Exploring Mechanisms Linking Signal Transduction and Cancer Stem Cell Expansion

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UNIVERSITY OF MIAMI

EXPLORING MECHANISMS LINKING SIGNAL TRANSDUCTION AND CANCER STEM CELL EXPANSION

By
Kibeom Jang

A DISSERTATION

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

Coral Gables, Florida

May 2017
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

EXPLORING MECHANISMS LINKING SIGNAL
TRANSDUCTION AND CANCER STEM CELL EXPANSION

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Increasing evidence indicates that cancer stem cells (CSCs) comprise a distinct self-renewing sub-population that can generate cancerous progeny with reduced replicative potency. CSCs are important therapeutic targets: they may not only initiate tumors but also mediate recurrence and metastasis. Most anti-cancer drugs kill the bulk cancer population. CSCs either proliferate too slowly for targeting by cycle active drugs, or escape chemotherapy by drug expulsion or greater DNA repair, leading to recurrence. The work in this thesis was undertaken to identify novel mechanisms leading to CSCs expansion.

My first project identified a novel mechanism whereby vascular endothelial growth factor A (VEGFA) promotes CSC expansion. VEGFA is widely known as an angiogenic factor. Here, we investigated the role of VEGFA as an ovarian cancer stem cell factor and mechanisms underlying its actions. We showed VEGFA mediated cancer stem cell actions via VEGFR2-dependent Src activation to upregulate Bmi1 and ALDH1 activity in primary human OVCA culture and OVCA lines. VEGFA stimulated sphere formation only in the ALDH1+ subpopulation, and increased OVCA-initiating cells and tumor formation in vivo through Bmi1. DNA methyl transferase 3A (DNMT3A) played a pro-oncogenic role downstream of Src to methylate miR-128-2, upregulating Bmi1 to increase stem like cells. DNMT3A knockdown prevented VEGFA-driven miR-128-2 loss, and the increase in
Bmi1 and tumor spheres. Analysis of over 1,300 primary human OVCAs revealed an aggressive subset in which high VEGFA is associated with miR-128-2 loss.

The second CSC pathway identified is C-terminally phosphorylated p27 dependent. p27 is known as a cell cycle inhibitor and a tumor suppressor. However, mis-regulated p27 has oncogenic effects. Previously, our group showed that double phosphomimetic p27 (p27CK-T157D/T198D) induced epithelial-mesenchymal transition (EMT) and metastatic potential of cancer cell lines. We also observed that C-terminally phosphorylated p27 increased CSC properties, including sphere formation, CSC markers and colony formation in soft agar in vitro. Transduction of a mutant p27 that cannot bind cyclin-CDKs and that bears phophomimetic mutations converting T157 and T198 to D (p27CK-DD) increased the expression of several embryonic stem cell transcription factors. These are known to drive embryonic stem cell self-renewal and to promote CSC expansion. A human phospho-kinase array showed that Pyk2 is activated by p27CK-DD. We found that Pyk2 activation and its binding to p27 are dependent on phosphorylation of p27 at T198 and T157. Treatment with a Pyk2 inhibitor, and PYK2-knockdown by siRNA, CRISPR or ShRNA revealed that Pyk2 is a key mediator of the increase in tumor spheres, ALDH1 activity and ES-TFs expression and of the increased abundance of tumor initiating stem cells in vivo in cancer cells expressing abundant C-terminally phosphorylated p27. These data reveal a novel mechanism whereby p27-driven Pyk2 activation promotes CSC expansion and tumor progression.
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### ABBREVIATIONS

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<th>Description</th>
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<tbody>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>Bmi1</td>
<td>B-cell specific moloney murine leukemia virus integration region 1</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ESA</td>
<td>Epithelial specific antigen</td>
</tr>
<tr>
<td>ES-TFs</td>
<td>Embryonic stem cell transcription factors</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>KIP</td>
<td>Kinase inhibitor protein</td>
</tr>
<tr>
<td>LSC</td>
<td>Leukemia stem cell</td>
</tr>
<tr>
<td>MaSC</td>
<td>Mouse mammary stem cell</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Non-obese diabetic/severe combined immunodeficiency</td>
</tr>
<tr>
<td>PRC1</td>
<td>Polycomb repressive complex 1</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>Sox2</td>
<td>Sex determining region Y-box2</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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CHAPTER 1: INTRODUCTION
1.1 THE CANCER STEM CELL HYPOTHESIS

There is expanding evidence that various tumors are hierarchically organized and maintained by a special subpopulation of tumorigenic cancer stem cells (CSCs). Within this heterogeneity, CSC are capable of self-renewal, tumor initiation, and mediate recurrence, invasion and metastasis. (Clarke et al., 2006; Nguyen et al., 2012; Kreso et al., 2014).

This cancer stem cell concept may underlie several poorly understood clinical challenges including resistance to radiation and chemotherapy, and the tumor relapse. Cancer stem cells may be able to survive these therapies which target rapidly replicating cells. Cancer stem cells proliferate slowly, or escape chemotherapy by drug expulsion or greater DNA repair, leading to recurrence (Magee et al., 2012).

Strong evidence for the cancer stem cell hypothesis was first provided by Dick and colleagues in acute myeloid leukemia (Bonnet and Dick, 1997; Lapidot et al., 1994). More recently, cancer stem cells have been demonstrated in diverse solid tumors (Visvader and Lindeman, 2008).

1.1.1 NORMAL STEM CELLS

To understand the biology of cancer stem cells, it is useful to review the unique characteristics of normal stem cells. Two important properties of normal stem cells are self-renewal ability and the ability to generate cells of multi-lineage differentiation. Stem cells divide by semi-conservative division that allows a stem cell to give rise to another identical stem cell and to differentiate to a daughter cell with reduced potential for self-renewal. Hematopoietic stem cells exist in a hierarchy with different ability of self-renewal.
Hematopoietic stem cells (HSCs) can be classified into three hierarchy: long-term self-renewing HSCs which produce short-term self-renewing HSCs which in turn give rise to multipotent progenitors that eventually form mature differentiated cells. Since HSCs develop from the long-term self-renewing pool to multipotent progenitors, they continuously lose their self-renewal ability creating more differentiated progeny (Reya et al., 2001).

The normal stem cells and cancer stem cells share the ability to self-renew and many pathways that regulate normal stem cell development have also been associated with cancer. The sonic hedgehog, Notch, and Wnt pathways and other key regulators include B lymphoma Mo-MLV insertion region 1 homolog (Bmi1), phosphatase and tensin homolog (PTEN) and p53 have been shown to play roles in both normal and malignant stem cell self-renewal (Molofsky et al., 2004; Molofsky et al., 2005). Although normal stem cells are able to self-renew, most normal stem cell populations are quiescent. They are capable to proliferate but mainly stay in a state of G0-like cell cycle arrest, until tissue damage or cell loss leads them into cell cycle to initiate tissue regeneration (Boyer and Cheng, 2008). Additionally, stem cells have an increased ability to export antineoplastic drugs and have increased DNA repair and anti-apoptotic abilities.

In the typical development of the mouse and human mammary gland, similar to that in the hematopoietic system, there are rare populations of stem/progenitor cells that show different degree of commitment with a hierarchy of stem and progenitor cells. The long-term self-renewing stem cells mediate interactions between these basal cells and the surrounding matrix through high levels of integrin expression. Experimental data also support that the short-term self-renewing stem cells exist downstream of the long-term self-
renewing stem cell (Visvader and Lindeman, 2006). As they become further committed, these become transit-amplifying progenitors that express bipotential markers which then differentiate into mature luminal or myoepithelial cells (Woodward et al., 2005).

Although it has not yet been possible to precisely characterize human mammary stem cells, mouse mammary stem cells have been described. A clonal analysis of epithelial cells first demonstrated that a single cell was able to reconstitute the entire mammary epithelium (Kordon and Smith, 1998). Microscopic single cell tracking implantation indicated that one out of 18 cells in the basal population have the capacity to regenerate an entire mammary gland (Shackleton et al., 2006). These cells have a CD49fhiCD29hiCD24+modSca1low phenotype (Shackleton et al., 2006; Stingl et al., 2006; Sleeman et al., 2007; Shehata et al., 2012). In the mouse mammary stem cells (MaSC) give rise to basal stem cells (BaSC) and luminal stem cells, (LuSc) which in turn generate committed luminal progenitors (Visvader and Lindeman, 2012a). A similar stem cell hierarchy is thought to exist in the human breast but is yet to be fully characterized.

Slug and Sox9 act as key determinants of the mammary SC state. Inhibition of either Slug or Sox9 prevents mammary SC activity in mouse primary mammary epithelial cells. Conversely, transient co-expression of exogenous Slug and Sox9 converts differentiated luminal cells into mammary SCs with long-term mammary gland-reconstituting ability (Guo et al., 2012). Since mice lacking the basal-restricted p63 transcription factor (p63-/-mice) lack rudimentary mammary glands, p63 has been identified as a master regulator of stem cells (Mills et al., 1999). The Stat3 transcription factor is required for mammary SC self-renewal. Deletion of Stat3 from basal cells results in reduced primary transplantation capacity and attenuated duct generation in mice
(Staniszewska et al., 2012). On the other hand, the tumor suppressor p53 play a critical role to restrict the self-renewal of mammary SCs regulating their asymmetric cell division (Cicalese et al., 2009; Chiche et al., 2013). It is not clear whether human breast cancers arise from mammary stem cells or from more differentiated progenitors that undergo genetic and epigenetic changes leading to carcinogenesis.

1.1.2 STOCHASTIC AND CANCER STEM CELL MODEL

Intense research in cancer biology aims to understand the cellular mechanisms underlying tumor heterogeneity. Various tumors have been shown to contain cells that exhibit different proliferation and differentiation abilities. Two models have been put forward to account for the heterogeneity present in solid tumors-the Stochastic/Clonal evolution model (Nowell, 1986) and the Cancer stem cell model (Clarke et al., 2006).

![Two models for tumor heterogeneity and propagation](image)

The stochastic model states that all cells in a tumor can proliferate and have similar capacity to form new tumors. The variable activities of tumor cells are only partially
determined by the environment in which the cells are found, but rather are determined by some stochastically varying intrinsic factors. The stochastic model depends on the premise that cancer is a disease defined by hyper-proliferation and sequential obtaining of genetic mutations in cell cycle genes that contribute to subsequent clonal expansions in an otherwise relatively quiescent normal adult somatic cell.

Several tumor types appear to adhere to the stochastic model, such as colorectal cancers and B cell lymphoblastic leukemia. However, this model spotlights on hereditary heterogeneity without considering that individual cells within genetically homogeneous sub-clones might still exhibit phenotypic variations because of various microenvironmental cues and in this way may not represent the heterogeneity in tumor initiation ability.

Early experimental assay observations in both liquid and solid tumors demonstrated that not all tumor cells have equal tumorigenic potential. Typically, under 1% of primary tumor cells can be cultured *in vitro* and even fewer cells can give rise to colonies in soft agar. Only very few cells can form tumors when transplanted into *in vivo* condition. These observations prompted to the idea that there may be a hierarchy in tumors were only a sub-population of cells within a tumor, called tumor-initiating stem cells or cancer stem cells is able to self-renew and to proliferate to give rise to a new tumor at a different site (Hamburger and Slamon, 1977). Thus, the cancer stem cell concept (Figure. 1.1B) suggests a hierarchical population of cells within the tumor, in which the CSC undergoes asymmetric division to give rise to a daughter CSC and a cell of limited proliferation. Both the stochastic and CSC tumor propagation models may have significance to human cancer but only the CSC model is hierarchical. It is important that the clonal evolution and stem
cell models are to some degree related. In the stem cell populations, clonal evolution may generate stem cells to increase invasion, metastasis and avoidance cancer therapies. (Visvader and Lindeman, 2008; Magee et al., 2012). CSCs themselves may give rise to progeny with more malignant potential by genomic instability and acquire genetic or epigenetic changes. If a mutation confers more aggressive self-renewal or growth, a more dominant daughter CSC can emerge (Barabe et al., 2007).

1.1.3 EVIDENCE FROM LEUKEMIA SUPPORTING MALIGNANT STEM CELLS

Evidence supporting a hierarchical model of cancer stem cells originated from leukemia. Acute myeloid leukemia (AML) is organized as a hierarchy of distinct, functionally heterogeneous classes of cells that are sustained by a small number of leukemia stem cells (LSCs). LSC can initiate human AML in NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice that have CD34+/CD38- phenotype (Lapidot et al., 1994) similar to normal HSCs in many respects (Bonnet and Dick, 1997). Disruption of pathways regulating self-renewal and differentiation through the acquisition of transforming mutations generates LSCs capable of sustaining growth of the leukemic clone in vivo. Moreover, leukemic transformation may occur in committed progenitor cells, and not only in the most primitive hematopoietic cells. Mouse experiments have shown that expression of transgenes in restricted progenitor populations can cause leukemia that is similar to chronic myeloid leukemia (Lagasse and Weissman, 1994). Therefore, leukemic stem cells have revealed heterogeneity in their potential to repopulate secondary
and tertiary recipients, indicating the presence of distinct classes of LSC with differing self-renewal capacity, like what is found in the normal HSC (Wang and Dick, 2005).

Cancer stem cells may arise from cell types other than normal stem cells. Differentiated cells may also give rise to cancer stem cells through a de-differentiation or a trans-differentiation process (Singh and Settleman, 2010). Weinberg and colleagues showed that differentiated populations of normal human breast cells can be made to undergo Epithelial to Mesenchymal transition (EMT) following sustained expression of the transcription factors Snail or Twist and yielding populations enriched for cells with cancer stem cells characteristics (Mani et al., 2008).

1.1.4 SOLID TUMOR STEM CELLS

Similar to what has been described in leukemia, multiple models suggest that human breast cancers are also generated from stem/progenitor populations that exhibit both self-renewal and different degrees of lineage commitment (Polyak, 2007; Visvader and Lindeman, 2008). Breast cancer stem cells were reported by Clarke and colleagues in 2003 (Al Hajj et al., 2003). The subpopulation of primary human breast cancer cells, that expressed CD44+ CD24neg/low epithelial specific antigen (ESA)+ surface markers and had very high capacity to form tumors following injection into NOD/SCID mice. While ten thousands of the CD44neg CD24+ cells did not form tumors, one hundred CD44+ CD24neg/low ESA+ cells were able to. After this study, cancer stem cell properties have been characterized in various types of human tumors, including brain tumors (Singh et al., 2004), colon cancer (Ricci-Vitiani et al., 2007), prostate cancer (Collins et al., 2005), head
and neck cancer (Prince et al., 2007), melanoma (Fang et al., 2005), pancreatic cancer (Li et al., 2007) and lung cancer (Eramo et al., 2008).

1.2 PATHWAYS GOVERNING CANCER STEM CELL BIOLOGY

Since both normal tissue and cancer stem cells must experience self-renewal, it has been hypothesized that they may share some molecular mechanisms that regulate this stem cell function. Multiple crucial pathways have been identified that govern self-renewal in both cancer stem cells and their normal counterparts.

1.2.1 BMI1

Since work of my thesis identified Bmi1 as a mediator of VEGFA-driven action to increase ovarian cancer stem cells, Bmi1 is briefly introduced here. The following and shown that. B-cell specific moloney murine leukemia virus integration region 1 (Bmi1) is a major component of the polycomb repressive complex 1 (PRC1) (Raaphorst et al., 2003). The PRC1 is made up of the polycomb group (PcG) proteins and serves to mediate transcriptional repression of target genes many of which direct differentiation (Mills, 2010). Bmi1 is a key mediator of self-renewal of leukemic and neural stem cells (Lessard and Sauvageau, 2003; Park et al., 2004). Bmi1 is expressed highly in primitive hematopoietic stem cells (HSCs) and is reduced when HSCs undergo differentiation (Hosen et al., 2007).

Bmi1 has been shown to play a role to maintain not only normal stem cells but also cancer stem cells, including ovarian cancer (Zhang et al., 2008a), breast cancer (Korkaya et al., 2011), and AML (Lessard and Sauvageau, 2003). In different models systems, cancer
stem cells have been shown to have greater chemo- and radio-therapy resistance (Vinogradov and Wei, 2012). In laryngeal squamous cell carcinoma (SCC), Bmi1 knock-down increased sensitivity to radiation treatment and to chemotherapy (Chen et al., 2011). Overexpression of Bmi1 led to cisplatin-resistance in osteosarcoma (Wu et al., 2011). Cisplatin resistant ovarian CSC populations show higher Bmi1 expression than in more differentiated tumor cells (Zhang et al., 2008a).

Bmi1 overexpression increased self-renewal in the immortalized human mammary epithelial cell line, HMLE, and in transformed NBLE mammary epithelial cells (Paranjape et al., 2014). Furthermore, Bmi1 downregulation in the self-renewing colorectal cancer cell population reduced colorectal tumor initiating stem cells \textit{in vivo} (Kreso et al., 2014).

Due to these Bmi1 effects on tumor progression, Bmi1 expression has been investigated as a prognostic marker in cervical (Tong et al., 2011; Tong et al., 2012), gastric (Liu et al., 2008), bladder cancer (Qin et al., 2009) and esophageal squamous cell carcinoma (Liu et al., 2010)

\subsection{1.2.2 DNMT3A}

Gene expression is importantly regulated by cytosine methylation. Since my thesis work of Chapter3 revealed a novel pro-oncogenic role for DNA methyltransferase (DNMT) 3A in ovarian cancer, the role of DNA methylation and DNMTs is briefly introduced here. The DNMT1 and the de novo methyltransferases DNMT3A and DNMT3B are required for mammalian development (Jin and Robertson, 2013). DNMT1 regulates basal DNA methylation while DNMT3A/DNMT3B mediate new methylation in response to various signals (Okano et al., 1999; Okano et al., 1998). The expression of
tumor suppressor genes is frequently decreased by DNA hypermethylation in cancer (Feinberg and Tycko, 2004).

