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Extracellular Pyruvate Kinase M2 regulates tumor angiogenesis

Liangwei Li
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EXTRACELLULAR PYRUVATE KINASE M2 REGULATES TUMOR ANGIOGENESIS

by

LIANGWEI LI

Under the Direction of Zhi-Ren Liu

ABSTRACT

Pyruvate kinase M2 (PKM2) has been studied for decades on its role in cancer metabolism. Recently, PKM2 is highlighted again for its new function: promoting gene transcription by acting as a protein kinase. Moreover, the PKM2 levels in patient circulation have been used as a diagnostic marker for many types of cancers. However, it remains unclear whether PKM2 in blood circulation has any physiological or pathological function. In my dissertation, I demonstrate that PKM2 released from cancer cells facilitates tumor growth by promoting tumor angiogenesis. Our experiments show that PKM2 promotes endothelial cell proliferation, migration and survival. Only the dimeric PKM2, not the tetrameric PKM2 possesses the activity in angiogenesis promotion. Our results further indicate that PKM2 regulates angiogenesis by integrin αvβ3 activation and integrin redistribution. I also found that PKM2 enhances drug resistance of cancer cells expressing integrin αvβ3.

INDEX WORDS: Pyruvate kinase M2, Cancer, Angiogenesis, Integrin
EXTRACELLULAR PYRUVATE KINASE M2 REGULATES TUMOR ANGIOGENESIS

by

LIANGWEI LI

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
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EXTRACELLULAR PYRUVATE KINASE M2 REGULATES TUMOR ANGIOGENESIS

by

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Georgia State University
May 2014
DEDICATION

At this very moment, I want to dedicate my dissertation to my grandfather: Deyuan Fang. He was a strict person and a great man in my memory. He taught me a lot when I was a little kid. I am the eldest among my cousins. He taught me how to become an honest man, a respectful man and a man with a sense of responsibility. He was not a talkative man himself and he actually showed me in person how to treat people well and how to deal with difficulty and adversity. In the last several hours in his life, he held my hands and tried to say something to me. But Parkinson’s symptoms denied this. I knew he wanted me to take care of all the younger cousins in my family when I grow up. I promised him. From that moment, I also made a promise in my mind that I will try to understand those diseases and how to cure them. By doing that, grandparents or parents would live longer with their children. Here I am, Grandpa. I am approaching to my promise step by step. I sincerely hope my grandfather is backing me up in heaven. He lives in my memory forever.

I also want to dedicate my dissertation to my parents: my mother, Chuanqin Fang and my father, Chunyu Li. They give me strong supports and trusts to help me complete my Ph.D. study.
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CHAPTER 1  GENERAL BACKGROUND

1.1  Cancer Metabolism and Warburg effect

Cancer progression requires high division rate. To accommodate themselves to this change, cancer cells produce more metabolic intermediates to synthesize cell components such as nucleotides, cell skeletons and other bio-macromolecules. Normal cells utilize glucose to produce three-carbon-intermediate Acetyl-CoA, which is transported to mitochondria for further metabolism, TCA cycle and oxidative phosphorylation. Major ATP producing sources are citric acid cycle (Three Carbon Acid cycle or TCA cycle) and oxidative phosphorylation. This occurs in normal cells with sufficient oxygen supply. However, cancer cells modify these metabolic pathways. Cancer cells elevate glucose and glutamine uptakes and produce ATP from glycolysis instead of TCA cycle and oxidative phosphorylation in mitochondria. Pyruvate produced by glycolysis is further metabolized to lactate in cytoplasm. Cancer cells produce ATP via glycolysis even when oxygen supply is sufficient (Hsu and Sabatini 2008). This pathological phenomenon was termed as Warburg effect, named after Nobel Laureate Dr. Otto Heinrich Warburg. Warburg effect is one of the manifest characteristics of cancer cells (Warburg 1956). Glycolysis only produces two moles of ATP per mole of glucose used, while TCA cycle produces 34 molecules of ATP. Why do cancer cells rely on glycolysis, a so inefficient pathway to produce energy? The most popular and acceptable explanation is that: cancer cells require huge amount of metabolic intermediates such as amino acids, nucleic acid and lipids to synthesize proteins, DNA/RNA and cell membrane for cancer rapid cell division (Soga 2013). Glycolytic intermediates provide the sources for synthesis of these bio-macromolecules. For instance, Fructose-6-phosphate can be used to synthesize nucleic acid via Pentose Phosphate Pathway (PPP); 3-phosphoglycerate is used to synthesize amino acid such as Glycine, Cysteine and Sphingolipids. Pyruvate kinase catalyzes the last reaction in glycolysis. Therefore, if pyruvate ki-
nase is impaired, all intermediates from glycolysis are retained for macromolecule biosynthesis. Another explanation for Warburg effect is that glycolysis is much faster than TCA cycle in producing ATPs. But this requires cancer cells to provide more glucose for glycolysis. The direct evidence for this is that cancer cells uptake more glucose than normal cells do. (Cairns, Harris et al. 2011). Warburg effect is caused by many metabolism alterations. For instance, cancer cells express Pyruvate Kinase M2 instead of Pyruvate Kinase M1 by alternative splicing; c-Myc and HIF-1 were reported to induce the expression of GLUT, a glucose transporter, on the cell membrane to facilitate glucose uptake; constitutive activation of signaling pathways in cancer cells manipulates metabolic enzymes and regulates their enzymatic activities. In conclusion, cancer cells modify metabolic pathways to support their rapid cell division.

1.2 Pyruvate kinase

1.2.1 Pyruvate kinase transcription and expression

Glycolysis is a critical metabolic pathway to convert glucose to pyruvate, which occurs in the cytosol. The last and rate-limiting step of glycolysis is catalyzed by an enzyme called pyruvate kinase to convert PEP and ADP to pyruvate and ATP. Pyruvate kinase family contains four isoenzymes in mammals: M2, M1, L and R types. PKL is expressed in liver, while PKR in red blood cells. PKM1 is expressed in most other cell types in adult tissues, mainly in muscle. PKM2 and PKM1 are both gene products of M gene (Noguchi, Inoue et al. 1986). M gene contains 12 exons and 11 introns. Alternative splicing leads to a 23 amino acid variation between PKM1 and PKM2 (between residues 378-434). Similarly, PKLR gene encodes both PKL and PKR. PKM2 is expressed in fetal tissues, and gradually replaced by other three isotypes during fetus maturation. It was noted for decades that PKM2 is expressed not only in embryonic cells but also in other highly proliferating cell types, such as cancer cells and immortal cell lines, MCF10A cells for example (Christofk, Vander Heiden et al. 2008). C-Myc has been reported to induce the conversion of PKM1 to PKM2 (David, Chen et al. 2010). PKM2 plays a crucial role in cancer proliferation.
and tumor progression. It was also reported that cancer cells can be converted to ‘normal-like’ or benign cells and that Warburg effect is partially reversed by knockdown of PKM2 and re-expression of PKM1 in cancer cells.

1.2.2 Pyruvate kinase structure

Since they are encoded by the same genes with one exon difference, human PKM1 and PKM2 share 96% identity in their amino acid sequences (Fig 1-1). Pyruvate kinases in different species are very conservative at the enzymatic active site, especially Glu271 and Asp295 residues (Larsen, Laughlin et al. 1994). Therefore, the major difference is from their quaternary structure. PKM1 forms homo-tetramers and each monomer is composed of four domains (N, A, B, C), while PKM2 has two quaternary conformations: dimers and tetramers. PKM2 tetrameric conformation possesses high binding affinity to its substrate phosphoenolpyruvate (PEP), but dimeric PKM2 has low affinity to PEP (Christofk, Vander Heiden et al. 2008) (Fig 1-2). The interfaces between each monomer can be defined as A-A’ (strong interaction interface) and C-C’ interface (weak interaction interface). The differences between the amino acid sequences of PKM1 and PKM2 locate in C-C’ interface and in FBP binding site (Dombrauckas, Santarsiero et al. 2005) (Fig 1-3).

PKM2 forms both homodimer and homotetramer conformations and the equilibrium of dimer and tetramer is regulated by numerous allosteric cofactors. Fructose 1, 6-Bisphosphate (FBP) has been reported to allosterically regulate PKM2 enzymatic activity (Ashizawa, McPhie et al. 1991). FBP binding site is located at a loop region from residue 402 to 407 and binding of FBP orientates the loop to an ordered structure (Jurica, Mesecar et al. 1998). In the absence of FBP, C domains from two subunits expel each other; upon the binding to PKM2, FBP induces a huge conformational change of C domains at the interface and exposes charged residues to form salt bridges interaction. The binding of FBP to PKM2 results in the interaction of two dimers by C domains to form tetrameric PKM2 and increase of PKM2 en-
zymatic activity. Besides FBP, metabolic intermediates such as amino acids L-Alanine, L-Leucine and L-Proline are also able to regulate PKM2 enzymatic activity. On the contrary to FBP, these factors inhibit PEP binding to PKM2 and suppress PKM2 activity physiologically (Mazurek, Boschek et al. 1997).

Besides the physiological regulations, cancer cells regulate PKM2 dimer/tetramer in different ways. Oncoprotein pp60v-src (Presek, Reinacher et al. 1988) and E7 of the human papilloma virus (Mazurek, Zwerschke et al. 2001) phosphorylates PKM2 and stabilizes dimeric PKM2. However, the detailed mechanism remains unclear in their studies. Recently, a number of studies have highlighted novel mechanisms of regulation of enzymatic activity of PKM2. PKM2 was also converted to dimer conformation by binding to certain synthesized phosphotyrosine peptides screened from a peptide library (Christofk, Vander Heiden et al. 2008). This peptide however does not belong to any existing protein as they discussed in their study, however, it is possible that peptide with similar sequence but not identical sequence could bind to PKM2. Other studies have confirmed this result that PKM2 binds to certain kinases in the cells. PKM2 is phosphorylated at tyrosine 105 residue by Fibroblast Growth Factor Receptor (FGFR) after ligand binding and phosphorylated PKM2 is induced to dimer conformation (Hitosugi, Kang et al. 2009). All the studies indicated that PKM2 is converted to dimers by different pathways in various types of cancers. We also reported that 399R mutated to Glutamic acid residue (E) in PKM2 mimics dimer conformation of PKM2. This mutation breaks down the salt bridge interactions between two dimers.

1.2.3 PKM2 localization

Glycolysis takes place in perinuclear region in cytosol. Pyruvate produced by glycolysis is transported to mitochondria and further oxidized to CO₂ by in TCA and produce more ATPs. Pyruvate kinase is associated with glycolytic enzyme complex with other glycolytic enzymes located in cytosol; therefore, the major PKM2 pool is cytosol.
However, non-canonical localizations of PKM2 have been reported in recent studies. Firstly, PKM2 translocates to nucleus. First report is that Interleukin-3 induces PKM2 nuclear localization which regulates cell proliferation (Hoshino, Hirst et al. 2007). Our lab also revealed that dimeric PKM2 has more nuclear localization than tetrameric PKM2 has (Gao, Wang et al. 2012). Our results indicated that mutation of residue Lysine 399 to Glutamic acid (R399E) converts recombinant PKM2 (rPKM2) from tetramer to dimer. Dimer rPKM2 translocates to nucleus to phosphorylate STAT3. Phosphorylated STAT3 further activates MEK5 gene transcription to promote cancer cell proliferation. PKM2 nuclear translocation might be regulated by ERK2. The detailed mechanism is studied by Yang and colleagues that activated ERK2 phosphorylates Pyruvate kinase M2 at Serine 37 residue. PIN1 recruits importin α5 binding to phosphorylated PKM2 and leads to PKM2 nuclear translocation (Yang and Lu 2013).

Furthermore, PKM2 is detected in body fluids from cancer patients. PKM2 has been used as a cancer biomarker especially for colorectal cancer, gastric cancer and lung cancer diagnosis. PKM2 is detected by diagnosis kit (ELISA, Enzyme-Linked ImmunoSorbent Assay kit) in patients’ blood sera or feces samples (Kumar, Tapuria et al. 2007). In vitro, PKM2 is released from colorectal carcinoma cell line colo205 and SW480 to culture medium (Wu, Chen et al. 2008). However, it remains unresolved how PKM2 is secreted and whether circulation PKM2 possesses any function. Many researchers have attempted to investigate the potential physiological or pathological functions of PKM2. Shin et al. found that PKM2 released by cancer cells is related with 5-fluorouracil resistance in colon cancer cell lines (Shin, Yoo et al. 2009). 5-fluorouracil resistance cell line secretes more PKM2 to the culture medium than 5-fluorouracil non-resistance cell line does.

PKM2 in cell culture medium is possibly released from exosomes or microvesicles (Meckes, Gunawardena et al. 2013). Exosomes and microvesicles are small vascular compartments and secreted by many cell types such as B lymphocytes, dendritic cells and cancer cells. The physiological roles of these microvesicles are not fully understood and exosomes are speculated to function in intercellular com-
munication or cell-matrix modification. Pyruvate kinase was reported to be present in exosomes and microvesicles identified by Mass spectrum. PKM2 was also reported to be included in neutrophil granules, which suggested another potential pathway of PKM2 secretion in immune cells (Lominadze, Powell et al. 2005). Our unpublished data also supported that PKM2 is secreted by neutrophils. In summary, non-canonical localizations of PKM2 are related to its novel biological functions from the enzymatic activity.

1.2.4 PKM2 biological functions

Pyruvate kinase catalyzes the last step of glycolysis to convert PEP to pyruvate and generate one mole of ATP. The active site locates in a pocket between the interface of A and B domains. Mg²⁺, K⁺ and H₂O molecules are required for PKM2 catalytic activity (Larsen, Benning et al. 1998). When oxygen is supplied, glucose is metabolized through glycolysis and followed by TCA cycle in mitochondria. Glucose is converted to pyruvate and lactate when oxygen in the cell environment is low. Cancer cells, however, produce lactate even with sufficient oxygen supply (Mazurek, Boschek et al. 1997). Metabolic enzymes form a protein complex termed as glycolytic enzyme complex, containing enolase, lactate dehydrogenase, pyruvate kinase and other glycolysis enzymes (Mazurek, Michel et al. 1997). Once PKM2 forms dimers, the quaternary structure changes and the binding affinity to its substrate PEP decreases. Meanwhile, dimeric PKM2 dissociates from the glycolytic enzyme complex. Due to these changes, metabolic intermediates in glycolysis are accumulated and utilized to synthesize amino acids, nucleotides and phospholipids for cancer cells (Mazurek, Boschek et al. 2005). PKM2 enzymatic activity is fully manipulated in cancer cells. Firstly, switch of PKM1 to PKM2 expression is one of strategies that cancer cells use to meet the demand for their high proliferation rate. As mentioned above, phosphorylation of PKM2 at several residues was reported to enhance PKM2 dimer formation and promote tumorigenesis. Anastasiou and co-workers found that reactive oxygen species (ROS) also inhibits PKM2 enzymatic activ-
The metabolic pathways, manipulated by cancer cells, down-regulate PKM2 enzymatic activity and support cancer cells to achieve Warburg effect.

