUCE AN EMT PROGRAM

Alexandra Besser

Approved:

Joyce M. Slingerland, M.D., Ph.D.
Professor of Medicine and of Biochemistry and Molecular Biology

Enrique Mesri, Ph.D.
Professor of Microbiology

Tan Ince, M.D., Ph.D.
Professor of Clinical Pathology

Xiang-Xi (Mike) Xu, Ph.D.
Professor of Cell Biology

Gary Danton, M.D., Ph.D.
Professor of Clinical, Diagnostic Radiology

Dean of the Graduate School
In normal cells, p27 regulates cell cycle and functions as an atypical tumor suppressor. Unlike typical tumor suppressors such as p16 and pRb, p27 gene deletions or mutations are rarely observed in human cancers. Instead, p27 is deregulated through either excess degradation or through key C-terminal phosphorylations, in human cancers. Phosphorylation at T157 or T198 by different PI3K effector kinases allows p27 to take on new binding partners and promote oncogenic transformation. This thesis addresses the functional contribution of T157 and T198-phosphorylated, deregulated p27 to cancer progression and metastasis.

By studying the effects of a transfected cell cycle defective (CK-) and double phosphomimetic p27 mutant (T157D/T198D) in transformed and non-transformed cells, we found that phosphorylation of p27 at T157 or T198 by PI3K/mTOR directly regulates tumor cell migration and invasion. Targeted inhibition of PI3K/mTOR impairs tumor cell motility and metastasis via modulation of p27. Furthermore, we uncovered a novel oncogenic function of p27 to promote 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 breast cancer cell lines. Knockdown of p27 in highly metastatic EMT-transformed cell lines with enriched p27pT157pT198 (p27pTpT) reverted EMT and impaired metastatic potential. Mechanistically, we showed phosphorylation of p27 at T157 and T198 promotes
the activation and induction of several EMT drivers and transcription factors to induce EMT. p27CK-DD activates STAT3 and facilitates its transactivation of *TWIST1* to induce EMT. Pharmacological inhibition of STAT3 or dominant negative STAT3 (STAT3DN) decreased *TWIST1* expression and reversed p27CK-DD-mediated EMT and tumor progression, while constitutively active STAT3 (STAT3CA) rescued the EMT phenotype and metastatic potential in p27 knockdown cells. We also identified a potential signaling feed-forward loop containing AKT activation, p27 phosphorylation, STAT3 activation and further AKT activation that might contribute to tumor progression.

This thesis work also provides novel evidence for p27 as a transcriptional co-regulator of c-Jun. We prove evidence that C-terminally phophorylated p27 binds and activates c-Jun, and forms a complex with c-Jun at an enhancer region upstream of the *TGF-β2* gene to upregulate TGF-β2. Not only does p27pTpT upregulate expression of the ligand, TGF-β2, p27pTpT is required for maximal TGF-β2-stimulated SMAD3 activation, *SNAI1* induction, and matrigel invasion. Following addition of exogenous TGF-β2 ligand, p27 forms a tripartite complex with c-Jun and SMAD3 on the *SNAI1* promoter, thus revealing an additional mechanism by which PI3K-activated, C-terminally phosphorylated p27 drives EMT and metastasis.

These findings reveal a novel, oncogenic function of p27 to promote tumor progression through EMT via STAT3-mediated induction of *TWIST1*, c-Jun-mediated *TGF-β2* induction, and c-Jun/SMAD3-mediated *SNAI1* induction. Combined inhibition of AKT, STAT3, and TGF-β2 in PI3K/mTOR activated, p27pTpT enriched human cancers may ultimately have therapeutic potential to limit p27-mediated EMT and cancer metastasis.
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CHAPTER 1: INTRODUCTION
1.1 CDK INHIBITORS – REGULATORS OF CELL CYCLE

The mammalian cell cycle is composed of four phases directed by the actions of Cyclin-Cyclin-dependent kinase (CDK) complexes. The intensity of mitogenic or anti-mitogenic signals received by the cell directs a binary decision to proliferate or not. A checkpoint occurs at a restriction point late in the first growth phase (G1), and is largely governed by the gatekeeper protein Rb. Once this checkpoint is passed, normal cells irreversibly commit to replicate their DNA and will divide if replication is successful and die if it is not. The Cyclin-CDK holoenzyme complex comprises a regulatory Cyclin and a catalytic CDK that, once active will phosphorylate its protein substrates. There are four major mammalian Cyclins: Cyclin E, A D and B, each of which has more than one isoform and the CDK family contains eleven members. Sequential, tightly regulated waves of increases in Cyclin RNA levels and protein stability and of Cyclin-CDK activation direct progression through the cell cycle. CDK protein and RNA levels remain largely constant across the cell cycle (Sherr and Roberts, 1999).

In response to mitogenic signals, such as EGFR or Ras activation, genes encoding D-type Cyclins are induced, and Cyclin D-CDK4/6 complexes assemble in the cytoplasm (Sherr, 1993). D-type Cyclins act as growth factor sensors to direct the cell to exit quiescence and proliferate under favorable conditions. In quiescent cells, hypophosphorylated Rb sequesters transcription factors E2F1-4 (Dyson, 1998) and inhibits induction of target genes required for DNA replication in S phase. Once Cyclin D-CDK4/6 complexes translocate to the nucleus they initiate phosphorylation and inactivation of pRb. Later in G1, activated Cyclin E-CDK2 complexes further phosphorylate pRb mediating release and activation of E2F to allow progression into S phase. During the second growth phase (G2), Cyclin A- and Cyclin B-CDK2 complexes maintain pRb in a
hyperphosphorylated state until the cell exits mitosis (reviewed in (Sherr and Roberts, 1999)).

In states of terminal cellular differentiation or unfavorable growth conditions, several inhibitors cooperate to halt cell cycle progression. There are two CDK inhibitor protein families, the inhibitor of CDK4/6 (INK) family, which includes homologous proteins p15\textsuperscript{INK4b} (Hannon and Beach, 1994), p16\textsuperscript{INK4a} (Serrano et al., 1993), p18\textsuperscript{INK4c} (Guan et al., 1994)(Hirai et al., 1995), and p19\textsuperscript{INKd} (Hirai et al., 1995)(Chan et al., 1995) and kinase inhibitor proteins (KIP), comprising p21, p27 and p57. INK4A family members act early in G1 to bind and inhibit Cyclin D-CDK4/6 complexes. Members of the CIP/KIP family of inhibitors have a broader target range and act largely to bind Cyclin E-CDK2 in G1 phase and Cyclin A-CDK2 in S phase, but may also inhibit Cyclin B/A-Cdc2 in G2 phase under situations of cellular stress or DNA damage (reviewed in (Sherr and Roberts, 1999)(Sherr, 1993)(Kim and Sharpless, 2006)(Gil and Peters, 2006).

My thesis work has investigated how deregulated p27 switches from a tumor-suppressive, anti-proliferative role to an oncogenic, tumor-promoting role. 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 p27 phosphorylation events, leading to its subsequent deregulation, in dictating how this atypical tumor suppression functions to regulate cell division and cell migration during tumor progression. The following briefly reviews the function and regulation of p27 in normal cells, and review our current understanding of how p27 becomes deregulated to contribute to cancer metastasis.
1.2 p27: A CDK INHIBITOR WITH DUAL ROLES IN TUMOR BIOLOGY

1.2.1 Normal Function

p27KIP1, hereafter p27, is encoded by CDKN2A and regulates the cell cycle by binding directly to both its cyclin partners, the E and A-type Cyclins, and to the catalytic cleft of its target CDKs, acting as a competitive inhibitor for CDK substrates. Both CDK and Cyclin binding motifs are located in the highly conserved N-terminal region of p27 (reviewed in (Sherr and Roberts, 1999)). Efficient CIP/KIP-mediated complex inhibition requires the inhibitor binding to both its target Cyclin and CDK simultaneously (Vlach et al., 1997)(Chu et al., 2007). In its C-terminus, p27 bears a conserved nuclear localization signal, ensuring that the newly translated protein is directed to CDK targets in the nucleus (Zeng et al., 2000)(Rodriguez-Vilarrupla et al., 2002)).

p27 was first identified as a CDK2-inhibitor in TGF-β-treated cells and is regulated by growth inhibitory cytokines and by contact-inhibition (Koff et al., 1993)(Polyak et al., 1994a)(Polyak et al., 1994b)(Slingerland et al., 1994; Hengst et al., 1994). p27 appears to play a critical role to govern tissue proliferation during development and is upregulated to mediate cell cycle arrest in normal cells. p27 is strongly expressed in non-proliferating cells and critically regulates both quiescence and G1 progression. Mice lacking p27 display multi-organ hyperplasia and increased body size, and haploinsufficient animals are susceptible to carcinogen-induced tumors. Thus, p27 acts to control both cell proliferation and tissue expansion (reviewed in (Vidal and Koff, 2000)). In mice expressing a Cyclin-CDK binding defective mutant p27 (p27CK-), increases in progenitor cell self-renewal in multiple tissues including pituitary, intestine, and bronchial epithelium become evident.
p27CK- knock-in animals have increased broncho-alveolar stem cells and develop spontaneous lung tumors (Besson et al., 2007).

1.2.2 Regulation

p27 is primarily regulated at the post-translational level, however, regulatory mechanisms controlling mRNA translation and stability do exist. While p27 mRNA levels are constant throughout G1-S phase, its translation decreases dramatically upon G0 exit (Chu et al., 2008). Key phosphorylation events have been identified that regulate p27 levels, activity, and intracellular localization. p27 acts in the nucleus to inhibit CDKs (Connor et al., 2003)(Cmielova and Rezacova, 2011). During G1 progression, different phosphorylation events regulate degradation of p27. There are at least two major pathways governing p27 proteolysis. In early G1, mitogens promote S10 phosphorylation of p27 (Boehm et al., 2002)(Deng et al., 2004), which facilitates CRM1-mediated p27 nuclear export (Connor et al., 2003)(Ishida et al., 2000)(Ishida et al., 2002)(Rodier et al., 2001) and subsequent degradation. The best-characterized proteolytic mechanism governing p27 is mediated by the SCF\textsuperscript{Skp2} (S-phase kinase associated protein 1 (SKP1) / Cullin / F-Box protein: S-phase kinase associated protein 2 (Skp2)) complex, whose binding to p27 is stimulated in late G1 by cyclin E or A-CDK2-mediated p27 phosphorylation at threonine 187 (T187) (Nakayama and Nakayama, 2006). While free active cyclin-CDK2 can phosphorylate CDK-bound p27 at T187 efficiently in vitro, p27-bound CDK2 is catalytically inactive (Larrea et al., 2008). p27 contains three tyrosines at residues 74, 88 and 89 within its CDK-inhibitory domain. Y88 is part of a 310-helix in p27 that inserts into the CDK2 catalytic cleft and displaces ATP (Russo et al., 1996). p27 phosphorylation by Src (Chu et al., 2007) and Abl (Grimmler et al., 2007) at tyrosine 74 (Y74), Y88 and Y89
causes its ejection from the catalytic cleft of CDK2 (Chu et al., 2007). Thus p27pY88-bound Cyclin E-CDK2 is no longer inhibited and can phosphorylate p27 at T187 to activate SCF^Skp2^-mediated p27 proteolysis in late G1/S (Chu et al., 2007) (Grimmler et al., 2007). While Y88 is highly conserved among all Cip/Kip family CDK inhibitors, including human p21 and p57 proteins, it is not clear whether Src family kinase-mediated phosphorylation governs p21 and p57 stability in an analogous manner.

C-terminal phosphorylations also critically regulate levels and localization of p27. p27 can be phosphorylated by PI3K-effector kinases, AKT, SGK, and RSK at threonine residues T157 and T198 (Wander et al., 2011b). T157 of p27 is located within its nuclear localization signal sequence and phosphorylation at this site delays its nuclear import, shifting the pool of p27 toward cytoplasmic localization (Wander et al., 2011b). Phosphorylation at T198 stabilizes p27 in the cytoplasm (reviewed in (Larrea et al., 2009b)). Interestingly, the highly homologous CDK-inhibitor p21 can also be phosphorylated by AKT within its nuclear localization motif at T145 (the site homologous to T157 in p27), leading to accumulation in the cytoplasm and loss of its CDK inhibitory action in the nucleus (reviewed in (Warfel and El-Deiry, 2013) and (Weiss, 2003)). Growing evidence suggests that the cytoplasmic mislocalization of both of these proteins, particularly p27, leads to pro-oncogenic gain of function (Wander et al., 2011b) (Besson et al., 2004a) (Besson et al., 2008). How p27 phosphorylation contributes to cancer progression has been the subject of my PhD work.
1.2.3 **CDK Independent Functions**

**D-type Cyclin/CDK4/6 assembly and nuclear import**

Paradoxically, p27 acts to enhance Cyclin-CDK complex formation and thereby promote proliferation. Cytoplasmic p27 increases assembly of Cyclin D-CDK4/6 and stabilizes these complexes (LaBaer et al., 1997). Since neither CDKs4/6 nor D-type Cyclins bear a NLS, the associated KIP is required for nuclear import of the complex (reviewed in (Warfel and El-Deiry, 2013) and (Weiss, 2003)). Cyclin D-bound and Cyclin E-bound p27 have different phospho-isoform patterns (Ciarallo et al., 2002) and p27 phosphorylation by AKT at T157 and T198 facilitates p27 assembly of Cyclin D-Cdk4/6 (Larrea et al., 2008)(James et al., 2007).

**Stem cell self-renewal**

p27 may contribute to regulation of stem cell populations. It is noteworthy that p27 null animals exhibit multi-organ hyperplasia and hypertrophy and spontaneous neoplasia (reviewed in (Chu et al., 2008)), suggesting p27 normally acts to limit organ size in embryogenesis. Notably, this is phenocopied in p27CK-knock-in mice, reflecting the fact that this function is independent of CDK inhibitory action. In addition, in p27CK-knock-in animals, loss of CDK inhibition and a CDK-independent gain of p27 function appear to perturb normal stem cell homeostasis and cause expansion of progenitor/stem cells in several tissues, increased bronchoalveolar stem cells and spontaneous lung tumor formation (Besson et al., 2007). These intriguing findings suggest that p27 may have cell cycle-independent roles to regulate stem expansion in many tissues (Besson et al., 2007).
Differentiation

p27 appears to regulate progenitor expansion and differentiation in various normal tissue types in a manner that may be largely independent of its cell cycle regulatory roles. It is shown to be critical for xenopus neuronal differentiation (Vernon et al., 2003)(Vernon et al., 2006)(Nguyen et al., 2006), and knockdown of Xic1, the p27 homologue in Xenopus, causes loss of muscle differentiation. Initially it was thought that p27 acts during differentiation to cause terminal growth arrest, however, Xic1 is required at a point prior to terminal muscle differentiation and cell cycle arrest and may act through modulation of transcription. Xic1 increases the activity of myogenic transcription factor, MyoD (Vernon and Philpott, 2003) to drive myogenic commitment through cell cycle independent actions (Vernon and Philpott, 2003)(Messina et al., 2005). Xic1 also plays a Cyclin-Cdk-independent role to drive cardiac myotome differentiation (Movassagh and Philpott, 2008). During bone differentiation, p27 cooperates with p130 to promote endochondral ossification (Yeh et al., 2007).

Transcriptional regulation

Increasing data suggests p27 may modulate gene expression by binding a number of transcription factors. p27 was recently shown to act as a transcriptional co-repressor in a complex with other transcriptional repressors p130, E2F4, HDAC1, SIN3A (Pippa et al., 2012). Putative p27-repressed target genes identified by ChIP/CHIP assays included targets that govern cell proliferation and tissue expansion (Pippa et al., 2012). Sox2 is a critical embryonic stem cell (ES) transcription factor that maintains ES self-renewal (Marson et al., 2008) and may also drive cancer stem cell expansion (Bass et al., 2009)(Leis et al., 2011). SOX2 is co-repressed by p27 (Li et al., 2012a) in that p27 binds
the main $SOX2$ regulator site, $SOX2$-SRR2, located 4KB downstream of the coding exon to co-repress $SOX2$ together with p130, E2F4 and SIN3A (Li et al., 2012a). Thus, p27 may carry out a spectrum of developmental roles via transcriptional actions. The gene targets of p27 that govern differentiation and how they may be disrupted in cancer remain to be elucidated. Work in this thesis further expands our knowledge of p27’s role in transcriptional regulation. We show that C-terminally phosphorylated p27 may act as a transcriptional co-activator at key EMT-inducing genes to drive PI3K-dependent metastasis. We postulate that AKT activation and phosphorylation of p27 may dislodge p27 from transcriptional repressive complexes in quiescent or differentiating cells, and allow p27 to bind new partners to activate pro-oncogenic and pro-self-renewal gene targets. Thus p27 may play a previously unappreciated role to shift entire gene programs.