DNMT3A has been shown to play either tumor suppressor or tumor promoting roles in various human malignancies. In leukemia, DNMT3A was identified as a tumor suppressor, since mutational inactivation of DNMT3A appears to drive progression of certain types of AML. DNMT3A null-mutations have been associated with poor outcome in AML (Thol et al., 2011) and can drive leukemic progenitor expansion (Fernandez et al., 2012). In contrast, in melanoma, DNMT3A is pro-oncogenic. Knockdown of DNMT3A inhibited tumor growth by increasing tumor suppressor PTEN expression, and it was shown that DNMT3A mediated PTEN promoter methylation (Deng et al., 2009). Recent work indicates that DNMTs regulate gene expression not only through DNA methylation, but also through methylation of microRNAs (miRNAs) (Suzuki et al., 2012a). Saito et al. showed that the expression of 17 out of 313 human mi-RNAs was increased at least 3 folds by the methylation inhibitor 5’azacytidine. Interestingly, tumor suppressing miRNAs have been shown to be downregulated by DNA methylation in cancers (Croce, 2009). miR-9, miR-34, and miR-200 families that play roles to oppose malignant progression are downregulated through DNA methylation (Suzuki et al., 2012a).

1.2.3 ROLE OF EMBRYONIC TRANSCRIPTION FACTORS IN CANCER STEM CELL EXPANSION

Several embryonic stem (ES) cell transcription factors (ES-TFs) have been demonstrated to stimulate and maintain ES self-renewal and to induce pluripotency. Expression of Nanog, Oct4, Klf4 and Sox2 is increased in poorly differentiated breast
cancers (Ben-Porath et al., 2008) and it has been demonstrated that ES-TFs also drive CSC (Li, 2010). The activation of ES-like transcriptional programs in diverse human epithelial cancers is a predictor of metastasis and death in the comparison of the transcriptional programs in ES cells between adult normal stem cells and human cancers (Wong et al., 2008; Rosen and Jordan, 2009). The oncogene c-Myc, but not Src, β-catenin, E2F3, and Ras, has been shown to be sufficient to activate this ES-like program and to increase the fraction of CSCs (Wong et al., 2008).

Since key ES-TFs, Oct4, Sox2, Nanog, Klf4, and c-Myc, closely cooperate to maintain pluripotency and self-renewal and are relevant to my thesis work, these will be briefly introduced below.

![Schematic representation of embryonic stem cell transcription factors interaction.](figure_1.2.png)
1.2.4 SOX2

Sex determining region Y-box2 (Sox2) is a member of the Sox family that consists of transcription factors with a single high-mobility group box DNA-binding domain and is part of the SOXB1 subgroup (Kamachi et al., 2000). Sox2 is expressed in the inner cell mass (ICM) and extraembryonic ectoderm of pre-implantation blastocysts (Avilion et al., 2003). Sox2 knock-out blastocysts could not form a pluripotent ICM. Besides, Sox2-knock-out mESCs differentiated primarily into trophectoderm, while the Oct4 overexpression recovered the pluripotency of Sox2-null mESCs (Masui et al., 2007). As a result, Sox2 is crucial for the maintenance of Oct4 expression and therefore the stem cell properties. Furthermore, Masui et al identified a synergistic characteristic of Sox2 and Oct4 for the activation of Sox-Oct enhancers, leading to the regulation of a variety of pluripotency genes, such as Sox2, Oct4 and Nanog (Masui et al., 2007).

Many studies have shown that re-expression of Sox2 plays a role in oncogenesis and particularly in CSCs. Sox2 is crucial for osteosarcoma cell self-renewal. Furthermore, it is implicated in the promotion of cell migration and invasion in ovarian cancer, through regulating fibronectin 1 (Lou et al., 2013). Studies in gastric cancer showed that inhibition of Sox2 effects in decrease of spheres formation and in expansion of apoptotic sphere cells (Lou et al., 2013). The contribution of Sox2 in pancreatic CSCs was suggested by the observation that it upregulates CSC properties (ALDH1, ESA and CD44) through the regulation of genes of G1/S transition and EMT (Herreros-Villanueva et al., 2013). In prostate CSCs, the inhibition of EGFR signaling led to the reduction of Sox2 expression and self-renewal of prostate CSCs. Moreover, knockdown of Sox2 reduces the capacity of prostate CSCs to develop in anchorage-independent conditions (Rybak and Tang, 2013).
1.2.5 NANOG

Nanog is a member of the pluripotency network in undifferentiated ESCs (Chambers et al., 2007). It is a homeodomain containing transcription factor that was found by a functional screening for pluripotency factors which allowed the maintenance of ESC (Mitsui et al., 2003). Oct4/Sox2 heterodimers bind the Nanog promoter and regulate Nanog expression in ESCs (Kuroda et al., 2005).

Nanog has been found to be overexpressed in various types of cancer, such as breast (Lu et al., 2014), ovarian (Amsterdam et al., 2013), kidney (Bussolati et al., 2008), and brain (Guo et al., 2011). Specifically, high expression of Nanog is correlated with a poor prognosis for ovarian serous carcinoma, colorectal, and breast cancer (Lee et al., 2012; Meng et al., 2010; Nagata et al., 2014). Several studies have indicated that cancer cells expressing stem cell surface markers have higher Nanog expression (Wang et al., 2013). CD133neg or CD44neg cancer cells express lower Nanog expression than CD133+ or CD44+ cells, respectively (He et al., 2012; Leung et al., 2010). In addition, Nanog confers a sub-population of colorectal cancer cells to acquire a stem-cell like phenotype (Wang et al., 2013).

1.2.6 KLF4

KLF4 is a critical transcription factor for reprogramming. Klf4 is a member of the Kruppel-like transcription factor family and has a crucial role in cell cycle regulation, somatic cell reprogramming and pluripotency (Takahashi and Yamanaka, 2006; Bourillot and Savatier, 2010). Klf4 expression is increased in mESCs and its expression is reduced during differentiation (Ivanova et al., 2006). The inhibition of Klf4 results in the
differentiation of ESCs, while Klf4 overexpression induces the Oct4 expression and self-renewal (Li et al., 2005).

Klf4 plays an important role in maintaining CSC populations. Klf4 is critical in maintaining breast CSCs and inducing cell migration and invasion (Wong et al., 2010). Klf4 expression is high in CSC populations in mouse primary mammary tumor and human breast cancer cell lines. The inhibition of Klf4 in the breast cancer cell line, MDA-MB-231 and MCF-7, results in reduction of the breast CSCs self-renewal and tumor formation in vivo (Yu et al., 2012).

1.2.7 C-MYC

c-Myc is included in the Myc family with other members such as N-Myc and L-Myc. c-Myc is an key regulator of cell growth, proliferation and differentiation. Importantly, it is crucial for stem cell pluripotency and proliferation (Cole and Henriksson, 2006; Meyer et al., 2006). Genome-wide analyses have demonstrated that Myc binds to and may regulate the transcription of at least 8,000 genes in ESCs leading to ESC pluripotency (Chen et al., 2008a; Sridharan et al., 2009). c-Myc regulates important oncogenes including KRAS and AKT, and tumor suppressors PTEN and p53 (Zheng et al., 2008; Ho et al., 2012). In glioma CSCs, decreasing Myc causes a decrease in neurospheres in vitro and in brain tumor formation in immunodeficient mice (Wang et al., 2008).
1.3 VASCULAR ENDOTHELIAL GROWTH FACTOR

Since my first thesis project identified a new role for vascular endothelial growth factor (VEGF) in ovarian cancer progression, the role of VEGFA in cancer is briefly reviewed here. Of the three isoforms of VEGF (VEGFA, B and C), VEGFA is arguably the best studied and appears to be the major mediator of tumor angiogenesis. VEGFA is a cytokine that regulates vascular development during embryogenesis and the formation of new blood vessel from pre-existing vascular networks (Olsson et al., 2006; Leung et al., 1989; Tischer et al., 1989). There are three receptors, VEGFR1, VEGFR1, 2, and 3. Of these, both VEGFR1 and VEGFR2 are more commonly expressed in cancers and in their supporting vasculature, while VEGFR3 expression is limited to blood vascular and lymphatic endothelial cells (Petrova et al., 2008). VEGFR2 appears to be most critical for angiogenesis and as mediator of VEGFA signals in cancer (Olsson et al., 2006). VEGFA is secreted by cancer and stromal cells to stimulate endothelial cell invasion and new blood vessel formation (Hicklin and Ellis, 2005). Without new blood vessel formation, tumor size is restrained due to limited nutrient and oxygen supply. VEGFA is expressed in a variety of tumors and its overexpression is associated with poor prognosis and death from metastasis (Goel and Mercurio, 2013; Berns et al., 2003; Manders et al., 2002). VEGFA functions are not restricted to vasculogenesis and angiogenesis (Senger, 2010). Autocrine VEGFA cooperates with EGFR to drive tumor development (Lichtenberger et al., 2010) and VEGFA has also been shown to drive tumor metastasis (Hanahan and Folkman, 1996; Hicklin and Ellis, 2005; Mercurio et al., 2005). Indeed, patients with metastatic breast cancer have higher circulating VEGFA levels than those without metastasis (Adams et al., 2000).
1.3.1 VEGFA IN ANGIOGENESIS

VEGFA was first identified as an endothelial cell specific mitogen (KECK et al., 1989) (Kieran et al., 2012). The VEGFA signaling pathway is initiated by VEGFA binding to VEGF receptor 1 (VEGFR1) or VEGF receptor 2 (VEGFR2). VEGFA binding to the extracellular domain of the receptor leads to dimerization of the intracellular domains of VEGF receptor tyrosine kinases. The conformational changes caused by ligand binding induce auto-phosphorylation of tyrosine residues activating the downstream signaling pathways. VEGFA binds more favorably to VEGFR2 than to VEGFR1 leading to vasculogenesis and angiogenesis (Olsson et al., 2006).

The definition of angiogenesis is the formation of new blood vessels from pre-existing vasculature. It is an important physiological process in normal development, tissue repair and various diseases including cancer. While many angiogenic factors are involved in this process, VEGFA governs the angiogenesis from start to the end (Kieran et al., 2012). VEGFA mediates proliferation, sprouting, migration, tube formation of endothelial cells (Ferrara, 2002). VEGFA induced angiogenesis that affects wound healing, ovulation, menstruation and pregnancy. VEGFA expression and its secretion are critical steps in vasculogenesis and angiogenesis (Shweiki et al., 1993; Jakeman et al., 1993).

1.2.2 VEGF IN CANCER PROGRESSION

VEGF contributes to cancer progression by inducing angiogenesis and vasculogenesis, required for in tumor growth, invasion and metastasis (Senger, 2010). VEGF overexpression has been correlated with tumor progression with poor patient
outcome in leukemia, lymphoma, multiple myeloma, breast and ovarian cancer (Goel and Mercurio, 2013; Berns et al., 2003; Manders et al., 2002; Bellamy, 2001; Bellamy et al., 2001; Padro et al., 2002; Yu et al., 2013). Thus, VEGF is highly expressed in various solid malignant tumors and associates with cancer progression (Bellamy, 2001; Bellamy et al., 2001; Padro et al., 2002; Yu et al., 2013), but it also plays an important role in hematopoietic malignancies (Goel and Mercurio, 2013; Berns et al., 2003; Manders et al., 2002). In addition, patients with metastatic breast cancer have higher VEGF levels compared to patients with localized disease, suggesting VEGF may be a prognostic marker for higher risk for distant metastasis or recurrence (Adams et al., 2000). The first part of my thesis revealed a novel role for VEGFA as a mediator CSC expansion in ovarian cancer.

1.4 CELL CYCLE REGULATOR – p27

Since the second project of my thesis has identified a novel role for the CDK inhibitor p27 in cancer stem cell maintenance or expansion, the role of p27 is briefly reviewed here. Cell cycle progression is regulated by cyclin dependent kinases (CDKs) that are activated by cyclin binding (Morgan, 1995; Sherr, 1994) and inhibited by the CDK inhibitors (Reed et al., 1994; Sherr and Roberts, 1999). The CDKs govern cell cycle regulatory pathways that promote mitogenic and growth signals and coordinate cell-cycle transitions (Hartwell, 1992; Murray, 1992). The G1 into S phase transition is regulated by the activities of Cyclin D-, Cyclin E-, and Cyclin A-associated CDKs. Cyclin B-associated kinases regulate the G2/M transition.

Two CDK inhibitor families negatively regulate the cell cycle (Sherr and Roberts, 1995; Slingerland and Pagano, 2000). The inhibitor of CDK4 (INK4) family (p15INK4B,
p16INK4A, p18, and p19) binds CDK4 and CDK6 and inhibit cyclin D association. Kinase inhibitor protein (KIP) family members, p21\textsuperscript{CIP1}, p27\textsuperscript{Kip1}, and p57\textsuperscript{Kip2}, bind and inhibit cyclin-CDKs binding. p27 can also promote assemble and activation of D-type cyclin-CDK complexes (Cheng et al., 1999; LaBaer et al., 1997; Zhang et al., 1994; Larrea et al., 2009b).

### 1.4.1 REGULATION OF P27 BY PHOSPHORYLATION

![Figure 1.3: Schematic representation of p27.](image)

p27 is rarely deleted or mutated in the malignant tumor but it is mainly regulated by proteosomal degradation or phosphorylation. P27 synthesis is maximal in G0 and early G1 and falls with G1 progression (Chu et al., 2007a). p27 contains several phosphorylation sites. Serine 10 of p27 can be phosphorylated by human kinase interacting stathmin (hKis) (Boehm et al., 2002). p27 is bound to the exportin CRM1 in early G1, and the binding is increased with G1 progression (Connor et al., 2003). p27 contains a nuclear export signal (NES) which is related with p27– Chromosomal Maintenance 1 (CRM1)
binding, nuclear exportation, and p27 degradation (Connor et al., 2003). Active CRM1-regulated nuclear export of p27 contributes to cytoplasmic proteolysis of p27 in early G1 phase. The ubiquitin ligase Kip1 ubiquitylation-promoting complex (KPC) also ubiquitylates cytoplasmic p27 (Nakayama, 2006).

Tyrosine (Tyr) phosphorylation of p27 by Abelson murine leukemia viral oncogene homolog 1 (Abl) and Src family kinases reduces p27-CDK2 inhibition and transforms p27 from inhibitor to substrate of cyclin-CDK2 complexes. Tyrosine phosphorylation of p27 by Abl and Src family kinases drives the transition of p27 from inhibitor of cyclin E-CDK2 in G0 to substrate of cyclin–CDK2 in G1. Overexpression of ABL or SRC increases p27pT187 and decreases p27 levels and stability. SRC phosphorylates both Tyr88 and Tyr74, whereas ABL and LYN kinases phosphorylate Tyr88 in vitro. SRC activation and SRC-p27 binding leads to loss of p27 during G1 progression. SRC-phosphorylated p27 binds less stably to cyclin E–CDK2 potentially due to loss of the interaction of p27pY74 with sites on the N-lobe of CDK2. Although Tyr88 phosphorylation only weakly reduces p27 affinity for CDK2, NMR structure analysis showed that Tyr88 phosphorylated p27 is removed from the catalytic cleft of cyclin–CDK2 (Grimmler et al., 2007; Chu et al., 2007b), opening up the ATP binding pocket of the kinase. Therefore, phosphorylation of p27 at Tyr88 results in loss of CDK2 inhibition by p27 and permits associated cyclin E–CDK2 to phosphorylate p27 at Thr187 to activate SKP2-mediated p27 proteolysis during the G1–S transition (Grimmler et al., 2007; Chu et al., 2007b).

Akt mediated phosphorylation of p27 on Thr157 (Liang et al., 2002; Shin et al., 2005; Sekimoto et al., 2004) delays nuclear import of monomeric p27 and increased stability of the cytoplasmic p27 (Kozar et al., 2004). PI3K-mediated activation of Akt
(Fujita et al., 2002), p90RSK (Fujita et al., 2003), mammalian target of rapamycin (mTOR), and serum and glucocorticoid inducible kinase (SGK) contribute to p27 phosphorylation on Thr157 and Thr198 and allow p27-cyclin D-Cdk assembly in multiple cell types (Larrea et al., 2008). Notably, our group showed p27pT157/pT198 (p27pTpT) binds to JAK2/STAT3 leading to STAT3-dependent TWIST1 induction, and EMT to promote cancer cell migration and invasion (Zhao D. et al., 2015b).

1.4.2 TRANSCRIPTIONAL REGULATION OF p27

p27 is largely regulated in human cancers through protein synthesis and stability (Chu et al., 2007b). The importance of transcriptional regulation of p27 to human cancers is unclear. p27 mRNA and protein are highly expressed in normal quiescent T cells and decline rapidly after T cell activation (Kwon et al., 1996). p27 mRNA expression is increased by androgen depletion in breast cancer cells (Menjo et al., 1998), in normal prostate tissue, and in benign prostatic hyperplasia (Cordon-Cardo et al., 1998). In melanoma cells, p27 mRNA expression is increased by interleukin-6 (IL-6) activated signal transduction and activators of transcription-3 (STAT3) (Kortylewski et al., 1999). p27 is regulated transcriptionally by several transcription factors including Sp1, cAMP-response element, Myb, NFκB, and acute lymphocytic leukaemia-1 fused gene from chromosome X (AFX). AFX, forkhead transcription factor, can activate p27 transcription (Medema et al., 2000). p27 transcription can be decreased by when AFX is phosphorylated and inactivated by AKT (Slingerland and Pagano, 2000).
1.4.3 REGULATION OF p27 LOCALIZATION

p27 localization is cell cycle dependent: p27 is observed in the nucleus during G0 and early G1 phase and transiently localizes in the cytoplasm at the G1/S transition (Connor et al., 2003). The nuclear import of p27 is governed by two nuclear localization signals in the carboxyl-terminal region of the protein (Zeng et al., 2000). Interaction between p27 and the nuclear pore protein NPAP60 (Guan et al., 2000; Muller et al., 2000) mediates p27 nuclear import and might also regulate p27 export (Guan et al., 2000). p27 is exported from the nucleus in response to mitogen-stimulated p27 serine 10 is phosphorylation (Boehm et al., 2002; Connor et al., 2003; Rodier et al., 2001). Serine 10 of p27 can be phosphorylated by human kinase interacting statmin (hKis) (Boehm et al., 2002). A nuclear export signal (NES) in p27 mediates its binding to exportin CRM1 in early G1, nuclear export and degradation (Connor et al., 2003). Active CRM1-RanGTP-derived nuclear exportation of p27 contributes to cytoplasmic proteolysis of p27 in early G1 phase. p27 can also be degraded in a SPK2 dependent manner in the nucleus (Chu et al., 2007b).