1.2.4.1 Pyruvate kinase M2 acts as a gene transcription activator

Early in 2008, Lee et al. reported that PKM2 interacts with transcription factor Oct-4 in vitro. This PKM2 and Oct-4 interaction was analyzed by affinity purification and mass spectrometry (Lee, Kim et al. 2008). The authors speculated that the interaction plays a role in regulation of gene transcription. Moreover, hypoxia inducible factor 1 (HIF-1) is well-known to be responsible for oncogene transcription activation. PKM2 has also been shown to enhance HIF-1α transcription activity by binding with prolyl hydroxylase 3 (PHD3) (Luo, Hu et al. 2011). The interaction is critical for regulation of HIF-1α transcription activity, thus it was proposed that PKM2 coordinates with HIF-1α in manipulating hypoxia response in cancer cells. PKM2 also plays an important role in β-catenin transactivation in tumor proliferation and malignancy. In quiescent cells, β-catenin forms complex with Axin, APC and GS3K. B-catenin is phosphorylated by GS3K and degraded through proteasome. Once cells are activated by Wnt ligand, β-catenin dissociates from the complex and translocates to nucleus to activated tumorigenesis-related-gene activation. PKM2 binds Y333 phosphorylated β-catenin and co-activates β-catenin downstream gene transcription (Yang, Xia et al. 2011). Our lab also reported that PKM2 regulates MEK5 gene transcription resulting in promoting cancer cell proliferation (Gao, Wang et al. 2012). It remains unclear that how PKM2 binds to those gene transcription factors. We proposed that PKM2 acts as a protein kinase to phosphorylate STAT3.

1.2.4.2 PKM2 is also a protein kinase

Small molecules PEP and ADP are the substrates of PKM2. The enzymatic active sites are only able to accommodate small molecules. However, proteins were also found as the substrates of PKM2. The first reported substrate is Histone H1 in Morris hepatoma 7777 tumor cell. Lysine residue in Histone
H1 is phosphorylated by utilizing PEP as phosphate donor (Ignacak and Stachurska 2003). Multifunctional protein prothymosin alpha (ProTα) is another target, which is phosphorylated by PKM2 (Diaz-Jullien, Moreira et al. 2011). Furthermore, Dr. Zhimin Lu’s group recently reported that PKM2 phosphorylated Histone H3 and this modification led to gene transcription and further promoted tumor growth. Our lab data also demonstrated that PKM2 phosphorylates STAT3. Transcription factor STAT3 is phosphorylated at Y705 by PKM2 using PEP as phosphate donor. Phosphorylated STAT3 leads to the enhanced tumor cell proliferation. However, PKM1 lacks this protein kinase activity (Gao, Wang et al. 2012). FBP regulates PKM2 protein kinase activity by promoting tetramer formation. It is likely that PKM2 protein kinase activity requires the conformational change because PKM2 crystal structure indicated that the enzymatic activity pocket does not provide enough space for protein substrates. Dimerization might induce conformational change to expose the enzymatic pocket to improve accessibility for protein substrates.

1.2.4.3 Extracellular PKM2

PKM2 in patients’ serum and stool samples was used as a biomarker in some inflammatory diseases as well as malignant tumors (Staib, Hoffmann et al. 2006; Aloysius, Zaitoun et al. 2009). PKM2 is a potential fecal marker in Crohn’s disease patients (Day, Judd et al. 2012). However, it was also reported that PKM2 level in inflammatory bowel disease (IBD) patients serum is not as high as in various cancer patients (Meng, Zhu et al. 2012). Colorectal cancer has elevated PKM2 concentration in blood serum (5-7 times higher than normal individuals); two-fold increase of serum PKM2 is in adenoma compared to health population. PKM2 secreted by cancer cells was reported to correlate with 5-fluorouracil resistance in colon cancer cell lines (Shin, Yoo et al. 2009). PKM2 in the culture medium is up-regulated in the cancer cell line with 5-fluorouracil resistance compared to the cell line without 5-FU resistance. However, the mechanism needs to be further studied. Similarly, another glycolytic enzyme: phosphoglycerate kinase 1 (PGK-1) is secreted to the extracellular region (Daly, Wind et al. 2004) and
regulates angiogenesis (Lay, Jiang et al. 2000). In our study, we found that PKM2 enhances angiogenesis
by promoting endothelial cell migration, adhesion and survival via interaction with integrin αvβ3.

1.2.5 **PKM2 inhibitor and activator in cancer therapy**

Since PKM2 is universally expressed in various types of cancers, researchers tried to develop small
compounds to inhibit PKM2 and induce cell apoptosis (Vander Heiden, Christofk et al. 2010). However,
cancer cell induces tetrameric PKM2 to form dimers either through binding phosphorylated peptide or
through being phosphorylated by tyrosine kinase. Decrease in enzymatic activity of pyruvate kinase fa-
vors the accumulation of metabolic intermediates in cancer cells. Therefore, PKM2 inhibitor may not be
successful due to this end. More recently studies focused on reversing the process by promote PKM2
enzymatic activity. Substitution of PKM2 by PKM1 decreases tumor sizes in mice model (Christofk,
Vander Heiden et al. 2008). PKM2 allosteric co-activator, FBP acts as an activator to promote PKM2 te-
tramer formation. However, it was demonstrated that FBP has no effect on the PKM2 phosphorylated
by FGFR in cancer cells (Hitosugi, Kang et al. 2009). Anastasiou and colleagues synthesized compounds
which specifically recognize PKM2 and convert dimeric PKM2 to tetrameric PKM2. These compounds
were termed as PKM2 activators (Anastasiou, Yu et al. 2012). PKM2 enzymatic activity was up-regulated
by PKM2 activators and these activators have been shown to inhibit mice tumorigenesis.

1.3 **Angiogenesis**

The closed blood circulation system is unique in vertebrates. Blood vessel growth is a vital physio-
logical process in embryonic development and wound healing. Blood vessels are formed by tube forming
endothelial cells, called endothelium. Endothelium is surrounded and covered by smooth muscle cell
layer. Elastic tissues and connective tissues provide support and protection to the blood vessel from
stress and physical damage (Adair and Montani 2010). Blood vessels without vessel wall or with thin wall
exist at the blood vessel frontier and those vessels are termed as capillaries or microvessels. Blood ves-
sels are formed by two stages: vasculogenesis and angiogenesis. Vasculogenesis is the process that blood vessels start growth from scratch. It is related to the differentiation of blood vessel precursor cells such as angioblasts (Patan 2004). It has been well-studied that blood vessel precursor cells differentiate into endothelial cells in response to growth factor or extracellular matrix, such as bFGF and VEGF (Kazemi, Wenzel et al. 2002); while angiogenesis is the process that blood vessels or capillaries grow or extend from the existing vessels. Angiogenesis involves endothelial cell proliferation, migration, survival and tube-like structure formation.

1.3.1 Angiogenesis and its regulation

Angiogenesis is tightly regulated in the body. Loss of the control of angiogenesis is related to a series of pathological conditions. Insufficient angiogenesis leads to Alzheimer’s disease, diabetes, chronic wound healing and etc. On the contrary, excessive angiogenesis also impairs the physiological orders and is involved in more than 20 diseases including cancer. Cancer is one of the most important disorders highly related to angiogenesis (Griffies and Molema 2000). Angiogenesis balance is maintained by two protein families: angiogenic activators and inhibitors (Mundel and Kalluri 2007) (Fig 1-4). Both angiogenic activators and inhibitors collaborate to regulate physiological angiogenesis. Angiogenesis activator family contains various growth factors (such as fibroblast growth factor, placental growth factor and vascular endothelial growth factor), angiopoietin 1, interleukin 8 and etc. Angiogenesis inhibitor family consists of a group of proteins or peptides which specifically inhibit angiogenesis, such as endostatin, interferon and thrombospondin 1 (Zetter 2008). The high proliferation rate of cancer cells requires enhanced nutrients transportation. Initial tumor growth depends on diffused oxygen and nutrients from adjacent stroma cells; while larger tumors need blood vessels to support their further growth (Folkman, Long et al. 1963). In clinical cases, tumor size is limited to 1-2mm in diameter without angiogenesis support (Folkman, Watson et al. 1989).
1.3.2 Angiogenesis and cancer therapy

1.3.2.1 VEGF-dependent and VEGF-independent angiogenesis

Vascular endothelial growth factor (VEGF) is the most important angiogenesis activator, which strongly stimulates vasculogenesis and angiogenesis. VEGF was identified in early 90s (Senger, Galli et al. 1983) and belongs to platelet derived growth factors VEGF/PDGF superfamily (Trelles, Leon et al. 2002). VEGF has at least 5 members: VEGF-A, B, C, D, E (Shibuya 2008). VEGF is a homodimeric glycosylated protein and contains two intermonomer disulfide bonds and three intramonomer disulfide bonds. VEGF has 5 isotypes: VEGF121, 145, 165, 189 and 206. All those isotypes are encoded by VEGF gene and produced by alternative splicing. VEGF has two subcellular forms: secreted VEGF and membrane-bound VEGF (Salvador, Li et al. 2008). VEGF is secreted by many types of cells, such as endothelial, fibroblasts and activated immune cells. VEGF is also expressed and secreted by cancer cells themselves or by cancer cell infiltrated stroma cells.

VEGF induced cellular response is mainly via VEGF receptors (VEGFRs). VEGFR is a tyrosine kinase receptor, which contains an extracellular domain, a transmembrane domain and a cytoplasmic tail. VEGFR family has 3 members, VEGFR-1 (flt-1), VEGFR-2 (flk-1 or KDR) and VEGFR-3 (flt-4). VEGFR-1 mainly is the receptor for VEGF-A and VEGF-B; VEGFR-2 is receptor for VEGF-A, C, D and E; VEGFR-3 is for VEGF-C and D. VEGFR-1 and VEGFR-2 is responsible for vasculogenesis and angiogenesis signaling transduction; while VEGFR-3 is conducting the signaling pathways related to lymph-angiogenesis (Hicklin and Ellis 2005). Binding VEGF to their receptors dimerizes VEGFR VEGFR c-terminal undergoes autophosphorylation. Phosphorylation of tyrosine residues leads to the activation of various signal pathways such as Ras, PLC, p38 and PI3K. Those downstream kinases regulate cell functions related to cell proliferation, survival and migration.
It is necessary to understand angiogenesis mechanism better in order to treat or diagnose cancer and other diseases with deregulated angiogenesis. Besides VEGF-dependent angiogenesis, VEGFR-independent angiogenesis is equally crucial. Other growth factors than vascular endothelial growth factor also regulate angiogenesis. It was reported that platelet-derived growth factor (PDGF) regulates angiogenesis through PDGF receptor by promoting endothelial cell proliferation and tube formation (Battegay, Rupp et al. 1994). EGF was also reported to modulate angiogenesis by increasing endothelial cell migration (Mehta and Besner 2007). Moreover, Angiopoietin regulates angiogenesis through Tie receptors. Angiopoietin family has four members: Ang-1, Ang-2, Ang-3 and Ang-4. Among all the members, Ang-1, Ang-3 and Ang-4 bind Tie2 receptor, while Ang-2 binds Tie1 receptor. After binding to receptors, Ang dimerizes Tie receptor and then autophosphorylates the intracellular domain of the receptor. Receptor activation phosphorylates their downstream signaling cascades, mainly through PI3K/Akt signaling pathway and STAT to regulate gene transduction of cell survival and cell proliferation (Mochizuki, Nakamura et al. 2002).

1.3.2.2 Angiogenesis inhibitor

Increase in VEGF secretion and/or VEGF receptor expression induces angiogenesis in cancer. Tumor vessels are different from vessels in normal tissues (Shojaei 2012). -Tumor blood vessels are not as organized as the normal vessels due to the defective vascular structure in cancer. Repressing angiogenesis is one of the important ways for cancer therapy. Different strategies have been explored for angiogenesis related cancer therapy.

1. Targeting VEGF. VEGF antibody was developed to specifically target VEGF and decreased the serum level of VEGF. Bevacizumab (Avastin) is the most successful case and was approved for clinical use in 2004 by U.S. Food and Drug Administration (FDA). Bevacizumab is also the first FDA approved biological drug for tumor angiogenesis. Furthermore, Bevacizumab has been used for conjugated with radio-
therapy chemicals or cocktailed with chemotherapy drugs to bring more benefits to cancer patients (Bhuvaneswari, Yuen et al. 2007; Jayson 2011).

2. VEGFR inhibitors. Two distinct inhibitors for VEGFR were exploited to inhibit angiogenesis. The first type of VEGFR inhibitor is anti-VEGFR antibody, which were developed to inhibit VEGF ligand binding to its receptor. DC101, an anti-VEGFR2 monoclonal antibody potentially inhibits prostate tumor growth and metastasis (Sweeney, Karashima et al. 2002). DC101 has also been shown to be used to enhance paclitaxel or radiology treatment (Inoue, Slaton et al. 2000; Verhoeff, Stalpers et al. 2009). Anti-bodies anti-VEGFR extracellular domain also induce cancer cell apoptosis (Sweeney, Karashima et al. 2002). The second type inhibitor is small compounds. Virtual Screening and computational modeling were used to design and screen the compounds for inhibiting VEGFR dimerization (Elgamacy, Shalaby et al. 2011).