**Cell Motility/Invasion**

C-terminally phosphorylated p27 in many PI3K-activated cancers appears to acquire a cell cycle-independent, oncogenic function to promote cancer cell motility and invasion (Slingerland and Pagano, 2000)(Chu et al., 2008). TAT-p27 protein transduction was shown to increase Rac-dependent cell motility (Nagahara et al., 1998). p27-/− 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., 2004b). 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., 2004b), 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
et al., 2007) (Wu et al., 2006) and promotes glioma cell invasion (See et al., 2010). Thus, while nuclear p27 inhibits CDK2 to restrain cell cycle in normal cells, deregulated p27 in the cytoplasm imparts pro-oncogenic effects to promote cancer invasion and metastasis in PI3K hyperactivated cells. Work in my thesis has aimed to further elucidate how phosphorylation of p27 by oncogenic signal transduction pathways, particularly PI3K, contributes to its pro-invasion and pro-metastatic actions in cancers, and what mechanisms p27 employs to induce these changes.

1.2.4 Oncogenic Signaling Pathways that Disrupt p27 Function

PI3K signaling is oncogenically activated in many human cancers (van der Heijden and Bernards, 2010) through receptor tyrosine kinase activation or amplification, PTEN loss, and activating mutations of PIK3CA or other downstream effectors (Wander et al., 2011a). PI3K signaling not only regulates cell motility, invasion, and survival, but also modulates the cell cycle via effects on cyclin D1, CDK2, p21 and p27 levels and action (Liang and Slingerland, 2003). At least three PI3K effectors (AKT, SGK and RSK) contribute to T157 and T198 phosphorylation of p27 (Liang et al., 2002b; Shin et al., 2002; Viglietto et al., 2002; Liang and Slingerland, 2003), which impairs import of p27 and increases p27-cyclin D-CDK4 assembly (Larrea et al., 2008). Phosphorylation at T198 stabilizes p27 (Liang et al., 2007; Kossatz et al., 2006), increases its cytoplasmic localization 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., 2002b; Kim et al., 2009; Motti et al., 2005; Min et al., 2004; Viglietto and Fusco, 2002; Shin et al., 2002). Our work provides evidence of a novel
mechanism by which p27 promotes PI3K-induced metastasis by inducing an EMT program and potentially promoting a stem-like phenotype.

Many human cancers have increased Src levels or activity (Mayer and Krop, 2010), which increases tyrosine phosphorylation of p27 to promote SCF\textsuperscript{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.

Constitutive activation of these different oncogenic pathways may contribute to loss of CDK-inhibiting nuclear p27 and increased deregulated p27 in the cytoplasm, both of which drive tumor growth and progression. Reversal of these effects and restoration of the cell cycle inhibitory action of p27 by targeted inhibition of these oncogenic pathways may contribute importantly to the efficacy of targeted therapies for cancer.

1.2.5 p27 as a Predictor of Efficacy for Anti-cancer Therapies

Tumor biomarkers assist in disease stratification by providing prognostic information about tumor progression. The most valuable biomarkers are those that reliably indicate response to treatment, acting as \textit{predictive markers}. p27 localization within tumor cells has proven to be a useful prognostic biomarker in certain tumors. p27
is primarily localized in the nucleus in normal tissues but predicts worse outcome when localized to the cytoplasm in breast (Wander et al., 2013), prostate (Li et al., 2006), pancreatic cancer (Fukumoto et al., 2004), and acute leukemia (Min et al., 2004). PI3K-induced phosphorylation of p27 stabilizes it in the cytoplasm, leading to diffuse cellular staining of p27 in tumor tissue. Therefore, aberrant p27 staining in a pre-treatment biopsy might prove to have predictive value and guide treatment decisions, as a pre-treatment biopsy showing increased cytoplasmic p27 may herald intratumoral PI3K/mTOR activation and identify tumors most likely to respond to inhibitors of this pathway. PI3K/mTOR inhibitor drugs should abrogate C-terminal phosphorylation of p27 and in responsive tumors, post-treatment biopsies should show a shift of p27 from the cytoplasmic to the nuclear compartment. Understanding the effects of aberrant signaling pathways that contribute to p27 deregulation, such as PI3K/mTOR signaling, may allow appropriate selection of targeted inhibitors, allowing physicians to better treat individuals based on their tumor characteristics.

1.3 PI3K/mTOR SIGNALING IN CANCER

PI3K/mTOR signaling is hyperactivated in many kinds of cancers and plays an important role to mediate tumor progression by regulating proliferation, survival and cell migration. My thesis aims to investigate how p27, when deregulated by PI3K/mTOR signalling, regulates metastasis through induction of a genetic program poising cells for escape from their primary environment, known as the EMT. The following provides a brief overview of this signaling pathway and its contribution to cancer metastasis.
1.3.1 PI3K Signaling in Cancer Metastasis

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). 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).

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).

Increasing data implicate PI3K/mTOR in tumor metastasis, yet the 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 of PI3K-mediated tumorigenesis and uncover its oncogenic role in promoting tumor cell migration and metastasis through induction of key EMT mediators.

1.3.2 PI3K Signaling Cooperates with TGF-β Signaling to Drive Oncogenesis and Metastasis

PI3K signaling cooperates with a variety of other signaling pathways to drive oncogenic events and promote tumor progression and metastasis. The TGF-β pathway is a strongly oncogenic in transformed cells, yet promotes cytostasis, differentiation, and apoptosis in quiescent cells. The effects of TGF-β signaling on target cells depends upon the context in which the signal is read, which may include the state of cellular differentiation, the type of cancer, and the surrounding extracellular cues. While these two pathways were originally thought to exert their effects in a linear, non-overlapping fashion, emerging evidence indicates that these pathways may in fact cooperate and collaborate to drive tumor progression (reviewed in (Zhang et al., 2013).
Cells that acquire hyper-activation of PI3K signaling also exhibit blunting of TGF-β cytostatic signaling. This dynamic may contribute to the switch of cellular response to TGF-β from a tumor suppressor to a tumor-promoter (Zhang et al., 2013), as NMuMG cells grown in matrix with increasing rigidity transitioned from a TGF-β-mediated apoptotic response to a TGF-β-mediated EMT response, and this was dependent upon PI3K/Akt signaling (Leight et al., 2012). In human hepatoma cells that apoptose in response to TGF-β signaling (Zhang et al., 2004), Akt sequesters unphosphorylated SMAD3 in the cytoplasm, thus preventing its interaction and phosphorylation at the TGF-βR (Conery et al., 2004; Remy et al., 2004). This also prevents SMAD3 interaction with another transcription factor, FOXO. SMAD interaction with FOXO proteins is necessary for transcriptional activation of p21, p15, and mediators of stress response in TGF-β signaling, thus inducing a cytostatic response (reviewed in (Tran et al., 2003). Additionally, Akt phosphorylates FOXO and impairs its import into the nucleus, further impairing TGF-β-mediated induction of CDK inhibitors to thwart cellular proliferation (Seoane et al., 2004; Tzivion et al., 2011).

Several studies show PI3K signaling enhances TGF-β activity through transcriptional and post-translational mechanisms. In MDCK cells, Akt phosphorylates Twist, which promotes transcriptional activity of its target gene TGF-β2. Increased expression of TGF-β2 leads to enhanced TGF-β pathway activation and subsequent PI3K hyperactivation (Xue et al., 2012). Akt can promote the stability of the TGF-βRI by phosphorylating the deubiquitinating enzyme USP4. USP4 normally resides in the nucleus, but Akt phosphorylation promotes its relocalization to the plasma membrane, where it de-ubiquitinates TGF-βRI, and stabilizes it at the plasma membrane (Zhang et al., 2012). Furthermore, PI3K/Akt signaling promotes SMAD3 protein stability by inhibiting GSK3-
β activity (Lim et al., 2012), which would normally phosphorylate SMAD3 and target it for proteasomal degradation. GSK3-β normally phosphorylates SMAD3, targeting it for destruction (Guo et al., 2008).

Conversely, TGF-β signaling can activate the PI3K/Akt pathway, through both direct and indirect mechanisms, to exert its oncogenic effects. The p85 regulatory subunit of PI3K constitutively associated with TGF-βRII. Upon ligand binding, TGF-βRII dimerization with TGF-βRI leads to activation of mTOR and rapid activation of Akt. Inhibition of TGF-βRI activity with an inhibitor prevented TGF-β-induced activation of Akt, suggesting that these pathways cooperate to exert their effects (Lamouille and Derynck, 2007; Yi et al., 2005). Tumors can also upregulate certain microRNAs that act as positive regulators for both TGF-β and PI3K signaling, suggesting that tumors can gain a significant advantage by upregulating these pathways simultaneously.

Hepatocellular carcinoma cells upregulate the miR 216a/217 cluster, which represses SMAD7 and PTEN, negative regulators of the TGF-β pathway and PI3K pathway, respectively (Xia et al., 2013). Additionally, TGF-β itself can lead to miR-216a/217 expression and miR-21 expression, another inhibitor of PTEN, further driving Akt activation (Kato et al., 2009; Dey et al., 2012). Finally, the catalytic activity of E3 ubiquitin ligase TRAF6 is essential for Akt activation, and recruitment of TRAF6 to the TGF-βR can also lead to Akt phosphorylation (Yang et al., 2009).

We found that C-terminally phosphorylated p27 induces TGF-β2 transcription and activates TGF-β pathway signaling to induce EMT, therefore, TGF-β signaling will be briefly reviewed below.
1.4 TGF-β SIGNALING IN CANCER

Signaling through the TGF-β pathway was originally identified as oncogenic in tumors of mesenchymal origin yet it plays a growth inhibitory role to mediate quiescence in normal epithelial cells (Roberts and Wakefield, 2003). During malignant tumor progression in many epithelial cancers, however, the role of TGF-β signaling switches from tumor suppressive to tumor promoter, thus allowing cells to undergo EMT and metastasize. Given its important role in mediating tumor progression, much work has been done to elucidate the mechanisms behind this oncogenic switch and how the context in which the signal is read and the functionality of downstream mediators play a role in this process. The following provides a brief overview of TGF-β signaling in normal and transformed cells and, given that p27 was initially discovered as a primary mediator of TGF-β-induced cytostasis (Polyak et al., 1994a; Slingerland et al., 1994), explores the possibility that deregulation of p27 might contribute to the oncogenic switch in cells reading TGF-β signaling.

1.4.1 Deregulation of TGF-β Signaling in Cancer

The TGF-β signaling pathway consists of transmembrane heteromeric Ser/Thr receptor kinases TGF-β receptor I (TGF-βRI) and TGF-β receptor II (TGF-βRII), which dimerize upon binding of extracellular TGF-β ligands. Upon dimerization, constitutively active TGF-βRII phosphorylates TGF-βRI, activating the intracellular SMAD family of proteins (Moustakas et al., 2002; Jakowlew, 2006). Upon TGF-βRI-mediated phosphorylation, the SMAD proteins oligomerize and translocate to the nucleus to direct gene expression with other available cofactors.
The TGF-β pathway is one of the most highly conserved signaling pathways in multicellular organisms and it directs the expression of vastly different gene patterns involved in a variety of cellular functions. Specificity for target genes depends on the context in which the signal is being delivered. Transcription factors, histone readers and modifiers, and chromatin remodelers that bind activated SMAD proteins determine what genes will be targeted by the signal transduction complexes and whether expression of the target genes will be positively or negatively regulated. SMAD proteins usually need other co-factors to bind DNA efficiently and this availability of binding partners directs gene expression in different contexts.

The context of TGF-β signaling can also begin extracellularly, with the binding of TGF-β ligands, TGF-β1, TGF-β2, or TGF-β3. While these three ligands share a high degree of sequence homology in their active domains, they display different binding affinities for TGF-βRII. TGF-β1 and TGF-β3 can directly associate with TGF-βRII and TGF-βRII with high affinity, but TGF-β2 can only associate with TGF-βRII and it does so with very low affinity. Expression of betaglycan (TGF-βRIII) seems to be required to present the TGF-β2 ligand to TGF-βRII, substantially increasing its affinity for binding this particular ligand (Lopez-Casillas et al., 1993). The role of TGF-β1 in tumorigenesis and metastasis has been well studied (Katsuno et al., 2013), but the roles played by TGF-β2 and TGF-β3 in this process remain unclear. Recent evidence suggests that TGF-β2-specific signaling is important in development of melanoma (Zhang et al., 2009; Reed et al., 1994; Van et al., 1996), glioma (Kjellman et al., 2000; Bodmer et al., 1989; Maxwell et al., 1992), pancreatic cancer (Friess et al., 1993; von et al., 2001), and breast cancer (Buck and Knabbe, 2006; Ouhtit et al., 2013), and the role of deregulated p27 in mediating TGF-β2-specific effects on tumorigenesis is explored in my thesis.
1.4.2  Cytostatic Effects of TGF-β Signaling in Development and Normal Cells

TGF-β fosters tissue growth and morphogenesis in embryological development, and activates cytostatic and cell death processes that maintain homeostasis in mature tissues. TGF-β signaling restrains cell proliferation in normal epithelial, hematopoietic, and neural cells by calling into action CDK inhibitors, and by repressing pro-proliferative mediators. To halt proliferation, p27 (Slingerland et al., 1994; Polyak et al., 1994a), p21 (Datto et al., 1995) and p15 (Hannon and Beach, 1994) are upregulated, and p27’s activity is directed away from pro-proliferative cyclin D-CDK4/6 assembly, to binding and inhibiting cyclin E-CDK2, an anti-proliferative function (Reynisdottir et al., 1995). Simultaneously, c-Myc activity is downregulated through a TGF-β-induced transcriptional complex containing Smad3/4, p107, E2F4/5, and C/EBP (Alexandrow and Moses, 1995). TGF-β signaling becomes oncogenic when cells lose their responsiveness to these homeostatic cellular controls.

1.4.3  Oncogenic Effects of TGF-β in Transformed Cells

Cancers that can selectively shut down the tumor-suppressive arm of the TGF-β signaling pathway are free to take advantage of its many pro-tumorigenic properties, as it prominently enhances cell invasion, migration, and evasion of immunity. Promotion of EMT and metastasis via TGF-β signaling are some of the final stages of this circumvention, and will be described in detail in the next section. To elude the cytostatic effects of TGF-β signaling, transformed cells can either inactivate core components of the pathway, such as TGF-β receptors, where they lose the signal to stop proliferating, apoptose, or differentiate, or they disable downstream effectors of this pathway.
These cells can also utilize TGF-β signaling to the prime tumor microenvironment, while leaving cell proliferation unrestrained.

TGF-β signaling can suppress immune surveillance allowing tumor cells to grow undetected. Through SMAD-mediated transcriptional repression, the production of cytolytic factors in CD8+ T cells, such as perforin, granzymes A and B, and IFNγ, are inhibited (Thomas and Massague, 2005). TGF-β can also impair immune activation by inhibiting the function of dendritic cells (Geissmann et al., 1999) and natural killer cells (Wallick et al., 1990; Arteaga et al., 1993). Through its effects on local angiogenic cytokine networks, TGF-β can induce a pro-angiogenic environment and stimulate vessel formation. CTGF is a TGF-β target gene that promotes vessel formation (Kang et al., 2003). Hypoxic conditions in the core of growing tumors stimulate Smad/HIF1-mediated transcription of the angiogenic factor VEGF (Sanchez-Elsner et al., 2001). TGF-β signaling also promotes the differentiation of myofibroblasts into mesenchymal precursors which produce pro-invasive matrix metalloproteases, IL-8, VEGF, and CXCL12 (De and Mareel, 2003). Secretion of these cytokines and chemokines promotes cancer cell proliferation, invasion, and angiogenesis around the tumor (Allinen et al., 2004).