1.4.4 p27 REGULATES CELL MIGRATION

In normal cells, nuclear 27 inhibits cyclin-CDKs and restrains the cell cycle. p27 is deregulated in almost all cancers. Many cancers show not only reduced nuclear 27 due to accelerated proteolysis, but also the presence of mislocalized cytoplasmic p27 (Slingerland and Pagano, 2000; Chu et al., 2008). Cytoplasmic p27 can promote cancer cell invasion in a manner that is independent of cyclin-CDK binding. Transduction of TAT-p27 protein increased Rac-dependent cell migration (Nagahara et al., 1998). p27 null mouse embryonic
fibroblasts (MEFs) show decreased motility compared to wild type MEFs, but transfection of either wild type p27 or a mutant p27 that cannot bind cyclin and CDKs (p27CK-) could rescue the reduced motility of p27 null MEFs (Besson et al., 2004). Thus, p27 induced cell motility is independent of its cyclin-CDK regulatory roles. p27 binds to RhoA in the cytoplasm to inhibit RhoA-ROCK, leading to loss of actomyosin stability and increased cell motility (Besson et al., 2004). In cancer, high cytoplasmic p27 promotes tumor invasion and metastasis in mouse models (Denicourt and Dowdy, 2004; Wu et al., 2006a) and increase glioma cell invasion (See et al., 2010). Our group recently showed that c-terminally phosphorylated p27 binds JAK2, to drive STAT3 activation and EMT through STAT3-mediated TWIST1 induction (Zhao D. et al., 2015b). In addition, p27CK- knock in into p27 null mice showed that p27CK- may have a stem cell actions to promote tumor formation: p27CK-knock-in mice showed increased lung epithelial progenitor cell self-renewal and lung tumor development (Besson et al., 2007).

1.4.5 p27 AS TRANSCRIPTIONAL REGULATOR

Accumulating evidence indicate that p27 can regulate gene expression by association with multiple transcription factors. Pippa et al. reported that p27 can act as a transcriptional co-repressor in a complex with p130, E2F4, HDAC1, SIN3A (Pippa et al., 2012). ChIP/CHIP assays revealed putative p27-repressed target genes targets that govern cell proliferation and tissue expansion (Pippa et al., 2012). Sox2 is a crucial embryonic stem cell (ES) transcription factor that maintains ES self-renewal (Marson et al., 2008) and also drives cancer stem cell expansion (Bass et al., 2009; Leis et al., 2011). p27 was shown to co-represses SOX2 via p27 binding with p130, E2F4 and SIN3A to the SOX2 regulatory
region 2 (SRR2), located 4Kb downstream of the SOX2 gene (Li et al., 2012). Therefore, p27 may play roles in transcriptional regulation to govern normal ES development or CSC self-renewal potential. The p27 target genes that may promote ES cell differentiation and how they may be de-regulated in cancer are yet unknown. This thesis has investigated further the potential role of p27 in cancer stem cell regulation. We postulated that C-terminal phosphorylation of p27 may lead to p27 binding to novel signaling partners that change its function as a transcriptional regulator. p27, when phosphorylated at T157 and T198 may acquire novel action as a transcriptional co-activator at key CSC-inducing genes to drive PI3K-dependent CSC expansion.

1.5 ROLE OF p27 IN DEVELOPMENT AND STEM CELL BIOLOGY

p27 knockout mice develop multi-organ hyperplasia and parathyroid tumors, suggesting that p27 controls both proliferation and differentiation (Nakayama et al., 1996; Kiyokawa et al., 1996; Fero et al., 1996). Although p27 knockout mice are not more prone to the development of de novo malignancies, allelic haplo-insufficiency for p27 is seen in many human tumors (Pietenpol et al., 1995; Kawamata et al., 1995; Ponce-Castaneda et al., 1995), and p27 haplo-insufficient mice form more radiation and chemical carcinogen-induced malignant tumors (Fero et al., 1998).

1.5.1 DIFFERENTIATION

p27 has been reported to regulate stem cell progenitor expansion and differentiation in many types of normal tissue in a way that may be independent of its cell cycle regulatory roles. p27 is critical for neuronal differentiation in Xenopus (Vernon et al., 2003).
et al., 2006) (Nguyen et al., 2006), and knockdown of Xic1, the p27 homologue in Xenopus, leads to decrease in muscle differentiation. While p27 effects on differentiation was initially thought to results from CDK inhibition and growth arrest, Xic1 is required before terminal muscle differentiation and cell cycle arrest, and may act through regulation of transcription. Xic1 increases activity of myogenic transcription factor, MyoD (Vernon and Philpott, 2003) resulting in myogenic commitment via 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 associates with p130 to promote endochondral ossification (Yeh et al., 2007).

1.5.2 STEM CELL BIOLOGY

p27 may regulates stem cell properties. It is notable that p27 knock-out animals showed multi-organ hyperplasia and hypertrophy and spontaneous neoplasia (reviewed in (Chu et al., 2008)), suggesting p27 acts to limit organ size in normal embryogenesis. In addition, in p27CK-knock-in animals, loss of CDK inhibition and a CDK-independent gain of p27 function appeared to stimulate normal stem cell homeostasis and caused expansion of progenitor/stem cells in several tissues, increased bronchoalveolar stem cells and spontaneous lung tumor formation (Besson et al., 2007). These results indicate that p27 may play an important role to regulate stem expansion, cell cycle-independently (Besson et al., 2007). My thesis work has identified a novel role for C-terminally phosphorylated p27pT157pT198 to promote cancer stem cell expansion (see Chapter 4 below).
1.6 PYK2 SIGNALING IN CANCER

In my thesis work, I identified Pyk2 is activated by C-terminally phosphorylated p27 and mediates the upregulation of cancer stem cells by p27. The following provides a brief introduction to Pyk2. Proline-rich tyrosine kinase 2 (Pyk2) is a non-receptor tyrosine kinase that is a member of the focal adhesion kinase (FAK) family (Behmoaram et al., 2008). The non-catalytic regions of Pyk2 consists of proline-rich residues which is the binding motifs for Src homology (SH) 3 domain-containing proteins including Crk-associated substrate, Pleckstrin and Arf-GAP proteins (Behmoaram et al., 2008). In addition, Pyk2 contains several tyrosine phosphorylation sites, including an autophosphorylation site (Y402), which acts as a binding site for the SH2 site of Src tyrosine kinases (Behmoaram et al., 2008).

Pyk2 has been shown to play a major role to regulate cell adhesion, motility, and invasion in various normal cell types and cancer models (Schaller, 2010). Pyk2 knock-out female mice exhibit a skeletal phenotype of higher bone mass and mineral density, suggesting Pyk2 normally acts to restrain bone differentiation (Schaller, 2010). Pyk2 controls cell polarization; Pyk2-null macrophages are not able to establish a polarized morphology in response to a chemotactic stimulus. Pyk2 also regulates macrophage motility; Pyk2-null macrophages show defective migration. (Okigaki et al., 2003) In macrophages, inhibition of Pyk2 expression prevents colony-stimulating factor-1 (CSF-1)-induced invasion (Owen et al., 2007). Pyk2-null fibroblasts and macrophages also show defective to chemotactic tail retraction, indicating Pyk2 appears to control migration and polarity. (Okigaki et al., 2003; Lim et al., 2008).
Pyk2 is highly expressed in the cytoplasm of brain cells, fibroblasts, platelets and other hematopoietic cells. Pyk2 can localize to the nucleus under suitable conditions in diverse cell types (Aoto et al., 2002; Schindler et al., 2007; Aoto et al., 2002; Genua et al., 2012). Pyk2 has been shown to translocate to the nucleus, suggesting that Pyk2 may regulate nuclear processes such as transcription.

Increased expression of Pyk2 was found in pulmonary metastatic nodules of FAK knock-out mice, suggesting Pyk2 may compensate for FAK loss (Fan and Guan, 2011).

Pyk2 may have a role to regulate mammary stem cells. Pyk2 upregulation was observed in mammary CSCs not in bulk mammary tumor cells in the mammary glands of FAK null mice (Fan and Guan, 2011). Pyk2 is overexpressed in tissues from early and advanced breast cancers (IHC) and is overexpressed with both FAK and epidermal growth factor receptor-2 (ErbB-2) in a subset of breast cancer cases. Inhibition of Pyk2 decreased mammary tumor development and metastasis (Behmoaram et al., 2008). Pyk2 inhibition in FAK-null mammary CSCs decreased in vitro tumor sphere formation and migration, and in vivo self-renewal, tumorigenecity and metastasis suggesting that Pyk2 plays a role to sustain mammary CSC self-renewal to compensate for loss of FAK. To date, there has not been demonstrated that Pyk2 governs mammary CSC in cells with normal function of FAK. Thus, Pyk2 induces breast cancer cell tumorigenecity and invasiveness (Behmoaram et al., 2008).

Pyk2 is highly expressed in neuroglioma, breast cancer and hepatocellular carcinoma (Schaller, 2010). Pyk2 mRNA, protein and pPyk2 (Y881) were higher in lung cancer than noncancerous tissues. Pyk2 and pPyk2 were highly expressed in 54.7% and 60.2% cases non-small cell lung cancer. In non-small cell lung cancer (NSCLC) study,
Pyk2 activates ERK1/2 to promote the progression of NSCLC. Pyk2 activation increased expression of ALDH1a1, ABCG2 and Bmi1. Pyk2 increased the number of colony formation in soft agar assay in A549 and H460 cells. FAK KO did not lead to Pyk2 compensation in FAK KO mice in NSCLC model (Kuang et al., 2013; Pylayeva et al., 2009).

In hematopoetic stem cells, FAK upregulates expression of the Wnt ligand, Frizzled-4, and phosphorylates Pyk2 to promote binding of Pyk2 to the Wnt5α/Frizzled-4/LRP5 endocytosis complex, leading to downstream activation of β-catenin. In hepatocellular carcinoma (HCC), Pyk2 mediates HCC progression and invasion. Pyk2 induces the activation of PI3K/AKT pathway to upregulate VEGF expression in HL-7702, SMMC-7721 and HepG2 cells. Clinically, high Pyk2 is associated with poor survival of hepatocellular carcinoma via regulation PI3K/AKT pathway (Despeaux et al., 2012; Cao et al., 2013). In glioma, Pyk2 plays an important role of signaling effectors. Pyk2 regulation may be determining factors in the temporal development of proliferation or migration. Pyk2 mediates heregulin/HER3 induced glioma migration and invasion (Lipinski et al., 2005).

Pyk2 is also involved in multiple signaling pathways related to cancer. Pyk2 induces epidermal growth factor (EGF) and c-Src-induced signal transducer and activator of transcription 3 (Stat3) activation (Shi and Kehrl, 2004a). In Jak-mediated IL-2 signaling pathway, Pyk2 is a downstream mediator of Jak. Pyk2 mediates the Jak-dependent activation of MAPK and Stat1 in IFN-γ, but not IFN-α. Cytoplasmic Pyk2 is also an effector of fibroblast growth factor receptor 3 (FGFR3) activation (Takaoka et al., 1999). In medulloblastoma, c-MET induces Pyk2 phosphorylation (Guessous et al., 2012). In
acute myeloid leukemia, expression of FAK induces Pyk2 phosphorylation (Recher et al., 2004).

Pyk2 can make complexes with several proteins to promote activation of itself or to activate the binding molecules. A literature search identified 50 different proteins that associate with Pyk2.

Table 1.1: Pyk2 binding proteins

<table>
<thead>
<tr>
<th>PYK2 Binding Protein</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>Integrin Signaling</strong></td>
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<tr>
<td>Integrin αVβ3</td>
<td>(Elsegood et al., 2006)</td>
</tr>
<tr>
<td>β1-integrins</td>
<td>(Melikova et al., 2004)</td>
</tr>
<tr>
<td>β3-integrins</td>
<td>(Butler and Blystone, 2005)</td>
</tr>
<tr>
<td>integrin alpha(M)beta(2)</td>
<td>(Duong and Rodan, 2000)</td>
</tr>
<tr>
<td>Tensin</td>
<td>(Benzing et al., 2001)</td>
</tr>
<tr>
<td><strong>Protein-tyrosine Phosphatase</strong></td>
<td></td>
</tr>
<tr>
<td>PTPN11</td>
<td>(Meyer et al., 2004)</td>
</tr>
<tr>
<td>Shp-1</td>
<td>(Okenwa et al., 2013)</td>
</tr>
<tr>
<td>PTPNM3</td>
<td>(Li et al., 2014)</td>
</tr>
<tr>
<td>PTP phi</td>
<td>(Pixley et al., 2001)</td>
</tr>
<tr>
<td><strong>Paxillin</strong></td>
<td></td>
</tr>
<tr>
<td>Hic-5</td>
<td>(Matsuya et al., 1998); (Osada et al., 2001); (Guignandon et al., 2006); (Srinivasan et al., 2008); (Arthur et al., 2011)</td>
</tr>
<tr>
<td>Paxillin</td>
<td>(Li and Earp, 1997); (Ostergaard et al., 1998); (Wang and DeFea, 2006); (St-Pierre et al., 2011)</td>
</tr>
<tr>
<td><strong>Membrane-Associated Guanylate Kinase</strong></td>
<td></td>
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<tr>
<td>PSD-95</td>
<td>(Sebold et al., 2003); (Ma et al., 2004); (Bartos et al., 2010)</td>
</tr>
<tr>
<td>SAP102</td>
<td>(Sebold et al., 2003)</td>
</tr>
<tr>
<td><strong>Ubiquitin-proteasome Pathway</strong></td>
<td></td>
</tr>
<tr>
<td>Cbl</td>
<td>(Bruzzaniti et al., 2005); (Elsegood et al., 2006)</td>
</tr>
<tr>
<td>Cbl-b</td>
<td>(Fan et al., 2014)</td>
</tr>
<tr>
<td>Nedd9 Ub ligase</td>
<td>(Sasaki et al., 2005)</td>
</tr>
<tr>
<td><strong>Glutamate-gated Ion Channel</strong></td>
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<td>NR2A</td>
<td>(Liu et al., 2003); (Ma et al., 2004); (Liu et al., 2005); (Hou et al., 2005)</td>
</tr>
<tr>
<td>NR2B</td>
<td>(Antion et al., 2010)</td>
</tr>
<tr>
<td><strong>Src Family Kinase Signaling</strong></td>
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</tr>
<tr>
<td>Src</td>
<td>(Preisig, 2007); (Block et al., 2010); (Wang and Brecher, 2001); (Espiritu et al., 2002); (Shi and Kehrl, 2004a); (Bruzzaniti et al., 2005); (Basile et al., 2005);</td>
</tr>
</tbody>
</table>
My thesis work reveals a novel role of Pyk2 as a mediator of p27-driven upregulation of cancer stem cells, tumor initiation and metastasis (see Chapter 4).
CHAPTER 2: MATERIALS AND METHODS
This chapter describes the materials and experimental approaches that were used for the work in this thesis. Section 2.1 contains a listing all of the relevant materials: Table 2.1 lists the cell lines used in this work, Table 2.2 lists the various reagents including antibodies and inhibitors. Table 2.3 lists the oligonucleotide sequences used for qPCR. Section 2.2 contains detailed descriptions of the various experimental methods that were utilized.

### 2.1 MATERIALS

#### Table 2.1: Cell Lines

<table>
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<tr>
<th>Name</th>
<th>Media</th>
<th>Description</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>MCF12A</td>
<td>DMEM/F12, 5% HBS, 1% Pen/Strep</td>
<td>Immortalized, non-tumorigenic human mammary epithelial cells</td>
<td>(Paine et al., 1992)</td>
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<tr>
<td>MDA-MB-231</td>
<td>DMEM, 10% FBS, 1% Glutamine, 1% Sodium Pyruvate, 1% Pen/Strep</td>
<td>Human breast cancer cell line, luciferase-tagged</td>
<td>(Minn et al., 2005)</td>
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<td>DMEM, 10% FBS, 1% Glutamine, 1% Sodium Pyruvate, 1% Pen/Strep</td>
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<td>(Minn et al., 2005)</td>
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<td>IMEM, 10% FBS</td>
<td>Human bladder cancer cell line</td>
<td>(Nitz et al., 2008)</td>
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<td>UMUC3-LUL2</td>
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<td>(Nitz et al., 2008)</td>
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<tr>
<td>PEO1R</td>
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<td>Human ovarian cancer cell line</td>
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<td>293T</td>
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Table 2.2: Reagents

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<td>Santa Cruz</td>
<td>Knockdown PYK2</td>
</tr>
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<td>Santa Cruz</td>
<td>Knockdown SRC</td>
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<td>Overexpression</td>
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<td>GeneCopoeia</td>
<td>Knockout PYK2</td>
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Table 2.2: Reagents (continued)

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<td>CD44</td>
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<td>p-AKT S473</td>
<td>Cell Signaling</td>
<td>Western (1:1000)</td>
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<td>AKT</td>
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<td>R&amp;D Systems</td>
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<td>p27</td>
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<td>Sox2</td>
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<td>IP</td>
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<td>Anti-rabbit IgG</td>
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<td><strong>INHIBITORS</strong></td>
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<td>PF-04691502</td>
<td>Pfizer</td>
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<td>PF-431396</td>
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<td>Pyk2/Fak inhibitor</td>
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<td>Bevacizumab</td>
<td>Genetech</td>
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<td>2C3</td>
<td>Santa Cruz</td>
<td>R. Brekken UT Southwestern</td>
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<td>AZD0530</td>
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<td>Src inhibitor</td>
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<td>5’ azacytidine</td>
<td>Sigma</td>
<td>DNA methyltransferase inhibitor</td>
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Continued Table 2.2: Reagents
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<thead>
<tr>
<th>Lenti-mcherry-shRNA control</th>
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<td>Midiprep kit</td>
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<td>Bradford Dye</td>
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<td>Western</td>
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<td>ECL (Standard)</td>
<td>Western Lighting</td>
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<td>ECL (Pico)</td>
<td>Thermo Scientific</td>
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<td>ECL (Femto)</td>
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<td>PVDF Membrane</td>
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<td>iQ SYBR Green Supermix</td>
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<td>qPCR</td>
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<td>Luc-Pairtm Duo-Luciferase Assay Kit 2.0</td>
<td>GeneCopoeia</td>
<td>Luciferase assay</td>
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<td>Stem Cell Technologies</td>
<td>ALDH1+ analysis</td>
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<td>QIAprep Miniprep Kit</td>
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<td>Nuclear/Cytoplasmic Fractionation kit</td>
<td>Thermo Scientific</td>
<td>Western</td>
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<td>Protein A/G beads</td>
<td>Santa Cruz</td>
<td>Immunoprecipitation</td>
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<td>pcDNA™3.1/V5-His TOPO® Expression kit</td>
<td>Life Technologies</td>
<td>Bisulfite Sequencing</td>
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**Table 2.3: Oligonucleotide Sequences**

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<th>Gene</th>
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<td>Human NANO5 Forward</td>
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<td>Human NANO5 Reverse</td>
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<td>Human SOX2 Forward</td>
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<td>Human SOX2 Reverse</td>
<td>TGGACAGTTAGCCGCACAT</td>
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<td>Human miR-128 Forward</td>
<td>TCACAGTGAAACCGTCCTTC</td>
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<tr>
<td>Human miR-128 Reverse</td>
<td>Universal reverse primer (Invitrogen)</td>
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<td>Human 18S Forward</td>
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<td>Human 18S Reverse</td>
<td>TGCCAGAAGTCTCAGTTCAGATCG</td>
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<tr>
<td>Human GAPDH Forward</td>
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<td>Human GAPDH Reverse</td>
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<td><strong>BISULFITE SEQUENCING</strong></td>
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<td>Human miR-128-2 Forward</td>
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<tr>
<td>Human miR-128-2 Reverse</td>
<td>TCAACAAAAATACACAAACCTTC</td>
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</tbody>
</table>
2.2 METHODS

2.2.1 Stable cell lines expressing p27 phosphomimetic mutants or stable p27 knockdown

The cell lines from different tissue origins, and their media are described in Table 2.1. MDA-MB-231, UMUC3, and MCF12A cell lines stably expressing phosphomimetic p27 mutant (Plvx-EGFP-p27CK-T157D/T198D) or EGFP-tagged p27CK were derived by Zhao et al and used in experiments of Chapter 4 (Zhao et al., 2014). Stable transfectants expressing similar EGFP-p27 levels were used for our studies. 4175, 1833 and UMUC3 cell lines with stable p27 knock-down of p27 (pGIPZ-ShCDKN1B, Open Biosystems) or corresponding lines with scrambled control ShRNA of CDKN1B (pGIPZ-scramble, Open Biosystems) were derived by Zhao et al and used in experiments of Chapter 4 (Zhao et al., 2014).