3. VEGFR signaling pathway inhibitors. VEGF receptor is one of the receptor tyrosine kinase family members. Therefore, the most common strategy to inhibit angiogenesis is to repress VEGFR signal transduction. Plentiful tyrosine kinase inhibitors (TKIs) are developed and utilized to treat different tumor types. Pazopanib is an FDA-approved medication for treatment of advanced renal cell carcinoma (Sloan and Scheinfeld 2008). Pazopanib is a synthetic chemical compound which specifically targets and inhibits tyrosine kinase sites of VEGFR and platelet-derived growth factor receptor (PDGFR).

Generally, VEGF-VEGF receptor induced angiogenesis has been well studied. However, inhibition of angiogenesis through VEGF-VEGFR pathway is not sufficient to treat cancer. Some important drawbacks cannot be ignored. Firstly, VEGF-VEGFR is not the only signaling pathway to regulate angiogenesis (Shibuya 2008). Secondly, inhibition of VEGFR pathway causes disease-related side-effects such as dysfunction or failure of major organs: heart, liver and renal system (Chen and Cleck 2009); repression on the wound healing; and hypertension (Bhargava 2009). The last but not the least, most VEGFR inhibitors
themselves cannot inhibit tumor growth and it is required to combine VEGF with other anti-cancer drugs for better clinical outcomes.

1.4 Integrins

Eukaryotic cells are constantly communicating with their microenvironment through both cell-matrix adhesion and cell-cell adhesion. Numerous molecules are involved in cell adhesion. Integrin family is one of the most important proteins involved in cell adhesion and migration. Specific integrin expression pattern is correlated to endothelial cell proliferation, spreading, migration and invasion. Integrins activate intracellular signaling pathways after interacting with extracellular ligands such as extracellular matrix proteins (ECM).

1.4.1 Cell adhesion

Cells interact with surrounding cells to form tissue layers or to transfer nutrient and cellular signals. Four major cell-cell adhesion complexes exist in epithelial and endothelial cells: tight junction, adherens junction, gap junction and desmosome.

Tight junction functions at anchoring adjacent cell together tightly and prohibiting fluids to diffuse through the gaps between epithelial/endothelial cells. Tight junction also maintains the polarity of epithelia or endothelia layer. Tight junction is composed of a series of transmembrane proteins such as claudin, occludin and tetraspanins. The interaction between these proteins from adjacent cells provides the molecular bases for tight junction (Gonzalez-Mariscal, Betanzos et al. 2003).

Adherens junction is mainly for holding epithelial and endothelial cells together and supporting the cell architecture. Cadherin and catenin family proteins are responsible for adherens junction formation. E-cadherin and N-cadherin are calcium binding transmembrane proteins and interact with cadherin molecules from adjacent cells. After two cadherin molecules interact, α- and β-catenins are activated to bind to actin filaments to sustain the cells architecture (Niessen 2007).
Gap junction is involved in cytoplasm component exchange. Gap junction is a hexamer formed by connexin family proteins. Gap junction close and open conformation switch is regulated by either calcium (Lurtz and Louis 2007) or post-translational regulation such as phosphorylation (Warn-Cramer and Lau 2004).

The last but not the least type of cell-cell junction is desmosome. Desmosome exists mainly in epithelial cells other than endothelial cells, but desmosome protein desmoplakin is expressed in cultured endothelial cells (Valiron, Chevrier et al. 1996). Desmoplakin interacts with cadherin or vimentin to regulate endothelial cell intercellular junction. In epithelial cells, the function of desmosome is to sustain the cells against shearing force. Desmosome complex forms plaque structure on the membrane across cells and the complex is constructed by a series of cadherin family proteins such as Desmoplakin, Desmocollin and Plakoglobin. The complex attaches to intermediate filaments inside of the cells, such as keratin (Bornslaeger, Corcoran et al. 1996).

Both epithelial cells and endothelial cells rely on the four types of cell junctions to interact or communicate with adjacent counterpart cell, to maintain and protect epithelia and endothelia tissues and to prohibit random diffuse. Besides the cell-cell interactions, epithelial and endothelial cells require the interaction with the extracellular matrix to survive and to perform various physiological functions.

1.4.1.1 Cell-matrix interaction

Basement membranes are thin layers under epithelia or surrounding endothelia. Basement membranes have fiber structure and are composed of various extracellular matrix proteins such as collagen, laminin and fibronectin. Basement membranes provide supports to epithelial and endothelial cell layers. Integrin and hemidesmosome are the two major receptors for cell-matrix interaction. Hemidesmosome shares the similar structure and components as the desmosome complex however, hemidesmosome functions differently with desmosome. Desmosome functions to connect epithelial
cells together while hemidesmosome is to attach epithelial cells to basement membranes or extracellular matrix. Hemidesmosome attaches to intermediate filament in the cells to regulate cellular functions (Zhang and Labouesse 2010). The other protein family for epithelial and endothelial attachment to basement membranes is the integrin. Integrins are heterodimeric transmembrane proteins, which regulate various types of cell functions such as cell adhesion, migration and proliferation through either inside-out or outside-in activation. Integrins are also involved in many pathological conditions such as angiogenesis. Since integrins are important molecules in my dissertation, I will discuss it in details.

1.4.2 Integrin structure

Integrins are membrane proteins with single alpha-helices transmembrane domain. Integrins are heterodimers formed by α subunits and β subunits. More than 20 different heterodimeric integrins are formed by 18 α subunits and 8 β subunits in mammals (Margadant, Monsuur et al. 2011). Extracellular domains of integrins are glycosylated and form functional domains to interact with ligands or other proteins. Integrin family can be divided into two subgroups by their structures difference: with or without an extra von Willebrand factor type A domain (αA or αI domain). Integrins can also be divided into four groups based on their ligands or binding partners: collagen-binding integrins; laminin-binding integrins; RGD dependent integrins and Leukocyte integrins. Each integrin subunit contains a bunch of specific domains. Integrin heterodimers are illustrated as a headpiece and ‘leg’ part (Springer 2002). Integrin ligand binding site is located in the headpiece.

1.4.2.1 Integrin conformation change

Integrins have two conformational structures: close and open conformation. In the close or ‘bent’ conformation, the headpiece of integrin heterodimers bends over to the ‘leg’ part. The ligand binding site is facing to the cell membrane, which results in the low binding affinity of integrin heterodimers to their ligands (Humphries, McEwan et al. 2003). Extensive studies have been carried out to un-
Understand the relationship between integrin conformation change and its function. Upon the binding of integrin to its ligand, integrin undergoes dramatic conformational change in its extracellular domain. This change makes integrins skewed towards clustering with adjacent integrin. The conformational change in the extracellular domain of integrins also induces a structure change in integrin transmembrane domain and intracellular domain (ICD), which separates integrin c-terminals of α and β subunits. C-terminal separation of integrin α and β subunits leads to the assembly of focal adhesion complex and activation of FAK and its further downstream signaling pathways (Askari, Buckley et al. 2009).

1.4.2.2 Integrin ligand

RGD-containing ligand is the largest protein family which binds integrins including αvβ3, α5β1, αIIbβ3, and etc. This integrin subfamily recognizes a consensus binding sequence: three amino acids RGD (R: Arginine, G: Glycine, D: Aspartic acid). Based on the analysis of the crystal structure, the binding site for RGD-containing ligand locates at the interface of integrin α and β subunits (Xiong, Stehle et al. 2002). Lysine residue interacts with β-propeller domain in α subunit while aspartic acid binds to von Willebrand factor A (vWF) domain in β subunit. Many extracellular matrix (ECM) components contain RGD sequence including fibronectin and vitronectin. Certain ECM proteins do not have accessible RGD sequences on the protein surface, but expose their cryptic RGD sequence to interact with RGD-specific integrins after being denatured by temperature or cleavage by the matrix metalloproteinase (Yamamoto, Yamato et al. 1995). For instance, Integrin α5β1 and αvβ3 are the typical RGD dependent integrins. But collagen I does not bind to either integrin α5β1 or αvβ3 due to the absence of any solvent accessible RGD sequence. However, after being partially denatured by increasing temperature or degraded by extracellular proteases (MMP-2 or MMP-9), collagen I unfolds and exposes its RGD sequence to interact with both α5β1 and αvβ3 integrins (Taubenberger, Woodruff et al. 2010). This mechanism is
related to cancer angiogenesis and metastasis (Eliceiri and Cheresh 1999). GRGDSP is a sequence from fibronectin and used as an angiogenesis inhibitor as to compete with RGD-containing native ligands.

In addition, some non-ECM proteins containing RGD sequence were also reported to interact with integrins. The human immunodeficiency virus (HIV-1) protein, transactivating factor (TAT) activates FAK through integrin αvβ3 and promote angiogenesis (Urbinati, Mitola et al. 2005). TAT interaction with integrin is inhibited by both RGD peptide competitive inhibitor and blocking antibody LM609, which indicates that the interaction is via RGD sequence in TAT protein. Another example is CD40 ligand or CD40L. Soluble CD40 ligand was reported to bind to integrin α5β1 and induce signaling transduction by activating MAPK and its downstream ERK1/2 in endothelial cells (Leveille, Bouillon et al. 2007). This interaction is through RGD sequence on the CD40L (Andre, Prasad et al. 2002).

Besides RGD sequence, KGD (Lysine-Glycine-Aspartic acid) is also recognized by integrins in certain cases. Integrin β3 is the major type of integrin for interacting with KGD, especially αIIbβ3. KGD plays important role in disintegrin binding to integrin. Disintegrin is a family of proteins in viper venom. Disintegrin inhibits platelet aggregation and causes hemorrhage through the interaction of KGD sequence with αIIbβ3 integrin (Lu, Lu et al. 2005).

A synergetic site might be important for integrin binding to RGD. RGD peptides are used for cancer therapy to target cancer angiogenesis. RGD peptide such as cilengitide a cyclic RGD pentapeptide has been applied as an angiogenesis inhibitor. However, the affinity of RGD peptide is 1000 fold lower than their natural ligand. One of the most important reason is that integrin ligand contains not only RGD sequence but also synergetic site. Pro–His–Ser–Arg–Asn (PHSRN) is a synergetic binding site in fibronectin for integrin α5β1 and this synergy site facilitates HUVEC cell attachment and spreading in vitro (Ochsenhirt, Kokkoli et al. 2006).

Other than RGD sequence, LDV is another major binding sequence for integrins. LDV mainly binds to β2, β1 and β7 integrins (Humphries, Byron et al. 2006). LDV has other derivative sequences
such as IDA or REDV (Mould and Humphries 1991). As mentioned earlier, a subgroup of integrins contain α domain, α1β1, α2β1, α10β1 and α11β1. Those integrin binds to specific residues other than RGD and LDV. GFOGER is the critical sequence on collagen to be recognized by αA-containing integrins. The glutamic acid residue in the recognition sequence is crucial to interact with the metal in the integrins (Emsley, Knight et al. 2000).

1.4.2.3 Metal binding vs. Ligand binding

Magnesium and calcium metal ions are crucial to maintain integrin proper structure and ligand binding activity. As described above, integrin can be divided into two subfamilies based on their structure similarity: αA integrins and non-αA integrins. αA integrins contain a von Willebrand factor type A domain. αA is responsible for mediating integrins binding to their ligands and α domain contains an ion binding site, named metal-ion-dependent adhesion site (MIDAS) (Michishita, Videm et al. 1993). MIDAS coordinates one divalent cation, usually Magnesium and MIDAS domain accepts one interaction from an acidic amino acid from integrin ligand (Lee, Rieu et al. 1995). MIDAS has even higher binding affinity for Manganese (Li, Rieu et al. 1998). However, Manganese is not a physiological ion. Although structure different exists between αA-containing and α-lacking Integrins, integrins without αA domain shows similar strategy for ligand binding. Integrin αvβ3 is an important αA-lacking integrin. αvβ3 contains an αA-like domain or βA/βI domain (Fig 1-5)(Luo and Springer 2006). Aspartic acid in RGD tripeptide attaches to the ion located at MIDAS site, while Arginine (R) residue interacts with a couple of negative charged residues at β propeller on α subunit. MIDAS on integrin β subunit and β propeller domain from α subunit forms the basic ligand binding pocket. Besides MIDAS at αA domain or A-like domain on integrin β subunit, other two metal binding sites locates adjacent to MIDAS: the ADMIDAS (adjacent to MIDAS) and LIMBS (ligand-associated metal binding site). Those sites favor calcium ion and provide additional regulation on ligand binding (Valdravidou, Humphries et al. 2008). Tumor endothelial marker 8 (TEM8) is a
transmembrane protein, which highly expresses in tumor endothelium. TEM8 also contains an MIDAS site and this MIDAS was reported to be involved in binding to the anthrax toxin antigen (Ramey, Villereal et al. 2010). The interaction of TEM8 and its ligand is much similar to the binding of integrin and RGD peptide. It was reported that PKM2 also could bind to TEM8 in vitro (Duan, Hu et al. 2007). This interaction indicates the possibility that PKM2 also binds to MIDAS on the integrin.

Integrin α subunit also contains 5 Calcium binding sites. 4 of them are in beta propeller loop region and the other one is at ‘genu’ region, which acts as a ‘knee’ for integrin conformational change (Arnaout, Goodman et al. 2007). Metal ions are required for integrin structure and integrin ligand binding. EDTA completely inhibits integrin function and detaches cells.

1.4.3 Integrin functions

Integrins transmit outside-in or inside-out signals atransmembrane proteins. The major physiological function of integrins is involved in cell adhesion and migration. Integrins are the main receptors for extracellular matrix proteins such as collagen, fibronectin and laminin. Binding of integrins with ECM proteins anchors the cells to basement membrane. After binding to ECM components, integrin recruits paxillin and talin to form focal adhesion complex in cytosol. Focal adhesion complex attaches to cytoskeleton: actin filament (Brakebusch and Fassler 2003). Ligand-bound integrins also activated Focal Adhesion Kinase (FAK). FAK phosphorylated and activated various downstream pathways, for example Src and PI3K/Akt pathways, which were well-studied to regulate cell proliferation, survival and migration (Yano, Mazaki et al. 2004; Caron-Lormier and Berry 2005).