### 1.4.4 Disrupted TGF-β Signaling in Human Cancers

Many of these mechanisms to thwart the cytostatic effects of TGF-β signaling are evident in human cancers. The TGF-βRII gene is mutated in colon, gastric, biliary, pulmonary, ovarian, esophageal, and head and neck cancers (Levy and Hill, 2006), and this gene is commonly mutated in tumors with microsatellite instability such as sporadic gastrointestinal and biliary carcinomas, lung adenocarcinomas, and glioma. TGF-βRI is
mutated in ovarian, esophageal, head and neck cancers, and it is epigenetic silenced in gastric cancer (Levy and Hill, 2006). Failed or altered presentation of ligands to receptors are also described in certain cancers. Mutations in betaglycans are documented in hemorrhagic telangiectasia syndrome and early onset Juvenile Polyposis Syndrome (Sweet et al., 2005). Overexpression of ligand traps that enhance sequestration of TGF-β in the extracellular matrix, such as follistatin, are associated with breast cancer bone metastasis (Kang et al., 2003). SMAD4/DPC mutations present in more than half of pancreatic carcinomas (Jaffee et al., 2002) and sporadic colorectal tumors (only those without microsatellite instability) and a high number of esophageal tumors (Sjoblom et al., 2006). Interestingly, mutations in Smad2/3 are rare, however, suggesting that tumorigenic selection pressure favors that maintenance of the intracellular network of TGF-β signaling, even when cytostatic signal is lost. Aberrant overexpression of TGF-β pathway inhibitors is also documented in certain cancers. Smad7, a TGF-βRI antagonist, is overexpressed in endometrial carcinomas and thyroid follicular tumors (Cerutti et al., 2003; Dowdy et al., 2005), and is associated with chronic inflammation and pre-tumorigenesis in immune cells within the colonic mucosa (Broderick et al., 2007).

1.4.5 TGF-β Signaling Leads to EMT and Metastasis

EMT is key in gastrulation and in the genesis of the neural crest, the somites, the heart, and the craniofacial structures, yet this process is aberrantly activated in tumorigenesis. During EMT, epithelial cells acquire mesenchymal characteristics that enhance their invasive properties. TGF-β is a potent inducer of EMT, both in normal development and tumorigenesis (Derynck and Akhurst, 2007; Thiery, 2003). SMAD-mediated induction of HMGA2 (high-mobility group A2) triggers the expression of key EMT transcription factors Snail, Slug, and Twist (Thuault et al., 2008). Independent of
SMAD-activity, TGF-βRII-mediated phosphorylation of Par6 promotes dissolution of cell junction complexes (Ozdamar et al., 2005). EMT is classically linked to an enhanced ability of cells to metastasize, and growing evidence implicates TGF-β in the promotion of distal metastasis.

TGF-β in the breast tumor microenvironment primes cancer cells for both lung and bone metastasis. SMAD-induced expression of ANGPTL4 (angiopoietin-like 4) in MDA-MB-231 cells directs metastasizing cells to disrupt capillary beds in the lung tissue, and blocking TGF-β signaling with either a dominant-negative TGF-βRI or SMAD4 knockout reduced lung tumor formation in mice (Padua et al., 2008). In the bone parenchyma, TGF-β signaling creates a permissive environment for establishment of secondary niches. Cytokines released by the cancer cells mobilize stores of TGF-β within the bone matrix. Kang et al (Kang et al., 2005; Kang et al., 2003) identified a bone metastatic signature, where several TGF-β-induced genes were determined to be important for the establishment of bone metastases, including PTHrP, IL11, and CTGF. TGF-β can therefore exert a pro-metastatic function to facilitate the establishment of metastatic lesions once tumors reach a secondary site. Understanding the mechanisms underlying how these cells are initially directed towards oncogenesis is crucial, as determining the players involved in the early EMT may offer novel targets in inhibiting the establishment of metastases.

1.5 EMT IN CANCER/MECHANISMS UNDERLYING TUMOR METASTASIS

The epithelial-mesenchymal transition (EMT) is a biological process in which epithelial cells undergo multiple molecular and cellular changes to acquire a 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), and this process plays a significant physiological role during wound healing (Kalluri and Weinberg, 2009). In addition, the EMT program is activated during organ fibrosis, cancer cell invasion and metastasis (Kalluri and Weinberg, 2009; Thiery, 2002; Micalizzi et al., 2010). Malignant cells undergoing EMT may also acquire stem-like 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.

1.5.1 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 demonstrate that epithelial cells undergo dramatic phenotypic changes when transforming into 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 type 3 regulates cancer progression and metastasis (Kalluri, 2009; Kalluri and Weinberg, 2009). Type 3 EMT is the focus of my thesis work, and is aberrantly initiated in neoplastic cells that have undergone genetic and epigenetic changes to favor clonal outgrowth and localized invasion from their primary site. This type of EMT primes tumorigenic cells to establish distant metastases and will be discussed in detail in the following section.
1.5.2 EMT in Cancer Development and Metastasis

Cancer metastasis, while responsible for the devastating consequences associated with the disease, is an inefficient process requiring cells to overcome a series of challenges to spread from primary to secondary organs (Sethi and Kang, 2011). Transformed cells must undergo multiple steps including local invasion, intravasation and extravasation from the vasculature, and organ colonization to form distant metastases (Fidler, 2003).

During early tumor invasion, epithelial cancer cells lose apico-basal polarity, dissolve cell-cell junctions, and break through the underlying basement membrane. This early step towards metastasis is believed to be initiated by reactivation of type 3 EMT (Micalizzi et al., 2010). Epithelial cancer cells are tightly associated with neighboring cells via E-cadherin-containing junctions, and progression to a mesenchymal phenotype begins when cells downregulate E-cadherins and upregulate mesenchymal markers such as N-cadherin and Vimentin. These cells, now poised for enhanced motility, are typically 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 an important role during the formation of metastasis (Thiery et al., 2009; Tsai et al., 2012).
1.5.3 EMT Markers and Transcriptional Regulators

A hallmark of EMT is loss of expression of E-Cadherin, a crucial “gate keeper” of the epithelial phenotype. 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 from E-Cadherin to N-Cadherin is a commonly used as a marker of EMT progression during embryonic development and cancer progression. Emergence of the cytoskeletal marker Vimentin is also commonly used to 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 guided by several EMT-inducing transcription factors, which include 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 coordinately 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). Twist1 is a basic helix-loop-helix protein that is transcriptionally active during osteoblast lineage determination and cell differentiation (Bialek et al., 2004). Snail is a zinc finger transcription factor active during gastrulation in the developing embryo (Nieto, 2002; Boulay et al., 1987). Both Twist1 and Snail can independently repress E-Cadherin, and Twist1 upregulates N-Cadherin while Snail upregulates Vimentin (Cano et al., 2000). As a master regulator of embryonic morphogenesis, Twist1 and Snail have been shown to promote cancer metastasis by regulating EMT (Yang et al., 2004; Nieto, 2002; Boulay et al., 1987). 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). Snail is aberrantly expressed in almost 50% of infiltrating ductal carcinomas and its expression correlates with higher tumor grade and worse outcome (Blanco et al., 2002).

1.5.4 Signal Pathways Governing EMT

Several extracellular signal pathways including TGF-β, Wnt/β-catenin, and 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., 2012). 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 factors. 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.

1.6 SUMMARY

PI3K signaling offers many potential targets with which to treat tumors or offer prognostic value. As reviewed above, dual PI3K/mTOR inhibitors deliver 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. 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. In the work described in Chapter 5, I describe an additional pathway whereby PI3K-activated, deregulated p27 drives metastasis by inducing TGF-β pathway activation and EMT by regulating transcription of $TGF-\beta2$ and $SNAI1$. 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
to also promote tumor progression and integrate EMT and metastasis by driving cancer stem cell self-renewal.
CHAPTER 2: MATERIALS AND METHODS
2.1 MATERIALS

This chapter describes the materials and experimental approaches that were utilized for the work in this thesis. Section 2.1 consists of an outline listing all of the relevant materials: Table 2.1 lists the cell lines used in this work, Table 2.2 lists the various reagents (antibodies, inhibitors, etc.). Table 2.3 lists the oligonucleotide sequences used for qPCR and ChIP assays. Section 2.2 contains detailed descriptions of the various experimental methods that were utilized.

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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, UMUC3, and MCF12A cells with lipofectamine. Stable transfectants expressing similar EGFP-p27 levels were selected for subsequent studies. Different p27 mutants were further subcloned into Lenti-AcGFP vector (Clotech) to generate Lenti-AcGFP-p27CK- and Lenti-AcGFP-p27CK-DD vectors for virus packaging to infect target cells.

2.2.2 Lentivirus Production and Infection

Lentivirus vectors encoding different shRNAs, scramble shRNAs (Open Biosystems) or p27 mutants were co-transfected with Delta VPR and CMV VSVG plasmids (Addgene) into asynchronous 293T with Lipofectamine Plus (Roche). Viral supernatants were collected at 48h and concentrated by ultracentrifugation for 2 h at 22,000 RPM at 4°C. Pellets were resuspended overnight at 4°C. Cells were infected twice in the presence of 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 to confluency in 60mm plates and grown for an additional 24h. A linear scratch/wound was made on cell monolayers with a sterile pipette. Photomicrographs were taken of live cells (10x objective) over time and distance migrated
measured using ImageJ software (1 μm = 1 pixel). Relative migration was calculated via the following formula: (initial wound distance – final wound distance) / initial wound distance. The values are plotted +/- SEM from three independent scratches.

2.2.4 Matrigel Invasion Assay

Transwell invasion of cells were assayed by seeding 10⁵ 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 using the Real-Time Cell Analysis (RTCA) system from Xcelligence. For transwell migration assays, the upper chambers of CIM plates were seeded with 20,000 cells in serum-free medium, which were subsequently allowed to migrate toward 10% FBS serum in the bottom chambers. For transwell invasion assays, the upper chambers of CIM plates were pre-coated with 5% matrigel and seeded with 40,000 cells, which were subsequently allowed to invade toward 10% FBS serum in the bottom chambers. Automated analysis of cellular density on the underside of the transwell membrane occurred every 30 minutes and was plotted as cell index +/- SEM for at least three wells per group.
2.2.6 **Quantitative Real-Time PCR (qPCR)**

qPCR analysis were performed as previously described (Lindley & Briegel, 2010; Rieger et al., 2010) using primers for indicated genes listed in table 2.3 (Lindley and Briegel, 2010). *GAPDH* was used as an internal control (Rieger et al., 2010). All samples were performed in triplicates and average Ct 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 using Millipore PVDF membrane and blocked for one hour in 5% non-fat milk solution. Primary antibody concentration and duration of exposure varied based on individual optimization to each antibody. Secondary incubation at a dilution of 1:5,000 was carried out for one hour. Chemiluminescent imaging was carried out with a variety of ECL reagents, listed in Section 2.2.

2.2.8 **Nuclear and Cytoplasmic Fractionation**

Nuclear-cytoplasmic fractionation was performed using a kit from Thermo-Scientific. Nuclei were isolated following centrifugation and removal of the cytosolic supernatant. Equal protein concentration in nuclear and cytoplasmic lysates were blotted for indicated proteins. RCC1 was used as a nuclear control and β-tubulin was used as a cytoplasmic 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 number (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 / Experimental Lung and Bone Metastasis Assay

Animal work was approved by the Institutional Animal Care and Use Committee. 5-week old female nude Balb/c mice (Charles River) were used for all xenograft studies. Mice were anaesthetized and injected intraperitoneally with 1.5 mg of d-luciferin (15 mg ml⁻¹ in PBS) and imaged with the Xenogen IVIS system (Xenogen). Photon flux values were normalized to the value obtained immediately after xenografting (day 0) so that all mice had an arbitrary starting BLI (Bioluminescence Intensity) signal of 100. For orthotopic tumor xenograft assays, 1x10⁶ cells were resuspended ex vivo in matrigel and then injected into the 4th mammary fat-pad in a volume of 100 microliters. Tumor growth was measured by twice-weekly calliper assessment and calculated by the following
formula: \((\text{long-side} \times \text{short-side}^2)/2\). 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.

For experimental lung metastasis assay, \(2 \times 10^5\) viable cells in 0.1 ml PBS were injected via tail vein into Balb/C nude mice (Minn et al., 2005a). Successful injection was verified by immediate IVIS imaging following injection. Bioluminescence was monitored by IVIS and quantified as normalized photo flux (Minn et al., 2005a). For lung metastasis BLI plots, a rectangular region of the thorax was utilized for each mouse. Lungs were photographed and surface tumors counted. For experimental bone metastasis assay, \(1 \times 10^5\) viable cells in 0.1 ml PBS were injected into the left ventricle via external visualization. Successful injection was verified by immediate IVIS imaging following injection. For bone metastasis bioluminescence (BLI) plots, systemic photon flux was calculated for each mouse. Values were normalized to the value obtained immediately after xenografting (day 0) so that all mice had an arbitrary starting BLI signal of 100. Representative individual mice were selected for each experiment and presented along with a standardized scale. Average normalized photon flux for each group is plotted over time +/- SEM.
2.2.12 Animal Studies for Drug Treatment

For bone metastasis assays, at least ten animals in each arm were injected with $10^5$ viable cells in 0.1 ml PBS 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). Systemic tumor bioluminescence was monitored at Day 0, 1, 2, 3, 7, 10, 14, and 21 for PI3K/TOR-KI studies and at Day 0, 7, 14, and 21 for shRNA p27 studies. 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. Animals were weighed twice-weekly. Leg and spinal tissues were recovered at necropsy at 3-4 weeks after injection for hemotoxylin and eosin (H & E) staining histopathology unless morbidity required earlier euthanization.

2.2.13 Immunohistochemistry and Analysis of Human Tumor Samples

For analysis of xenograft tumors, animal tissues were recovered at necropsy and immediately placed in 10% neutral buffered formalin for 24 hours. Soft tissues were subsequently paraffin embedded, while bone tissues were first decalcified and then paraffin embedded. Tumor sections were cut at 4uM and the lower-most section was stained with H+E. p27 and Ki67 immunostaining were as described (Catzavelos, 1997). Sections were counterstained with hematoxylin.
For 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 UMSCCC Tissue Core Facility. p27 staining was 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 Fluorescence Immunocytochemistry

Fluorescence immunocytochemistry on cultured cells was performed as previously described (Hong et al., 2008). Incubation with primary antibodies against E-cadherin (1:1,000), N-cadherin (1:500), Vimentin (1:500), p-STAT3 (1:1,000), and Twist1 (1:500), were 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).
2.2.15 Immunoprecipitation

UMUC3-LuL2 and MDA-MB-231-1833 cells were treated +/- PF1502 for 48 hours, and MDA-MB-231-1833 cells were additionally treated with 10nM TGF-β2 for 30 minutes before immunoprecipitation. Cells were lysed and 500 μg lysate was incubated with 1 μg of p27 (BD Transduction or Santa Cruz Biotechnology), p27pT198 (Research and Development) or SMAD3 (Cell Signaling) antibodies, collected on protein A- or G-agarose beads and washed three times with IP buffer. Samples were then analyzed by SDS-PAGE followed by western analysis. Antibody-alone controls were run with all immunoprecipitations.

2.2.16 Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assays for the TWIST1 promoter, TGF-β2 promoter or SNAI1 promoters were as described (Assou et al., 2007). Briefly, soluble chromatin was prepared from a total of $2 \times 10^7$ asynchronously growing MDA-MB-231-4175 cells, UMUC3 cells, or MDA-MB-231-1833 that were pre-treated with 30 min 10nM TGF-β2. The chromatin solution was diluted 10-fold with ChIP dilution buffer (1.1% Triton X-100, 1.2 mm EDTA, 167 mm NaCl, 16.7 mm Tris-HCl, pH 8.1, 0.01% SDS, plus protease and phosphatase inhibitors), and pre-cleared with protein, and blocked with 2 μg of sheared salmon sperm DNA and pre-immune serum. The pre-cleared chromatin solution was divided and utilized in immunoprecipitation assays with either an anti-STAT3, anti-p27, anti-c-Jun, anti-SMAD3, or an anti-IgG antibody. Following multiple washes, the antibody-protein-DNA complex was eluted from the beads by resuspending the pellets in 1% SDS, 0.1 m NaHCO₃ at room temperature for 20 min. After reversal cross-link
incubation at 67 °C, protein and RNA were removed by incubation with 10 µg of proteinase K and 10 µg of RNase A at 42 °C for 3 h. Purified DNA was subjected to qPCR with primers specific for the STAT3 bindings sites upstream of the transcriptional start sites in the TWIST1 promoter, and e-Jun binding sites upstream of the transcriptional start site in the TGF-β2 promoter and SNAI1 promoter. The primers for CHIP analysis are listed in Table 2.3.