2.2.2 Lentivirus production and infection

Two µg of either scrambled ShRNA (psi-LVRU6MH-scramble, Genecopoeia) or shPYK2 (psi-LVRU6MH-ShPTK2B, Genecopoeia) were co-transfected with Delta VPR (1.5 µg) and CMV-VSVG (0.5 µg) plasmids (Addgene) into asynchronous 293T (2x10^6 cells/10cm plate) with Lipofectamine Plus (Roche). Viral supernatants were collected at 48h and concentrated by ultracentrifugation for 2 h at 22,000 RPM at 4°C. Pellets were resuspended overnight at 4°C. Cells were infected twice in the presence of polybrene (10 µg/ml), and treated with hygromycin (500 µg/ml), and then analyzed 3-5 days post infection via both RFP visualization and Western blotting.
2.2.3 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 or 18S were used as internal controls (Rieger et al., 2010). All samples were performed in triplicates and average C\textsubscript{t} values were normalized to the values of GAPDH or 18S. A comprehensive list of primers used for qPCR in this thesis is included in Table 2.3.

2.2.4 Western blotting

Westerns were as described (Sandhu et al., 1997). A comprehensive list of antibodies used during Western blotting (along with the company from which they were purchased) is included in Table 2.1. All Western blots used 20-50 μg protein lysate/lane. Proteins were resolved on 10-12 % SDS PAGE gels. Then, proteins were transferred to 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 of each antibody. The source of each antibody and the dilutions used for Western Blotting are indicated in Table 2.2. Secondary incubation at a dilution of 1:5,000 was carried out for one hour. Chemiluminescent imaging was carried out with a variety of ECL reagents, listed in Table. For IP-Westerns, 1 μg of antibody was used for protein precipitation of from 1 mg protein lysate.
2.2.5 miRNA RT-PCR

miRNA isolation used miRNeasy (Qiagen) +/- prior VEGFA, saracatinib or 5’ azacytidine (Sigma-Aldrich). cDNA synthesis used Ncode miR First Strand cDNA synthesis kit (Invitrogen). QPCR of miR-128 used miR-128 forward: 5’-TCACAGTGAACCGGTCTCTTT-3’ and Universal reverse primer (Ncode miR First Strand cDNA synthesis kit, Invitrogen).

2.2.6 Luciferase assay

PEO1R, OVCAR8, OCI-C5X cells were treated with VEGFA 10 ng/ml for 4 days prior to transfection with 500 ng pEZX-MT01-BMI1-3’UTR plasmid (Genecopoeia) followed by 72 hours further VEGFA treatment. Firefly and Renilla luciferase reporter activity were measured using Luc-Pair™ Duo-Luciferase Assay Kit 2.0 (Genecopoeia) per manufacturer.

2.2.7 Cell proliferation assay

In vitro cellular proliferation was assessed by plating equal cell number (n=100,000) in a 12well culture plate at time = 0. Triplicate samples were plated in parallel and cells were harvested and counted at day 2, 4, 6 and 8. Average cell counts were calculated and plotted +/- SEM.

2.2.8 Cell cycle analysis

Flow cytometry for cell cycle distribution was conducted as in (Sandhu et al., 1997). Prior to plating of sphere assays, the cells were shown to have similar cell cycle
distributions. Cell cycle assays were done immediately prior to plating sphere assays or injection into nude mice for tumor initiation stem cell (T-ISC) limiting dilution assays for both Chapters 3 and 4. In work of Chapter 4, since saracatinib 1μM (AZD0530) caused partial G1 arrest, all groups were subjected to two days washout without drug or cytokine prior to sphere assays. Cells returned to asynchronous cycling two days after saracatinib washout. Cell cycle distribution was assayed after 7 days of VEGFA followed by 2 days without cytokine (VEGFA), or following 7 days VEGFA with AZD0530 added for 48 hrs (days 6 and 7) followed by 2 days washout without cytokine or AZD0530 (AZD0530 + washout). siBMI1 cells were transfected with siBMI1 48 hrs prior to VEGFA treatment for 7 days and followed by 2 days without cytokine.

Prior to sphere assays and T-ISC quantification in Chapter 4, the cells in the following experimental groups (231-pLVX-EGFP-psi-LVRU6MH-scramble, 231-p27CK-DD-psi-LVRU6MH-scramble, 231-p27CK-DD-ShPYK2, 1833-psi-LVRU6MH-pGIPZ-scramble, 1833-psi-LVRU6MH-Shp27) were shown to have similar cell cycle distribution by flow cytometry. All cells were proliferating asynchronously at plating.

2.2.9 In vivo tumor-initiating Stem Cell Assays

The University of Miami (UM) Animal Care and Use Committee approved all animal work. For the chapter 3, for limiting dilution xenograft assays, PEO1R was transduced or not with siBMI1 or scrambled siRNA (Santa Cruz Biotech) controls for 48 hours and then treated +/- VEGFA 10 ng/ml for 7 days. One hundred and twenty four five week old, female NOD/SCID mice were injected with cells from four experimental groups (untreated scrambled siRNA controls, VEGFA-treated scrambled siRNA controls,
VEGFA-treated siBmi1 transduced and Bmi1siRNA transduced). Animals were injected in the mammary fat pad with 100 (10 mice/group), 1,000 (8 mice/group), 10,000 (8 mice/group) or 100,000 (5 mice/group) cells in 100 µl matrigel (BD Biosciences) as in (Ginestier et al, 2007). Tumors were measured 2X/week. Mice without tumors were followed at least 9 months per UM Animal Care and Use Committee standards.

For the work of chapter 4, MDA-MB-231 was transduced or not with pLVX-EGFP-control vector or with pLVX-EGFP-p27CK-DD. The same EGFP-vector control transduced line was also transfected with the scrambled shRNA corresponding to the vector used for knockdown of Pyk2 or ShPYK2. 1833 was transduced or not with scrambled shRNA or Shp27. One hundred and fifty, five weeks old, female nude mice were injected with cells from six experimental groups (231 GFP and scrambled ShRNA controls, 231 p27CK-DD and scrambled ShRNA, 231 p27CK-DD and ShPYK2, 1833 scrambled ShRNA, 1833 Shp27). Animals were injected in the mammary fat pad with 10 (12 mice/group), 100 (10 mice/group), or 1,000 (8 mice/group) cells in 100 µl matrigel (BD Biosciences) as in (Ginestier et al, 2007). Tumors were measured 1X/week. Mice without tumors were followed at least 3 months per UM Animal Care and Use Committee standards.

2.2.10 Immunoprecipitation

UMUC3-LuL2 and MDA-MB-231-1833 cells were lysed and 500-800 µg lysate was incubated with 1 µg of p27 antibody (BD Transduction) or Normal mouse/rabbit IgG overnight, then collected on 30µl protein A/G-agarose beads (100mg/ml) for 2 hours and washed three times with IP buffer. Samples were then resolved by 10 % SDS-PAGE
followed by Western analysis. Antibody-alone controls were run with all immunoprecipitations.

### 2.2.11 Sphere Formation

Sphere assays were as in (Dontu et al., 2003). Cells were seeded for sphere assays into a serum-free mammary epithelial growth medium (MEGM, BioWhittaker), supplemented with B27 (Invitrogen), 20 ng/mL EGF and 20 ng/mL bFGF (BD Biosciences), and 4 μg/mL heparin (Sigma).

In chapter 3, ovarian cancer lines were seeded into spheres with or without a prior 7 days VEGFA exposure (R&D) 10 ng/ml. For limiting dilution sphere assays, cells were seeded in the following numbers: PEO1R; 1,000 and 500 cells, OVCAR8; 2,500 and 1,250 cells, OCI-C5X; 4,000 and 2,000 cells, as in (Ginestier et al., 2007). Spheres > 75 μM were counted after 14-21 days by visualization with direct microscopy. VEGFA was renewed every 48 hrs over 7 days. Where indicated, bevacizumab (100 μg/ml, Genetech, CA, USA) or the 2C3 VEGFR2-blocking, antibody (15 μg/ml, provided by R. Brekken, UT Southwestern USA) were added immediately before VEGFA and renewed every 2 days. Where indicated, the Src inhibitor, saracatinib (AstraZeneca) 1μM was added for the final 48 hrs of the 7 day VEGFA pre-treatment.

In chapter 4, sphere assays were carried out using MCF12A-EGFP, MCF12A-p27CK-DD, MDA-MB-231-EGFP, MDA-MB-231p27CK-DD, 4175-ShRNAscramble, 4175-Shp27, 1833-ShRNAscramble and 1833-Shp27 cells. 2,000 cells were seeded for sphere assays into sphere media as described previously. Where indicated, PF431396 (500
nM, Sigma) was added for 48 hrs followed by 2 days washout without PF431396, prior to sphere assays.

2.2.12 siRNA experiments for Bmi1

2x10^5 cells/well cells were seeded in a six well plate in 2ml antibiotic free medium. Cells were incubated at 37°C in a CO₂ incubator until cell confluency is 80%. 8 μl of siRNA (80 pmols) is transfected with Lipofectamine (Thermo Fisher). Cells were incubated with 5 hours at 37°C in a CO₂ incubator. Cells were transfected with siBMI1 for 48 hrs prior to VEGFA treatment for 7 days and followed by 2 days without cytokine. Knock-down of BMI1 is verified immediately before sphere formation assays or T-ISC assay.

2.2.13 Viability assays

To assay cell viability, 1x10^5 cells were stained by Trypan Blue dye (VWR) with 1:1 dilution of the cell suspension using a 0.4% Trypan Blue solution. Within minutes, non-viable cells stain blue, viable cells are unstained. Stained cells were counted by TC20™ Automated Cell Counter (Bio-rad).

2.2.14 Aldefluor Assay and flow Sorting of ALDH1+ or ALDH1- Cells

The ALDEFLUOR kit (StemCell Technologies, Durham, NC, USA) was used to identify the population with a high ALDH enzymatic activity in both our breast cancer and ovarian cancer models as in (Ginestier et al., 2007). Cells were suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, 1 mmol/l per 1x10^6 cells) and incubated during 40 min at 37°C. As negative control, for each sample of cells an aliquot was treated
with 50 mmol/l diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. ALDEFLUOR was excited at 488 nm, and fluorescence emission was detected using a standard fluorescein isothiocyanate (FITC) 530/30-nm band-pass filter.

To sort ALDH1+ and ALDH1- cells, 3 x 10^6 unsorted PEO1R cells were stained using the ALDEFLUOR kit (Stem Cell Technologies, Durham, NC) as above and stained cells were aliquoted to 3 tubes with 1x10^6 cells in 1 ml of ALDEFLUOR assay buffer and then, cells were transferred to FACSaria II (BD Biosciences) machine with incubation on ice. ALDH1+ or ALDH1- cells flow sorted by FACSaria II for 2 hours in the University of Miami Flow cytometry core facility. (Ginestier et al., 2007).

2.2.15 Colony formation assay

Soft agar was prepared as follows. Agar was dissolved into complete culture medium 10 % FCS (3.5 mg/ml). Cells were suspended in soft agar (6 X10^3 cells/ml agar solution/1 cm well) and incubated in at 37 °C C02 incubator and then colonies were counted at 4 weeks.

2.2.16 Bisulfite Sequencing

Bisulfite conversion was performed with Epitect Fast DNA Bisulfite Kit (Qiagen) using genomic DNA from the VEGFA, saracatinib or 5’ azacytidine treated PEO1R and OCI-C5X cell lines. DNA (1 μg) was incubated with bisulfite solution (100 μl) and bisulfite DNA conversion performed using the following thermal cycler conditions: Denaturation 5 min at 95 °C, Incubation 10 min at 60 °C. Primers (Forward: TGTTTTTAAGGTTAGGGAATTAAATTAG, Reverse: TCAACAAAAATAACACACAA
ACCTCTC) amplified miR-128-2 in bisulfite converted genomic DNA. PCR products were gel purified and cloned with pcDNA™3.1/V5-His TOPO® Expression kit (Life Technologies). PCR product (4 μl) was incubated with 1 μl salt solution, 2 μl sterile water and 1 μl TOPO vector for 5 min at room temperature. TOPO Cloning reaction (2 μl) was added to a vial of One Shot® TOP10 competent E. coli. Competent cells were incubated on ice for 30 mins and then, heat shocked for 30 seconds at 42 ºC and transferred to ice. Cells were incubated into Super Optimal Catabolite (Thermo Fisher) for 1 hour at 37 ºC with shaking and 50 μl of cell suspension were cultured on LA plate overnight at 37 ºC. DNA from bacterial colonies was subjected to bisulfite sequencing.

2.2.17 Survival Analysis

Publicly available ovarian cancer datasets from the National Center for Biotechnology Information (GSE14764, GSE9891, GSE51373, GSE3149, GSE30161, GSE27651, GSE26712, GSE26193, GSE23554, GSE19828, GSE18520, GSE15622) and the ovarian cancer dataset from The Cancer Genome Atlas (TCGA) (using cBioPortal [http://www.cbioportal.org/], downloaded 4-10-15) were analyzed using KM plotter (http://kmplot.com/analysis/) to create Kaplan Meier (KM) curves for PFS and OS versus VEGFA expression (Gyorffy et al., 2012; Mihaly et al., 2013). The KM curves for disease free survival and OS with miR-128-2 expression used TCGA data. OVCA with high VEGFA and high or low miR-128-2 expression from TCGA were compared by cBioPortal to create Kaplan Meier curves (http://in-silico.net/tools/statistics/survivor).
2.2.18 Statistical Analysis

All results are expressed as mean ± SEM. Comparisons of > two groups used one way analysis of variance (ANOVA) followed by Student-Newman-Keul's post hoc analysis or two way ANOVA followed by Bonferroni post hoc tests. Comparisons of two groups used Student’s t-test. Tumor initiating cell frequency was calculated by L-Calc Limiting Dilution Software (http://www.stemcell.com/en/Products/All-Products/LCalc-Software.aspx) from STEMCELL Technologies.
CHAPTER 3

VEGFA ACTIVATES AN EPIGENETIC PATHWAY UPREGULATING OVARIAN CANCER INITIATING CELLS

¹: A version of this chapter is accepted and will be published in Embo Molecular Medicine.
3.1 SUMMARY

The angiogenic factor, VEGFA, is a therapeutic target in ovarian cancer (OVCA). VEGFA can also stimulate stem-like cells in certain cancers but mechanisms thereof are poorly understood. Here, we investigated the role of VEGFA as an ovarian cancer stem cell factor and mechanisms underlying its actions. We show VEGFA mediates stem cell actions via VEGFR2-dependent Src activation to upregulate Bmi1, tumor spheres and ALDH1 activity in primary human OVCA culture and OVCA lines. VEGFA stimulates sphere formation only in the ALDH1+ subpopulation, and increases OVCA-initiating cells and tumor formation in vivo through Bmi1. In contrast to its action in hemopoietic malignancies, in ovarian cancer, DNA methyl transferase 3A (DNMT3A) plays a pro-oncogenic role downstream of Src to methylate miR-128-2, upregulating Bmi1 to increase stem like cells. DNMT3A knockdown prevents VEGFA-driven miR-128-2 loss, and the increase in Bmi1 and tumor spheres. Analysis of over 1,300 primary human OVCAs revealed an aggressive subset in which high VEGFA is associated with miR-128-2 miR-128-2 loss. Thus, VEGFA stimulates OVCA stem like-cells through Src-DNMT3A driven miR-128-2 methylation and Bmi1 upregulation.

3.2 INTRODUCTORY REMARKS

Developing cancers rapidly outstrip the diffusion capacity of nutrients and oxygen and must form new blood vessels via angiogenesis (Bergers and Benjamin, 2003). They must also maintain self-renewal capacity despite adverse conditions of sub-optimal pH, nutrient and oxygen availability. Vascular endothelial growth factor A, VEGFA, is a key angiogenic factor (Bergers and Benjamin, 2003) that alters the endothelial cell niche to
promote new vessel formation (Beck et al., 2011). In various cancers, VEGFA stimulates not only angiogenesis but also tumor growth, metastasis and survival (Hu et al., 2007; Paez-Ribes et al., 2009; Wu et al., 2006b).