1.4.3.1 Integrin activation

Integrins are activated via two different ways: inside-out and outside-in pathways. The inside-out activation of integrin has been well studied. Without stimuli, integrins rest in the inactive (bent form) and have low affinity to its ligand. Cytoplasmic tails of α and β integrin are close together and in-
teract with each other. When the cells receive stimulation from growth factors such as VEGF or EGF, cells initiate signal transduction. One important regulator, talin is activated by phosphorylation. The detailed mechanism of talin activation is not fully understood. Activated talin exposes its FERM domain (Pearson, Reczek et al. 2000) and binds to integrin intracellular domain. Binding of talin to integrin carboxy-terminus induces a conformational change of cytoplasm domain and transmembrane domain of integrins. This change leads to a further conformational change of integrin extracellular domain: integrin ECD switch from bent form (low affinity to ligand) to extended form (high affinity to ligand). Inside-out activated integrin facilitates cell to attach to ECM (Qin, Vinogradova et al. 2004). The conformational change of bent and extended form is visualized by electron microscopy (Weisel, Nageswami et al. 1992).

Outside-in activation is more straightforward. Ligands bind to integrin ECDs and cause integrins conformational change, which leads to integrin activation. Ligand binding also induces integrin clustering to enlarge integrin activation. Activated integrins (extended or open conformation) cluster together on the cytoplasm membrane to form a patch-like structure. Integrin clustering provides stronger binding capability of integrins to ECM by holding a bunch of integrins at one single spot. Those structures then form focal adhesion (Kinashi 2005). Upon focal adhesion complex assembled, integrins regulate cell attachment and migration through this outside-in manner.

1.4.3.2 Integrrn endocytosis and recycling activation

In order to attach to or migrate on ECM proteins or basement membrane, cells require assembly and disassembly of focal adhesion complex rapidly. Therefore, integrins need to be transported from the rear region to the migrative leading edge. This is achieved by integrin endocytosis and recycling. Integrin endocytosis is initiated by binding to integrin ligand or synthetic peptide such as RGD peptide Cilengitide (Reynolds, Hart et al. 2009). Growth factors could also induce integrin endocytosis and recycling. After initiation, endocytotic integrins are delivered to endosomes and transported back to cytoplasm mem-
brane for reuse (Bretscher 1996). Integrins retrieved from the rear of the moving cells is mediated by Rab4 and Rab5; while integrin delivered to the frontier of the cell is mediated by Rab11 and Arf6 (Margadant, Monsuur et al. 2011).

1.4.3.3 **Integrin regulates cell adhesion**

Cell adhesion regulates various physiological events, such as embryonic development, cancer metastasis, inflammation, homeostasis and wound healing (Edelman and Crossin 1991). Endothelial cells and epithelial cells have to line on the extracellular matrix to form tissues. Integrin family members show specificity to different ECM substrates. For example, integrin α5β1 has highest affinity to fibronectin, αvβ3 has higher affinity to vitronectin and α1β1 and α2β1 mostly bind to collagens and laminins. Upon the binding of integrins to their ECM, integrins convert from bent form to extended form and cluster together. Integrin activation and clustering recruit a group of focal adhesion proteins such as talin, paxillin and vinculin. Focal adhesion complex attaches to actin filaments to regulate cell attachment to ECM. Other than integrins, desmosome and other proteoglycans also provide cell-matrix adhesion. However, integrins are the most dominant and universal adhesion molecules. Cancer cells alter integrin patterns to dysfunction cell adhesion and deregulate cell migration.

1.4.3.4 **Integrin dictates cell migration and invasion**

Migration is described as directional cell movement. This movement is generally divided into several steps including (1) Lamellipodium extension at the leading edge; (2) detachment on the rear side of moving cells; (3) make new contact at the front side; (4) cell contract to move forward. Integrins play vital roles at both front and rear sides of moving cells. Integrin endocytosis and recycling is responsible for redistribution of integrins. The intracellular domain of integrins contains a motif for clathrin-dependent endocytosis (Gawaz, Besta et al. 2001). After endocytosis, Rab GTPase family proteins such as Rab11 and Rab4 mediates integrin trafficking. Integrin recycling is involved in two pathways: short-
loop and long loop. Rab4 GTPase is involved in short-loop recycling and integrins enter early endosomes and are rapidly transported to the plasma membrane. On the other hand, long-loop recycling is mediated by Rab11 small GTPase. Integrins are translocated to perinuclear recycling compartment (PNRC) first and then transported back to plasma membrane (Caswell and Norman 2006).

It was also reported that integrin α4β1 regulates SPARC activity, which regulates extracellular matrix remodeling (Gerson, Shearstone et al. 2012). MMP-2 and MMP-9 were commonly studied to regulate cancer cell invasion by degrading ECM components. Both MMP-2 and MMP-9 were reported to be modulated by integrin directly and indirectly (Chen, Wei et al. 2009; Morozevich, Kozlova et al. 2009). Cancer cells frequently mutate integrin genes to facilitate cell migration.

1.4.3.5 Integrin signaling mediates cell proliferation and cell survival

In early 1990’s, Integrins were proved to be responsible for cell proliferation upon cell attachment on the fibronectin-coated surface. The proliferation is associated to Mitogen-activated protein kinase activation, ERK1 and ERK2 (Zhu and Assoian 1995). Various types of integrin expressions were found to be associated with endothelial and cancer cell proliferation. The main mechanism is that ligand binding to integrins activates FAK and its downstream signaling pathway. Meanwhile, it was also reported that integrin α5β1 regulates epithelial cell proliferation through interaction with epidermal growth factor receptor (Kuwada and Li 2000). This suggested an alternative pathway for integrins to regulate cell proliferation. Another membrane-bound receptor, Urokinase-type plasminogen activator receptor (uPAR) was reported to interact with intergrins to regulate cell proliferation (Ossowski and Aguirre-Ghiso 2000).

In certain extracellular environment, integrins were shown to be able to promote cell survival or induce cell apoptosis (Desgrosellier and Cheresh 2010) (Fig 1-6). Integrin activation caused the phosphorylation and activation of Focal Adhesion Kinase (FAK). FAK phosphorylation activates many cell sur-
vival signaling pathways, such as PI3K-Akt, NF-κB and Bcl-2. Among them, Bcl-2 is the member of anti-apoptotic Bcl family. Bcl-2 binds Bax or Bak in resting cells and BH-3 only proteins compete with Bcl-2 to release Bax or Bak. Bax or Bak forms pores on the mitochondria membrane leading to the release of Cytochrome c to cytosol. It was reported that activation of integrin α5β1 and αvβ3 increased Bcl-2 on both mRNA and protein level (Matter and Ruoslahti 2001). This increase is FAK-activation and Src-activation dependent. Immobilized vitronectin (VN, an ECM component and secreted by cancer cells) conferred resistance of cancer cells to drug treatments (Uhm, Dooley et al. 1999). This is due to the increase of Bcl-2 and Bcl-XL induced by α5β1 and αvβ3 activation. Recently, PKM2 was also suggested to be related to drug resistance (5-FU resistance) (Shin, Yoo et al. 2009). This might be explained by the fact that PKM2 binds and activated integrins, and increased anti-apoptotic protein expression. Similar result was reported that integrins prevents breast cancer cells from apoptosis by activating PI3K-Akt signaling pathway (Aoudjit and Vuori 2001). It was also reported that integrin activates Scr/Akt pathway and mediates anti-cancer drug resistance in cancer cells (Kanda, Kawahara et al. 2013). Scatena and colleagues had shown that NF-κB is responsible for integrin suppression of endothelial cell apoptosis induced by integrin αvβ3 during angiogenesis (Scatena, Almeida et al. 1998).

Pyruvate Kinase M2 has been studied for decades on its role in cancer metabolism. Recently, PKM2 is highlighted again for its new function: promotion gene transcription by acting as a protein kinase. Moreover, it is well known that PKM2 is released into the circulation of cancer patients. The PKM2 levels in patient circulation have been exploited as a diagnostic marker for many types of cancers. However, it is not known how the glycolytic enzyme is released into circulation, and whether the circulative PKM2 has any physiological function(s) in tumor progression. In my dissertation, I demonstrated that PKM2 in the blood circulation facilitates tumor growth by promoting tumor angiogenesis. My data have shown that PKM2 promotes tumor angiogenesis by increasing endothelial cell proliferation, migration and survival. These angiogenic responses are induced by the interaction of PKM2 with αvβ3 integrin.
PKM2 increased FAK phosphorylation and activated focal adhesion complex. I also found that PKM2 re-distributed integrin to the leading edge of endothelial cells, which might be involved in endothelial cell migration. In my dissertation, I also discussed PKM2 dimer/tetramer issue. Dimer PKM2, other than tetrameric PKM2 possesses the activity in promoting tumor angiogenesis. I screened several PKM2 monoclonal antibodies, one of which has been proved to prevent PKM2 from binding to integrin and repress PKM2-induced angiogenesis response. Our data suggested that PKM2 in blood circulation could be used as a therapeutic target for cancer treatment. In addition, I found that PKM2 enhances drug resistance of cancer cells with high integrin αvβ3 expression. I also discussed a cooperative study on investigating a compound, RX-5902 which specifically interrupts the interaction of phosphorylated p68 RNA helicase and β-catenin.
1.5 Figures

Figure 1-1 PKM1 and PKM2 sequence alignment

‘+’ indicates similar charged residues. Blank indicated completely different residues. Human PKM1 and PKM2 have 10 residues difference and 12 residues with similar charges.
PKM2 has two isoforms: dimer and tetramer. Tetrameric PKM2 (also termed as Tumor M2-PK) has high affinity to its substrate PEP, while dimeric PKM2 has relatively low affinity. In cancer cells, oncoproteins binding or modification leads to the conversion of tetramer PKM2 to dimer PKM2. Therefore, all intermediates above pyruvate kinase are accumulated and utilized for other macromolecules synthesis, which facilitates cancer cell proliferation.
Human pyruvate kinase crystal structure was illustrated. Pyruvate kinase M1 and M2 difference was shown in yellow and red.
Angiogenesis balance is maintained by levels of angiogenesis activator and angiogenesis inhibitor. Impropropriate amount of activator or inhibitor leads to dysfunction of angiogenesis and angiogenesis related diseases.

### Activators

- VEGF-A,-B,-C,-D
- PLGF
- Ang1, Tie2
- PDGF-BB
- TGF-β1
- TGF-β receptors
- FGF, HGF, MCP-1
- Integrin αvβ3, αvβ5, α5β1
- VE-Cadherin
- PECAM (CD31)
- Ephrins
- Plasminogen Activator
- MMPs
- NOS, COX-2
- G-CSF, GM-CSF

### Inhibitors

- VEGFR-1
- Ang2
- PAI-1,-2
- TIMP-1,-2,-3,-4
- Angiostatin
- Endostatin
- Vasostatin
- Tumstatin
- Arresten, Canstatin
- Thrombospondin-1,-2
- MMP Inhibitors
- PF-4
- Prolactin
- IFN-α,-β,-γ, IP-10
- IL-4, IL-12, IL-18

Figure 1-4 Angiogenesis balance
a. Functional domain of integrin α and β chains

b. Activation of integrin with I-domain

c. Activation of integrin without I-domain

Figure 1-5 Integrin activation
Integrin regulate cell survival signals upon binding to ECM. Integrin & ECM interaction activates PI3k-Akt and NFκB signaling pathways and its downstream survival response. Unligated integrins induce cell apoptosis though Caspase 8.
CHAPTER 2 Pyruvate Kinase M2 in Blood Circulation Facilitates Tumor Growth by Promoting Angiogenesis

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Running title: PKM2 promotes angiogenesis
2.1 Abstract

It is long known that pyruvate kinase isoform M2 (PKM2) is released into the circulation of cancer patients. The PKM2 levels in patient circulation have been used as a diagnostic marker for many types of cancers. However, it is not known how the PKM2 is released to blood circulation, and whether the circulating PKM2 has any physiological function(s) in tumor progression. In this report, we demonstrate that PKM2 in the blood circulation facilitates tumor growth by promoting tumor angiogenesis. Our experiments show that PKM2 promotes tumor angiogenesis by increasing endothelial cell proliferation, migration, and cell-ECM adhesions. Only the dimeric PKM2 possesses the activity in promoting tumor angiogenesis, which is consistent with the observations that the PKM2 in circulation of cancer patients is a dimer form. Our results further show that PKM2 directly interacts with integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$. PKM2 promotes endothelial cell migration by activating the integrin signals.
2.2 Introduction

An important molecular signature of tumor development is that a shift in expression of isoenzymes of pyruvate kinases occurs to the tumors of almost all types. The tissue specific isoform (L, R, or M1) disappears in many tissue types. In replacement, PKM2 is expressed in cancer cells [1-3]. In tumor cells, growth stimulations convert the pyruvate kinase active tetramer PKM2 to a pyruvate kinase inactive dimer form [4]. It is believed that during tumor development the demands for biosyntheses, especially syntheses of nucleotides and amino acids, are high. One main source of the carbon frame and reducing power of NADPH for the biosynthesis comes from metabolites of glycolysis. Thus, the inactive dimeric PKM2 actually provides a metabolic advantage to supply precursors for biosynthesis. Activation of pyruvate kinase activity is actually unfavorable for tumor growth [5, 6]. Christofk, HR. and colleagues demonstrated that PKM2 is important for cancer cell metabolism and tumor growth [7]. Interestingly, a number of recent studies showed that PKM2 is functionally involved in multiple cellular processes in different subcellular locations, including metabolism control, transcription regulation, and chromatin package [8-12].