2.2.17 Reverse Phase Protein Array (RPPA) Analysis of Human Tumors

RPPA assay was performed at the Functional Proteomics Reverse Phase Protein Array Core facility at MD Anderson to test expression of 178 proteins and phosphoproteins in 747 breast cancers from The Cancer Genome Atlas (TCGA) project as described (Hennessy et al., 2010). Briefly, the tissue samples were washed twice in ice-cold PBS and then homogenized in RPPA lysis buffer [1% Triton X-100, 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L NaP[Pi], 10% glycerol, 1 mmol/L Na₃VO₄, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 µg/mL aprotinin]. After centrifugation, the supernatant was collected, and the protein concentration was determined by routine (e.g., Bradford) assays and then adjusted to 1–1.5 mg/ml by addition of lysis buffer. The tissue lysates were mixed with 1/4 volume of 4× SDS sample buffer containing 40% glycerol, 8% SDS, 0.25 M Tris-HCl (pH 6.8), and 10% (v/v) 2-mercaptoethanol (freshly added). Two-fold serially diluted tissue lysates (from undiluted to 1:16 dilution) were printed on nitrocellulose-coated slides (Grace Biolab) by using a Aushon 2470 arrayer (Aushon BioSystems), along with corresponding positive and negative controls prepared from the
dilution buffer. Each slide was probed with a validated primary antibody plus a biotin- 
conjugated secondary antibody. The signal was amplified using a DakoCytomation 
catalyzed system (Dako) and visualized by 3,3’-diaminobenzidine tetrahydrochloride 
colorimetric reaction. The slides were scanned, analyzed, and quantified using 
customized software, (ArrayPro), to generate spot intensity. Signals from each dilution 
were fitted with the non-parametric model developed by the Department of 
Bioinformatics and Computational Biology at MD Anderson (Hennessy et al., 2010). The 
protein concentrations of each set of slides were then normalized and corrected across 
samples by the linear expression values, using the median expression levels of all 
antibody experiments to calculate a loading correction factor for each sample. Since 
RPPA is intrinsically a batch approach, each tumor type was run on a single batch to limit 
batch effects except the breast cancer set. We merged different batches of RPPA data 
using a novel algorithm, called Replicates Based Normalization (RBN), which reduces 
batch effects. (Nature Methods: doi:10.1038/nmeth.2650Supplementary Methods)

2.2.18 Statistical Analysis

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

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.
CHAPTER 3

TARGETED PI3K/mTOR INHIBITION IMPAIRS TUMOR CELL MOTILITY AND BONE METASTATIC OUTGROWTH VIA MODULATION OF p27
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 p27pT157pT198 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 p27pT157pT198 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 p27T157DT198D caused resistance to the anti-invasive effects of PF04691502. Pretreatment 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,
Figure 3.1: Low-dose PF1502 inhibits PI3K/mTOR activity in MDA-MB231 and 1833 without affecting proliferation or caspase activation. (A) Cells were treated 48 hrs with 250nM 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 hr (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 250nM PF1502 or vehicle control for 48 hrs (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 doses for 48hrs. Drug treatment did not yield any sub-G1 fraction. (E) Caspase 3 cleavage assay after 250nM PF1502 for 48hr (left) or 7 days (right). Positive control in far right lane shows MDA-MB-231 after 72 hrs of paclitaxel 100nM.
231 and 1833, Figure 3.1D). 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).

### 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

![Figure 3.2: Low-dose PI3K/mTOR inhibition attenuates tumor cell adhesion, motility, and invasion.](image)

(A) Cell adhesion in 1833 cells +/- drug (mean +/- SEM, * T-test for final time points vs control, p<0.002) (B) Relative migration 6 hrs after wounding of a confluent cell monolayer (graphed +/- SEM, treated vs untreated T-test * p=0.005). (C) Mean transwell matrigel invasion +/- SEM (T-test of treated vs control * p=0.02).
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.

### 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 hrs with 250nM PF1502 or DMSO prior to Westerns of: (A) Total p27, p27pT157 and p27pT198 and (B) p27 in cytosolic (C) and nuclear (N) fractions (right). 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 24h after wounding of cell monolayer (mean +/-SEM T-test* p=0.01 for 1833 – vs +Shp27). (F) Relative transwell matrigel invasion over 24h (mean +/-SEM T-test* p<0.05 for 1833 – vs +).
3.2.4  **p27CK-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-

![Figure 3.4: Expression of a phosphomimetic, cell-cycle inert p27 mutant conveys resistance to PF1502.](image)

**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 vs. 231 control p<0.0001). (C-D), Matrigel invasion of 231 (C) and 1833 cells (D) transduced with p27CK-DD, with and without 48hr PF1502 250nM and graphed +/- SEM. (T-tests as indicated: *p=0.003, ‡p=0.068 (left); *p=0.0002, ‡p=0.064 (right)). (C,D) Representative photomicrographs demonstrate differential cell invasion with and without p27CK-DD expression in 231 and 1833.
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.

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 (data not shown), 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 (data not shown).
Figure 3.5: Low-dose PI3K/mTOR inhibition abrogates bone metastatic outgrowth in vivo. 231 and 1833 were cultured +/- 250nM 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 vs time (comparative analysis of growth curves, *p=0.04, ‡p=0.70). (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 +/- 250nM PF1502 for 7 days prior to mammary fat pad injection (‡p>0.50). Animals received no drug therapy. See also supplemental Fig S2 showing nodal metastasis from orthotopic primary tumors arising from drug-pretreated cells or controls.
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). 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 vs. cytoplasmic positive tumors (N=21 and N=73, respectively) and disease-free survival. (D) Kaplan-Meier analysis of nuclear only vs. 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 (Akcakanat 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., 2004b). 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., 2002b; 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\textsuperscript{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 p27\textsuperscript{CK-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 \textit{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 \textit{in vitro}, and did not reduce primary orthotopic tumor growth,
significantly impaired the subsequent outgrowth of bone metastatic tumors after
intracardiac injection \textit{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., 2002b; 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., 2002b), 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

C-TERMINALLY PHOSPHORYLATED p27 PROMOTES TUMOR METASTASIS THROUGH TWIST1-MEDIATED EPITHELIAL-MESENCHYMAL TRANSITION
4.1 SUMMARY

p27 restraints normal cell growth, but PI3K dependent C-terminal phosphorylation of p27 at T157 and T198 promotes cancer cell invasion. Here, we describe an oncogenic feed-forward loop in which p27pT157pT198 binds JAK2 promoting STAT3 recruitment and activation. STAT3 induces TWIST1 to drive a p27 dependent EMT and further activates AKT contributing to acquisition and maintenance of metastatic potential. p27 knockdown in highly metastatic PI3K-activated cells reduces STAT3 binding to the TWIST1 promoter, TWIST1-promoter activity and TWIST1 expression, reverts EMT and impairs metastasis, while activated STAT3 rescues p27 knockdown. Cell cycle-defective phosphomimetic p27T157DT198D (p27CK-DD) activates STAT3 to induce a TWIST1-dependent epithelial-mesenchymal transition (EMT) in human mammary epithelial cells and increases breast and bladder cancer invasion and metastasis. Data support a mechanism in which PI3K-deregulated p27 binds JAK2, to drive STAT3 activation and EMT through STAT3-mediated TWIST1 induction. Furthermore, STAT3, once activated, feeds forward to further activate AKT. 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 p27pT157pT198 enriched human cancers may effectively inhibit p27-mediated EMT and metastasis.
4.2 RESULTS

4.2.1 Overexpression of Phosphomimetic p27CK-DD Induces/Enhances EMT in Human Mammary Epithelial and Cancer Cells

Prior work showed mutations converting T157 and T198 to aspartate in p27 are phosphomimetic. (Larrea et al., 2009a; Hong et al., 2008; Fujita et al., 2003) To test if negative charges at both sites cooperate to drive these effects, single and double phosphomimetic mutations (T157D, T198D or DD) were inserted into a p27 mutant that cannot bind either cyclins or CDKs (CK-) (Data not shown). (Denicourt et al., 2007; Vlach et al., 1997) To test if C-terminally phosphorylated p27 may contribute early in the process of malignant transformation, these different phosphomutant p27 vectors were transduced into the immortalized, non-transformed human mammary epithelial cell line MCF-12A (MCF-12A-p27CK-DD). While expression of each single phosphomimetic p27 mutant significantly increased cell migration, p27CK-DD enhanced MCF-12A migration most significantly and caused these cells to acquire the ability to invade matrigel (Figures 4.1A, B).

Similarly, in MDA-MB-231 cells, while p27CK- alone had a very modest effect, each phosphorylation site appears to contribute to cytoplasmic p27 localization and p27CK-DD caused the greatest increase in cell motility, invasion and p27 mislocalization (Data not shown). Neither p27CK- nor any of the CK- mutants bearing single or double phosphomimetic mutations affected the cell cycle (Data not shown). Relative levels of endogenous p27 and p27CK-DD are shown in Supplementary Figures S1G, H for the 231 model and in Supplementary Figures S2A, B for MCF-12A.
Notably, p27CK-DD overexpressing MCF-12A cells underwent a progressive, morphological switch from a typical, cobble-stone-like epithelial appearance to an elongated, spindle-like, mesenchymal shape over the next four weeks (Figure 4.1C),
indicative of EMT. p27CK-expressing cells retained their cobblestone morphology, suggesting that the C-terminal phosphorylation of p27 is required for its action on the EMT (Figure 4.1C). Relative expression of endogenous and transfected p27CK-DD are shown in Supplementary Figure S2A,B. p27CK-DD overexpressing MCF-12A showed decreased levels of the epithelial marker, E-cadherin, and increased mesenchymal markers, N-cadherin and Vimentin (Figures 4.1D, F). In MCF-12A cells, expression of vector alone or of the p27CK- vector lacking T157D and T198D mutations did not upregulate EMT drivers and mesenchymal markers (Data not shown). Thus, introduction of the CK- mutations and loss of cell cycle inhibitory p27 function are not sufficient to drive p27-mediated EMT, and p27 phosphorylations at T157 and T198 appear to be required.

Similar findings were observed in the hTert-immortalized, normal human mammary epithelial HME3 line, (Ince et al., 2007) which showed a significant increase in mesenchymal marker expression, and enhanced cell migration and invasion following stable p27CK-DD transduction (Data not shown). Thus, overexpression of a p27 protein defective for CDK inhibition and bearing phosphomimetic mutations at T157 and T198 induces an invasive EMT phenotype in non-tumorigenic human mammary epithelial cells.

The EMT program is initiated by EMT-inducing transcription factors including Snail, Slug, zinc finger E-box binding homeobox 1 (Zeb1), and Twist. (Puisieux et al., 2014) To elucidate which transcription factors contribute to p27CK-DD-mediated EMT, several were screened by QPCR in MCF-12A-p27CK-DD and control cells. While p27CK-DD modestly increased \textit{SNAI1} (encoding Snail), \textit{SNAI2} (encoding Slug) and \textit{ZEB2} expression, \textit{TWIST1} expression increased by 20 fold (Figure 4.1E), suggesting that \textit{TWIST1} may play a critical role during p27CK-DD-induced EMT. p27CK-DD also increased
Twist1 protein and its nuclear localization was confirmed by direct immunofluorescence (Figures 4.1D, F). Indeed, *TWIST1* knockdown significantly attenuated the EMT phenotype, causing re-expression of E-cadherin, loss of mesenchymal markers N-cadherin and Vimentin (Figure 4.1G) and loss of the excess motility and invasive potential of MCF-12A-p27CK-DD cells (Figure 4.1H, I), supporting the notion that *TWIST1* induction is essential for the p27CK-DD-induced EMT phenotype in immortalized human mammary epithelial cells.

p27CK-DD overexpression in the luminal A, MCF-7 breast cancer line also induced a morphological change compatible with EMT, with increased expression of mesenchymal markers (N-cadherin and Vimentin) and *TWIST1*, and an increase in MCF-7 cell migration and invasion (Data not shown). Moreover, the effects of C-terminally phosphorylated p27 on EMT are not exclusive to breast cancer, as p27CK-DD significantly enhanced mesenchymal features of a human bladder cancer cell line, UMUC3 (Data not shown). Together these data support a novel, oncogenic role in which p27 promotes EMT in human mammary epithelial cells and in weakly or non-metastatic breast and bladder cancer models.

### 4.2.2 Loss of p27 Decreases Mesenchymal Characteristics in PI3K-Activated, Metastatic Lines

To assay if C-terminal phosphorylation of endogenous p27 contributes to EMT maintenance, the weakly metastatic MDA-MB-231 (231) breast cancer line and a derivative with enhanced lung metastatic ability, MDA-MB-231-4175 (4175) (Minn et al., 2005a) were compared. 4175 cells showed strong PI3K pathway activation: activating
Figure 4.2: Loss of p27 decreases mesenchymal characteristics in PI3K-activated, metastatic lines. (A) Western of PI3K activation and p27 in MDA-MB-231 (231) and MDA-MB-231-4175(4175) cells. (B) Lysates containing equal amounts of p27 were immunoprecipitated for p27pT157 or p27pT198 or total p27, followed by p27 blotting in 231 and 4175 cells. (C) Western of p27 and EMT markers in 4175 cells with or without p27 knockdown (Scramble control or Shp27). (D) RT-PCR of mRNA for p27, E-cadherin and Vimentin in 4175-Shp27 and controls. (E) Time-course analysis of RT-PCR for Twist1 expression in 4175 cells treated with sip27. (F) Time-course of E-cadherin expression following p27 knockdown by siRNA against p27 (sip27) in 4175 cells. (G) Cell cycle analysis of 4175-Scr and 4175-Shp27 cells. (H) Transwell migration of 4175-Scr and 4175-Shp27 cells. (I) Transwell invasion of 4175-Scr and 4175-Shp27 cells. (J) Western analysis of total and phosphorylated PI3K effector kinases in UMUC3 and UMUC3-LUL2 (UMUC3-L) cells. (K) Western for p27, N-cadherin and Twist1 in UMUC3-L cells with or without stable p27 knockdown (Shp27). (L) RT-PCR of p27, N-cadherin and Vimentin expressed in UMUC3-L cells with or without Shp27. (M) RT-PCR for p27 and Twist1 mRNA in UMUC3-L cells treated with siRNA against n27 (sin27). All data graphed represent mean of at least 3 repeats +/- SEM. *signifies p < 0.05.
phosphorylations of AKT, SGK, and PDK1 were increased while total kinase levels were similar and p27 was increased relative to parental 231 (Figure 4.2A). When equal amounts of cellular p27 were loaded from each line, 4175 cells showed a marked increase in both p27pT157 and p27pT198 compared to 231 (Figure 4.2B, upper panel). Subcellular fractionation followed by immunoblotting showed greater cytoplasmic p27 in 4175 compared to 231 (Figure 4.2B, lower panel). Stable p27 knockdown in 4175 increased E-cadherin, and decreased Vimentin and Twist1 (Figures 4.2C, D). In 4175 cells, migration and matrigel invasion were both markedly decreased following p27 knockdown (Figure 4.2E). Stable p27 knockdown in 4175 cells did not alter cell cycle distribution (Figure 4.2F). Notably, siRNA-mediated p27 knockdown significantly reduced TWIST1 expression within 8 hrs, followed by an increase in E-cadherin protein by 48 hrs (Figures 4.2G, H).