The importance of VEGFA in angiogenesis and its frequent upregulation in human cancers (Goel and Mercurio, 2013) stimulated development of VEGF- and VEGF receptor-targeted therapies. The monoclonal antibody, bevacizumab, blocks VEGFA interaction with receptors VEGFR1 and 2 (Ferrara, 2004). Despite initial promise, VEGF-targeted therapies have shown limited efficacy, with short responses in most solid tumors (Eskander and Tewari, 2014). Hypoxia resulting from inhibition of angiogenesis, upregulates VEGFA expression, contributing to aggressive disease recurrence and angiogenic therapy failure (Paez-Ribes et al., 2009; Ebos et al., 2009). In ovarian cancer, bevacizumab significantly increases progression free survival (PFS) compared to chemotherapy alone in advanced disease, and more recently VEGFR targeting agents were shown to significantly increase PFS (Eskander and Tewari, 2014) and overall survival (OS) (Witteveen A et al., 2013). Bevacizumab is approved for use with non-platinum chemotherapy in Europe and the USA for platinum-resistant ovarian cancer, however improvements are of short duration and resistance rapidly emerges (Eskander and Tewari, 2014).

Increasing evidence indicates that cancer stem-like cells (CSCs) comprise a distinct self-renewing sub-population that can generate cancerous progeny with reduced replicative potency (Dalerba et al., 2007). CSCs are important therapeutic targets: they may not only initiate tumors but also mediate recurrence and metastasis (Magee et al., 2012). Most anticancer drugs kill the bulk cancer population. CSCs either proliferate too slowly for targeting by cycle active drugs, or escape chemotherapy by drug expulsion or greater DNA
repair, leading to recurrence (Magee et al., 2012). CSC share properties with normal tissue stem cells, expressing discrete surface markers and forming spheres when seeded at single cell density (Visvader and Lindeman, 2012b). Subpopulations of many malignancies, including OVCA, with aldehyde dehydrogenase 1 activity (ALDH1+) are enriched for CSC properties \textit{in vitro} and the ability to initiate tumors in immunocompromised mice (Ginestier et al., 2007; Landen, Jr. et al., 2010). Several surface markers have been proposed to characterize OVCA stem-like cells (Shah and Landen, 2014). The ALDH1+ subpopulation of primary ovarian cancers (Stewart et al., 2011) and in OVCA lines (Shah and Landen, 2014; Silva et al., 2011) is enriched in tumor initiating cells \textit{in vivo} and by prior chemotherapy exposure (Landen, Jr. et al., 2010). While certain cytokines increase CSC and enhance tumor initiation \textit{in vivo} (Zhao et al., 2014), extracellular growth factors and signaling pathways that stimulate CSC expansion are poorly characterized. A greater understanding of pathways governing CSC may permit the design of more effective anticancer treatments.

VEGFA is not only a potent angiogenic factor, it also stimulates stem-like cells in both normal and cancer tissues. VEGFA maintains normal stem cell populations in hemopoietic (Gerber et al., 2002), endothelial (Kane et al., 2011), neuronal (Calvo et al., 2011) and adipose tissues (Gerber et al., 2002). VEGFA was recently shown to increase tumor-initiating stem-like cells in certain human malignancies (Beck et al., 2011; Zhao et al., 2014). Pathways activated by VEGFA that increase cancer stem-like cells and tumor initiation are largely uncharacterized. Since VEGFA is frequently overexpressed in OVCA (Yu et al., 2013) and VEGF/VEGFR-targeted therapies have significant activity in this cancer (Eskander and Tewari, 2014), we investigated whether VEGFA drives ovarian CSC
expansion and sought to identify targetable pathways mediating these effects.

MicroRNAs (miRNAs) are increasingly implicated in CSC regulation (Takahashi et al., 2014). These small non-coding RNAs bind the 3’ untranslated region (3’ UTR) of target genes to inhibit gene expression. miRNAs regulate targets essential for normal and malignant stem-like cell self-renewal (Takahashi et al., 2014) and are often misregulated in cancer (Croce, 2009). Oncogenic miRNAs target tumor suppressors and increase drug resistance and metastasis. In contrast, tumor suppressor miRNAs are frequently down-regulated in cancer (Croce, 2009). Here, we investigated the role of VEGFA as a driver of stem-like cell expansion in OVCA. This work reveals a novel pathway linking VEGFA to miRNA-dependent CSC regulation. We show VEGFA activates Src, and induces DNMT3A to methylate miR-128-2, leading to increased Bmi1 and OVCA stem-like cell expansion.

3.3 RESULTS

3.3.1 VEGFA increases sphere formation and ALDH1 activity in OVCA populations

While VEGFA is an angiogenic agent and therapeutic target in OVCA, the possibility that the limited effects of VEGF-targeted therapies and emergence of resistance might be due, in part, to VEGFA effects on ovarian CSC has not been evaluated. To investigate VEGFA stimulates ovarian CSCs, we used three models. Since > 60% of OVCA express the estrogen receptor α (ER), we used the well-established ER+ line, PEO1R, derived from human OVCA ascites (Langdon et al., 1994) and validated results in the ER- human OVCAR8 line (Simpkins et al., 2012). Since OVCA lines diverge from primary tumors over time (Ince et al., 2015), results were also validated using early passage OCI-C5X, a direct primary OVCA culture. OCI-C5X, faithfully representing the molecular
and cellular phenotype of the original patient tumor, is one of twenty-five new ovarian cancer cultures established by Ince by immediate culture of primary cancer in Ovarian Carcinoma Modified Ince medium, OCMI (Ince et al., 2015).

Sphere formation from a single CSC seeded in low adhesion conditions measures stem-like cell abundance in vitro (Visvader and Lindeman, 2012b). Prior work showed VEGFA and a network of pro-inflammatory cytokines increase breast CSC abundance, but required prolonged exposure for full effect (Zhao et al., 2014; Picon-Ruiz et al., 2016). VEGFA (10 ng/ml) effects were assayed over short and long term exposures (1, 3 and 7 days). A seven day exposure, but not shorter intervals, significantly increased sphere formation by PEO1R, OVCAR8 and OCI-C5X cells seeded without further VEGFA. All sphere assays were carried out in limiting dilutions and sphere formation could not be accounted for by aggregation. VEGFA blocking antibody, bevacizumab, and 2C3 antibody that blocks VEGFR2, both inhibited VEGFA-stimulated sphere formation (Figure 3.1A). Neither antibody alone decreased baseline sphere formation, suggesting VEGFA does not drive basal CSC self-renewal, but augments CSC recruitment in these OVCA models. VEGFA was not a mitogen in these models. Cell cycle profiles were not changed by VEGFA exposure for 48 hours in 2D and were not affected by 7 days in sphere culture (representative data from OVCAR8, Figure 3.2A) and VEGFA did not increase cell numbers over time in unsorted cells (Figure 3.2B).

The ALDH1+ population of OVCA lines (Shah and Landen, 2014; Silva et al., 2011) and primary tumors (Stewart et al., 2011) is enriched for CSC in vitro and tumor initiating cells in vivo. VEGFA exposure increased ALDH1+ cell abundance in all three models,
including early passage OCI-C5X culture (Ince et al., 2015) (Figure 3.1B). Thus, prolonged VEGFA exposure increases the abundance of sphere forming and ALDH1+.

**Figure 3.1:** VEGFA increases sphere forming and ALDH1+ positive OVCA cells. (A) Indicated cells were pre-treated for 7 days with either 10 ng/ml VEGFA, VEGFA + 50 µg/ml bevacizumab, VEGFA + 15 µg/ml 2C3 or no cytokine control and plated into sphere assays. Spheres >75 µM were counted at 14 days for PEO1R and at 21 days for OVCAR8 and OCI-C5X. All assays were performed in triplicate biologic repeats with at least 3 technical repeats within each assay. Graphed data represent mean +/- SEM. Differences between multiple treatment groups were compared by ANOVA. **p<0.01, ‡p<0.001** (B) Indicated cells were treated ±VEGFA for 7 days and the proportion of aldefluor positive (% ALDH1+) assayed by flow cytometry and mean +/-SEM graphed for repeat assays. #p<0.0001. ALDH1+ cells in controls vs VEGFA treated cells were compared by Student’s T test.
3.3.2 VEGFA increases expression of the stem-like cell regulator, Bmi1

Several embryonic stem cell transcription factors (ES-TFs) govern embryonic stem cells (ES) self-renewal, induce pluripotency in skin fibroblasts (Li, 2010) and have been implicated in CSC sel-renewal in a number of different cancers (Zhang et al., 2008b). B cell-specific Moloney murine leukemia virus integration site 1 (Bmi1) is part of the polycomb repressive complex 1 (PRC1) that regulates chromatin remodeling during development (Siddique and Saleem, 2012). Bmi1 promotes normal hematopoietic stem cells expansion (Park et al., 2003), in part through changes in ES-TFs (Molofsky et al., 2005; Siddique and Saleem, 2012) and can upregulate malignant stem-like cells (Lessard and Sauvageau, 2003; Siddique and Saleem, 2012). Bmi1 is has been shown to be upregulated in cancers, including OVCA (Siddique and Saleem, 2012) but its role in ovarian CSC expansion has not been established.

Figure 3.2: VEGFA is not mitogen (A) The cell cycle distributions of OVCAR8 cells treated without (control) or with VEGFA in 2D culture over 7 days (top) or assayed from dissociated OVCAR8 spheres formed +/- VEGFA (bottom panel). Data is graphed as mean +/- SEM of triplicate data from 3 biologic repeat experiments. Comparison of the mean % S phase cells with and without prior VEGFA exposure showed no difference in the % S phase, with p>0.05, not statistically significant by Student’s T test. (B) Equal numbers (10,000 cells) of PEO1R and OVCAR8 cells were plated into 2D culture +/- VEGFA and viable cells were counted every 2 days. Triplicate repeat data show no difference in population growth between groups over 8 days as calculated using comparative analysis of growth curves (http://bioinf.wehi.edu.au/software/compar-eCurves), with p>0.05, not statistically significant.
Figure 3.3: VEGFA increases OVCA sphere formation via Src mediated Bmi1 upregulation. VEGFA effects on indicated proteins, Bmi1 (A), and total and Y416-phosphorylated Src (pSrc) (B), at times indicated. (C) Cells were treated with or without 7 d VEGFA, +/- Src inhibition by 1 μM saracatinib (AZD0530) during the last 48 hrs. Westerns show Src, pSrc and Bmi1 levels. (D) Cells were transduced with siRNA BMI1 or scrambled controls 48 hrs prior to VEGFA treatment for 7 d and then recovered for Western. (E) Cells were transduced with either siBMI1 or control siRNA for 48 hrs prior to VEGFA addition for 7 d (+/- siBMI1) or treated with VEGFA for 7 d with or without Src inhibitor (AZD0530, 1μM) during the last 48 hrs followed by a 2 day washout without drug or cytokine prior to plating of spheres into limiting dilution sphere formation. All graphed data show means +/- SEM for at least 3 different biologic repeat assays. Differences between multiple treatment groups were compared by ANOVA.

*p<0.05, **p<0.01, ‡p<0.001, #p<0.0001.
To assay their potential involvement in VEGFA-stimulated ALDH1 activity and sphere formation, we tested VEGFA effects on Bmi1 and ES-TFs in PEO1R, OVCAR8 and OCI-C5X. Basal Bmi1 levels were increased by 6 hrs and remained elevated at 7 days (Figure 3.3A, left). Densitometry of Westerns on repeat assays showed Bmi1 protein increased by 2.0 +/- 0.02 fold in PEO1R, 4.7 +/- 0.1 fold in OVCAR8 and 1.9 +/- 0.09 fold in OCI-C5X after 7 days of VEGF exposure. Notably, while VEGFA also increased cMyc levels (by 3.5 +/- 0.1 fold by day 4), Oct4 (2.2 +/- 0.06 fold, day 5) and Klf4 (4.1 +/- 0.3 fold day 5) (Figure 3.4), these rose after the increase in Bmi1. Thus, we assayed the role of Bmi1 in VEGFA-mediated ovarian CSC effects.

### Figure 3.4: VEGFA effects on embryonic stem cell transcription factors

Western shows VEGFA effects on indicated proteins in PEO1R cells at indicated times in hours (h) or days (D).

#### 3.3.3 Increased sphere formation after VEGFA exposure is Src- and Bmi1-dependent

Src is frequently overexpressed in human OVCA (Simpkins et al., 2012) and promotes tumor growth (Kim et al., 2009). Since Src was recently shown to mediate cytokine driven CSC upregulation (Picon-Ruiz et al., 2016), we tested its potential as a mediator of effects of VEGFA on stem-like cells. VEGFA caused sustained Src activation with an increase in pSrc of 4.1 +/- 0.42 fold in PEO1R, and 4.8 +/- 0.08 fold in OVCAR8 and 1.8 +/- 0.04 fold in OCI-C5X within 7 days (Figure 3.3B). Src inhibition by saracatinib
(AZD0530) down-regulated basal Bmi1 levels. Furthermore, saracatinib addition during the last 48 hours of a 7 day VEGFA exposure prevented the increase in Bmi1 by VEGFA in all three ovarian models (Figure 3.3C). To test effects of Src inhibition on the sphere forming population, cells were treated with AZD0530 in the last 48 hrs of a 7 day VEGFA exposure, followed by a 2 day washout to allow recovery of asynchronous cycling prior to seeding into sphere assays (Figure 3.5A). Src inhibition followed by drug wash-out and

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**Figure 3.5:** Cell cycle distribution and viability of cells used in sphere assays and/or in tumor initiating stem cell assays. (A) PEO1R cell cycle distribution was assayed immediately prior to plating into sphere formation or prior to injection into nude mice for limiting dilution stem cell assays. Cells were recovered for cell cycle distribution after either 7 days of VEGFA followed by 2 days without cytokine (VEGFA), or after 7 days treatment with VEGFA with AZD0530 added for 48 hrs (days 6 and 7) prior to a 2 day washout without cytokine or AZD0530 (AZD0530 + washout). siBMI1 cells were transfected with siBMI1 for 48 hrs prior to addition of VEGFA for 7 days and followed by 2 days without cytokine. While AZD0530 (1μM) over 48 hrs caused partial G1 arrest (AZD0530), cells return to asynchronous cycling after a two day washout without AZD0530 (AZD0530 + washout). (B) PEO1R, OVCAR8 and OCI-C5X cell viability was measured by Trypan blue staining. Cell viability was not changed by one week of VEGFA exposure with or without either Src inhibition in the last 48 hrs of treatment, or by prior knockdown of Bmi1 48 hrs prior to addition of VEGFA. All graphed data show means +/- SEM for at least 3 different biologic repeat assays.
prior \textit{BMI1} siRNA knock-down (Figure 3.3D) each decreased sphere formation below that of scrambled siRNA and DMSO treated controls and prevented the VEGFA-mediated increase in sphere formation in both lines, and in OCI-C5X primary culture (Figure 3.3E). This loss of sphere formation could not be attributed to changes in cell cycling or viability, since neither \textit{BMI1} knock-down nor Src inhibition followed by wash out affected cell cycle profiles or viable cell numbers of cells prior to seeding (Figure 3.5A-B). Thus, Src kinase action appears to govern basal Bmi1 expression and both are required for the VEGFA-mediated increase in sphere formation.

\textbf{3.3.4 VEGFA increases ovarian tumor initiating cells via Bmi1 in vivo}

Effects of VEGFA and Bmi1 on ovarian tumor initiating cell abundance were further investigated \textit{in vivo}. Limiting dilution tumor initiating cell assays, injecting between 100 and 100,000 cells showed that sustained VEGFA exposure over 7 days prior to injection increased PEO1R tumor-initiating cell abundance. Ex-vivo exposure to VEGFA decreased tumor latency and more animals formed tumors from VEGFA-exposed cells than from cells without VEGFA pre-treatment. \textit{BMI1} knock-down prevented the VEGFA-mediated increase in tumor initiating cell abundance (Figure 3.6A). Note that VEGFA was not a mitogen in this model (Figure 3.2), and Bmi1 siRNA did not impair proliferation or viability (Figure 3.5). The tumor initiating cell frequency in VEGFA exposed cells was 1/2,018, compared with 1/21,607 in non-VEGFA exposed cells and 1/20,313 in VEGFA exposed cells pre-treated with siRNA to \textit{BMI1}, as calculated by L-Calc™ Limiting Dilution Software (Figure 3.6B). Thus, VEGFA increases tumor initiating OVCA cell abundance \textit{in vivo} and this is Bmi1 dependent.
3.3.5 *VEGFA repression of miR-128-2 is Src dependent*

Bmi1 is regulated by miR-128, a 21 nucleotide (ucacaguaaccgcguucu) that targets the *BMI1* 3’ UTR (Zhu et al., 2011; Jin et al., 2014; Godlewski et al., 2008). Mature miR-128 is encoded by two miRs, *miR-128-1* and *miR-128-2*. QPCR with primers

<table>
<thead>
<tr>
<th>Group</th>
<th>Limiting Dilution Tumors / Implant</th>
<th>T-ISC Freq. (1 in/...)</th>
<th>P value (vs ctrl)</th>
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<tr>
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<td>4 / 5</td>
<td>4 / 7</td>
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<tr>
<td>VEGFA</td>
<td>5 / 5</td>
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<td>siBmi1</td>
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that distinguish pre-miR-128-1 and pre-miR-128-2 showed VEGFA significantly reduced miR-128-2 but not miR-128-1 expression after 7 days (Figure 3.7). Since pre-miRs are unstable and less abundant, subsequent work used primers to detect mature miR-128. VEGFA downregulated miR-128 in all OVCA models (Figure 3.8A). To test if VEGFA relieves miR-128 targeting of the BMI1 3’ UTR, VEGFA-exposed and PBS treated control cells were transfected with a BMI1 3’ UTR luciferase reporter. VEGFA increased BMI1 3’ UTR luciferase reporter activity in both OVCA lines and in OCI-C5X (Figure 3.8B). Thus, Bmi1 up-regulation by VEGFA results from decreased inhibitory occupancy of the BMI1 3’ UTR.