High serum levels of PKM2 have long been observed in cancer patients of many types, including gastrointestinal cancer, pancreatic cancer, renal cell carcinoma (RCC), lung cancers, and ovarian cancer [13-16]. Studies show that there is a strong correlation between the serum levels of PKM2 and tumor progression. Thus, it is proposed that serum levels of PKM2 can be used as an important molecular marker for cancer diagnosis/prognosis. PKM2 is a glycolytic enzyme. The forms and the mechanism of its release into the circulation of cancer patients are not known. It is also not known whether the circulating PKM2 has any physiological function in tumor progression. In present study, we provide evidence to show that the circulating PKM2 facilitates tumor growth by promoting angiogenesis. PKM2 promotes tumor angiogenesis by increasing endothelial cell proliferation, migration, and cell-ECM adhesions.
2.3 Results

2.3.1 PKM2 in blood circulation facilitates tumor growth

We sought to investigate the physiological function(s) of circulating PKM2 in tumor progression. We first tested whether PKM2 was also released to the blood circulation in the xenograft model of human colon cancer cell SW620. After four week tumor growth in nude mice, blood samples from the mice with or without tumor implantation were collected. PKM2 levels in the blood samples were analyzed by immunoblot and ELISA of the serum. It was evident that the PKM2 levels in blood of the SW620 tumor mice were very high. As a control, negligible PKM2 levels were detected in blood of mouse without tumor inoculation (Fig. 1A). We also examined the PKM2 levels in the cell culture medium of SW620 cells. In consistent, we observed high levels of PKM2 in the medium (Fig. S1A). We questioned what would be the effects if the PKM2 in the mouse blood circulation was neutralized using an antibody against PKM2. We used an in-house developed rabbit polyclonal antibody raised against full length recombinant PKM2 (Ref to as PabPKM2). Antibody screening indicated specific recognition of PKM2 in the cell extracts (Fig. S2A). The recognition of cellular PKM2 was completely abolished by the bacterially expressed PKM2 (Fig. S2B). The antibody did not recognize any protein in serum of nude mouse (Fig. S2C). IgGs were purified from the antisera of the PabPKM2 (Ref to as IgGPK) by an affinity column of rPKM2 (Fig. S2D). The purified IgGPK or the IgGs purified from pre-immune serum by protein A/G beads (Ref to as IgGCon) was i.p. injected into nude mice that carried SW620 xenograft tumor every two days for 8 days. The administered IgGPK was detectable in mouse serum (Fig. S1B). It was clear that the IgGPK inhibited the tumor growth, while administration of the IgGCon did not exhibit any significant effects on the growth of the same tumor (Fig. 1 B, C, and D). The results suggest that PKM2 in the blood circulation is critical important for the tumor growth.
We next tested whether addition of PKM2 to blood circulation would promote tumor growth. We employed the bacterially expressed recombinant PKM2 (ref to as rPKM2) and its isoenzyme PKM1 (ref to as rPKM1) as a control. Since PKM2 is secreted from cancer cells, presumably, the protein should be present in the extra-cellular space of tumors. Thus, the purified rPKM2 and rPKM1 were pre-mixed with cancer cells at concentration of 2 µM. The mixtures were then s.c. implanted into nude mouse. The purified recombinant proteins were also subsequently i.p. injected (5 mg/kg) to the tumor-bearing nude mice every other days for 8 days. The first injection started 5 days post tumor inoculation. Clearly, the SW620 tumors that were treated with the rPKM2 experienced over 2 folds higher growth rates compared to the tumors that were treated with the rPKM1 and buffer. The tumors treated with the rPKM1 and buffer had almost similar growth rates (Fig. 2 A, B, and C). Fructose-1,6-bisphosphate (FBP) is an allosteric activator of PKM2 [17]. We therefore examined whether addition of FBP would affect the activity of the rPKM2 in promoting tumor growth. Apparently, there is a decrease in the activity of the rPKM2 in promoting tumor growth with addition of FBP (Fig. 2 A, B, and C). To test whether the observed effects of rPKM2 was specific to the SW620 tumor, we employed another xenograft model, human prostate cancer PC-3 cells, by the same treatment schedule. PKM2 was detected in the cell culture medium of PC-3 cells (Fig. S1A). Evidently, administration of the rPKM2 facilitated PC-3 tumor growth (Fig. 3 A, B, and C).

2.3.2 PKM2 promotes angiogenesis

It is intriguing that cancer cells release PKM2 to the blood circulation and the circulating PKM2 promotes cancer growth. We questioned what the functional role of the circulating PKM2 is in promoting tumor growth. One possibility is that extracellular PKM2 promotes cancer cell proliferation. Tissue section stains using an antibody against Ki-67 indeed indicated that the tumors treated with the IgGPK had reduced proliferation rates, while the tumors treated with rPKM2 had higher proliferation rates (Fig.
The representative H&E stains of SW620 tumor tissue sections are shown in figure S1C. However, addition of the IgGPK, IgGCon, rPKM2, rPKM1, and rPKM2 + FBP into SW620 and PC-3 cell culture medium did not lead to any significant change in cell proliferation (Fig. 4 A&B). Thus, the tumor growth promotion by the rPKM2 and inhibition by the IgGPK were unlikely due to their actions on cancer cells. The other possibility is that the PKM2 in circulation promotes angiogenesis to facilitate tumor growth. To test this conjecture, we carried out histology analyses with the tumor tissue sections using antibody against mouse CD31, a marker for endothelial cells. It was clear that treatment of mouse with the IgGPK dramatically reduced blood vessels in the SW620 tumor (Fig. 1 F&G). Reversely, treatment of mouse with the rPKM2 led to substantial increases in blood vessels in both PC-3 and SW620 tumors. The rPKM2 + FBP had reduced effects, while the rPKM1 had no significant effects (Fig. 2 F&G, Fig. 3 D, E, &F). Our analyses strongly suggested that the PKM2 in blood circulation promotes angiogenesis.

To further verify the role of PKM2 in angiogenesis, we employed the in vitro tube formation assay using HUVEC cells. The rPKM2, rPKM2+FBP, rPKM1, and buffer alone were added to the culture medium of the cells. Formation of endothelial tubes was analyzed. The rPKM2 strongly promoted endothelial tube formation (Fig. 5 A&B). The time required for formation of the tubes was substantially shortened, and the tubes were maintained much longer time (data not shown). The rPKM2 + FBP had less effects compared to that of the rPKM2. The rPKM1 had only marginal effects, while saline had no effects (Fig. 5 A&B). We subsequently tested the effects of the PKM2 antibody on the tube formation by coculture the medium collected from SW620 cell cultures in which PKM2 was immunodepleted by the IgGPK with HUVEC cells. Immunoblots indicated that PKM2 in SW620 cell culture medium was completely removed by the IgGPK (Fig. S3A). Clearly, the antibody depletion greatly reduced the endothelial tube formation in the co-culture of SW620 medium with HUVEC cells (Fig. S3 B&C). These in vitro tests supported our notion that PKM2 promotes angiogenesis.
2.3.3 Dimer PKM2 promotes angiogenesis

It is believed that the PKM2 in the cancer patient blood circulation exists as a dimer [18, 19], while the protein in cancer cells exists as a mixture of tetramer and dimer [17, 20] [8]. Thus, an interesting issue is whether the dimer and tetramer status of PKM2 have different effects in promoting tumor growth. We first examined the tetramer and dimer status of the administered rPKM2/rPKM1 in the mouse serum. Chromatography analyses followed by immunoblots indicated that the rPKM2 existed mostly as dimer in the circulation, while the rPKM1 was mostly tetramer (Fig. 6A). Using an ELISA analysis, we estimated that the concentration of the i.p. injected rPKM2 and rPKM1 (at dose of 5 mg/kg) in the mouse blood was around 400 – 800 nM 4 hours after the administration. We also analyzed the tetramer and dimer status of the rPKM2/rPKM1 in vitro. Chromatography profiles indicated that the rPKM2 existed as a mixture of tetramer and dimer (with tetramer to dimer ratio at around 80% to 20%) at concentration of 12 µM, while the rPKM1 was almost completely tetramer at the same concentration (Fig. 6B). The purified proteins possessed pyruvate kinase activity (Fig. 6C). Interestingly, dilution of the rPKM2 led to conversion of tetramer to dimer with the rPKM2 became almost completely dimer at around 1 µM (Fig. 6 D&G). This concentration is very close to the concentration of the administered rPKM2 in mouse blood. Most of the rPKM1 still existed as tetramer at this concentration (Fig. 6 F&G). Addition of 3 mM FBP converted the rPKM2 to the tetramer, even at concentration as low as 1 µM (Fig. 6E). Consistently, a large portion of rPKM2 was tetramer in mouse blood circulation when the protein was co-administered with 3 mM FBP (Fig. 6A). Clearly, the observed activity of FBP in facilitating the conversion of the rPKM2 from dimer to tetramer is consistent with the results that addition of FBP to the rPKM2 reduced the effects of the protein on promoting tumor growth (Fig. 2 A, B, and C, Fig. 3 A, B, and C). Both support a conclusion that introduction of the dimeric PKM2 to blood circulation promoted tumor angiogenesis. Can FBP also convert the circulating dimer PKM2 to a tetramer therefore inhibit tumor angiogenesis? To answer this question, we collected the serum samples from the mice that had
been implanted tumors. FBP was added to the serum samples. The PKM2 in the serum was analyzed by the same chromatography procedure. Clearly, addition of FBP did not convert the circulating PKM2 to tetramer (Fig. S4). The observation is consistent with previous report that FBP could not convert the nuclear dimer PKM2 to a tetramer [8].

### 2.3.4 PKM2 facilitates endothelial cell migration

How does circulating PKM2 promote tumor angiogenesis? To address this question, we analyzed cell proliferation, migration, and adhesion of HUVEC cells. Addition of rPKM2, to the cell culture medium led to a marginal increase in cell proliferation (Fig. 5C). It is unlikely this small effect would be the sole factor that confers the in vivo effects on tumor growth. On the other hand, boyden chamber assays showed that addition of the rPKM2 led to a strong increase in cell migration, and the effects were substantially reduced by addition of FBP. The rPKM1 had almost no effects (Fig. 5D). The increases in cell migration were not observed with SW620 and PC-3 cells (Fig. S5A). We noted a strong attachment of HUVEC cells to the culture plates with rPKM2 coating (Fig. 5E). In addition, the attachment of HUVEC cells to the ECM coated plate was substantially strengthened by addition of SW620 cell culture medium, while the enhancement was abolished by addition of the antibody PabPKM2 (Fig. S5B). We suspected that the rPKM2 might affect endothelial cell adhesion to ECM. Indeed, the cell attachment assays showed that the HUVEC cell adhesion to vitronectin and fibronectin, matrix molecules was strongly enhanced upon addition of the rPKM2 to the culture medium (Fig. 5F). The effects were not observed with rPKM1. In consistent, we observed a strong effect on the HUVEC cell spreading by addition of rPKM2 into the cell culture medium, while this effect was not observed with rPKM1 (Fig. S5C). The effects of PKM2 on cell adhesion to ECM and cell migration was not observed with epithelial cancer cells (Fig. S5 A&D), indicating that the effects were endothelial cell specific. Thus, we conclude that PKM2 promoting angiogenesis by facilitating endothelial cell migration and cell adhesions to ECM.
PKM2 enhanced the attachment of endothelial cells to ECM. We therefore examined whether PKM2 interacts with integrins by Biacore using recombinant integrins αvβ3 and α5β1. PKM2 interacted with both integrin pairs with stronger affinity for αvβ3 (Fig. 7A). We further confirmed the interaction by an antibody blocking experiments. HUVEC cells were attached on microscopic slides with rPKM2 coating. The cells were detached by addition of antibody against αvβ3 and the IgGPK (Fig. 7B). To verify whether the PKM2 and integrin interaction had any functional relevance, we examined HUVEC cell migrations on fibronectin and vitronectin in the presence of rPKM2 and rPKM1. Evidently, migrations of the cells on vitronectin and fibronectin were significantly increased by addition of rPKM2, but not rPKM1. The increases in migration were diminished by addition of the PabPKM2 and the antibody against αvβ3 (Fig. 7C). Immunostaining of F-actin indicated that addition of rPKM2 led to an increased stress fiber of actinfilament accompanied by increased accumulation of vinculin at the tips of stress fibers at early time of ECM attachment (Fig. 7D). In consistent, a strong increase in activation of FAK was observed upon addition of PKM2. The activation of FAK was diminished by the antibody IgGPK (Fig. 7E). Thus, our results indicate that PKM2 target integrins to promote angiogenesis.

2.4 Discussion

A very high percentage of cancer patients of different cancer types have elevated levels of PKM2 in their blood circulation [21]. We show that the circulative PKM2 plays a critical role in facilitating tumor growth by promoting tumor angiogenesis. An obvious question is why tumors release this glycolysis enzyme to the circulation to promote angiogenesis. One plausible explanation is that dimeric PKM2 is a sensor for the glycolysis status and glucose demands in cancer cells for high proliferation. It is demonstrated by many laboratories that PKM2 is converted to dimer in high proliferation cells with high glycolysis activity [20, 22]. Thus, it is possible that dimeric PKM2 is a signal that reflecting nutrition requirement, therefore a signaling for angiogenesis. Most recently, Luo, W. and co-workers reported that PKM2
co-activates the transcription factor HIF1α [10]. It is conceivable that PKM2 may also be an important molecular factor that response to hypoxia condition for angiogenesis stimulation. Interestingly, PKM2 is the main pyruvate kinase isoform during fetus development. It is well known that angiogenesis is active during organ development and maturation. Is PKM2 also secreted into blood circulation of fetus for promoting angiogenesis?

Another open question is why PKM2 but not PKM1 in the circulation promotes tumor angiogenesis. PKM2 and PKM1 differ by only 23 amino acids resulted from alternative pre-mRNA splicing. These two isoforms share almost identical structure. However, structural analyses revealed that the segment with different amino acid sequence is mostly localized at the dimer-dimer interface [4]. Thus, it is conceivable that the dimer interface is engaged in interaction with the integrins, and consequently promotes angiogenesis. This notion is consistent with the results that the dimer PKM2 is effective in promoting angiogenesis, while tetramer is inactive. We previously demonstrated a PKM2 mutant R399E that is a dimer and less active in pyruvate kinase activity [8]. The R399E mutant is fully functional (slightly more active) in promoting HUVEC cell attachment to ECM (Fig. S6), suggesting that the pyruvate kinase activity is not required and the exposed dimer-dimer interface is required, which also supports the notion that the dimer interface may be engaged in interaction with the target(s) consequently promotes angiogenesis. Apparently, inhibition of tumor growth by the PKM2 antibody reveals a potential new anti-angiogenesis target for cancer patients with high circulative PKM2. One great advantage of targeting PKM2 is that the levels of circulative PKM2 would be a prognosis marker to predict the possible outcomes.
2.5 Materials and Methods

2.5.1 Reagents, cell lines, antibodies, and protein expression/purifications

Antibodies against β-actin, mouse CD31, Ki-67 were purchased from Cell Signaling, SantaCruz, and Abcam respectively. Antibody against PKM2 was raised using recombinant PKM2 expressed/purified from *E. coli*. as an antigen. IgGs were purified from the rabbit anti-serum over a protein G column. Integrins α<sub>v</sub>β<sub>3</sub>, α<sub>5</sub>β<sub>1</sub> were purchased from R&D System, and were directly used without further treatment. Cell lines SW620 and PC-3 were purchased from ATCC, and HUVEC cells were purchased from Invitrogen. The cells were cultured by following the vendor’s instructions. The cDNAs that encode human PKM2 and PKM1 were purchased from Adgenes. The cDNAs were subcloned into bacterial expression vector pEG-32a. The recombinant proteins were purified from bacterial lysates by a two column procedure.