To validate these findings in a cancer from a different organ, we compared the UMUC3 bladder cancer line with a highly metastatic sister cell line, UMUC3-LuL2 (UMUC3-L), derived following selection and expansion of lung metastases from serial tail vein injections. (Nitz et al., 2008) As in the breast cancer models, the highly metastatic UMUC3-L line showed significant PI3K pathway activation and increased p27 compared to parental UMUC3 (Figure 4.2I). Similarly, p27 knockdown significantly reduced mesenchymal characteristics, decreasing Vimentin, N-Cadherin and Twist1 expression (Figures 4.2J, K) and reduced cell motility (Figure 4.2L). Thus, in both metastatic breast and bladder cancer models with oncogenic PI3K activation, p27 appears to maintain an EMT program with increased cell motility and invasion.
4.2.3  p27CK-DD Overexpression Activates STAT3 to Induce TWIST1 and Activate EMT

To elucidate the oncogenic signaling pathway(s) involved in p27CK-DD-induced EMT, we screened for signaling kinases known to contribute to EMT. Y705-phosphorylated- and total STAT3 protein and STAT3 mRNA were increased in MCF-12A-p27CK-DD cells compared to control MCF-12A (Figure 4.3A), as was nuclear STAT3 localization (Figure 4.3B). Nuclear STAT3 was minimal in control MCF-12A and in MCF-12A-p27CK-DD cells treated with a STAT3 inhibitor. Prolonged STAT3 inhibition over two weeks in culture reversed the mesenchymal phenotype of MCF-12A-p27CK-DD cells, shown by morphological change from spindle-shaped mesenchymal cells to a cobble-stone epithelial appearance (Figure 4.3B), increased E-cadherin, and decreased mesenchymal markers and Twist1 expression (Figure 4.3C). STAT3 inhibitor treatment reduced the intense nuclear Twist1 expression in MCF-12A-p27CK-DD to levels seen in MCF-12A controls (Figure 4.3D). Interestingly, p27CK-DD overexpression markedly increased AKT activation, and this was attenuated by STAT3 inhibitor treatment (Figure 4.3E), suggesting that AKT activation in p27CK-DD is STAT3 dependent. Transduction of dominant negative STAT3 (STAT3DN) into MCF-12A-p27CK-DD cells phenocopied pharmacological STAT3 inhibition, with reversion of EMT (Figure 4.3F), and marked attenuation of p27CK-DD-induced cell migration and invasion (Figures 4.3G, H). Thus, p27CK-DD-induced EMT is reversible in immortalized, non-malignant mammary epithelial cells and STAT3 signaling is critical for p27-dependent TWIST1 induction and maintenance of this EMT phenotype.
Figure 4.3: p27CK-DD drives STAT3 activation to induce EMT. (A) Western of pSTAT3 (Y705) and total STAT3 in vector control MCF-12A, C, and MCF-12A-p27CK-DD (p27CK-DD) cells (left) and QPCR for STAT3 mRNA in control MCF-12A and MCF-12A-p27CK-DD cells (right). (B) Immunofluorescence (top) and phase-contrast (bottom) images of pSTAT3 in control MCF-12A and MCF-12A-p27CK-DD cells with or without treatment for two weeks with STAT3 inhibitor (STAT3-I). (C-E) Control MCF-12A and MCF-12A-p27CK-DD cells treated with or without STAT3 inhibitor (STAT3-I) for two weeks were assayed for: Twist1 and EMT markers by Western (C), Twist1 protein by indirect immunofluorescence (D) and total and activated Stat3 and Akt levels by Western. (F-H) MCF-12A vector only controls and MCF-12A-p27CK-DD cells with or without dominant negative STAT3 (STAT3DN) transduction were compared for Twist1 and EMT markers expression (F), transwell migration (G) and transwell invasion (H). All data graphed as mean +/- SEM. *signifies p<0.05 by Student’s T test.
4.2.4 p27 Scaffolds JAK2, Increases JAK2-STAT3 Complexes and Enhances STAT3 Transcriptional Activity at the TWIST1 Promoter

We next assayed effects of p27 on transcriptional activity at the TWIST1 promoter. As was the case for TWIST1 expression (Figure 4.2C, E), TWIST1 luciferase activity was also attenuated by p27 knockdown but not by scrambled shRNA controls in 4175 (Figure 4.4A). Overexpression of constitutively active STAT3 (STAT3-CA) restored TWIST1 promoter activity in p27 knockdown cells. STAT3 inhibition reduced TWIST1 promoter activity in 4175 to the same extent as p27 knockdown (Figure 4.4A).

Quantitative ChIP assays revealed that STAT3 occupancy of the TWIST1 promoter was increased in 4175 compared to 231 cells and attenuated by p27 knockdown, while p27CK-DD transduction increased STAT3 binding to the TWIST1 promoter in 231 cells (Figure 4.4B). p27CK-DD expressing UMUC3 showed increased STAT3 binding to the TWIST1 promoter and this was reversed by STAT3DN; and p27 knockdown in UMUC3-L decreased STAT3 occupancy of the TWIST1 promoter (Figure 4.4C), supporting a model in which C-terminally phosphorylated p27 promotes STAT3 activation, driving STAT3 binding to the TWIST1 promoter and TWIST1 activation. Notably, ChIP assays failed to reveal co-occupancy of p27 with STAT3 on the TWIST1 promoter.

We next assayed if p27pT157pT198 might bind to JAK2-STAT3 to promote STAT3 activation. p27 has been shown to bind JAK2 via both its catalytic and FERM domains. (Jakel et al., 2011) Treatment of 4175 with a dual PI3K/mTOR inhibitor, PF-04691502 (PF) at 250 nM reduced pAKT to parental 231 levels by 48 hrs (Figure 4.4D, top panel). This drug dose, which reduces p27pT157 and p27pT198 levels (Wander et al., 2013) also reduced total and activated pSTAT3, while JAK2 was unchanged (Figure 4.4D,
Figure 4.4: C-terminally phosphorylated p27 binds JAK2, increases JAK2-bound STAT3 and enhances STAT3 transcriptional activity at the \textit{TWIST1} promoter. (A) Relative \textit{TWIST1}-promoter luciferase expression in 4175 scrambled shRNA controls (scr), 4175shp27 (shp27), 4175shp27 + STAT3-CA, and 4175 treated with a STAT3 inhibitor (Stat-I). (B) ChIP assays show relative STAT3 binding at the \textit{TWIST1} promoter in 231, 231-p27CK-DD (231-DD), 4175, and 4175shp27 cells. (C) ChIP assays show relative STAT3 binding to the \textit{TWIST1} promoter in parental UMUC3, UMUC3-p27CK-DD, UMUC3-p27CK-DD + STAT3DN, the highly metastatic variant UMUC3-LuL2, and in UMUC3-LuL2shp27 cells. (D) Immunoprecipitation of JAK2 in 231, 4175, and 4175 cells treated with the PI3K/mTOR inhibitor PF1502 (PF) shows associated STAT3 and p27 (left panel). Westerns show inputs (right panel). (E) Densitometry from (D) for both p27 (top) and STAT3 (bottom) comparing total protein (input) and protein bound to JAK2. (F) Westerns show input levels of STAT3 and p27pT198 in 4175 (right) and STAT3-bound to immunoprecipitated p27pT198 (left). (G) Westerns show input levels of JAK2, STAT3, p27-GFP, and total p27 levels in UMUC3 and UMUC3-p27CK-DD cells (left). JAK2 associated STAT3 and p27-GFP were detected in JAK2 precipitates (right).
Notably, JAK2 bound STAT3 and p27 were increased in 4175 compared to 231 (Figure 4.4D, bottom panel). Although total and activated STAT3 were modestly increased in 4175 compared to 231 and PF-treated 4175, densitometric quantitation showed the increases in JAK2-bound STAT3 and p27 in 4175 were greater than predicted by their abundance alone (Figure 4.4E), suggesting that p27 binding may facilitate recruitment of STAT3 to JAK2. p27pT198 precipitation using a T198-phospho-specific antibody revealed associated STAT3 in 4175 cells (Figure 4.4F). While these complexes were detected in 231, the p27pT198 and associated STAT3 levels were too low to permit accurate quantitation (not shown).

Notably, when p27CK-DD was stably expressed in the weakly metastatic UMUC3 bladder cancer line, a trimeric JAK2/p27CK-DD/STAT3 complex was detected, JAK2-bound STAT3 was increased and STAT3 was activated (Figure 4.4G).

4.2.5  **STAT3 Inhibition Attenuates p27CK-DD-Induced Cancer Metastasis**

Since both induction and maintenance of an EMT program are critical for metastatic tumor progression (Kalluri and Weinberg, 2009; Thiery et al., 2009; Micalizzi et al., 2010), we next assayed effects of p27CK-DD overexpression with and without STAT3 inhibition on invasion and metastasis in our breast and bladder cancer models. p27CK-DD transduction into 231 increased STAT3 levels and STAT3 activation, increased Twist1, decreased E-cadherin (Figure 4.5A), and markedly increased cell migration and invasion (Figures 4.5B, C) over control 231 cells. Transduction of STAT3DN into 231-p27CK-DD cells reverted the mesenchymal phenotype, causing loss
of Twist1, increased E-Cadherin, and reversed the p27-driven increase in cell migration and invasion (Figures 4.5A-C).

Upon tail vein injection into nude mice, both the number and size of lung metastasis formed from p27CK-DD-expressing 231 cells were significantly increased, as was cumulative tumor bioluminescence compared to parental 231 controls (Figures 4.5D-F). The p27CK-DD-mediated gain of metastatic potential was STAT3 dependent: STAT3DN transduction attenuated lung tumor formation by 231-p27CK-DD cells to levels similar to those of parental 231 (Figures 4.5D-F).

The pro-metastatic effect of p27CK-DD was confirmed in the UMUC3 bladder cancer model. p27CK-DD overexpression in UMUC3 parental cells activated STAT3, modestly increased STAT3 mRNA and induced a STAT3-dependent increase in N-Cadherin and Twist1, and increased cell migration and invasion (Figures 4.5G-I). Moreover, p27CK-DD overexpression caused widespread multi-organ tumor metastasis in vivo, detected by direct immunofluorescence of metastatic tumor that was not observed in parental UMUC3, and this was attenuated by co-expression of STAT3DN (Figure 4.5J). These data provide strong evidence that STAT3 activation is required for p27CK-DD-induced EMT and enhanced metastasis in these models.
Figure 4.5: STAT3 inhibition decreases p27CK-DD-induced cancer metastasis. (A) The effects of STAT3DN on Twist1 and E-cadherin expression in 231-p27CK-DD cells. (B) Real time migration of 231 cells expressing the indicated vectors. (C) Real time matrigel invasion of 231 cells expressing indicate vectors. (D) Bioluminescence/time of experimental lung metastasis following tail vein injection of 231 cells expressing the indicated vectors. (E) Representative bioluminescence images of mice from indicated groups. The color scale depicts the photon flux (photons per second) emitted. (F) Representative images of lungs from indicated 231 groups. (G) The effects of STAT3DN on Twist1 and N-cadherin expression in UMUC3 (low metastatic) cells expressing indicated vectors. (H) Real time migration of UMUC3 cells expressing indicated vectors. (I) Real time matrigel invasion of UMUC3 cells expressing indicate vectors. (J) Representative immunofluorescence images of multi-organ (lung, liver and kidney) GFP positive metastasis from mice that received intravenous injection of UMUC3 cells expressing indicate vectors. All data graphed represent mean of at least 3 repeats +/- SEM, *signifies p<0.05, **signifies p<0.01.
4.2.6  *p27 Knockdown Reduces Metastasis in vivo and This is Reversed by STAT3-CA*

Since loss of p27 reversed the EMT phenotype in the highly metastatic 4175 and UMUC3-L, (Figure 4.2), we next assayed effects of p27 knockdown on their metastatic potential *in vivo*. In 4175, while p27 knockdown markedly reduced pSTAT3, total STAT3 mRNA and protein levels were less notably reduced (Figure 4.6A-C). This is consistent with the known action of pSTAT3 to transactivate the STAT3 gene. An increase in STAT3 mRNA expression was also observed in p27CK-DD transduced cells (Figures 4.3A). As observed *in vitro* for cell migration and invasion (Figure 4.2), loss of p27 also significantly decreased experimental lung metastasis formed by 4175 (p<0.001) (Figure 4.6D-F). Similarly, in UMUC3-L, p27 knockdown decreased pSTAT3 (Figure 4.6G) and decreased experimental lung metastasis (p<0.001) (Figure 4.6H-J). The loss of metastatic ability could not be attributed to a cell cycle effect for either cell model (Figure 4.2G). Since STAT3 activity was decreased by p27 knockdown, a constitutively active STAT3 (STAT3-CA) was introduced into 4175shp27 cells to test if restoration of STAT3 activity could rescue the loss of metastasis caused by p27 knockdown. In 4175shp27 cells, STAT3-CA expression partially restored loss of metastasis following p27 knockdown (Figures 4.6D-F), suggesting that STAT3 serves as a critical mediator of the pro-metastatic effects of p27pT157pT198 *in vivo*. 
Figure 4.6: p27 knockdown reduces metastasis in vivo and is reversed by STAT3-CA. (A) Effects of p27 knockdown on STAT3 and pSTAT3 levels in 4175. (B) Graphs of relative densitometry values from (A) for both pSTAT3 and total STAT3 in 4175 compared to 231. (C) QPCR for STAT3 mRNA in 4175 control and 4175shp27 cells. (D) Bioluminescence/time of experimental lung metastasis from xenograft mice that received intravenous injection of 4175 cells expressing the indicated vectors. (E) Representative bioluminescence imaging from indicated 4175 groups. The color scale depicts photon flux (photons/second) from xenografted mice. (F) Representative images of lungs from indicated 4175 groups. (G) Effects of p27 knockdown on STAT3 and pSTAT3 in UMUC3-LuL2. (H) Bioluminescence/time of experimental lung metastasis from xenograft mice that received intravenous injection of UMUC3-L cells expressing indicated vectors. (I) Representative bioluminescence imaging from UMUC3-L groups. The color scale depicts photon flux (photons/second) from xenografted mice. (J) Representative images of lungs from indicated UMUC3-L groups. All data are graphed as mean +/- SEM, *signifies p<0.05, **signifies p<0.01.
4.2.7  p27pT157 Correlates with STAT3 and PI3K/mTOR Activation in Human Cancers

Proteomic analysis of 747 primary human breast cancers from The Cancer Genome Atlas (TCGA)/The Cancer Proteome Atlas (TCPA) dataset showed p27pT157 and p27pT198 are highly correlated (correlation coefficient R=0.411, p=0) and both are strongly associated with activation of kinases downstream of PI3K, p70pT389, p90pT359, S6pS235 and S6pS240 (R=0.13-0.41, all p values <9 E-08) and correlate negatively with PTEN (R=-0.24, p=8.9 E-11 and R=-0.14, p=0.0002, respectively) (data shown for p27pT157 in Figure 4.7A). Box plots show elevated p27pT157 (Figure 4.7A) and pSTAT3 (Figure 4.7B) correlated significantly with levels of indicated proteins above the mean. Elevated pSTAT3p705 levels correlate significantly with both p27pT157 (R= 0.102, p=0.007) and with activation of PI3K/mTOR effectors pAKT (pT308 and pT473), p70pT389, p90pT359, S6pS235 and S6pS240 (R=0.103 0.428; all p values between 0.006 and 1.3 E-10) and correlated inversely with PTEN (R=-0.10, p=0.009) (Figure 4.7B). Due to the recent collection of TCGA samples, correlation with patient outcome was not possible. The correlation between pSTAT3 and PI3K/mTOR pathway activation was validated in a second dataset of 712 primary breast cancers (AKTpS473, R=0.137, p=0.0003; mTORpS2448, R=0.172, p=3.95E-06). Unfortunately, p27pT157 and p27pT198 data were not available in the second breast cancer dataset.

The strong correlation between activation of STAT3 and activation of multiple PI3K/mTOR pathway effector kinases was confirmed in over one thousand bladder, renal and lung cancers from TCGA data (Figure 4.7C). Patient follow-up time for these datasets was also too short to permit outcome analysis.
4.3 DISCUSSION

Distant metastases are the cause of most cancer patient deaths. (Valastyan and Weinberg, 2011) Despite substantial progress in our understanding of the complex process of human tumor metastasis, the lack of knowledge of molecular mechanisms underlying metastasis has limited our ability to specifically target tumor spread. (Chiang and Massague, 2008) Cytoplasmic p27 plays an oncogenic role via RhoA-Rock inhibition to increase cell migration, and this may contribute to increased tumor metastasis. (Besson et al., 2004b;
Besson et al., 2007) Present data indicate that in addition to facilitating RhoA binding, (Larrea et al., 2009a) p27 phosphorylations at T157 and T198 link PI3K activation with a STAT3 driven metastatic cascade in vivo in human tumors. Knockdown of cellular p27 in PI3K-activated cancer models attenuates metastasis, while phosphomimetic p27CK-DD causes non-transformed mammary epithelial cells to acquire invasive ability in vitro and increases metastasis of cancer cells in vivo. Furthermore, we identify a mechanism in which C-terminally phosphorylated p27 drives tumor metastasis in vivo via STAT3 activation, which induces TWIST1 and EMT.

To initiate metastasis, tumor cells must transgress cell-cell junctions and acquire the ability to invade beyond surrounding basement membranes. (Chaffer and Weinberg, 2011) Reactivation of EMT is thought to initiate this early step in the metastatic cascade. (Micalizzi et al., 2010; Puisieux et al., 2014) Present data demonstrate that p27CK-DD is sufficient to induce a reversible, EMT-like phenotypic switch in two immortal, non-tumorigenic mammary epithelial lines, and to upregulate this process in tumorigenic cancer cells. Conversely, in PI3K-activated, metastatic 4175 breast and UMUC3-LuL2 bladder cancer models with high endogenous p27pT157pT198, p27 knockdown reverted the EMT phenotype, reduced cell invasion and substantially reduced formation of experimental lung metastasis. The loss in metastatic ability following p27 knockdown in these models could not be attributed to effects on cell cycle progression or proliferation. Thus, PI3K activation is accompanied by an oncogenic gain of function in which p27pT157pT198 promotes metastasis in part by trigging EMT-like changes. Cancer cells appear to gain an advantage by subverting a CDK inhibitor to promote tumor progression. This may explain why p27
is rarely completely lost, despite the decreased nuclear p27 observed in a majority of human cancers. (Besson et al., 2008; Wander et al., 2011b)

EMT is initiated by several transcription factors, including Twist1, Snail, and Slug. (Ansieau et al., 2010; Yang et al., 2004) Twist1 can act independently of Snail and Slug to repress E-cadherin and upregulate N-cadherin. (Thiery et al., 2009) As a master regulator of embryonic morphogenesis, Twist1 has been shown to promote cancer metastasis by activating EMT. (Yang et al., 2004) Here, we show p27 knockdown in p27pT157pT198-enriched highly metastatic lines decreased \textit{TWIST1} expression, while p27CK-DD transduction led to \textit{TWIST1} overexpression in low metastatic cells. \textit{TWIST1} knockdown reversed the p27CK-DD-induced mesenchymal phenotype, indicating \textit{TWIST1} upregulation is key to p27CK-DD-induced EMT.