**Figure 3.6:** The VEGFA-mediated increase in OVCA initiating stem-like cell abundance in vivo is Bmi1-dependent. (A) Tumor formation from limiting dilutions of inoculated PEO1R cells (100,000, 10,000, 1,000, 100 cells) is graphed as % of tumor free animals/time (weeks). (B) Tumor initiating cell frequency was calculated by L-Calc Limiting Dilution Software (http://www.stemcell.com/en/Products/All-Products/LCalc-Software.aspx) from STEMCELL Technologies.

**Figure 3.7:** VEGFA decreases miR-128-2 expression but not that of miR-128-1. PEO1R cells were treated with VEGFA for 7 days and then miR-128-1 and miR-128-2 levels assayed by QPCR. All graphed data show means +/- SEM for at least 3 different biologic repeat assays. ‡p<0.0001. Differences between two groups were compared by Student’s T test.
To test if Src activation drives VEGFA-mediated miR-128 loss, cells were treated with or without VEGFA, and with or without saracatinib (AZD0530). Saracatinib increased basal miR-128 and abrogated miR-128 down-regulation by VEGFA in all 3 models (Figure 3.8C). Since Saracatinib effectively inhibits several Src kinase family members, the requirement for cSrc was verified using Src knockdown by SiRNA, with three different oligonucleotides. Src knock-down increased basal miR-128 expression, and Src SiRNA for 48 hrs prior to addition of VEGFA abrogated the VEGFA driven loss of miR-128 expression in both OVCA lines and in the primary OCI-C5X culture (representative data, Figure 3.8D). Thus, Src appears to govern basal miR-128 expression and Src activation is required for miR-128 repression following VEGFA exposure.

3.3.6 VEGFA repression of miR-128 requires DNA methyltransferase activity

VEGFA exposure had effects long after VEGFA withdrawal to increase OVCA tumor-initiation in vivo (Figure 3.6), thus we speculated that VEGFA might regulate miR-128-2 epigenetically. The extent to which miRNAs are epigenetically regulated is not fully understood (Suzuki et al., 2012b). Tumor suppressor miRs are downregulated in cancers (Croce, 2009) through DNA methylation, since their repression is relieved by the methylation inhibitor 5’azacytidine (Saito et al., 2006; Suzuki et al., 2012b). While such miRNAs are methylation sensitive, the methyltransferases governing miRNA expression have not been characterized. 5’ azacytidine increased miR-128 expression in PBS treated controls and blocked miR-128 downregulation by VEGFA in PEO1R and OCI-C5X, (Figure 3.8E). Thus, VEGFA downregulation of miR-128 requires DNA methyltransferase action.
3.3.7 VEGFA upregulates Bmi1 through Src-mediated DNMT3A induction and miR-128-2 methylation

DNA methyltransferase (DNMT) 3A plays an important role to restrain gene expression and is deregulated in human malignancies (Fernandez et al., 2012). To test
potential DNMT involvement in VEGFA-driven miR-128-2 repression, and since DNA methyltransferases DNMT3A, DNMT3B, DNMT3-1 are often co-regulated, and elevated in cancer stem-like cells (Yang et al., 2015), we assayed all 3 methyltransferases. VEGFA increased DNMT3A but not DNMT1 and DNMT3B expression (Figure 3.9A). Src inhibition decreased DNMT3A expression in untreated cells and prevented the increase in DNMT3A by VEGFA (Figure 3.10A, left). DNMT3A protein was increased by 2.6 +/- 0.11 fold in PEO1R and by 1.6 +/- 0.08 fold in OCI-C5X after 7d VEGFA exposure (Figure 3.10A, B). Addition of saracatinib (AZD0530) abolished the VEGFA-stimulated DNMT3A increase and decreased basal DNMT3A protein levels (Figure 3.10A, right). Notably, siRNA mediated Src knockdown decreased basal DNMT3A and Bmi1 levels, and prevented the VEGFA-driven upregulation of both DNMT3A and Bmi1 in PEO1R and OCI-C5X (Figure 3.10B).

DNMT3A knock-down modestly decreased baseline sphere formation and prevented the increase in sphere formation by VEGFA (Figure 3.10C). DNMT3A was required for both VEGFA driven loss of miR-128 and for the increase in Bmi1 protein (Figs 5D, and 5E). Thus, VEGFA-driven miR-128 repression, Bmi1 upregulation and stem-like cell expansion appear to be DNMT3A-dependent. miRNA-128-2 DNA
methylation was analyzed by genomic bisulfite sequencing in PEO1R and in early passage OCI-C5X. The miR-128-2 region contains ten CpG sites in a 347-bp sequence (nucleotide -166 to +181) (Figure 3.10F). VEGFA increased the frequency of methylated CpGs in miR-128-2 in both PEO1R and OCI-C5X (Figure 3.10G and Figure 3.9B) consistent with its downregulation of miR-128 expression.

Figure 3.9: VEGFA effects on DNMT levels and methylation of miR-128-2 (A) DNMT1 and DNMT3B expression levels are not affected by VEGFA treatment over 7 days in PEO1R. (B) Results of bisulfite sequencing of the miR-128-2 region in OCI-C5X. Open circles indicate unmethylated and filled circles indicated methylated CpG sites. The individual rows represent one of the cloned alleles sequenced from individual bacterial colonies. (C) Expression levels of miR-128 in PEO1R and OCI-C5X are graphed. Data are graphed as mean +/- SEM for at least 3 different biologic repeat assays.

Saracatinib (AZD0530) and 5’ azacytidine both decreased baseline methylation and prevented the VEGFA-mediated increase in methylated CpGs in miR-128-2 (Figure 3.10G)
and Figure 3.9B). Notably, baseline miR-128 expression was lower and miR-128-2 methylation was higher in PEO1R than in early passage OCI-C5X (Figure 3.10G and Figure 3.9B, C), supporting the notion that miR-128-2 expression is governed by DNA
methylation. DNA methylation of miR-128-2 was strongly inversely correlated with its expression in both PEO1R (R2=-0.7513) and the primary OCI-C5X models (R2= -0.9559) (Figure 3.10H).

3.3.8 VEGFA confers self-renewal of ALDH1+ cells

Our initial assays showed VEGFA increased the proportion of ALDH1+ cells. We next assayed Src activation, DNMT3A levels and Bmi1 in cells with different ALDH1 activity. When ALDH1 positive and negative populations were isolated by flow cytometry, ALDH1+ PEO1R cells showed higher Bmi1 (5.1 +/- 0.08 fold), Src activation (pSrc, 1.8 +/- 0.02 fold), and DNMT3A levels (1.5 +/- 0.07 fold) than ALDH1- cells, supporting the notion that Src and Bmi1 are drivers of ovarian CSCs in this model (Figure 3.11A). Furthermore, ALDH1+ population also expressed lower miR-128 than ALDH1- cells (Figure 3.11B).

The ALDH1+ population contained more sphere forming cells than ALDH1- cells (Figure 3.11C). VEGFA treatment of sorted populations for 7 days significantly increased the % of sphere forming cells only in the ALDH1+ (p=0.0072), not the ALDH1- subpopulation.
Thus, prolonged VEGFA exposure increases the abundance of sphere forming and ALDH1+ cells, but these effects are limited to an ALDH1+ target population. To further investigate VEGFA effects on ovarian CSCs, ALDH1 positive and negative cells were isolated by flow cytometry and grown with or without VEGFA over 8 days. The number and phenotype of their progeny was assayed every 2 days. The progeny

**Figure 3.11: Effect of VEGFA on ALDH+ population maintenance** (A-E) ALDH1 positive and negative cells were flow sorted. (A) Westerns show Bmi1, pSrc, Src, and DNMT3A level in sorted ALDH1+ and ALDH1- PEO1R cells. (B) miR-128 expression in ALDH1+ and ALDH1- PEO1R cells. Data are graphed as mean +/- SEM for at least 3 different biologic repeat assays. Differences between two groups were compared by Student’s T test. **p<0.01. (C) Sorted ALDH1+ and ALDH1- PEO1R cells were plated into sphere assays +/- VEGFA. Spheres >75 μm were counted at 14 days. Data are graphed as mean +/- SEM for at least 3 different biologic repeat assays. Differences between two groups were compared by Student’s T test. **p<0.01. (D) Sorted ALDH1+ and ALDH1- cells were reseeded into culture. The proportion of ALDH1- or ALDH1+ cells arising from ALDH1+ cells over the next 8 days in culture was serially assayed and graphed as mean% +/-SEM. (E) Growth curves of ALDH1+ progeny arising from ALDH1+ cells over 8 days in culture with or without added VEGFA. All experimental data is derived from at least 3 biological repeat assays.
of both ALDH1+ and ALDH1- cells increased at similar rates in 2D culture (Figure 3.12A).

The growth of either ALDH1+ or ALDH1- populations over the next 8 days was similar with or without VEGFA exposure (Figure 3.12B and C). Untreated ALDH1+ cells gave
rise to ALDH1-negative cells over time, compatible with asymmetric cell division (Figure 3.11D). VEGFA exposure increased the number of ALDH1+ cells generated from ALDH1+ cells compared to vehicle treated controls: the fraction of ALDH1+ cells remained higher and VEGFA decreased the generation of ALDH1- from ALDH1+ precursors (Figure 3.11D). That VEGFA-treated ALDH1+ cells produced more ALDH1+ progeny over time with VEGFA treatment (Figure 3.11E), is compatible with a VEGFA-induced shift from asymmetric division toward self-renewal of ALDH1+ ovarian CSC. Notably, sorted ALDH1- cells yielded only ALDH1- progeny over time (Figure 3.12D). Taken together, our data are consistent with baseline activation of Src governing $DNMT3A$ expression and Bmi1 in the ALDH1+ population. This pathway appears to be further activated following VEGFA stimulation.

3.3.9 High VEGFA and decreased miR-128-2 associate with poor OVCA outcome

High intra-tumor VEGFA correlates with poor outcome for various cancers, but OVCA studies have been limited by small numbers and not all show significance (Yu et al., 2013). A recent OVCA meta-analysis indicated high VEGFA associates with poor outcome, but reported heterogeneity among studies and a greater prognostic import for VEGFA in early than in late stage disease (Yu et al., 2013). We evaluated VEGFA expression by Kaplan-Meier analysis in over one thousand primary OVCA (n=1,305 for progression free survival, PFS; and n=1,581 for overall survival, OS) using http://kmplot.com/analysis. OVCAs with reduced VEGFA expression had significantly better PFS, shown for the lowest quartile of VEFGA [HR 1.37, 95% CI (1.18-1.6), p = 3.9e-05] and below the median [HR 1.23, 95% CI (1.08-1.41), p = 0.0019] (Figure 3.13A).
Figure 3.13: Prognostic value of VEGFA and miR-128-2 expression in ovarian cancer patients. (A) Kaplan-Meier plot of progression free survival (PFS) and overall survival (OS) of OVCA patients, stratified by VEGFA expression using KM Plotter (http://kmplot.com/analysis/). Patients with available clinical data: PFS, n=1305; and OS, n=1581. (B) Kaplan-Meier plot of PFS and OS of TCGA high grade serous ovarian cancer (HGSOC) patients, stratified by miR-128-2 expression using cBioPortal (http://www.cbioportal.org/). Patients with available clinical data: DFS, n=338; and OS, n=350. (C) Workflow for TCGA data classification. Clinical data for HGSOC disease free survival (DFS) were obtained from cBioportal are graphed by VEGFA and miR-128-2 levels.
Among four hundred and eighty five primary high grade serous ovarian cancers (HGSOC) in The Cancer Genome Atlas (TCGA) database with gene and miRNA expression, and outcome data available, tumors with the lowest miR-128-2 associated with the worst disease free survival (DFS, n=396, \( p=0.038 \)) and overall survival (OS, \( n=485 \), \( p=0.023 \)) (Figure 3.13B). HGSOC in the top \( VEGFA \) expression quartile with the lowest quartile of miR-128-2 expression showed a significantly worse disease free survival (DFS) than those with high miR-128-2 [DFS 14.8 vs 21.6 mo; HR 1.98, 95% CI (1.04-3.75), \( p=0.034 \)] (Figure 3.13C), supporting the notion that a subset of aggressive OVCA have high VEGFA-driven loss of miR-128-2.

### 3.4 DISCUSSION

Although increasing data suggest a cancer stem-like cell subpopulation mediates treatment resistance and disease recurrence, the extracellular growth factors and pathways that mediate stem-like cell expansion are still too poorly defined to permit specific targeting. Present data provide novel evidence that VEGFA, produced by OVCA cells and their stroma, works through VEGFR2 to stimulate Src activation and increase CSC. Rapidly growing tumors experience central hypoxia as growth outstrips new vessel formation. VEGFA, whose expression is induced in hypoxia, and would support both angiogenesis and expansion of stem-like cancer cells. While VEGFA may not be required for basal CSC maintenance in Src-activated OVCA, hypoxia would permit VEGFA to further stimulate Src, induce \( DNMT3A \) above basal levels required for ongoing CSC maintenance, leading to hypermethylation and silencing of miR-128-2, increasing Bmi1 to expand CSC. Bmi1 is part of the multiprotein PRC complex and modest increases in its levels can change
complex stoichiometry with significant epigenetic consequences. VEGFA-driven Bmi1 upregulation is followed by increases in cMyc, KLF4, and Sox2, all major drivers of CSC self-renewal, consistent with observations that Bmi1 regulates ES-TFs, including Sox2 and Klf4 (Molofsky et al., 2005; Siddique and Saleem, 2012).

Tumor suppressor miRs are frequently extinguished in cancers. For example, miR-148a, miR-34b/c and miR-9 downregulate oncogenes, including c-MYC, E2F3, CDK6 and TGIF2 and are often reduced in cancers. 5’azacytidine restored miRNA expression, indicating that DNA methylation repressed these tumor suppressor miRNAs to increase metastasis (Suzuki et al., 2012b). Methylation also downregulates miR-129-2 to induce SOX4 (Croce, 2009), and miR-143 to upregulate the MLL-AF4 oncogene in leukemia (Dou et al., 2012). While many such miRNAs are methylation sensitive, the specific methyltransferases governing their expression have not been identified. Present work links a cytokine, VEGFA, with Src pathway activation and identifies DNMT3A as a novel regulator of site-specific miR-128-2 methylation, Bmi1 upregulation and expansion of stem-like OVCA cells. Our data are consistent with recent findings that DNA methyltransferase inhibition by SGI-110 reduced the abundance of OVCA cells with stem-like properties including ALDH1+ and tumor initiating cells, and re-sensitized cisplatinum-resistant A2780 cells to platinum therapy (Wang et al., 2014). In both the PEO1R line and primary OCI-C5X culture, miR-128 expression was strongly inversely correlated with miR-128-2 methylation (R2 = -0.7513 and R2 = -0.9559, respectively). Interestingly, the early passage primary OVCA culture, OCI-C5X showed considerably less miR-128-2 methylation and higher miR-128-2 expression than the extensively passaged, highly
tumorigenic PEO1R line, potentially reflecting greater malignancy of the original PEO1R tumor in vivo or progression during culture.

Present data suggest that in contrast to hematologic malignancies where inactivating *DNMT3A* mutations reveal a tumor suppressor role (Yang et al., 2015; Fernandez et al., 2012), DNMT3A plays an oncogenic role as a key effector of VEGFA-driven ovarian CSC expansion. Epigenetic regulators, including EZH2 and DNA methyltransferases appear to have dual roles, acting as tumor suppressors or oncogenes in different malignancies, depending on the differentiation state of cells undergoing malignant change (Fernandez et al., 2012). Present work suggests DNMT3A also plays a dual role. In addition to its tumor suppressor in leukemia, DNMT3A also mediates stem-like cell expansion in ovarian cancers. VEGFA induced *DNMT3A*, but not related *DNMT3B* or *DNMT1*, and *DNMT3A* knockdown prevented VEGFA-mediated miR-128 loss and the increases in Bmi1 expression and OVCA tumor sphere formation in vitro. *BMII* knockdown abolished VEGFA-dependent increases in sphere formation and in ovarian tumor initiating cells in vivo. DNMT3A appears to act downstream of Src, which is constitutively activated in over 70% of OVCA (Simpkins et al., 2012), and is further stimulated by VEGFA to promote tumorigenesis via miR-128-2 methylation, increasing Bmi1 and CSC. Bmi1 thus emerges as a critical mediator of VEGFA-driven ovarian CSC expansion. Notably, Bmi1 overexpression induces chemotherapy and radiation resistance in various cancers (Siddique and Saleem, 2012) and platinum resistance in OVCA (Wang et al., 2011) compatible with a role for Bmi1 to drive treatment resistant ovarian CSC.

OVCA is usually diagnosed late, and despite initial chemo-responsiveness, resistant subpopulations emerge rapidly and lead to patient demise (Winter, III et al., 2007). In
contrast to other cancers, where bevacizumab has been less encouraging, several clinical trials support the use of bevacizumab with chemotherapy for advanced or recurrent ovarian cancer (for review, see (Eskander and Tewari, 2014)). The GOG218 trial of 1,873 advanced OVCA patients, showed bevacizumab added to carboplatinum and paclitaxel delayed disease progression (Burger et al., 2011). In earlier stage OVCA, bevacizumab also prolonged the disease free interval before progression (Perren et al., 2011). Two trials for recurrent OVCA, showed bevacizumab also delayed disease progression in this context (Eskander and Tewari, 2014). Ongoing Phase 3 trials are evaluating other VEGFA/VEGFR targeted therapies. Of these, the ICON6 trial of chemotherapy with or without the VEGFR inhibitor, cediranib, for platinum sensitive recurrent OVCA is notable, since it is the first antiangiogenic trial to report improvement of both progression free [HR 0.57, 95% CI (0.45-0.74)] and overall survival [HR0.70, 95%CI (0.51-0.99)] (Eskander and Tewari, 2014). Despite these promising findings, the median extension of the disease free interval is <6 months (Eskander and Tewari, 2014) and disease progression is inevitable.

Recurrent cancers are enriched for stem-like cells. OVCA cells bearing stem-cell like markers CD44 and CD133, and ALDH1 activity are increased after chemotherapy (Shah and Landen, 2014). A recent meta-analysis showed elevated intratumor and, more significantly, serum VEGFA in OVCA both significantly associate with drug resistance and death (Yu et al., 2013). Our analysis of over one thousand OVCA, largely comprised of the most aggressive HGSOC subtype, shows those with the lowest intratumor \textit{VEGFA} expression have significantly improved survival. Among HGSOC in the highest VEGFA expression quartile, those with decreased miR-128 levels show a significantly worse outcome, supporting the existence of an aggressive HGSOC subset with VEGFA-driven
miR-128 repression. There are few datasets with gene, miRNA and clinical OVCA data available. The present analysis provides important in vivo human data to confirm the molecular pathway identified herein. Taken together, present findings link high VEGFA to treatment resistance and death through stimulation of ovarian CSCs.