2.5.2 Mice xenografts and treatments

All animal experiments were carried out in accordance with the guidelines of IACUC of Georgia State University. Nude mice were subcutaneously injected with 5 × 10<sup>6</sup> of SW620 or PC-3 cells. Tumor formation and volumes were assessed every 2 days. Tumor volumes were measured by two perpendicular diameters of the tumors with the formula 4π/3 × (width/2)<sup>2</sup> × (length/2). The tumor bearing mice were subjected to the i.p. injections of appropriate agents once every other days for eight days. The treatments started five days post tumor inoculations. The tumors were collected and weighed at the end of the experiments. Tissue sections were prepared from harvested tumors, and stained using commercially available antibodies against Ki-67 or mouse CD31. Statistical analyses were done in comparison to the control group with Student’s t test.
2.5.3 Boyden chamber and cell proliferation assays

QCM™ 24-Well Fluorimetric Cell Migration Assay kit (ECM) was used to measure the migration of different cells. The test cells were first treated under the different conditions (indicated in figure legends) in regular cell culture plates. The treated cells were re-suspended into optimum medium (without serum) and seeded into the inner chamber of the migration assay kit. The culture medium with 10% FBS was added to the outer chambers. After overnight incubation, medium in the inner chamber was removed and the cells attached to the outer bottom side were detached using the cell detachment buffer (included in the kit). The detached cells were then lysed using the cell lysis buffer (included in the kit). The amounts of the migrated cells were determined by measuring the fluorescence using λex=485nm and λem=535nm.

For analyses of cell proliferation, a cell proliferation ELISA kit that measures BrdU incorporation was used. Briefly, cells were incubated for appropriate time in the presence of 10 µM BrdU under different conditions (indicated in figures). The cells were fixed after incubation and washed 3 times. The fixed cells were detected by anti-BrdU-POD antibody and secondary antibody. The nuclei incorporations of BrdU were measured by chemiluminescence emission (Victor 3TM, PerkinElmer). Cell proliferation was also measured by cell number counting. Cells were incubated for appropriate time under appropriate conditions. Cell numbers was counted before and after the indicated time of culture by five independent cell counting.

2.5.4 Endothelial tube formation and cell attachment assays

Endothelial tube formations were carried out with the endothelial tube kit. Briefly, HUVEC cells were seed in culture plat coated with martigel. After 30 minutes incubations, agents, e.g. FBS, proteins, or cancer cell culture medium, were added to the HUVEC. The cells were further cultured for additional
16 hours. The formed endothelial tubes were analyzed under light microscope. For the tube formation with supplement of SW620 culture medium, no FBS was added to the HUVEC cell culture.

For cell attachment, cells were cultured overnight under standard conditions. Next days, different cells (with appropriate cell numbers) were transferred to a new plate with wells that coated with different proteins (indicated in the figures) with fresh medium with addition of appropriate agents in the medium (indicated in the figures). The cells were further cultured for 2 hours and washed gently. The attached cells were either directly counted or lysed. The cell lysates were then measured to determine the amounts of attached cells.

### 2.5.5 Size-exclusion chromatography

Size exclusion chromatograph was performed with a Superdex 200 10/300GL column. The samples of mouse serum (2–8 mg/ml of total protein), the rPKM2 (~15 µM), the rPKM1 (~15 µM) were prepared in tris-HCl buffer with/without FBP. 100 µl of the sample was loaded into the column and eluted with elution buffer (50 mM phosphate, 0.15M NaCl pH7.2). The fraction of 300 µl was collected, and 20 µl of each fraction was analyzed by immunoblot. The elution profiles were compared to that of a size exclusion chromatograph calibration kits (GE Healthcare) under identical conditions. The elution profile was plotted against LogMW according to vendor’s instructions.

### 2.5.6 Pyruvate kinase activity

Pyruvate kinase activity was analyzed by following the experimental procedure similar to that was described by Christofk and colleagues [22].

### 2.5.7 Biacore binding analyses

The interactions of rPKM2 and rPKM1 with integrins were analyzed by Surface Plasmon Resonance (Biacore). The purchased integrins α5β1 and αvβ3 was diluted in 50 mM acetate buffer to a concen-
tration of 100 µg/ml. The integrins were immobilized on the CM5 chips to show response units (RU) of 1980 and 2030 for α5β1 and αvβ3 respectively. The first channel with full ethanol amine block was used as negative control. The rPKM2 and rPKM1 were diluted to 100 µg/ml in the running buffer (10 mM HEPES, 150 mM NaCl, 5 mM MgCl2, pH7.4). The flow rate of rPKM2/rPKM1 in running buffer was 20 µl/min and the binding time was 16 min. The dissociated time is 30 min. The Kds were obtained by the fitting the binding data as 1:1 ratio of integrin/PKM binding.

2.6 Conflict of interest

The authors declare no conflict of interest.

2.7 Acknowledgments

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2.8 References


Figure 2-1 Antibody against PKM2 inhibits tumor growth.

(A) The levels of PKM2 in the serum of mouse blood collected from tumor bearing nude mouse (SW620) and non-tumor nude mouse (Con) were examined by immunoblot of PKM2 (IB:PKM2). Coomassie stain (CBS: M-Albumin) of serum albumin is a loading control. (Bottom) The PKM2 levels in serum of nude mouse with/without SW620 tumor implantation and with/without i.p. administration of rPKM2 (48 hours after injection) were determined by sandwich ELISA using an in-house developed rabbit monoclonal antibody against PKM2 and a commercially available goat polyclonal antibody against PKM2. The PKM2 concentration was presented as the range of high and low levels (ng/ml) in serum samples from five mice per group tested.
Figure 2-1B Growth curve of SW620 tumor.

Growth of SW620 tumor under the treatment of purified IgGs of IgGPK (IgGPK) or pre-immune serum (IgGCon) was monitored by growth curve measuring tumor volumes every two days.

The *p values in (B) and (D) are calculated using unpaired two-tailed Student t-test. The error bars in (B) are standard deviations from measurement of five mice.
PKM2 antibody inhibits tumor growth.

Figure 1C&D PKM2 antibody inhibits tumor growth.

(C) and (D) indicates the growth of SW620 tumor under the treatment of purified IgGs of IgGPK (IgGPK) or pre-immune serum (IgGCon) was monitored by; (C) endpoint pictures and (D) endpoint weights of the harvested tumors with 8 days treatment (treatment started 5 days post tumor inoculation).
Figure 2-1E Ki-67 staining of SW620.

The top panel is representative images of immunostaining of tissue sections prepared from the harvested tumors using antibodies against Ki-67 (Red). The blue is DAPI stain of cell nucleus. The bottom panel indicates the quantization of Ki-67 staining signals of the tissue sections. The standard deviations of the mean values were measured from randomly selected 4 fields in randomly selected 3 sections from each tumor.
Figure 2-F&G  CD31 staining of SW620 tumors.

(F) is DAPI stain of cell nucleus. The proliferation index was percentage of the positive nucleus staining by counting the nucleus number with ki-67 positive staining in randomly selected three fields in each slide from randomly selected three slides from each tumor. (G) Quantitative analyses of vessel lengths, densities, and branch points (manually counting) of the CD31 staining of the tumor tissue sections using software imaging-J. The quantization was statistical mean values of randomly selected 4 fields in randomly selected 3 sections from each tumor.
**A**

- Saline
- rPKM1
- rPKM2
- rPKM2+FBP

**B**

Saline
rPKM1
rPKM2
rPKM2+FBP

**C**

- *p = 0.09
- *p = 0.013
- *p = 0.46
Figure 2-2 Recombinant PKM2 promotes SW620 tumor growth. (A), (B), and (C) Growth of SW620 tumor under the treatment of different recombinant proteins (indicated) was monitored by; (A) growth curve by measuring tumor volumes every two days, (B) endpoint pictures and (C) endpoint weights of the harvested tumors with 8 days treatment (treatment started 5 days post tumor inoculation). The *p values in (C) are calculated using unpaired two-tailed Student t-test. The error bars in (A) are standard deviations from measurement of five mice.
Figure 2-2D Ki-67 staining of SW620 tumor administered by recombinant proteins.

Representative images of immunostaining of tissue sections prepared from the harvested tumors with antibodies against Ki67 (Red). The blue color indicates nucleus staining by DAPI. Photos were taken by Confocal Microscope.
Figure 2-2E quantization of Ki-67 staining of SW620 tumor.

(E) indicates quantization of Ki-67 staining signals of the tissue sections. The proliferation index was percentage of the positive nucleus staining by counting the nucleus number with ki-67 positive staining in randomly selected three fields in each slide and randomly selected three slides from each tumor. The standard deviations of the mean values are from measurements of randomly selected 4 fields in randomly selected 3 sections from each tumor.
Figure 2-F CD31 staining of SW620 tumor treated with recombinant proteins. (F) is representative images of immunostaining of tissue sections prepared from the harvested tumors with antibodies against mouse CD31 (Green). The blue color is DAPI stain of cell nucleus. Photos were taken by Confocal Microscope.
G

<table>
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<th>Saline</th>
<th>rPKM1</th>
<th>rPKM2</th>
<th>rPKM2+FBP</th>
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<td>MVD (per mm$^2$)</td>
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<td>46.2 ± 11.9</td>
<td>90.6 ± 19.5</td>
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<td>Vessel Length (µm)</td>
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<td>933.3 ± 66.2</td>
<td>742.2 ± 92.8</td>
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<td>Branch points</td>
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<td>4.3 ± 3.1</td>
<td>9.4 ± 4.5</td>
<td>8.0 ± 3.5</td>
</tr>
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Figure 2-2G Quantization of CD31 staining of SW620 tumors.

Quantitative analyses of vessel lengths, densities, and branch points (manually counting) of the CD31 staining of the tumor tissue sections using the software imaging-J. The quantization was statistical mean values of randomly selected 4 fields in randomly selected 3 sections from each tumor.
A

Days post implantation

B

Saline

rPKM1

rPKM2

rPKM2 + FBP

Saline

rPKM1

rPKM2

rPKM2 + FBP

Days post implantation

Tumor volume mm$^3$
Figure 2-3 Recombinant PKM2 promotes PC-3 tumor growth.

(A), (B), and (C) Growth of PC-3 tumor under the treatment of different recombinant proteins (indicated, one tumor in the rPKM1 treatment group did not grow) was monitored by; (A) growth curve by measuring tumor volumes every two days and the tumor volumes were calculated by formula; Tumor volume = \( \pi/6 \times (\text{width})^2 \times \text{length} \), (B) endpoint pictures and (C) endpoint weights of the harvested tumors after 13 days growth with 8 days treatment. The *p values are calculated using unpaired two-tailed Student t-tests.
Figure 2-3D CD31 staining of PC-3 tumor treated with recombinant proteins.

Representative images of immunostaining of tissue sections prepared from the harvested tumors with antibodies against mouse CD31 (Green). The blue color is DAPI stain of cell nucleus. Photos were taken by Confocal Microscope.
**E**

![Bar graph showing microvessel density](image)

**F**

![Bar graph showing numbers of branch points](image)
Figure 3E&F Quantization of CD31 staining of PC-3 tumor.

(E) & (F) Quantitative analyses of vessel densities (E) and branches (F) (by manually counting) of the CD31 staining of the tumor tissue sections using the software imaging-J. The quantization was statistical mean values of randomly selected 4 fields in randomly selected 2 sections from each tumor. The error bars in (E) and (F) are standard deviations of the mean values from measurements of randomly selected 4 fields in randomly selected 2 sections from each tumor.
A

![Bar graph showing relative proliferation of SW620 and PC-3 cells with different treatments: Saline, IgGPK, and IgGCon.](image)

B

![Bar graph showing relative proliferation of SW620 and PC-3 cells with different treatments: saline, rPKM1, rPKM2, and rPKM2+FBP.](image)
Figure 2-4 PKM2 and anti-PKM2 antibody does not affect cell proliferation of SW620 and PC-3 cells.

(A) and (B) Cell proliferations of SW620 (filled bars) and PC-3 (open bar) cells in the presence of rPKM2, rPKM2 + FBP, and rPKM1 (5 µg/ml) (A) or the antibody IgGPK (IgGPK) and the IgGCon (IgGCon) (B) were analyzed by a commercial BrdU proliferation kit. The cell proliferations are presented as relative proliferation by defining the proliferation of buffer saline treated cells as 100. The error bars in (A) and (B) are standard deviations from five repeating experiments.
Figure 2-5 PKM2 affects endothelial tube formation, migration and attachment.

(A) Representative light microscopic images of endothelial tubers formed by HUVEC cells in the presence of rPKM2, rPKM2 + FBP, and rPKM1, or (B) Quantitative analyses of the branch points in the formed HUVEC tubes. The quantization was the mean average of branch point counts of randomly selected four fields from each slide and with five repeating experiments (slides).
Figure 2-5C&D PKM2 promotes endothelial cell proliferation and migration.

(C) & (D) Cell proliferation (C) and migration (D) of HUVEC cells in the presence of rPKM2, rPKM2 + FBP, rPKM1, and buffer saline were analyzed by a commercial BrdU proliferation kit and boyden chamber assay respectively. The cell proliferation and migration are presented as relative proliferation (C) or relative migration (D) by defining the proliferation of buffer saline treated cells as 100 and cell migration of rPKM2 treated cell as 100.
Figure 2-5E Endothelial cells bind to PKM2.

Cell attachment of HUVEC cells to cell culture plate on which rPKM2, rPKM1, or BSA was coated respectively.
Figure 2-5F PKM2 enhances endothelial cells to bind to Extracellular Matrix.