STAT3 is aberrantly expressed and activated in many cancers including breast cancer (Lieblein et al., 2008) and plays critical roles in malignant transformation and tumor progression. (Marotta et al., 2011a; Devarajan and Huang, 2009; Xiong et al., 2012) p27 knockdown in p27pT157pT198-enriched cancer cells decreased total and phosphorylated STAT3. Conversely, p27CK-DD expression significantly increased total and phosphorylated STAT3 (Y705) in immortal mammary epithelial cells and cancer cells. STAT3 inhibition or STAT3DN reversed p27CK-DD-mediated EMT, reverted mesenchymal to epithelial morphology, upregulated epithelial markers, and attenuated the p27CK-DD-mediated increases in cell invasion and cancer metastasis. Thus, STAT3 appears to be critical for maintenance of p27-driven EMT and metastasis.

In cancer cells expressing high endogenous p27pT157pT198, we observed high \textit{TWIST1}-luciferase activity and \textit{TWIST1} expression. STAT3 inhibition and p27
knockdown in 4175, both attenuated TWIST1 promoter activity and TWIST1 expression, consistent with reports that TWIST1 is induced by STAT3. (Cheng et al., 2008; Lo et al., 2007) In both breast and bladder cancer models, p27CK-DD increased, while p27 knockdown decreased STAT3 occupancy and activation of the TWIST1 promoter. p27 can act as a transcriptional co-repressor with p130 and E2F4 to form a repressive complex at target promoters (Li et al., 2012a; Pippa et al., 2012) and p27 has been shown to form part of a TWIST1 repressor complex. (Menchon et al., 2011) While it is tempting to speculate that C-terminal phosphorylation of p27 might sterically disrupt repressive complexes at certain promoters and convert p27 from a co-repressor to a co-activator at novel gene promoters, we were unable to detect p27 in complex with STAT3 on the TWIST1 promoter.

p27CK-DD transduction led to formation of a tripartite p27/JAK2/STAT3 complex and increased both JAK2-bound STAT3 and STAT3 activation. In addition, JAK2/STAT3/p27 complexes were increased in 4175 compared to 231 and were attenuated by PI3K inhibition. These data suggest a model in which p27pT157pT198 binds JAK2 to facilitate STAT3 recruitment and activation, and STAT3-dependent TWIST1 induction.

Present data also reveal a potential feed-forward loop between PI3K, p27 and STAT3. p27CK-DD-expressing cells showed a STAT3 dependent increase in pAKT. Our proteomic analysis of two independent breast cancer data sets comprising over 1400 primary cancers, and additional TCGA/TCPA datasets of over 1000 primary cancers of bladder, kidney and lung all show correlations between pSTAT3 and PI3K pathway activation, supporting our in vitro and in vivo findings. Thus, PI3K activation in human cancer would increase p27 phosphorylation at T157 and T198 and contribute to the acquisition of metastatic potential through a STAT3/TWIST1-dependent EMT program. In
addition, deregulated p27pT157pT198 would also drive STAT3-dependent AKT activation, to further phosphorylate p27, amplify STAT3 activation and drive EMT, contributing thereby to the acquisition and maintenance of metastatic potential.

In summary, we have uncovered a novel oncogenic function of p27 to drive tumor metastasis through activation of EMT in both breast and bladder cancer models. Cytoplasmic p27 is observed in many human cancers (Chu et al., 2008) and is correlated with adverse outcome in prostate cancer (Li et al., 2006) renal cell cancer (Kruck et al., 2012) glioma (Piva et al., 1999) and high-grade astrocytomas (Hidaka et al., 2009). In breast cancer, cytoplasmic p27 staining correlates with AKT activation (Liang et al., 2002a; Shin et al., 2002; Viglietto et al., 2002) predicts early disease relapse (Liang et al., 2002a) and is associated with increased lymph nodal metastasis and poor overall survival (Wander et al., 2013). Our analysis of proteomic data in primary human breast cancers also links PI3K/mTOR activation with high p27pT157 and reveals a novel association with activated pSTAT3. Taken together these data support an oncogenic feedforward loop in which oncogenic PI3K activation would increase p27pT157pT198 to increase STAT3 activity, further activate AKT, induce STAT3-dependent TWIST1 expression and drive EMT, contributing thereby to the acquisition and maintenance of metastatic potential. Combined inhibition of PI3K/mTOR and JAK2/STAT3 in p27pT157pT198 enriched human cancers may ultimately have therapeutic potential to limit p27-mediated EMT and cancer metastasis.
CHAPTER 5

C-TERMINALLY PHOSPHORYLATED p27 FORMS A TRANSCRIPTIONAL CO-ACTIVATOR COMPLEX WITH c-JUN TO DRIVE TGF-β2 AND SNAI1 EXPRESSION
5.1 SUMMARY

In normal cells, p27 restrains the cell cycle by inhibiting cyclin-CDKs. In human cancers, p27 is deregulated by PI3K-dependent phosphorylations at T157 or T198 that increases cell motility by RhoA-ROCK inhibition and actin destabilization. Here we show p27 acts as a c-Jun coactivator to drive EMT transcriptional programs and metastasis. p27 knockdown in bone-tropic, metastatic breast cancer MDA-MB-231-variant 1833 (1833) cells caused re-expression of metastasis-suppressor genes, and reverted an EMT gene signature. p27 knockdown in three PI3K-activated, metastatic lines reduced expression of EMT drivers including SNAI1, SNAI2, ZEB2, and TGF-β2, but not TGF-β1, while transduction of a cell cycle defective (CK-), double phosphomimetic p27 T157D/T198D mutant, p27CK-DD, into less metastatic or non-transformed lines increased their expression. Notably, p27CK-DD over-expression in human mammary epithelial cells increased c-Jun activity and this was decreased with p27 knockdown in 1833. In silico analysis revealed AP-1 binding sites upstream of the TGF-β2 promoter, not present in that of TGF-β1. p27pT157pT198 bound JNK/c-Jun in the cytoplasm but only c-Jun in the nuclear fractions and co-localized with c-Jun at an AP-1 motif -15KB upstream of the TGF-β2 transcriptional start site. p27CK-DD also increased TGF-β2 promoter activity, suggesting that p27 co-activates c-Jun to induce TGF-β2 expression. p27pT157pT198 was also required for TGF-β2-mediated increases in SMAD3 activation, SNAI1 expression, and cell motility. p27pT157pT198 increased recruitment of c-Jun to SMAD3 and localized to an AP-1 motif together with c-Jun on the SNAI1 promoter following TGF-β2 stimulation. These data support a model in which PI3K-driven p27pT157pT198 promotes EMT and
metastasis through coactivation of c-Jun to induce TGF-β2, and of c-Jun-SMAD3 at the SNAI1 promoter to upregulate EMT.

5.2 RESULTS

5.2.1 p27 Downregulates Putative Metastasis Suppressor Genes and Induces an EMT Program

Our prior work showed p27 mediates PI3K-induced metastasis in breast and bladder models, and that this requires C-terminal p27 phosphorylation (Wander et al., 2013; Zhao D. et al., 2015). To further elucidate effects of p27 on metastasis, the gene expression profiles of parental low MDA-MB-231 (hereafter 231), a line with low metastatic ability, and its bone-tropic metastatic derivative line, MDA-MB-231-1833 (hereafter 1833) were compared. These differentially expressed genes were compared to the “bone metastatic signature” (BMS) gene expression profile generated by Kang et al (Kang et al., 2003). Expression of BMS genes was compared using gene expression microarrays in parental 231, 1833, and in 1833shp27, in which p27 was stably knocked down (Figure 5.1A). Of note, a profile of BMS genes that were reduced during the transition from 231 to 1833 was upregulated following p27 knockdown in 1833 (Figure 5.1A). Thus, loss of p27 promotes re-expression, in 1833 cells, of a subset of putative bone metastasis suppressor genes.

Since aberrant EMT activation is implicated in cancer metastasis, we investigated if p27 may orchestrate aspects of an EMT. While 231 shows features of an EMT-like transformation (Taube et al., 2010), we postulated that mesenchymal characteristics might be further enhanced in 1833. To further explore the effects of p27 loss, genes differentially expressed in 1833 versus 1833shp27 were compared to a previously
established “EMT core signature” derived by over-expression of master EMT regulators in immortalized human mammary epithelial cells (Taube et al., 2010). p27 knockdown in 1833 significantly altered the expression of forty genes in this EMT core signature “away from” an EMT phenotype (Figure 5.1B and 5.1C top). EMT core upregulated genes and core downregulated genes are shown in Figure 5.1C (bottom). Genes upregulated in the EMT core signature were reduced by p27 knockdown in 1833, and genes downregulated in the EMT core signature were increased in 1833 with p27 knockdown. Thus, p27 knockdown reverts expression of an EMT profile, supporting the notion that p27 may drive metastasis, in part, through activation of an EMT transcriptional program.

To confirm that p27 knockdown drives key EMT markers, E-cadherin, N-cadherin, and Vimentin levels were compared in parental 231 and metastatic 1833 cells with and without stable p27 knockdown. Indeed, 1833 expressed lower E-cadherin and higher N-cadherin and Vimentin than 231 (Figure 5.1D). Stable p27 knockdown increased $\text{CDH1}$ expression in 1833 (Figure 5.1E top), and acute siRNA-mediated p27 knockdown increased E-cadherin protein by 48-72 hours (Figure 5.1E bottom). Thus, loss of p27 reverts a cellular phenotype away from EMT, decreases EMT markers and reverts elements of the EMT phenotype.
Figure 5.1: p27 shifts an EMT program. (A) Gene expression analysis of parental 231, metastatic 1833, and 1833shp27 identifies a gene pattern in a bone metastatic signature mediated by p27 (left). (Right) Gene set analyses for BMS downregulated genes in 1833 scr vs. 1833 shRNA p27. Shown are ordered gene scores for each gene in the line plot and the average fold change in the heatmap (orange indicates high expression in 1833 shRNA p27 and blue is low). Average fold gene expression changes are indicated by bar graphs. (B) Genes changed with p27 knockdown in the BMS revert away from a previously established EMT core signature. (C) Flow chart shows comparison of p27-regulated genes in both signatures (top). Graphs show changes in individual gene scores with p27 knockdown compared to EMT core signature (bottom). (D) Stable p27 knockdown in metastatic 1833 cells reverts the expression of key EMT markers. (E) In 1833 cells, p27 knockdown increases E-cadherin mRNA (top) and protein (bottom) re-expression within 48 hours.
5.2.2 Key EMT Transcription Factors are Regulated in a p27pT157pT198-Dependent Manner

The EMT program is orchestrated by the expression of master transcriptional regulators (EMT-TFs) including Snail, Slug, Twist1, and Zeb2. MDA-MB-231-4175 (4175) (Minn et al., 2005b) and MDA-MB-231-1833 (1833) (Kang et al., 2003) are lung and bone-tropic derivatives of the breast line MDA-MB-231, respectively. UMUC3-LuL2 is a metastatic derivative of the bladder carcinoma line UMUC3 (Overdevest et al., 2011). All three of these lines are PI3K-activated and express high endogenous p27pT157pT198 (Zhao D. et al., 2015). p27 knockdown by siRNA acutely downregulated EMT-TF expression within 12-24 hours (Figure 5.2A).

We previously showed that transduction of a phosphomimetic p27 mutant protein defective in cyclin-CDK binding (p27CK-DD) can induce EMT (Zhao D. et al., 2015). To evaluate effects of C-terminal p27 in more epithelial models, non-transformed mammary epithelial line MCF12A and lines with low metastatic potential, 231 and UMUC3, were transduced with p27CK-DD. In all models tested, p27CK-DD upregulated EMT-TFs.

The TGF-β pathway is a major driver of EMT in transformed cells. Notably, TGF-β2 expression was significantly downregulated 4-8 hours following acute p27 loss in all three metastatic models, while that of TGF-β1 was unchanged (Figure 5.2C). Indeed, a time course following acute p27 knockdown in 4175 cells showed loss of p27 protein after one hour, followed by a significant reduction in TGF-β2 protein within 4 hours (Figure 5.2D).
Figure 5.2: Key EMT transcription factors and drivers are regulated in a p27\_T157p\_T198-dependent manner. (A) Acute p27 knockdown reduces several EMT transcription factors in PI3K-activated, metastatic lines. (B) p27CK-DO overexpression increases EMT transcription factors in epithelial and weakly metastatic cells. (C) Acute p27 knockdown reduces TGF-\(\beta\_2\) mRNA, and not TGF-\(\beta\_1\) mRNA, in PI3K-activated, metastatic lines. (D) A time course following acute p27 knockdown in lung-tropic, metastatic 4175 cells shows TGF-\(\beta\_2\) protein reduced around 4 hours following a drop in p27 protein levels one hour after addition of p27-
In all models tested, TGF-β2 expression was increased by p27CK-DD transduction, while that of TGF-β1 was unchanged (Figure 5.2E). Thus, C-terminally phosphorylated p27 may turn on an EMT program by first inducing upregulation of the ligand, TGF-β2, with subsequent induction of EMT-TF expression to drive a metastatic phenotype. Since C-terminally phosphorylated p27 enhances TGF-β2 expression, and not TGF-β1, we next investigated how p27 directs this specificity.

5.2.3 c-Jun Activation and its Binding to p27 are p27pT157pT198-Dependent

A phosphoprotein array comparing proteins and kinases differentially activated in MCF12A cells with and without p27CK-DD revealed that activated c-Jun (c-JunpS63) is significantly increased in p27CK-DD-expressing cells (Figure 5.3A). c-Jun is a transcription factor that forms part of the heterodimeric AP-1 complex. c-Jun contributes to oncogenic transformation (reviewed in (Hess et al., 2004)). An in silico search revealed that the genomic region upstream of the TGF-β2 coding sequence contains several putative AP-1 binding sites, while the TGF-β1 promoter has none (Figure 5.3B).

p27CK-DD expression in non-metastatic UMUC3 significantly increased activated c-JunpS63, while p27 knockdown in metastatic UMUC3-LuL2 cells reduced c-JunpS63 (Figure 5.3C left). p27 immunoprecipitation revealed c-Jun binds cellular p27, and this complex was significantly increased in p27CK-DD-transduced cells (DD vs GFP, Figure 5.3C right). Treatment with PI3K/mTOR inhibitor, PF1502 (250nM), inhibits AKT activation and reduces p27 phosphorylation at T198 (Figure 5.3D left) and T157 (Zhao D. et al., 2015). In UMUC3-LuL2 cells, PF1502 treatment for 48 hours decreased p27-c-Jun complexes (Figure 5.3D right).
Figure 5.3: c-Jun activation and its binding to p27 are p27pT157pT198-dependent. (A) A kinome array screen identifies increased c-JunS63 in MCF12A cells expressing p27CK-DD. (B) A query of SA Biosciences’ online database, DECODE, identifies putative AP-1 sites in the TGF-β2 promoter. A TPA-response element (TRE) is located approximately 15kb upstream of the TGF-β2 transcriptional start site (C) Western blots show c-JunS63 is increased in UMUC3 parental cells expressing p27CK-DD and metastatic UMUC3-LuL2 cells compared to controls. p27 knockdown in UMUC-LuL2 significantly reduces c-JunS63 (left). Immunoprecipitation of total p27 in UMUC3 control and UMUC3-DD cells show associated c-Jun (right). (D) Immunoprecipitation of p27 in UMUC3-LuL2 cells treated for 48 hours with or without PI3K/mTOR inhibitor PF1502 (PF) show associated c-Jun (right). Western blots show inputs (left). (E) Nuclear-cytoplasmic fractionation of UMUC3-LuL2 protein lysate shows c-Jun in the nucleus and JNK in the cytoplasm (left). Immunoprecipitation with a p27 antibody shows association with JNK and c-Jun in the cytoplasm, with a more abundant c-Jun/p27 complex in the nucleus (right) (F) Immunoprecipitation with a p27 antibody specific for p27pT198 shows associated c-Jun in the nucleus in fractionated UMUC3-LuL2 lysate, and not in the cytoplasm even with 4x lysate loaded (Cx4).
Thus, p27-c-Jun binding in PI3K-activated cells may require p27 C-terminal phosphorylation.