Present work illuminates the limited efficacy of bevacizumab and other antiangiogenic/chemotherapy regimens. VEGFA blockade, by interrupting angiogenesis, creates tumor hypoxia. Hypoxia stimulates HIF-1α dependent VEGFA induction (Goel and Mercurio, 2013), which would stimulate the most aggressive hypoxia tolerant, chemoresistant CSC to reseed local and metastatic niches. Indeed tumors surviving bevacizumab were shown to have increased CSC, due to the effects of hypoxia to upregulate VEGFA (Conley et al., 2012). VEGFA can act via VEGFR2 (Zhao et al., 2014) and via the neuropilin receptor (Goel and Mercurio, 2013) to drive VEGFA induction and CSC expansion in breast models. The present study reveals a novel rationale and a potential strategy for targeting VEGFA pathways in OVCA. While bevacizumab generates a resistance cycle, combined use of drugs that inhibit VEGFA signaling at multiple levels, such as inhibitors of VEGFR, Src and/or DNA methyltransferases, might hold greater promise as stem-like cell-directed treatments.
CHAPTER 4

p27 PHOSPHORYLATION DRIVES PYK2-DEPENDENT EXPANSION OF CANCER STEM CELLS VIA ACTIVATION OF A NOVEL p27 REGULATED TRANSCRIPTIONAL PROGRAM
4.1 SUMMARY

p27 is known as a cell cycle inhibitor and a tumor suppressor. However, evidence is accumulating that mis-regulated p27 has oncogenic effects. Previously, our group showed that double phosphomimetic p27 (p27CK-T157D/T198D) upregulates epithelial-mesenchymal transition (EMT) and the metastatic potential of cancer cell lines. In addition to its action to promote EMT, p27 appears to promote CSC expansion and or maintenance. Here we observed that C-terminally phosphorylated p27 increased CSC properties, including self-renewal, CSC markers and colony formation in soft agar. p27CK-DD increased the expression of several embryonic stem cell transcription factors (ES-TFs), including SOX2, NANOG and cMYC, which are known drivers of ES self-renewal. A human phospho-kinase array showed Pyk2 is activated in p27CK-DD expressing MCF-12A. This was confirmed in other lines and we showed p27-knockdown reduced Pyk2 activity, tumor spheres, ALDH1 activity and ES-TF expression. Treatment with a Pyk2 inhibitor, and PYK2-knockdown by siRNA or ShRNA revealed that Pyk2 is a key mediator of the increase in tumor spheres, ALDH1 activity and ES-TFs expression and of the increased abundance of tumor initiating stem cells in vivo in cancer cells expressing abundant C-terminally phosphorylated p27. These data reveal a novel mechanism whereby p27-driven Pyk2 activation promotes CSC expansion and tumor progression.

4.2 INTRODUCTORY REMARKS

p27Kip1 binds and inhibits Cyclin E and A bound Cyclin dependent kinase 2 (Cdk2) and also assembles cyclin D-Cdks (Cheng et al., 1999; LaBaer et al., 1997). p27 integrates mitogenic and growth inhibitory signaling networks to govern cell cycle exit (Chu et al.,
p27 levels and Cdk inhibitory activity are increased by differentiation signals and growth arrest signals (Hengst et al., 1994; Liang and Slingerland, 2003; Sherr and Roberts, 1999) in normal epithelial cells. In G0 and early G1, p27 translation and protein stability are maximal and it inactivates Cyclin E-Cdk2 (Sherr and Roberts, 1999; Hengst et al., 1998). p27’s Cdk-inhibitory activity is controlled by its levels, localization and phosphorylation (Chu et al., 2008). p27 translation falls rapidly on G0 exit (Gillies and Lorimer, 2007; Gopfert et al., 2003) and its proteolysis increases dramatically in late G1/S (Nakayama et al., 1996; Pagano et al., 1995), permitting Cyclin E-Cdk2 and Cyclin A-Cdk2 activation and G1-S progression (Nigg, 1993). The most potent p27 proteolytic pathway involves ubiquitination by E3 ligase S-phase kinase associated protein, Cullin, F-box complex (SCF^Skp2), whose binding to p27 is stimulated by p27 phosphorylation at T187 by Cyclin E or A-Cdk2 (Nakayama and Nakayama, 2006). Previously, we showed that p27 phosphorylation by Src (Chu et al., 2007b) and Abl (Grimmler et al., 2007) at tyrosine 74 (Y74), Y8 and Y89 in early G1 causes its ejection from the catalytic cleft of Cdk2 (Chu et al., 2007b) to activate SCF^Skp2-mediated p27 proteolysis in late G1/S (Chu et al., 2007b; Grimmler et al., 2007). We and others showed p27 proteolysis is hyperactivated in cancers (Chu et al., 2008).

p27 localization is regulated by phosphorylation. In normal quiescent cells, p27 is exclusively nuclear (Connor et al., 2003). In early G1, mitogens activate p27 S10 phosphorylation (Boehm et al., 2002; Deng et al., 2004) to promote CRM1-mediated p27 nuclear export (Connor et al., 2003; Ishida et al., 2002; Ishida et al., 2000). In early G1, p27 phosphorylation by AKT at T157 within its nuclear localization signal (NLS) (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002) delays p27 nuclear import (Liang et
al., 2002), and T198 phosphorylation by AKT (Fujita et al., 2002; Fujita et al., 2003) stabilizes p27 (Kossatz et al., 2006; Liang et al., 2007), leading to accumulation in both the nucleus and cytoplasm.

p27 is aberrantly regulated by several oncogenic signaling pathways in cancer (Chu et al., 2008). While p27 is rarely completely lost, it is frequently deregulated: nuclear p27 protein is reduced and/or p27 is mislocalized to the cytoplasm in a majority of human cancers. Normal epithelial cells express high nuclear p27 (Catzavelos et al., 1997), but human cancers often have reduced nuclear p27 (Slingerland and Pagano, 2000; Tan et al., 1997; Tsihlias et al., 1998) due to increased p27 proteolysis, associated with poor prognosis (Singh et al., 1998; Slingerland and Pagano, 2000). Consitutive activate ot the PI3K pathway leads to constitutive phosphorylation of p27 at T157 and R198 and p27pTpT has been shown to bind a number of non-cyclin-CDK partners to drive cell cycle independent pro-oncogenic actions contributing to cancer progression and metastasis. p27 phosphorylation at T198 promotes binding to RhoA, inhibition of RhoA-RACK, destabilization of actin and increased cell motility (Besson et al., 2004; Larrea et al., 2009a). p27 is a major mediator of metastasis downstream of activated PI3K (Wander et al., 2013; Denicourt and Dowdy, 2004; Wu et al., 2006a). p27 also appears to drive the activation of the epithelial to mesenchymal transition (EMT). Recently, we showed p27pTpT binds to JAK2, recruits STAT3 and drives STAT3 activation, TWIST1 induction, EMT and metastasis in vivo (Zhao D. et al., 2015b).

Just as normal tissues are maintained by stem cells, many cancers appear to be sustained by tumorigenic cancer stem cells (CSC) that self-renew and yield heterogenous progeny (Dalerba et al., 2007). CSCs may mediate chemotherapy resistance, recurrence
and metastasis (Takebe et al., 2011). CSCs from breast cancers form tumor spheres in 3D culture (Kakarala and Wicha, 2007). Breast cancer cells with aldehyde dehydrogenase activity (ALDH1+) and/or surface CD44+CD24low/- expression are enriched for tumor initiating stem cells (Al Hajj et al., 2003; Ginestier et al., 2007). Recent data connect EMT and CSCs, overexpression of EMT transcription factors increases CSC abundance, suggesting that EMT may promote both the tumor dissemination and CSC self-renewal needed for metastasis (Mani et al., 2008; Morel et al., 2008; Wellner et al., 2009).

In Xenopus, the p27 homologue, Xic1 serves non-cell cycle dependent functions to promote normal tissue differentiation during myogenesis (Messina et al., 2005; Vernon and Philpott, 2003) and cardiogenesis (Movassaghi and Philpott, 2008). In p27CK-knock in mice, p27CK- accumulates to high levels and 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 the EMT process has been shown to be closely linked to acquisition of stem-like properties in cancer populations, and p27 mediates activation of EMT, we tested its potential role as a driver of cancer stem cell expansion. Here we identify Pyk2 as a mediator of p27pTpT-driven upregulation of tumor sphere formation and ALDH1 activity, expression of CMyc, Sox2, Oct 4 and KLF4 to increase the tumor initiating stem cell population and tumor initiation in vivo and tumor metastasis. Thus, PI3K-driven C terminal phosphorylation of p27 not only activates EMT via STAT3 and cJun activation, it also induces genes that mediate cancer stem cell expansion to promote tumor formation and metastasis.
4.3 RESULTS

4.3.1 p27CK-DD increases sphere formation and cancer stem cell surface marker.

While p27 is known as a tumor suppressor in breast cancer, we found that c-terminally phosphorylated p27 can promote EMT, cell migration and invasion through JAK/STAT3 pathway activation (Zhao D. et al., 2015b). Since recent work links acquisition of EMT with an increase in cancer stem cells (Mani et al., 2008), we hypothesized that p27 c-terminal phosphorylation might also mediate breast cancer stem cell expansion.

Since the stem cell and transcriptional regulatory actions of p27 do not appear to require Cyclin Cdk binding, we generated p27CK-DD mutant which has phosphor-mimic aspartic acid instead of threonine at 157 and 198 amino acids, and has mutational activation of CDK-cyclin binding potential (Vlach et al., 1997).

p27CK-DD was introduced into breast cancer or normal breast cell lines and its effects on sphere formation assayed. p27CK-DD significantly increased sphere formation in MCF12A, MCF7 and MDA-MB-231 cells (Figure 4.1A). In MCF12A, p27CK-DD transduction increased expression of CD44, a CSC marker in many human cancers and increased the proportion of cells with stem cell markers CD44+CD24−/low (Figure 4.1B). The oncogenic potential of p27CK-DD was also shown by acquisition of anchorage-independent growth in two immortal human mammary epithelial lines, MCF-12A and HME3 (Figure 4.1C). We next assayed if p27 loss would decreased CSC in cell lines with high metastatic potential. MDA-MB-231-4175 (hereafter, 4175) was derived from MDA-MB-231 (231), through selection for increase lung metastatic ability following tail vein injection (Minn et al., 2005). The 4175 line has hyper-activation of PI3K/AKT signaling.
causing more endogenous p27pT157pT198 (p27pTpT) (Zhao D. et al., 2015b). Loss of p27pTpT through p27 knockdown decreased sphere formation in the highly metastatic 4175 cells (Figure 4.1D). Thus, p27 c-terminal phosphorylation increases cancer stem cell properties including abundance of sphere forming cells, CD44+CD24−/low populations, and colony forming cells. Similar loss of sphere forming ability was demonstrated for MDA-MB-231-1833, a line with high metastasis to bone (Kang and Pantel, 2013).
4.3.2  **p27CK-DD increases embryonic stem cell transcription factor expression.**

As mentioned earlier, several embryonic stem cell transcription factors (ES-TFs) govern embryonic stem cells (ES) self-renewal, induce pluripotency in skin fibroblasts (Li, 2010) and have been implicated in CSC self-renewal in a number of different cancers (Zhang et al., 2008b). To assay their potential involvement in p27 c-terminal phosphorylation derived cancer stem cell expansion, we tested p27CK-DD effects on MCF12A and MDA-MB-231. p27CK-DD transduction in MCF12A also increased expression of embryonic stem cell transcription factor (ES-TF) **NANOG**, a major driver of ES self-renewal (MCF12A in Figure 4.2A). Notably, while in normal ES cells, p27 co-represses **SOX2** with p130, E2F4, SIN3A (Li et al., 2012), in 231, the phosphomimetic p27CK-DD caused induction of **SOX2**, **NANOG** and **MYC**, key drivers of ES and CSC self-renewal (Figure 4.2B). Conversely, loss of p27pTpT through p27 knockdown in highly metastatic 4175 decreased **SOX2**, **NANOG** and **MYC** expression (Figure 4.2C). Findings in breast cancer models were confirmed in a second set of sister cell lines with high and low metastatic potential derived from a human bladder cancer. A highly lung metastatic variant of the bladder cancer line, UMUC3, was derived through serial tail vein injections in nude mice. The highly lung tropic metastatic variant UMUC3Lul2 is also PI3K activated and expresses high p27pTpT (Zhao D. et al., 2015b). p27 knockdown decreased SOX2 and MYC expression in highly metastatic bladder cancer cell line UMUC3Lul2 to levels similar to those seen in parental UMUC3 (Figure 4.2D). Thus, p27pTpT appears to drive ES-TF expression.
4.3.3 *p27*CK-DD induces Pyk2 activation which increases cancer stem cell properties.

A kinome screen showed p27CK-DD expression in MCF12A activates multiple kinases including pSTAT3, PLCγ and Pyk2 (Screen data published in Besser et al. unpublished). Pyk2 is a mediator of cell motility in many normal cell types (Schaller, 2010) and can drive cancer invasion and metastasis (Kuang et al., 2013). We next tested if Pyk2 mediates effects p27CK-DD on CSCs. p27CK-DD overexpression caused Pyk2 activation in 231, and loss of phosphorylated p27pTpT through ShRNA mediated p27 knockdown
attenuated Pyk2 activation in 4175 (Figure 4.3A). To test the effects of Pyk2 inhibition on sphere forming population, cells were treated with Pyk2 inhibitor, PF431396, for 48 hours, followed by 48 hours washout to allow recovery of asynchronous cycling prior to seeding into sphere assays. Pyk2 inhibition decreased sphere formation from DMSO treated 231 controls and abolished the p27pTpT-mediated increase in sphere formation in 4175 cells compared to DMSO treated 231 controls (Figure 4.3B, C). siRNA Pyk2 knockdown abolished the increased sphere formation mediated by p27CK-DD overexpression in 231 cells (Figure 4.3D, E). Furthermore, Pyk2 knockdown prevented the p27CK-DD mediated increase in ALDH1+ cell abundance in MDA-MB-231 cells (Figure 4.3F). Thus, Pyk2 kinase action is required for the p27CK-DD-mediated increase in the abundance of both sphere forming cells and in ALDH1+ cells.

Figure 4.3: p27CK-DD induces Pyk2 activation which increases cancer stem cell properties. (A) p27CK-DD expression increases Pyk2 phosphorylation in MDA-MB-231, MCF12A and UMUC3 cells, and knockdown of p27 reduces Pyk2 phosphorylation in highly metastatic cells. (B-C) Pyk2 inhibitor decreases sphere formation. Data in bar graphs represent mean +/-SEM for at least 3 different biologic repeat experiments. Differences between two groups were compared by Student’s T test. *p<0.05. (D-E) Pyk2 knockdown decreases p27CK-DD mediated sphere formation. Data in bar graphs represent mean +/-SEM for at least 3 different biologic repeat experiments. Differences between two groups were compared by Student’s T test. *p<0.05. (F) Pyk2 knockdown decreases p27CK-DD mediated increase in ALDH1+ population. Data in bar graphs represent mean +/-SEM for at least 3 different biologic repeat experiments. Differences between multiple groups were compared by ANOVA. *p<0.05.
4.3.4 Increased ES-TFs expression by p27CK-DD is Pyk2 activation dependent.

To further investigate the potential involvement of Pyk2 in the p27CK-DD mediated increase in cancer stem properties, we tested Pyk2 effects on ES-TFs in MDA-MB-231 using shRNA of PYK2. As noted earlier, p27CK-DD transduction increased NANOG, SOX2, and MYC expression (see Figure 4.4). ShPYK2 abrogated the p27CK-DD-mediated increase in Sox2, Nanog and MYC expression in mRNA and protein levels (Figure 4.4A, B). Loss of c-terminal phosphorylation of p27 following 48 hrs treatment with a PI3K/mTOR inhibitor PF1502 also decreased Sox2 and Myc expression (Figure 4.4C). Thus, Pyk2 appears to govern basal ES-FTs expression and is required for the p27CK-DD-mediated increase in ES-TFs expression in the 231 model.

Figure 4.4: Increased ES-TFs expression by p27CK-DD is Pyk2 dependent. (A) Loss of PYK2 decreases SOX2, NANOG, and cMYC expression in 231 p27CK-DD cells. Data in bar graphs represent mean +/-SEM from at least 3 different biologic repeat experiments. Differences between multiple groups were compared by ANOVA. *p<0.05, ‡p<0.001, #p<0.0001. (B) PYK2 knockdown in MDA-MB-231 reverses p27CK-DD mediated Sox2, Nanog and cMyc upregulation. (C) Akt inhibition in 1833 reverses p27pTpT mediated Sox2, cMyc upregulation.
4.3.5 C-terminally phosphorylated p27 increases breast tumor initiating cells via Pyk2

Effects of C-terminally phosphorylated p27 and Pyk2 on breast tumor initiating cell abundance were further investigated in vivo. Limiting dilution tumor initiating cell assays were carried out, injecting between 10 and 1,000 cells with or without p27 CK-DD

Figure 4.5: The C-terminally phosphorylated p27 mediated increase in tumor initiating stem-like cell abundance in vivo is Pyk2 dependent. (A) Tumor formation from limiting dilutions of inoculated cells (1,000, 100, 10 cells) is graphed as % of tumor free animals/time (weeks). (B) Tumor initiating cell frequency was calculated by L-Calc Limiting Dilution Software (http://www.stemcell.com/en/Products/All-Products/LCalc-Software.aspx) from STEMCELL Technologies.
transfection in 231 and with or without PYK2 knockdown in 231 cells. In addition, T-ISC formation in 1833 compared to the 231 and we assayed effect of p27 knockdown in 1833. These showed that C-terminal phosphomimetic p27CK-DD increased 231 tumor-initiating stem cell (T-ISC) abundance. The PI3K activated 1833 that have high endogenous p27pT<TpT (Zhao D. et al., 2015b) showed higher T-ISC frequency (1/14) than parental 231 pLVX-EGFP-scrambled-ShRNA (1/1,032) and knockdown of p27 decreased both T-ISC frequency (1/402) and tumor latency.