Cell attachment of HUVEC cells to cell culture plate on which fibronectin (open bars) or vitronectin (filled bars) was coated and the indicated proteins were added to the culture medium. The cell attachments are presented as relative attachment by defining the cell attachment in the plate on which rPKM2 was coated as 100 in (E) or buffer saline was added to the culture medium as 100 in (F). In (A), (B), (C), and (D), buffer saline is a control. The error bars in (B), (C), (D), (E), and (F) represent standard deviations from five repeating experiments.
Figure 2-6 tetramer or dimer formation of PKM2 and PKM1.

(A) (Upper) Immunoblot analyses of the chromatography fractions of the mouse serum collected from mice that were administered rPKM2, rPKM2 + FBP, and rPKM1 using the antibody PabPKM2. (Bottom) The chromatography profiles of the standard molecular weight calibration kit.
Figure 2-6B Recombinant PKM2 forms tetramer at high concentration.

Chromatography profiles of rPKM2 (Blue) and rPKM1 (Red) at concentration of 12 µM. Elution volumes equivalent to tetramer and dimer are indicated by arrows.
Figure 2-6C PKM2 has low enzymatic activity.

Pyruvate kinase activity of the rPKM2, rPKM2 + FBP, and rPKM1 (5 µg/ml) was analyzed by the method described by Christofk and colleagues. The pyruvate kinase activity was expressed as relative pyruvate kinase activity by defining the activity in the rPKM1 as 100.
Figure 6D, E, F and G rPKM2 forms dimer at low protein concentration (D), (E), (F), and (G) The chromatography profiles of different concentrations (indicated) of rPKM2 (D), rPKM2 + FBP (E), and rPKM1 (F), as well as 1 µM of rPKM2/rPKM1 (G). The dimer and tetramer ratios (T/D ratio) in (B), (D), (E), and (F) were calculated by the areas under the dimer and tetramer peaks. In (A), (B), (D), (E), (F), and (G) the y-axis is the absorbance of the elution at 280 nm. The numbers in the X-axis are the elution volumes. The error bars in (C) are standard deviations from five repeating experiments.
Figure 2-7 PKM2 interacts with integrins to activate integrin signaling.

(A) (Upper) the representative binding curves of binding of PKM2 to integrins αvβ3 and α5β1 that were monitored by Biacore with integrins that were immobilized on the Biacore chip. (Bottom) The deduced disassociation constants (kd) of PKM2 to integrin αvβ3 and α5β1 from the Biacore binding analyses.
Figure 2-7B endothelial cell binding to PKM2 is inhibited by integrin αvβ3 antibody.

Cell attachment of HUVEC cells to microscopic chamber slides on which rPKM2 was coated and the indicated agents were added to the slides. LM609 is an antibody against integrin αvβ3. The cell attachments are presented as relative attachment by defining the cell attachment in the slide on which the rabbit IgG purified from pre-immune serum (IgGCon) was added to the culture medium as 100. The error bars in (B) and (C) represent standard deviations from five repeating experiments.
Figure 2-7C Integrin mediates PKM2-induced HUVEC migration.

Migration of HUVEC cells in the presence of rPKM2, rPKM2 + FBP, rPKM1, and buffer saline (Upper) and with addition of antibodies against PKM2 (IgGPK) or integrin αvβ3 (Bottom) were analyzed by boyden chamber assay. The ECM vitronectin (VN) or Fibronectin (FN) was always added to the migration assays. The cell migrations are presented as relative migration by defining the cell migration of buffer saline treated cells as 100.
Figure 2-7D PKM2 promotes focal complex assembly and stress fiber forming.

Representative microscopic images of immune-staining of HUVEC cells with antibody against F-actin (Red) and Vinculin (Green). The cells were seeded on microscopic chamber slides with ECM coating. The indicated proteins (at 5 µM) were added to the slides. The cells were fixed 45 minutes after seeding on the chamber slides and stained using the indicated antibody. The numbers indicate the percent of cells have typical or similar staining pattern as showed in the images after analyses of randomly selected 4 view fields from each slide and three independent experiments.

62.5 ± 15.4%  16.3 ± 6.8%  8.7 ± 3.0%
Figure 2-7E PKM2 activates FAK by tyrosine 397 phosphorylation.

Activation of FAK was analyzed by immunoblot of cell extracts of HUVEC cells that were seeded on ECM coated culture plate with addition of the indicated proteins (5 µM) for 60 minutes using antibody against phosphorylated FAK (IB:p-FAK397). Immunoblot of extracts using antibody against FAK (IB: FAK) is a control indicate the cellular levels FAK and loading control.
Figure 2-8 PKM2 was actively released or secreted to cell culture medium.

Left panel: PKM2 and β-actin were detected in SW620 whole cell lysate (SW620 Lysate), SW620 cell culture medium (SW620 CM) and controlled culture medium (Con) respectively. The data indicated that PKM2 is actively released to the culture medium since β-actin was not present in the SW620 culture medium.

Right panel: PKM2 were present in both colon cancer cell line SW620 and prostate cancer cell line PC-3. Meanwhile, SW620 cells released 3-5 fold more PKM2 than PC-3.
Figure 2-S1B administered PKM2 antibody in mouse blood serum.

Immunoblot indicates PKM2 antibody (IgGPK) can be detected in mice blood serum. SW620 tumor bearing mice were administered by IgGPK.
Figure 2-S1C H&E staining of SW620 tumors.

Hematoxylin and eosin staining indicates the structure of SW620 tumors treated with Saline, rhPKM1, rhPKM2 and rhPKM2+FBP respectively.
Figure 2-S 1 specificity test of anti-PKM2 antibody.

Left panel (S2A): Single band was detected in H146 and H460 whole cell lysate by western blot. The band located in around 60KDa Protein ladder band is PKM2. BSA was used as control.

Right panel (S2C): PKM2 antibody (IgGPK) only recognized PKM2 band in the blood serum of SW620 tumor bearing mice, but not the control mice. Control antibody (IgGCon) did not recognized any protein in the blood serum of control mice.
Figure 2-S2B recombinant PKM2 competes with cellular PKM2 to bind to IgGPK.

Immunoblot indicates the recognition of cellular PKM2 was completely abolished by bacterially expressed recombinant PKM2.
Figure 2-S2D PKM2 antibodies purification.

Anti-PKM2 antibody, IgGPK and normal rabbit IgG, IgGCon were purified by Protein A sepharose beads.

Purified antibodies were analyzed by SDS-PAGE.
A

IB: PKM2
IB: IgG HC

B

620CM ConCM+IgGPK
620CM+IgGCon

C

Numbers of branch points

620CM
conCM+IgGPK
620CM+IgGPK
620CM+IgGCon
Figure 2-9 PKM2 secreted by SW620 cell promotes endothelial cell tube formation.

(A) PKM2 in SW620 culture medium was removed by anti-PKM2 antibody, IgGPK. Immunoblot has shown that PKM2 level was significantly reduced after removal by IgGPK conjugated to Protein A sepharose beads. IgGCon was taken as a control. IgG heavy chain was blotted as an internal loading control.

(B) Representatives of endothelial tube formation assay show that PKM2 removal from SW620 culture medium significantly reduced tube formation compared to SW620 culture medium treated with IgGCon.

(C) Quantization of branch points in endothelial tube formation assay. The error bars in branch points are standard deviations of the mean values from measurements of randomly selected four wells.
Figure 2-10 FBP does not convert circulating PKM2 to tetramer.

Mice blood sera were analyzed chromatography and immunoblots. PKM2 mainly forms dimer with absence or presence of FBP.
PKM2 does not affect SW620 and PC-3 cell migration.

Cell migration of SW620 (filled bars) and PC-3 (open bar) cells in the presence of rPKM2, rPKM2+FBP, and rPKM1 were analyzed by Boyden chamber assay. The cell migration is presented as relative migration by defining the migration of buffer saline treated cells as 100. The error bars are standard deviations from three repeating experiments.
SW620 cell culture medium enhanced endothelial cells attaching to ECM coated surfaces and this attachment is abolished by anti-PKM2 antibody, IgGPK, but not by normal rabbit IgG, IgGCon. The cell attachments are presented as relative attachment by defining the cell attachment in the well in which the control culture medium (ConCM) was added as 100. The error bars represent standard deviations from five repeating experiments.
Figure 2-S5C rhPKM2 enhanced endothelial cell spreading.

Top left, top right and bottom left panels illustrated endothelial spreading upon treatment of saline, rhPKM1 and rhPKM2 respectively.

Bottom right panel: statistically analysis of percentage of spreading endothelial cells among all attached cells. RhPKM2 treatment significantly increased numbers of spreading cells compared to rhPKM1 and BSA control.
Figure 2-S5D PKM2 does not affect SW620 and PKM2 adhesion to ECM.

SW620 cell attachment on ECM in the presence of rPKM2, rPKM2 + FBP, and rPKM1 were analyzed cell binding assay. The cell attachments are presented as relative attachment by defining the cell attachment in the plate on which saline was added as 100. The error bars represent standard deviations from five repeating experiments.
Figure 2-12 PKM2 dimer is required for promoting endothelial cell attaching to ECM.

Endothelial cell attachment on ECM in the presence of rPKM2 or PKM2 R399E mutant was analyzed cell binding assay. The cell attachments are presented as relative attachment by defining the cell attachment in the plate on which rPKM2 was added as 100. The error bars represent standard deviations from three repeating experiments.
CHAPTER 3 Circulative pyruvate Kinase M2 mediate cancer drug resistance by activation of integrin αvβ3 signaling

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Running title: PKM2 promotes cancer drug resistance
3.1 Abstract

Pyruvate kinase isoform M2 (PKM2) is released into the circulation of cancer patients. The PKM2 levels in patient circulation are used as a diagnostic/prognostic marker for many types of cancers. We previously demonstrated that PKM2 in blood circulation facilitates tumor growth by promoting tumor angiogenesis. Here, we report that the circulative PKM2 mediates cancer cell drug resistance. Antibody raised against PKM2 render cancer cells sensitive to chemotherapies. Our results demonstrate that PKM2 mediates drug resistance by activating integrins αvβ3 signaling. Evidently, the extracellular PKM2 activates survival signal Akt via activation of the integrin signaling. Our study uncovers a novel molecular mechanism by which cancers acquire drug resistance by secreting PKM2, revealing a potential target for developing cancer treatment.
3.2 Introduction

An important molecular signature of tumor development is that a shift in expression of isoenzymes of pyruvate kinases occurs to the tumor of almost all types. The tissue specific isoform (L, R, or M1) disappears. In replacement, PKM2 is expressed in cancer cells [1-3]. In tumor cells, growth stimulations convert the pyruvate kinase active tetramer PKM2 to a pyruvate kinase inactive dimer form [4]. It is believed that during tumor development the demands for biosyntheses, especially syntheses of nucleotides and amino acids, are high. One main source of the carbon frame and reducing power of NADPH for the biosynthesis comes from metabolites of glycolysis. Thus, the inactive dimeric PKM2 actually provides a metabolic advantage to supply precursors for biosynthesis. Activation of pyruvate kinase activity is actually unfavorable for tumor growth [5, 6]. Christofk, HR. and colleagues demonstrated that PKM2 is important for cancer cell metabolism and tumor growth [7]. Interestingly, a number of recent studies showed that PKM2 is functionally involved in multiple cellular processes in different subcellular locations, including metabolism control, transcription regulation, and chromatin package [8-12].

High serum levels of PKM2 have long been observed in cancer patients of many types, including gastrointestinal cancer, pancreatic cancer, renal cell carcinoma (RCC), lung cancers, and ovarian cancer [13-16]. Studies show that there is a strong correlation between the serum levels of PKM2 and tumor progression. Thus, it is proposed that serum levels of PKM2 can be used as an important molecular marker for cancer diagnosis/prognosis. How PKM2 is released into patient blood circulation was an intriguing question. Recent studies showing that cultured cancer cells actively secrete PKM2 to the culture medium suggesting that the circulative PKM2 may not be a result of nature turn-over of cancer cells, rather it is an active mechanism by which cancer cells protect themselves. Several studies noted that the levels of PKM2 in patient blood circulation correlate well with patient drug resistance to several anti-cancer drugs. It is not known whether and how the circulative PKM2 mediate cancer drug resistance.
We previously demonstrated that the circulative PKM2 facilitated cancer growth by promoting tumor angiogenesis. The extracellular PKM2 promoted angiogenesis by acting on integrin $\alpha_v\beta_3$. We showed that PKM2 directly interacted with the integrin and activated the integrin signals. It is well known that activation of integrin $\alpha_v\beta_3$ activates cell survival signals. Activation of integrin $\alpha_v\beta_3$ contributes a major mechanism for cancer cell drug resistance. In present study, we provide evidence to show that extracellular PKM2 mediates cancer cell drug resistance. Antibody raised against PKM2 sensitizes cancer cells to apoptosis induction by anticancer drugs. Extracellular PKM2 mediates cancer cell drug resistance by activating integrins $\alpha_v\beta_3$ signaling and subsequent activation of survival signals. Our study uncovers a novel molecular mechanism.