We recently showed that p27 promotes JAK2/STAT3 complex formation to enhance STAT3 activation and STAT3-mediated Twist induction (Zhao D. et al., 2015). To test if p27 may facilitate c-Jun recruitment and activation by c-Jun N-terminal kinase (JNK), p27 binding to these proteins was assayed in fractionated cell lysates. In UMUC3-LuL2, JNK was largely cytoplasmic, while both total and phosphorylated c-Jun localize predominantly in the nucleus (Figure 5.3E left). Immunoprecipitation of p27 in fractionated lysate allowed detection of a low level tripartite JNK-c-Jun-p27 complex in the cytoplasm and a more abundant p27-c-Jun complex in the nucleus (Figure 5.3E right). To test if C-terminal phosphorylated p27 binds c-Jun, p27 was precipitated with an anti-p27pT198 antibody in fractionated UMUC3-LuL2 lysates. A p27pT198-c-Jun complex was detected in the nuclear but not in the cytoplasmic fraction (Figure 5.3F). Taken together, these data support a model in which a p27-JNK-c-Jun complex forms transiently in the cytoplasm, followed by translocation of activated c-JunpS63-p27pT157pT198 to the nucleus.

5.2.4 c-Jun and p27 Form a Transcriptional Complex at the TGF-β2 Promoter

Recent data suggest that p27 may play a role as a co-regulator of other transcription factors (Pippa et al., 2012). To further investigate the mechanism of p27 activated TGF-β2 expression, we next assayed if the nuclear p27-c-Jun complex might bind AP-1-c-Jun binding motifs upstream of the TGF-β2 promoter. We tested two
putative c-Jun binding sites, identified by our in silico search to contain TRE (TPA-response element) motifs approximately 15kb upstream of the TGF-β2 gene transcriptional start site (Figure 5.3A). Chromatin immunoprecipitation (ChIP) assays showed both c-Jun and p27 bind to the TRE located approximately 15kb upstream of the TGF-β2 gene transcription start site (basepairs 218500872-79) in both 1833 and UMUC3 parental cells. (B) Quantification of % DNA bound relative to input for both c-Jun and p27 are shown for UMUC3. (C) Quantification of % DNA bound relative to input for p27 in UMUC3 parental compared to UMUC3-DD cells.

Figure 5.4: c-Jun and p27 form a transcriptional complex at the TGF-β2 promoter. (A) Chromatin immunoprecipitation with a c-Jun and p27 antibody show DNA amplification at the TRE located approximately 15Kb upstream of the TGF-β2 transcriptional start site in 1833 and UMUC3 parental cells. (B) Quantification of % DNA bound relative to input for both c-Jun and p27 are shown for UMUC3. (C) Quantification of % DNA bound relative to input for p27 in UMUC3 parental compared to UMUC3-DD cells.

These data suggest that p27pTpT may enhance c-Jun-driven TGF-β2 gene induction.
5.2.5 TGF-β2-Driven Cell Motility, SMAD3 Activation, and SNAI1 Induction are p27pT157pT198-Dependent

In addition to increasing TGF-β2 ligand expression, p27pTpT appears also to be required to mediate downstream signaling by TGF-β2. TGF-β2 increases motility in transformed cells (Massague, 2008) and this is also the case in 4175 cells (Figure 5.5A). Pretreatment of 4175 cells with exogenous TGF-β2 ligand increased motility above baseline in a wound closure scratch assay, and this was decreased in 4175shp27 cells, suggesting that p27 is required for TGF-β2-mediated cell motility (Figure 5.5A).

TGF-β pathway induction leads to SMAD3 phosphorylation and recruitment of co-SMAD, SMAD4 (Zhang et al., 1996)(reviewed in (Massague et al., 2005). This SMAD3/4 heterodimer then translocates to the nucleus to direct target gene transcription. SMAD3 activation has been implicated in numerous TGF-β pathway-mediated oncogenic or transforming events (Tarasewicz and Jeruss, 2012). Since SMAD3 mediates TGF-β-driven cell motility (Luwor et al., 2015), we tested if C-terminally phosphorylated p27 is required for SMAD3 activation. p27CK-DD-transduced UMUC3 cells showed higher SMAD3pS423,S425 than GFP controls. pSMAD3 levels were higher in UMUC3-LuL2 than UMUC3 and p27 knockdown reduced pSMAD3 in metastatic UMUC3-LuL2 (Figure 5.5B), suggesting that C-terminal p27 phosphorylation is important for SMAD3 activation following TGF-β receptor activation.

SNAI1 overexpression in highly PI3K-activated 4175, 1833 and UMUC3-Lul2 cell lines is p27pT198pT157-dependent (Figure 5.2A & B, and Figure 5.5C). Snail expression in these models is TGF-β2-dependent (Figure 5.5D). We next assayed if TGF-β2 activates SNAI1 induction in a p27-dependent manner. A time course shows
Figure 5.5. TGF-\(\beta\)-2-driven cell motility, SMAD3 activation, and SNAI1 induction are p27-dependent. (A) TGF-\(\beta\)-2 treatment of 1833 cells enhances wound closure in a scratch assay over time compared to controls, and this is dependent on p27 expression. UMUC3 cells expressing p27CK-DD and PI3K-activated UMUC3-Lul2 cells show increased SMAD3 activation (B) and Snail protein (C) compared to controls, and p27 knockdown in UMUC3-Lul2 reduces SMAD3 activation back to baseline. (D) Acute siRNA-mediated knockdown of TGF-\(\beta\)-2 decreases SNAI1 expression in 24 hours. (E) Time course of 1833 cells treated with TGF-\(\beta\)-2 shows maximal SMAD3 activation at 30 min – 1 hour. (F) 1833 cells were treated for 30 min with vehicle control, TGF-\(\beta\)-2 ligand, or TGF-\(\beta\)-1 ligand. TGF-\(\beta\)-2-, but not TGF-\(\beta\)-1-mediated SNAI1 induction is dependent upon p27 expression.
maximal SMAD3 phosphorylation at 30 min to one hour following TGF-β2 treatment of 1833 cells (Figure 5.5E). SNAI1 expression falls within 12 hours after siRNA-mediated acute p27 knockdown (Figure 5.5F and Figure 5.2A). Pretreatment with TGF-β2 ligand for 30 minutes significantly enhanced SNAI1 expression and this was abolished by p27 knockdown. Interestingly, while pretreatment with TGF-β1 also increased SNAI1 expression, this effect was not p27-dependent. Thus, C-terminally phosphorylated p27 directs a rapid, TGF-β2-specific increase in SNAI1 expression. Since SNAI1 is a known target for SMAD3-mediated transcription, we postulated that p27 might cooperate with SMAD3/4 to induce SNAI1 transcription.

5.2.6 p27-c-Jun Recruit SMAD3 to the SNAI1 Promoter Following TGF-β2 Treatment

While the SMADs regulate gene expression upon TGF-β pathway activation, they are weak DNA binders and usually form complexes with other factors to activate transcription (reviewed in (Massague et al., 2005). SMAD3 has been shown to cooperate with AP-1 transcription factors following TGF-β activation (Sundqvist et al., 2013). Therefore, we postulated that TGF-β activation might cause c-Jun-p27 to bind SMAD3 to increase SNAI1 induction. Within 30 min after pretreatment with TGF-β2, p27 immunoprecipitates show associated c-Jun and SMAD3 in 1833 (Figure 5.6A). To determine whether p27 may promote SMAD3-cJun interaction, 1833 cells were treated with or without p27 siRNA and complex formation assayed. SMAD3 immunoprecipitation shows SMAD3-bound c-Jun was decreased by p27 knockdown (Figure 5.6B). To test the importance of p27pTpT for complex formation, SMAD3 complexes were assayed 48 hours after PF1502 pretreatment of 1833 cells.
Figure 5.6: p27/c-Jun forms a complex with SMAD3 following TGF-β2 treatment and c-Jun/SMAD3 binding is dependent of p27pT157pT198. (A) Immunoprecipitation in 1833 cells with a p27 antibody shows associated c-Jun and SMAD3 following 30 min TGF-β2 treatment. Immunoprecipitation in TGF-β2-treated 1833 cells with a SMAD3 antibody shows abrogation of c-Jun binding to SMAD3 following (B) acute p27 knockdown and (C) 48h pre-treatment with a PI3K/mTOR inhibitor. (D) Chromatin immunoprecipitation with a c-Jun and SMAD3 antibody show DNA amplification on the SNAI1 promoter in 1833 (top). Quantification of % DNA bound relative to input for both c-Jun and SMAD3 are shown in the bottom panel.
Notably, loss of p27pT198 decreased recruitment of c-Jun to SMAD3 in Figure 5.6C.

We next assayed whether p27-c-Jun-SMAD3 complexes are recruited to AP-1 binding sites on the SNAI1 promoter. Our in silico search revealed a putative c-Jun binding site -12kb upstream of the SNAI1 transcriptional start site. Chromatin immunoprecipitation of c-Jun and SMAD3 showed that both c-Jun and SMAD3 were recruited to this putative SNAI1 enhancer motif within 30 min of TGF-β2 stimulation but not in its absence (Figure 5.6D).

These data support a mechanism whereby PI3K/Akt-mediated C-terminal phosphorylation of p27 at T157 and T198 promotes assembly of a JNK-c-Jun complex with p27 in the cytoplasm. Activated c-Jun-p27 complexes would translocate to the nucleus to direct TGF-β2 transcription. Upregulated TGF-β2 expression would then stimulate TGF-β receptor activation in an autocrine or paracrine manner. In addition to upregulation of TGF-β2 ligand, p27 also appears to promote TGF-β2 downstream signaling. p27 appears to facilitate complex formation between activated SMAD3 and formation of a p27-c-Jun-SMAD3 complex to drive c-Jun-SMAD3-dependent induction of SNAI1, to promote EMT and metastasis (Figure 5.7).
5.3 DISCUSSION

Most cancer-related deaths are attributable to the formation of distant metastases. Despite substantial progress in our understanding of the complex process of human tumor metastasis, the lack of knowledge of molecular mechanisms underlying metastasis has limited our ability to specifically target tumor spread. C-terminally phosphorylated p27 is required for PI3K-mediated metastasis (Zhao D. et al., 2015). p27 phosphorylation at T198 promotes its binding and inhibition of RhoA activity to increase motility (Larrea et al., 2009a). Furthermore, p27pTpT drives JAK2-mediated STAT3 activation leading to...

Figure 5.7: Diagram of p27pT157pT198-mediated induction of TGF-β2 expression, TGF-β pathway activation, and SNAI1 induction. PI3K/Akt-mediated C-terminal phosphorylation of p27 at T157 and T198 promote scaffolding of a JNK/c-Jun complex with p27 in the cytoplasm. Activated c-Jun with bound p27 translocates the nucleus to direct TGF-β2 transcription. Expression of TGF-β2 ligand stimulates TGF-β activation in autocrine or paracrine manner. TGF-β-mediated SMAD3/4 activation recruits the p27/c-Jun complex onto the SNAI1 promoter, leading to EMT and metastasis.
STAT3-mediated *TWIST1* induction and EMT (Zhao D. et al., 2015). Present data indicate that p27pT157pT198 also promotes metastasis by facilitating c-Jun-dependent *TGF-β2* induction and by cooperating with both c-Jun and SMAD3 to induce *SNAI1* induction following TGF-β2 stimulation. p27 knockdown reverts an EMT program in PI3K-activated, metastatic lines, and abrogates *TGF-β2* and *SNAI1* gene expression. p27pT157pT198 binds c-Jun and both are found to co-occupy a *TGF-β2* enhancer site to induce *TGF-β2* expression. Finally, p27 also acts downstream of TGF-β2 pathway activation to promotes a tripartite p27/c-Jun/SMAD3 complex on the *SNAI1* promoter. These data suggest that p27 drives EMT by integrating oncogenic signals from both activated PI3K and TGF-β pathways.

EMT is one of the first, crucial steps required in the metastatic process, and key cellular drivers are needed to set this process in motion. Extracellular TGF-β is a critical driver of EMT in transformed cells (reviewed in (Heldin et al., 2012)). Present data demonstrate that p27 knockdown in PI3K-activated, metastatic 1833 and UMUC3-LuL2 cells with high endogenous p27pT157pT198 reduces TGF-β2 ligand expression and attenuates TGF-β pathway activation by decreasing SMAD3 activation. Conversely, p27CK-DD expression in epithelial cells upregulates *TGF-β2* mRNA and enhances SMAD3 activation. While much of the work in studying TGF-β signaling has been carried out using the TGF-β1 isoform, a growing body of evidence has suggested that the TGF-β2 isoform may play an important role in driving tumorigenesis. TGF-β2 has been implicated as a mediator of metastasis in a number of different tumor types including glioma (Kingsley-Kallesen et al., 2001), melanoma (Zhang et al., 2009), pancreatic cancer (Schlingensiepen et al., 2011; Cui et al., 2014), and breast cancer (Beisner et al.,...
Beisner et al (Beisner et al., 2006) showed that increased TGF-β2 promoter activity enhances breast cancer lymph node metastasis. The present work also suggest that TGF-β2 is an important mediator of metastasis in breast and bladder carcinogenesis. An antisense oligonucleotide specific for TGF-β2 has shown therapeutic efficacy in early clinical trials (Jaschinski et al., 2011).

Recent evidence of p27 transcriptional activity describes a role for p27 as a co-repressor on pleuripotent genes to promote differentiation in quiescent cells (Pippa et al., 2012; Li et al., 2012a; Menchon et al., 2011). It is tempting to speculate that the activity of p27 in a transcriptional complex may be dependent on its integration of upstream signaling processes. As a result of aberrant PI3K mediated p27 C-terminal phosphorylation, the repressive complex may be disrupted and p27 may bind other transcriptional regulators in the nucleus to activate genes that drive EMT. c-Jun is aberrantly activated in many cancers and has critical roles in malignant transformation and tumor progression (Hess et al., 2004). p27 knockdown in p27pT157pT198-enriched cancer cells decreased phosphorylated c-Jun (S63). Conversely, p27CK-DD expression significantly increased phosphorylated activated c-Jun in low metastatic cells and immortal mammary epithelial cells. Here we show that p27pT157pT198 scaffolds a cytoplasmic JNK/c-Jun complex and translocates with c-Jun to the nucleus where it forms a transcriptional co-activator complex with c-Jun on the TGF-β2 promoter. p27 is a central signaling node for both the PI3K/mTOR and TGF-β pathways. Although, p27 was initially discovered as a mediator of TGF-β-induced cytostasis, our data suggest that C-terminally phosphorylated p27 and may be involved in switching a cell’s response to TGF-β signaling from cytostatic to oncogenic.
We previously identified a p27-driven EMT mechanism whereby p27 scaffolds a JAK2/STAT3 complex to enhance STAT3 activation and subsequent TWIST1 induction (Zhao D. et al., 2015). Snail is a critical driver of EMT and SNAI1 is a downstream transcriptional target of TGF-β pathway-mediated SMAD3/4 activity (Thuault et al., 2008). Knockdown of p27 in highly PI3K-activated metastatic breast and bladder cancer models strongly induced TWIST1 (Zhao D. et al., 2015), but also induced both TGF-β2 and SNAI1. TGF-β signaling via SMAD3 activation had been shown to cooperate with AP-1 activity to enhance invasion in a MCF10A breast model (Sundqvist et al., 2013).

We propose that in addition to STAT3-mediated TWIST1 induction, PI3K-activated p27 may drive an EMT through TGF-β pathway activation to turn on the master EMT regulator Snail. Present in vitro data suggest that p27pTpT not only drives TGF-β pathway activation through c-Jun-dependent TGF-β2 ligand upregulation, but p27pTPT also facilitates SMAD3 recruitment of c-Jun and co-activation of SNAI1 to drive cancer metastasis.
CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS
6.1 SUMMARY

Work of my thesis has provided new insights into how oncogenic deregulation of
the PI3K pathway disrupts p27 function. While most studies have evaluated PI3K and/or
mTOR inhibitor 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 provided 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 cancers expressing high p27pT157pT198.