(Figure 4.5A). The tumor initiating cell frequency in 231-p27CK-DD was 1/175, compared with 1/1,032 in parental 231 pLVX-EGFP-scrambled-ShRNA cells and decreased to 1/2,845 in 231-p27CK-DD cells with shRNA to PYK2, as calculated by L-Calc™ Limiting Dilution Software (Figure 4.5B). Thus, C-terminally phosphorylated p27 increases tumor initiating breast cancer cell abundance in vivo and this is Pyk2 dependent.

This loss of the tumor initiating cell frequency could not be attributed to changes in cell cycle, since neither Pyk2(PTK2B) knock-down nor p27(CDK1B) knock-down affected cell cycle profiles prior to assay (Figure 4.6).
4.3.6 C-terminally phosphorylated p27 associates with Pyk2

While we could confirm that p27 driven CSCs expansion is Pyk2 dependent, the mechanisms whereby p27 activates Pyk2 is not clear. Pyk2 activation is mediated by many different associated proteins in different cell contexts (Table 1.1). p27 immunoprecipitation revealed Pyk2 binds cellular p27 in parental control 231 pLVX-EGFP cells, and this complex was increased in p27CK-DD-transduced cells (Figure 4.7A and B). Treatment with the PI3K inhibitor, PF1502 250nM for 48 hr decreased AKTp4S473 and p27pT198 (Figure 4.7C, Western blot on left). p27pTpT is required for p27-Pyk2 binding, since PI3K decreased p27pT198 and decreased the amount of p27-bound Pyk2 on p27 IP blot (Figure 4.7C). These data indicate that p27 phosphorylation is PI3K dependent in UMUC3LuL2 and is required for Pyk2 binding.

Figure 4.6: Cell cycle distribution of cells used in sphere assays and/or in tumor initiating stem cell assays. Cell cycle distribution was assayed immediately prior to plating into sphere formation or prior to injection into nude mice for limiting dilution stem cell assays. Data in bar graphs represent mean +/-SEM for at least 3 different biologic repeat experiments. Differences between multiple groups were compared by ANOVA. *p>0.05.
4.4 DISCUSSION

The tumor initiation and cancer stem cell expansion are thought to be the main cause of tumor recurrence, drug resistance and metastasis. There has been significant progress in recent decades in our understanding of cancer stem cells. Until recently, the lack of knowledge of molecular mechanisms underlying cancer stem cell expansion has limited development of therapies.

Here, we provide a noble evidence that deregulated p27 plays a cell cycle-independent, oncogenic role to drive a cancer stem cell expansion in human breast and bladder cancer models. Overexpression of p27CK-DD, which mimics p27 phosphorylation at T157 and T198 by PI3K/AKT signaling in human cancers, increased cancer stem cell properties (tumor spheres, ALDH1 activity, and ES-TF expression). In contrast, p27 knockdown in highly metastatic cells enriched for p27pTpT decreased cancer stem cell properties. Furthermore, our xenograft experiments revealed that the C-terminal phosphorylated p27 is a critical driver of cancer stem cell expansion via activation of Pyk2 and drives tumor initiation in vivo.

It is thought that CSC, but not the bulk population of a solid tumor, can initiate tumor formation and maintain the malignant population through self-renewal and generation of more differentiated progeny with a more limited self-renewal ability. Our findings demonstrate that expression of p27CK-DD is sufficient to mediate cancer stem cell expansion in vitro and in vivo. In MCF12A, an immortal but non-malignant breast epithelial line, and the MCF7 and MDA-MB-231 breast cancer models, p27CK-DD increased sphere formation, cancer stem cell surface marker, and soft agar colony formation. Conversely, in the highly PI3K-activated metastatic 1833 and 4175 breast
cancer cell line with high p27pTpT, p27 knockdown reverted the CSC self-renewal and ES-TFs expression.

CSC self-renewal is maintained by several ES-TFs including Sox2, Nanog and c-Myc. These ES-TFs govern CSC properties both independently and together. Here, we showed that overexpression of p27CK-DD in breast normal or cancer cells markedly increased one or more of Nanog, Sox2 or c-Myc at both mRNA and protein levels, in the different models assayed. Furthermore, p27 knockdown in the p27pTpT-enriched highly metastatic cancer lines, 4175, decreased ES-TFs. Similar findings were observed in the UMUC3-LuL2 bladder cancer model. Highly metastatic 1833, 4175 and UMUC3-LuL2 lines have higher PI3K activation and higher total and p27pTpT than their low metastatic counterparts (Zhao D. et al., 2015b; Wander et al., 2013) and p27 knockdown substantially reduced the ES-TFs expression in both breast and bladder models.

Aberrant expression and activation of Pyk2 occurs in many types of cancers including breast cancer (Behmoaram et al., 2008; Kuang et al., 2013). Activated Pyk2 plays critical role in the malignant transformation and tumor progression. Recent data suggest that constitutive activation of Pyk2 also contributes to CSC expansion and breast cancer metastasis in Her2+ 231 cells. However, this study did not investigate CSC properties in multiple aspects (Behmoaram et al., 2008). We found that p27binds Pyk2 in a Pi3K-dependent and p27 phosphorylation dependent manner. Both total and phosphorylated Pyk2 (Y402) were significantly increased by p27CK-DD in normal epithelial cells and in cancer cells. Conversely, knockdown of p27 in p27pTpT enriched cancer cells decreased total and phosphorylated Pyk2. Pharmacological inhibition of Pyk2 or knock-down of Pyk2 reversed p27CK-DD-mediated CSC self-renewal, indicating Pyk2 was critical for p27
induced CSC self-renewal. Pyk2 inhibition attenuated p27CK-DD induced ES-TFs expression. Furthermore, Pyk2 inhibition significantly attenuated the p27CK-DD-mediated increase in tumor initiation \textit{in vivo}. Thus Pyk2 signaling appears to play a critical role in the maintenance of p27-driven CSC self-renewal and tumor initiation.

Notably, the PI3K inhibitor significantly reduced Pyk2 activation. The loss of Pyk2 activity with PI3K inhibition reflects the loss of p27pTpT-mediated Pyk2 activation.

We previously identified a p27-driven EMT mechanism whereby p27 scaffolds a JAK2/STAT3 complex to enhance STAT3 activation and subsequent \textit{TWIST1} induction (Zhao D. et al., 2015a). Here, we show p27pTpT binds another novel pro-oncogenic partner, Pyk2. p27pTpT is required for p27-Pyk2 binding, since PI3K inhibition decreased p27pT198 and decreased p27-binding to Pyk2.

In summary, we have uncovered a novel oncogenic function of p27 to induce CSC expansion in both breast and bladder cancer models. p27pTpT is observed in human cancers including breast, esophageal, colon, and prostate cancers (Chu et al., 2008) and has been correlated with adverse outcome in prostate cancer (Li et al., 2006), glioma (Piva et al., 1999) and high-grade astrocytomas (Hidaka et al., 2009). In breast cancer, mislocalized p27 staining correlated with AKT activation (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002) and predicted decreased time to disease relapse (Liang et al., 2002) and was associated with increased lymph nodal metastasis and poor overall survival (Wander et al., 2013). Here we extend the understanding of p27 deregulation in cancer: p27pTpT activates Pyk2 to increase ES-TFs expression, in a manner critical for CSC expansion. Present data also raise the possibility that p27 may also bind to Pyk2 as a consequence of C-terminal phosphorylation by AKT. Combined inhibition of both AKT and Pyk2 in PI3K/mTOR
activated, p27pTpT enriched human cancers may ultimately have therapeutic potential to target cancer stem cell.
CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS
5.1 SUMMARY

Increasing evidence suggest that diverse solid tumors are hierarchically organized and sustained by a distinct subpopulation of tumorigenic cancer stem cells (CSC) with self-renew and tumor initiating ability (Dalerba et al., 2007). CSCs appear to play a critical role in tumor invasion, metastasis, drug resistance, and disease recurrence (Takebe et al., 2011). Work of this thesis in Chapter 3 provides evidence that VEGFA mediates stem cell promoting actions via VEGFR2-dependent Src activation to upregulate Bmi1, tumor spheres and ALDH1 activity in primary human OVCA culture and OVCA lines. DNA methyl transferase 3A (DNMT3A) appears to play a pro-oncogenic role downstream of Src to methylate miR-128-2, upregulating Bmi1 to increase stem like cells. Analysis of over 1,300 primary human OVCAs revealed an aggressive subset in which high VEGFA is associated with miR-128-2 miR-128-2 loss. Thus, these findings elucidate the limitations of current anti-angiogenic therapy in ovarian cancer, and provide a novel rationale for further preclinical and clinical investigation of antitumor efficacy of dual inhibition of VEGFA and either Src or DNMT3A in ovarian cancers. Chapter 4 provides evidence that C-terminally phosphorylated p27 increased CSC properties, including self-renewal, CSC markers, colony formation, and tumor initiation. p27CK-DD increased the expression of several embryonic stem cell transcription factors (ES-TFs), including \textit{SOX2}, \textit{NANOG} and \textit{cMYC}, which are known as an embryonic stem cell self-renewal driver. Our human phospho-kinase array revealed that Pyk2 is activated in p27CK-DD expressing cells and Pyk2 is decreased by p27 knockdown in highly metastatic p27pTpT enriched cell lines. We found that Pyk2 activation and its binding to p27 are dependent on C-terminal
phosphorylation of p27. Pyk2 inhibitor treatment or \textit{PTK2B} knockdown experiments showed Pyk2 is mediator of C-terminally phosphorylated p27 driven increase in CSC self-renewal and ES-TFs expression.

5.2 \textbf{FUTURE DIRECTIONS}

p27 is a globular protein whose C-terminal phosphorylation mediates novel protein-protein interactions (Larrea et al., 2009a; Besson et al., 2004; Larrea et al., 2008). We showed p27 co-precipitates with cellular STAT3 (PMID25684140) and Pyk2 (Figure 4.6). Here, we will test if p27pTpT promotes and STAT3 binding to and activation by Pyk2.

5.2.1 \textit{Effect of p27pTpT on cellular p27-Pyk2-STAT3 complexes}

Earlier, we showed p27pTpT promotes JAK2 binding to and activation of STAT3 (PMID25684140) (Zhao D. et al., 2015b). STAT3 can also be activated by Pyk2 (Shi and Kehrl, 2004a) and Pyk2 can translocate to the nucleus (Schindler et al., 2007; Aoto et al., 2002; Genua et al., 2012) and modulate gene expression (Schindler et al., 2007; Verma et al., 2015; Selitrennik and Lev, 2015). Pyk2 binds p27 in highly metastatic cell line models (Figure 4.6B) and p27-mediated STAT3 activation depends in part on Pyk2 activity (not shown). To test if tripartite p27-Pyk2-STAT3 complex forms \textit{in vivo}, we will test if IP of each protein can co-precipitate the other two. We will also test if p27 loss (Shp27) or loss of p27pTpT (by PI3K inhibition with PF1502, 250 nM (Wander et al., 2013), as in Figure 4.6B) decreases STAT3-Pyk2 binding on Co-IP in highly metastatic 4175, 1833 and UMUC3-Lul2. Conversely, we will test if p27CK-DD transduced into low metastatic lines
increases tripartite complex formation, and if p27CK-DD-STAT3 binding is decreased by Pyk2 knockdown (ShPyk2) or Pyk2 inhibitor (PF431396 500 nM).

5.2.2 **Effect of p27pTpT on ES-TF upregulation in primary breast cancers**

The Cancer Genome Atlas (TCGA) contains gene expression and The Cancer Proteome Atlas has corresponding proteomic data on over 740 primary human breast cancers and similar datasets exist for other solid tumors. We previously used these data to show p27pT198 and p27pT157 strongly associate with high levels of activated pAKT, pRSK and pSGK and pSTAT3 in breast cancer (Zhao D. et al., 2015b). Here, we will analyze these datasets to test for correlations between PI3K activation, p27pT157/p27pT198 levels, pPyk2 and increased RNA/protein expression levels of ES-TFs. Evidence for a strong correlation between these pathways would support *in vivo* relevance to human cancers and stimulate future pre-clinical efforts to test if either Pyk2, STAT3 inhibitors augment therapeutic efficacy of PI3K/mTOR inhibitors.

5.2.3 **Transcriptional targets of p27 activating CSC programs.**

A limited survey of p27 binding to gene promoters by ChIP/Chip in NIH3T3 cells recently showed p27 is recruited to p130-E2F4-bound promoter sites with HDAC1 and SIN3A to repress target genes (Pippa et al., 2012). This survey did not include all potential DNA binding sites (Pippa et al., 2012). p27 acts as a transcriptional co-repressor with p130, E2F4 and SIN3A to repress *SOX2* in quiescent normal ES cells (Li et al., 2012). We posit that p27 may switch from a transcriptional co-repressor in normal cells to a co-activator in cancer cells. p27CK-DD increased *SOX2*, *NANOG* and *MYC* expression in 231, and in
PI3K-activated 4175 cells with high endogenous p27pTpT, p27 knockdown decreased these ES-TFs (Figure 4.2). We will test if p27 acts as a transcriptional co-regulator, whose phosphorylation regulates transcription factor binding to co-repress or co-activate target genes.

Since we postulate that T198 and T157 phosphorylation switches p27 from a co-repressor to a co-activator, we will analyze expression of p27-bound genes in 4175, with or without 250 nM PF1502, a PI3K/mTOR inhibitor that potently decreases p27pTpT (Wander et al., 2013). We will carry out a comprehensive analysis combining chromatin immunoprecipitation (ChIP) with high throughput sequencing using the Illumina HiSeq platform to identify expression of stably p27-bound genes (ChIP-Seq). ChIP-Seq analysis will assess p27 gene occupancy with or without PF1502 (Wander et al., 2013) to test how C-terminal p27 phosphorylation affects p27-DNA binding profiles. Genome-wide p27 binding sites will be mapped in 4175 and 4175+PF1502.

These assays will identify which p27-bound genes (detected on ChIP-Seq) are differentially expressed in 4175 +/- PF1502 and in MCF-12A vs MCF12Ap27CK-DD. Gene expression differences in different lines/conditions will be compared to the genome-wide p27 occupancy studies to gain a comprehensive picture of how p27pTpT binding relates to gene expression. Loss of p27pTpT with 250nM PF1502 treatment (Wander et al., 2013) is expected to decrease both p27 binding to and activation of target genes driving CSC.

Embryonic stem cell transcription factors (ES-TFs) are master regulators of ES self-renewal that are also implicated in cancer (Puisieux et al., 2014). Since we postulate p27pTpT may induce ES-TFs to expand CSC, putative p27-target gene candidates to be
validated by promoter-/enhancer analysis below will be prioritized according to CSC self-renewal. These include ES-TFs that are increased by p27CK-DD: SOX2, NANOG, and MYC. Since p27pTpT activates STAT3 in a Pyk2-dependent manner, we will also compare p27 genomic binding with published STAT3 ChIP-Seq data (Durant et al., 2010; Yang et al., 2011; Zhang et al., 2013; Chen et al., 2008b). Genes co-occupied by both STAT3 and p27 will be selected and evaluated further for potential co-regulation.

5.2.4 Role of p27 as a co-repressor and co-activator of key drivers of stem cell self-renewal

p27 knockdown in highly metastatic 4175 decreased SOX2 MYC and NANOG expression (Figure 4.2C, D), and p27CK-DD transduction in 231 increased these ES-TFs (Figure 4.2A, B). Although these p27 effects may be indirect, p27 is known to bind the SOX2 regulator, SOX2-SRR2, as co-repressor with p130/E2F4/SIN3A (Li et al., 2012). Here, we will test if p27 shifts from a p130/E2F4/SIN3A co-repressor in serum starved cells or in lines with low endogenous PI3K activity, to a co-activator of SOX2 expression (and potentially of other ES-TFs) in cells with high PI3K activity and high p27pTpT. ChIP and ChIP-re-ChIP assays will test if cellular p27 binds with p130/E2F4/SIN3A to repress the SOX2-SRR2 in quiescent MCF-12A, 231 and MCF-7 and if this is lost in p27CK-DD transduced sister lines. We anticipate that these p27 co-repressor complexes would be lost after EGF/insulin stimulation of cellular PI3K and p27pTpT upregulation in parental lines, and in p27CK-DD transduced lines. We will focus this analysis on SOX2 and other putative p27-target genes (p27-TG) implicated by Figure 4.2 (ES-TFs: SOX2, NANOG, and MYC)
or on other CSC regulators identified in ChIPSeq/RNASeq above, particularly those also bound by STAT3.

5.3 CONCLUDING REMARKS

In cancers, p27 loss relieves Cyclin-Cdk inhibition to promote tumor progression. p27 also acquires pro-oncogenic protein-protein interactions to promote cancer invasion and metastasis when phosphorylated at T157 and T198 by AKT, SGK and RSK. We showed p27pTpT binds JAK2/STAT3 to activate STAT3 and induce TWIST1 and EMT. p27pTpT also increases spheres, CSC markers and ES-TF induction in vitro and may promote CSC self-renewal in vivo. p27pTpT binds to Pyk2 and STAT3, both of which promote EMT and metastasis, leading us to posit that p27pTpT may drive transcription programs governing EMT and CSC self-renewal. Experiments described above in future direction will test further how p27 increases CSC through Pyk2 activation. p27pTpT may serve as a scaffold for Pyk2/STAT3 signaling complexes to activate transcription. We will survey genomic p27 binding sites and prioritize p27 bound genes driving stem cell mediators for further investigation. We will test if p27pTpT shifts p27 from a co-repressor to co-activator, to activate transcriptional programs driving CSC. Finally, we will test if inducible transgenic p27CK-DD expands stem cell/progenitor populations during murine development to promote tumorigenesis in mice. This work may show that p27pTpT has pro-oncogenic effects beyond cytoskeleton disruption and reveal a novel, broader p27 function as a phosphorylation-dependent transcriptional regulator with implications for differentiation and cancer. Although most human cancers have PI3K pathway activation, PI3K and mTOR inhibitors have shown limited efficacy. Importantly, these experiments
may illuminate the modest efficacy PI3K/mTOR inhibitor drugs as monotherapies in humans, and provide a novel rationale for anticancer therapies combining PI3K inhibitors together with STAT3 or Pyk2 inhibiting drugs.
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