Oncogenic activation of PI3K/mTOR signaling, through C-terminal
phosphorylation of p27 at T157 and T198, increases tumor cell invasiveness and drives
progression in a variety of cancers (Larrea et al., 2009b). However, the mechanisms
whereby C-terminal phosphorylation of p27 by PI3K/mTOR signaling kinases contributes
to human cancer 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 provided novel evidence that this deregulated p27 promotes cancer metastasis through EMT by promoting JAK2-STAT3 binding to activate STAT3-mediated *TWIST1* upregulation. We identified a signaling feed-forward loop in which PI3K/AKT activation drives p27 phosphorylation leading to STAT3 activation, which in turn activates AKT to contribute to tumor progression. These findings suggest that combined inhibition of both AKT and STAT3 in PI3K/AKT activated, p27pT157pT198 enriched human cancers may ultimately have therapeutic potential to limit p27-mediated EMT and cancer metastasis.

Although C-terminally phosphorylated p27 exhibits delayed import into the nucleus, our data show a detectable pool of p27pT157pT198 in the nucleus. Recent work has suggested that p27 can act as a regulator of transcription. Interestingly, the C-terminus of p27 has been shown to be required for its interaction in a multiprotein transcriptional complex (Pippa et al., 2012). In **Chapter 5**, I describe an additional pathway whereby PI3K-activated, deregulated p27 drives metastasis, TGF-β pathway activation, and EMT by upregulating transcription of *TGF-β2* and *SNAI1*. I provide evidence that C-terminally phosphorylated p27 binds a JNK/c-Jun complex, to enhance c-Jun activation and p27 accompanies c-Jun to activate transcription of *TGF-β2*. p27pTpT further promotes TGF-β pathway activation and forms a tripartite transcriptional complex with c-Jun and SMAD3 to drive *SNAI1* transcription. TGF-β2-mediated metastasis has been implicated in several different tumor types and a novel TGF-β2 specific, antisense oligonucleotide (Trabedersen, AP-12009) has shown promise in early clinical trials (Schlingensiepen et al., 2006). My work revealed a new rationale for treating patients with high p27pTpT-expressing tumors with dual therapy targeting both the PI3K and TGF-β2 pathways.
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 metastasis, but to also promote tumor progression by driving cancer stem cell self-renewal. In summary, the work in this thesis has revealed novel oncogenic function of p27 downstream of PI3K/AKT as a molecular driver of cell migration, EMT and cancer metastasis. We identify that p27 drives STAT3-mediated TWIST1 induction, c-Jun-mediated TGF-β2 induction, and c-Jun/SMAD3-mediated SNAI1 induction as critical mediators of tumor cell invasion and progression. These findings expand our knowledge of molecular mechanisms underlying cancer metastasis and provide insights for developing novel treatment for inhibiting cancer metastasis.

Due to high selection pressure and rapid evolution of cells within the tumor population, targeted therapies using a single drug almost invariably fail to prevent tumor progression. Taken together, our data suggest that dual therapies targeting PI3K/mTOR signaling together with either JAK2/STAT3, or TGF-β signaling, or even triple therapy targeting all three, may prove effective in PI3K-activated tumors exhibiting elevated p27pT157pT198 to thwart relapse of a responding tumor. Not only might this allow lower dosing of each inhibitor, leaving non-tumorigenic cells less affected, but oncogenically addicted cells will have fewer tumor-driving signaling pathways to exploit to evade targeted therapy. Immunohistochemical p27 staining of newly diagnosed patient tumors might serve a dual role as an initial prognostic factor, and subsequently as a predictive factor, in which a shift of p27 from cytoplasm to tumor nuclei identifies response to combined inhibitors of PI3K/Akt, TGF-β, and STAT3.
6.2 FUTURE DIRECTIONS

Our work and that of others have shown that deregulated, C-terminally phosphorylated p27 associates with new binding partners to direct oncogenic events independent of its CDK-binding role. Preliminary data from our phosphoproteome screen indicate that p27 may activate additional kinases to induce EMT and may also promote stem cell self-renewal. Increasing evidence suggests that diverse solid tumors are hierarchically organized and sustained by a distinct subpopulation of tumorigenic cancer stem cells (CSC) with self-renewal 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$^+$CD24$^{low/-}$ 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 C-terminally phosphorylated 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 was shown to be elevated, constitutively phosphorylated at T157, and localized in the cytoplasm (Chu et al., 2010). In addition, p27CK- knock in mice exhibit an expanded population 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 a mesenchymal phenotype to promote dissemination as well as self-renewal properties for metastatic tumor initiation (Mani et al., 2008). The STAT3 signaling pathway, that we found to be required for p27-driven induction of TWIST1 and tumor metastasis, has also been shown to be required for the self-renewal of CD44+CD24− stem cell–like breast cancer cells (Marotta et al., 2011b). A kinome screen showed p27CK-DD expression in MCF12A not only activates pSTAT3, but also activates PLCγ (Choi et al., 2007; Tam et al., 2013) and Pyk2 (Wendt et al., 2013), two kinases that have been implicated in EMT, tumor invasion, and/or metastasis. Based on these findings, we hypothesize that p27CK-DD may activate these additional kinases to drive transcription programs governing EMT and CSC self-renewal in the cancer. In the following sections, I present future work that stems from the findings of my thesis.

### 6.2.1 p27CK-DD Activates Kinases that Drive EMT and Metastasis

**Preliminary data:** Our microarray data showed that C-terminally phosphorylated p27 mediates a global EMT program by repressing putative anti-metastatic genes and activating metastasis-promoting genes (Fig. 5.1 A and B). While we have identified key components of this p27pTpT-regulated EMT program, including extracellular driver TGF-β2, and transcription factors c-Jun, STAT3, Snail, and Twist, it is possible the p27 may regulate additional components of an EMT program to drive PI3K-induced metastasis.
A kinome screen comparing MCF12A and MCF12A-p27CK-DD showed p27CK-DD increases activating phosphorylations in PLCγ and Pyk2 (Figure 6.1A and B).

PLCγ (Choi et al., 2007; Tam et al., 2013), and Pyk2 (Wendt et al., 2013) (non-receptor tyrosine kinase FAK-family member), have been implicated in EMT, tumor invasion, and/or metastasis, and PLCγ can activate both STAT3 (Zhang et al., 2011) and Pyk2 (Tam et al., 2013; Sala et al., 2008; Choi et al., 2007). I showed that p27 knockdown in metastatic 4175 cells reduces Pyk2 activation (Figure 6.1C). In the highly PI3K-activated/high p27pTpT bone metastatic 1833 line, PLCγ and Pyk2 activation are greater than in parental 231 (not shown). Additionally, I found that p27 co-precipitates with PLCγ (Figure 6.1D), thus suggesting that p27 binding may promote PLCγ catalytic activity by inducing a conformational change in this kinase.

**Proposed experiments:** p27pTpT will be tested to analyze whether it serves as a scaffolding factor to promote assembly and activation of PLCγ/Pyk2 complexes. We will test if p27WT binds GST tagged recombinant PLCγ and Pyk2 in vitro, and if this is increased with either pre-treatment of recombinant p27 with AKT or by using

![Figure 6.1: p27CK-DD activates PLCγ and Pyk2. (A-B) A kinome screen in MCF12A shows p27CK-DD increases PLCγ and Pyk2 activation vs control (GFP vector), both drivers of EMT and CSC. (C) p27 knockdown in 4175 decreases pPyk2 (Y410). (D) Co-IP show p27 binds PLCγ in 4175 cells.](image-url)
phosphomimetic p27pT157pT198 vs p27WT. We will then test if these p27-bound kinases more effectively recruit and phosphorylate their substrates in vitro.

We will also further test if p27 binding promotes complex formation between PLCγ and Pyk2 using recombinant purified His-tagged p27WT, single phosphomimetic mutants p27pT157D and p27pT198D, and double phosphomimetic mutant p27pT157DpT198D. Since PLCγ is known to activate STAT3, this work may show that p27pTppT recruits multiprotein kinase complexes to activate one or more STAT-like molecules. Notably, STAT1 and STAT4 were also up-regulated in MCF12Ap27CK-DD-expressing cells in the kinome screen. PLCγ and Pyk2 have also been implicated as potential drivers of cancer stem cell self-renewal and may promote association and activation of these complexes to promote stem cell self renewal in a p27pTppT-dependent manner. This will be further described in the next section.

6.2.2 p27 as a Putative Mediator of Cancer Stem Cell Self Renewal

As noted above, CSCs may be critical drivers of cancer metastasis and ultimately lead to treatment failure. CSCs from breast cancers form tumor spheres in 3D culture (Kakarala and Wicha, 2007), and express aldehyde dehydrogenase activity (ALDH1+) and/or CD44+CD24low/ surface marker expression (Al Hajj et al., 2003). 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).
High surface CD44 is a general marker for CSCs in many types of human cancers including lung, colon, liver and pancreas (Wicha, 2008). 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 metastases (Yang et al., 2008; Shipitsin et al., 2007). Recently, CD44 expression was shown to be 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).

Self-renewal is tightly regulated in normal embryonic stem cells, in part, by master regulators called embryonic stem cell transcription factors (ES-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 (ES-TFs), such as SOX2, KLF4, NANOG, and MYC have been shown to be critical for the maintenance of CSCs in various cancer models (Leis et al., 2011; Yu et al., 2011; Cavaleri and Scholer, 2003; Kim et al., 2010). Additionally, mammosphere formation is an in vitro assay used to identify 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. Cells that form mammospheres exhibit higher expression of the ES-TFs SOX2, NANOG, KLF4, and MYC, and recent data have linked the upregulation of EMT drivers with the cancer stem cell phenotype coincides with an EMT phenotype (Mani et al., 2008).

Over-expression of EMT transcription factors increases CSC abundance, suggesting that EMT may promote both tumor dissemination and CSC self-renewal needed for metastasis (Mani et al., 2008; Morel et al., 2008; Wellner et al., 2009). In p27CK- knock in mice, p27 plays a CDK-independent role to expand progenitors/stem cells in several tissues, increase bronchoalveolar stem cells and spontaneous lung tumors, suggesting that p27 has cell cycle-independent roles to regulate normal stem cell expansion (Besson et al., 2007). Since p27pTpT activates STAT3 to induce TWIST1 and drive tumor metastasis, and since STAT3 is a known driver of breast cancer stem cell self-renewal (Marotta et al., 2011a), we tested if p27 drives CSC expansion.

**Preliminary data:** In MCF12A, p27CK-DD transduction increased expression of CD44 and increased cells with surface CD44+CD24−/low (Fig 6.2A). p27CK-DD increased mammosphere formation (a proxy for stem cell function) in both MCF12A (Fig 6.2B) and in MCF-7 breast cancer cells (not shown). p27CK-DD transduction in MCF12A also increased expression of embryonic stem cell transcription factor (ES-TF) NANOG, a major driver of ES self-renewal (Fig 6.2D). Notably, while in normal ES cells, p27 co-represses SOX2 with p130, E2F4, SIN3A (Li et al., 2012b), in MDA-MB-231 (231), the phosphomimetic p27CK-DD increased sphere formation (Fig 6.2C) and caused induction of SOX2, NANOG and MYC, key drivers of ES and CSC self-renewal (Fig 6.2E).
Conversely, loss of p27pTpT through p27 knockdown in highly metastatic 4175 reduced sphere formation (Fig 6.2C), and decreased SOX2, NANOG and MYC expression (Fig 6.2F). Notably, in 4175shp27, transduction of STAT3-CA only partially rescued SOX2 expression but did not affect that of NANOG or MYC (Fig 6.2F). Thus, these data suggest that p27pTpT drives ES-TF expression to up-regulate CSC self-renewal in part through p27pTpT activation of STAT3, although other mediators are likely involved. We hypothesize thatp27pTpT drives STAT3-dependent and STAT3-independent programs to promote stem cell self renewal.

Proposed experiments:

To test the hypothesis that deregulated p27 regulates cancer stem cell properties, future research will be directed to validate the effects of p27CK-DD to up-regulate stem

![Image of Figure 6.2](image_url)

**Figure 6.2. p27 increases a CSC phenotype.** In MCF12A cells, p27CK-DD increases CD44+/CD24low marker expression (A) and sphere formation (B). In 231, p27CK-DD up-regulates sphere formation and p27 knockdown in 4175 decreases sphere formation (C). p27CK-DD increases NANO expression levels in MCF12A (D) and SOX2, NANO, and MYC mRNA levels in 231 (E) by qPCR. In 4175, p27 knockdown decreases SOX2, NANO, and MYC mRNA levels, and SOX2 expression is partially rescued by STAT3-CA (F).
cell markers (CD44+CD24−/low), stem cell transcription factors (SOX2, NANO, MYC), and mammosphere formation in additional cell lines, and to test if tumor initiation ability, assayed in vivo using limiting dilution tumor formation assays, is dependent of p27pTpT. 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.

**Test the Effect of p27 on CSC Markers, ES-TFs, and Mammosphere Formation**

Our preliminary data indicate that p27CK-DD markedly increases the proportion of stem cells with surface marker CD44+CD24−/low in non-tumorigenic MCF12A cells (Fig. 6.2A) and that MDA-MB-231 cells have fewer sphere forming cells compared to the highly PI3K activated 4175 cells with high p27pTpT (Fig. 6.2C). We will further test whether p27CK-DD transduction can increase the proportion of CD44+CD24−/low cells in additional, tumorigenic 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 proportions of CD44+CD24−/low and ALDH+ cells.

Preliminary data also indicate that the ES-TFs, SOX2, NANO, and MYC are regulated in a p27-dependent manner in 231 and 4175 cells (Fig. 6.2E and F), and that p27CK-DD drives NANO expression in MCF12A cells (Fig. 6.2C). We will further assay the expression of these transcription factors in additional cell lines and assess their dependence on p27pTpT expression. Furthermore, we will knockdown these transcription factors to test their functional contribution to p27CK-DD-mediated stem cell regulation. Our previous data show that over-expression of p27CK-DD significantly increases the formation of mammospheres in MCF12A cells (Fig. 6.2B). We will further test whether
loss of p27 in p27pT157/pT198-enriched metastatic cells will decrease their self-renewal ability to form mammospheres.

**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 siRNA-mediated loss of p27 will result in a decrease of mammosphere formation and tumor initiation ability.

**Test the Effect of p27 on Tumor Initiation in Human Mammary Epithelial Cells and Cancer Stem Cell Frequency**

Accumulating evidence suggests that CSCs are responsible for tumor initiation. Our previous data in MCF12A cells suggest that p27CK-DD induces transformation, as its expression causes formation of transformed foci in 2D culture and mediates clonogenic growth in soft agar (not shown). However, whether C-terminally phosphorylated, deregulated p27 contributes to initial tumor formations remains unknown. To test this, MCF12A-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.
A 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 tumors at higher frequency and initiate tumors from fewer cells when injected into immunodeficient mice. The effects of deregulated p27 on breast cancer stem cell frequency will be tested in both p27CK-DD-over-expressing cancer cells and following p27 knockdown in p27pT157/pT198-enriched cells. We speculate that p27CK-DD will increase, while p27 knockdown will decrease the abundance of cells with tumor initiating ability in limiting dilution xenograft assay, supporting the notion that deregulated p27 regulates CSC properties.

6.4 CONCLUDING REMARKS

The cell cycle regulator 27 is frequently deregulated in human cancers. Phosphorylation at T157 or T198 by different PI3K effector kinases allows p27 to associate with new binding partners and promote oncogenic transformation. While it is well appreciated that C-terminally phosphorylated p27 has a cell-cycle independent role to regulate cell migration, the underlying mechanisms whereby PI3K-deregulated p27 modulates an invasive and metastatic phenotype remain poorly understood. My thesis work demonstrated that targeted inhibition of PI3K/mTOR by a dual catalytic-site inhibitor impairs tumor cell motility in vitro and metastatic dissemination in vivo. We identified p27pT157pT198 as a critical driver of PI3K/mTOR-dependent tumor cell invasion in vitro and metastasis in vivo. Furthermore, we uncovered a novel, oncogenic function of p27 to promote invasion and metastasis by up-regulating an EMT program that involves STAT3-mediated TWIST1 up-regulation. My work also provided initial evidence that c-Jun
mediates $TGF-\beta 2$ induction, and c-Jun/SMAD3 may induce $SNAI1$. Our data suggest that p27, when C-terminally phosphorylated and act in the cytoplasm as a scaffold to promote recruitment and activation of oncogenic transcription factors that are critical EMT drivers.

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 and/or TGF-β2 signaling with AKT inhibition in PI3K/mTOR activated, p27pT157/pT198-enriched human cancers may ultimately offer therapeutic potential to limit p27-mediated EMT and cancer metastasis.
REFERENCES


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