### 3.3 Results

Our previous studies showed that PKM2 in cancer patient blood circulation facilitated tumor growth by promoting tumor angiogenesis. The extracellular PKM2 promotes tumor angiogenesis by activating integrin $\alpha_v\beta_3$ signals in angiogenic endothelial cells. Since it is well known that activation of integrin $\alpha_v\beta_3$ signaling constitutes a major mechanism by which cancer cells acquire apoptosis resistance, we reasoned whether secretion of PKM2 constituted an important mechanism for cancer cells that express integrin $\alpha_v\beta_3$ to develop drug resistance. To test our hypothesis, we carried out screening to select our study system. We examined several cancer cell lines for integrin $\alpha_v\beta_3$ expression and PKM2 secretion. Evidently, melanoma SK-MEL-28 cells have high levels of integrin $\alpha_v\beta_3$ expression and relative high level of PKM2 secretion, while colon cancer SW620 cells do not express the integrin pair but has high levels of PKM2 secretion. PC-3 cells do not have high levels of integrin $\alpha_v\beta_3$ expression nor PKM2 secretion (Fig. 1A). Based on the screening, we selected SK-MEL-28 and SW620 cells as our study cell lines. We examined whether the cells with high levels of integrin $\alpha_v\beta_3$ expression and high levels of PKM2 secretion would be resistant to drug treatments. We used oxaliplatin as an example. Clearly, both SK-Mel-28 and
SW620 cells were strongly resistant to the drug induced apoptosis, with SK-Mel-28 had stronger resistance (Fig. 1B). To verify whether the extracellular PKM2 levels play a role in the apoptosis resistance, we first employed the rabbit monoclonal antibody against PKM2 (ABPK) that is known to block the PKM2 and integrin αvβ3 interaction. The SK-Mel-28 and SW620 cells were treated with oxaliplatin in the presence of ABPK or control rabbit IgG. It was clear that the SK-Mel-28 cells were more sensitive to the drug induced apoptosis in the presence of ABPK. As a control, rabbit IgG did not have such effects, while the antibody almost had no effects on the drug sensitivity of SW620 cells (Fig. 2A). To further test whether PKM2 indeed mediates the drug resistance, we examined whether addition of the recombinant PKM2 (rPKM2) into culture medium would increase the apoptosis resistance with the integrin αvβ3 expressing cells. SK-Mel-28 cells have high levels of integrin αvβ3 expression, while SW620 cells do not express integrin αvβ3. Treatment of SK-Mel-28 cells with rPKM2 strongly increased resistance to apoptosis induced by oxaliplatin, as a control, rPKM1 did not have such effects. On the other hand, both rPKM2 and rPKM1 did not increase the apoptosis resistance with SW620 cells (Fig. 2B). The observations suggest PKM2 mediate cancer cell apoptosis resistance via integrins αvβ3. To prove that the effects of PKM2 on increasing cancer cell drug resistance act via integrin αvβ3, the integrins were exogenously expressed in SW620 cells (Fig. 3A). The integrin expressing cells were treated by oxaliplatin in the presence and absence of rPKM2 and in the presence of IgGPK or IgGCon. Clearly, cell apoptosis induced by the drug became rPKM2-dependent (Fig. 3B). These experiments suggest that the secreted PKM2 mediates cancer cell apoptosis resistance via activation of integrin αvβ3.

Preceding experiments demonstrate that cancer cells secret PKM2 to feedback act on their cell surface integrin molecules to promote survival. We next question whether the effects of the extracellular PKM2 would also be true in vivo with animal models of cancer. To this end, we employed xenograft of SK-MEL-28 melanoma cells. As demonstrated early SK-MEL-28 cells express high levels of integrin αvβ3. The cells also secret relative high levels of PKM2 to culture medium. We first examined whether
PKM2 was secreted to blood circulation of xenograft mouse. The tumors were implanted. Blood samples from tumor-bearing animals were collected. Analyses of serum samples indicated that PKM2 levels were high (Fig. 4A). We then carried out the treatments with oxaliplatin, ABPK, and oxaliplatin + ABPK. As our previous observations, ABPK alone had marginal effects in inhibition of tumor growth, likely due to inhibition of tumor angiogenesis, while oxaliplatin had almost no effects. Interestingly, combination of ABPK and oxaliplatin had much strong effects in inhibiting tumor growth (two tumors regretted in size) (Fig. 4 B, C, D). The experiments indicate that PKM2 secreted by the SK-MEL-28 tumor plays a role in drug resistance.

We asked what the molecular mechanism by which extracellular PKM2 mediated cancer cells resistance to apoptosis induction was. We first examined whether the extracellular PKM2 activated the integrin signaling. Thus, we analyzed the activation of FAK upon addition of rPKM2 to the culture medium of SK-MEL-28 cells. Immunoblot of S397 phosphorylated FAK in the cell extracts indicated that addition of rPKM2 into culture medium strongly activated FAK, and addition of rPKM1 could not lead to the FAK activation. Addition of rPKM2 into culture medium of cells without integrin αvβ3 expression (SW620) did not result in FAK activation (Fig. 5A). It is well established that activation of integrin αvβ3 signaling consequently leads to several survival signal activations. Typical examples are activation of AKT and ERK kinases. We therefore examined whether presence of extracellular PKM2 would lead to activation of AKT and/or ERK inside of cells. We still employed SW620 and SK-MEL-28 cells. Addition of rPKM2 into the culture medium of SK-MEL-28 cells led elevation of phosphorylated/activated AKT, while the effects were not observed with SW620 cells (Fig. 5B). The results suggest that extracellular PKM2 mediates cancer cell apoptosis resistance by activation of AKT signaling pathway. To verify indeed AKT pathway mediated the effects of extracellular PKM2, we used a commercially available AKT inhibitor triciribine. Evidently, in the presence of AKT inhibitor, the effects of PKM2 in promoting SK-MEL-28 apoptosis resistance were abrogated (Fig. 6).
3.4 Discussion

Cancer cells actively secret PKM2 into extracellular space and patient blood circulation. Measurements of the secreted PKM2 have been suggested as an important marker for cancer diagnosis and prognosis. Several studies have revealed that serum PKM2 levels have a close correlation with cancer drug resistance. On the other hand, a number of studies revealed that knockdown of PKM2 expression abrogate cancer cell apoptosis resistance. Expression of PKM2 in cancer cells is shown to be associated with cell survival. All these studies suggested a functional role of intracellular/extracellular PKM2 in promoting cancer cell survival. Consistently, our study demonstrated that extracellular PKM2 promotes cancer cell apoptosis resistance, thus confer the cancer cells drug resistance. Apparently, sequestration of the function of extracellular PKM2 in mediating cancer survival may represent a new target to develop anti-cancer treatment. Clearly, our experiments with PKM2 antibody treatment provided proof of concept showing that antibody that neutralize the function of extracellular PKM2 sensitize cancer cells to apoptosis induction. A very important potential advantage of application of the PKM2 antibody in treatment is that the levels of circulative PKM2 would be a prognosis marker to predict the possible outcomes. Since secretion of PKM2 is relatively disease associated, it would also be expected that the effects of the PKM2 antibody would be more cancer specific. We tested the effects of PKM2 antibody and rPKM2 in combination with oxaliplatin in this study. It will be interesting to examine a number of other anti-cancer drugs to see whether the PKM2 antibody have effects to enhance the apoptosis induction by other anti-cancer drugs.

Our experiments showed that the effects of PKM2 and the PKM2 antibody are integrin αvβ3 pair expression dependent. The extracellular PKM2 mediates apoptosis resistance by activation of integrin αvβ3 signaling, subsequently activation of Akt survival signal. It is well known established that cancer cells very frequently express this pair of integrin, and activation of integrin αvβ3 signaling is one of major mechanisms by which cancer cells promote survival under various stress conditions. Integrin activation
involves the engagement of the integrin receptor with the specific type of ECM molecule, which signaling the attachment of cancer cells to ECM. Apparently, PKM2 does not belong to any ECM families, the extracellular PKM2 more acts like a soluble molecule. It is intriguing how the extracellular PKM2 is able to activate the integrin signaling. Answers to this question will certainly require a comprehensive understanding the interaction between PKM2 and the integrin $\alpha_v\beta_3$, including binding site and binding mode.

Another interesting issue is how the PKM2, a glycolytic enzyme, secreted to extracellular space, and whether cancer cells are the only cell types that secret PKM2. An inhibition of conventional exocytosis process did not prevent secretion of PKM2 to extracellular space, while a number of studies indicated that PKM2 exist in the exosome of several different cell types. These observations suggest that PKM2 may be secreted by non-canonical pathways that response to the stress conditions, such as hypoxia and apoptosis induction agent treatment. We observed that hypoxia conditions promote the PKM2 secretion (data not shown), which support this speculation.

3.5  Materials and Methods

3.5.1  Cells, reagents, and antibodies

Human melanoma cells SK-MEL-28 and colon cancer cells SW620 were from ATCC, and were cultured by following vendor’s instruction. Oxaliplatin, MTT formazan, and triciribine were purchased from Sigma-Aldrich and Millipore respectively. Antibodies against Akt, phospho-Akt, Erk 1/2, phospho-Er 1/2, FAK, phosphor-FAK, integrin $\alpha_v$, integrin $\beta_3$, and GAPDH were purchased from Cell Signaling and Abcam. Rabbit monoclonal antibody against PKM2 was generated by Epitomic-Abcam.
3.5.2  **Plasmids for integrins and transient transfection**

The cDNA encoded integrin αv and β3 were purchased from Addgen and cloned into pEF1 and pcDNA3.1 vectors for integrin overexpression in mammalian cells. The DNAs were verified by DNA autosequencing.

SW620 cells were seeded 60 mm cell culture dishes and cells were transfected at 60-70% confluence. Briefly, 3 µg of each integrin αv and β3 plasmids and 10 µL Lipofectamine 2000 was mixed in 100 µL Opti-MEM at room temperature for 20min. Then Opti-MEM containing plasmid and Lipofectamine 2000 were added to SW620 cell culture. The cells were transfected for 24 hours and then trypsinized and reseeded into 96 well plates for future analyses.

3.5.3  **Cell viability and TUNEL assays**

After cells were treated, 20 µL of 5mg/mL MTT reagent was added to each well of 96 well plates. Cells were continuously cultured for 2-4 hours. Culture medium containing MTT reagent was removed and cells were rinsed with PBS twice. 100 µL DMSO was added to each well to dissolve MTT crystals and then absorbance was measure at 570nm using spectrophotometer.

SK-MEL-28 cells were seeded on glass coverslips in 6 well plates. After treatment, culture medium was discarded and cells were washed with TUNEL equilibration buffer (from Biotium) for 5min. 1 µL TdT enzyme diluted to 50 µL TUNEL reaction buffer was added on to each coverslip. The coverlips were incubated at 37°C for 60min, followed by twice washes with TPBS (0.1% Triton X-100 in PBS). Coverslips were mounted with anti-fade mounting medium containing DAPI for further fluorescence microscopy analysis (excitation/emission: 593/614nm).

3.5.4  **Mice xenografts and treatments**

All animal experiments were carried out in accordance with the guidelines of IACUC of Georgia State University. Nude mice were subcutaneously injected with $5 \times 10^6$ of SK-MEL-28 cells. Tumor for-
mation and volumes were assessed every 2 days. Tumor volumes were measured by two perpendicular diameters of the tumors with the formula $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$. The tumor bearing mice were subjected to the i.p. injections of appropriate agents at appropriate time intervals. The treatments started five days post tumor inoculations. The tumors were collected and weighed at the end of the experiments. Statistical analyses were done in comparison to the control group with Student’s t test.

3.6 Acknowledgments

We thank Dr. Hua Yang for her assistant in the in vitro and in vivo angiogenesis analyses. We are grateful to Birgit Neuhaus for her assistance in microscopic imaging. This manuscript is greatly improved by comments from Jenny J. Yang and Ritu Aneja and Yuan Liu. This work is supported in part by research grants from National Institute of Health (CA118113) and Georgia Cancer Coalition to Zhi-Ren Liu.
3.7 Reference


Figure 3-1 integrin expression and PKM2 secretion in SK-MEL-28 and SW620 cells.

(Left panel) Expression of integrins $\alpha_v\beta_3$ in indicated cancer cells were examined by immunoblot of cell extracts using antibodies against integrin $\alpha_v$ (IB: integrin $\alpha$) and integrin $\beta_3$ (IB: integrin $\beta$). (Right panel) The levels of PKM2 in medium (CM) of the indicated cancer cells were analyzed by immunoblot of PKM2 (IB: PKM2). Immunoblot of GAPDH (IB: Actin) in whole cell lysate is loading controls.
Cell viabilities of SK-MEL-28 (Upper) and SW620 (Bottom) cells under the indicated concentration of oxaliplatin treatments were measured by MTT assays. The viabilities were expressed as percent of viability by defining the cells treated with buffer as 100%.
Figure 3-2 Effects of extracellular PKM2 on the cell viability and apoptosis.

Cell viability of SK-Mel-28 and SW620 cells under treatments of oxaliplatin (20 µM) and in the presence of antibody against PKM2 (OXA+IgGPK) or control rabbit IgG (OXA+IgGCon). The viabilities were expressed as percent of viability by defining the cells treated with buffer as 100%.
Cell viability of SK-Mel-28 and SW620 cells under treatments of oxaliplatin (20 µM) and in the presence of either rPKM2 (OXA + hrPKM2) or rPKM1 (OXA+ hrPKM1), were measured by MTT assays. The viabilities were expressed as percent of viability by defining the cells treated with buffer as 100%.
Figure 3-2C PKM2 antibody induces oxaliplatin sensitivity of SK-MEL-28 cell sensitivity.

(Upper) Apoptosis of SW620 cells under the indicated treatments was measured by TUNEL assays. The cell apoptosis is presented as % cell apoptosis by defining the apoptosis of buffer treated cells as 1%.

(Bottom) Representative cell images of the TUNEL assays. Red is Terminal Transferase (TdT) stains. Blue is DAPI stains.
Figure 3-3 Apoptosis resistance mediated by extracellular PKM2 is integrin αvβ3 dependent.

Exogenous expression of integrin αv and integrin β3 in SW620 cells were analyzed by immunoblot of the cell extracts using antibodies against the integrins (IB: integrin αv, IB: integrin β3). Immunoblot of GAPDH (IB: GAPDH) is a loading control.
Apoptosis resistance mediated by extracellular PKM2 is integrins αvβ3 dependent.

Cell viability of SW620 cells with transient transfection of integrins αvβ3 or empty vector under treatments of oxaliplatin (20 µM) and in the presence of rhPKM1, rhPKM2, IgGCon and IgGPK; indicated agents were measured by MTT assays. The viabilities were expressed as percent of viability by defining the cells treated with buffer as 100%.
Figure 3-4 The effects of antibody against PKM2 on tumor drug treatment.

The levels of PKM2 in the serum of mouse blood collected from tumor bearing nude mouse (SK-MEL-28) and non-tumor nude mouse (Con) were examined by immunoblot of PKM2 (IB: PKM2). Coomassie stain (CBS: M-Albumin) of serum albumin is a loading control.
Figure 3-4 Effects of antibody against PKM2 on tumor drug treatment.

Growth of SK-MEL-28 tumor under the indicated treatments (Buffer, oxaliplatin – OXA, the antibody IgGPK – IgGPK, oxaliplatin plus IgGPK – OXA + IgGPK) was monitored by (C) endpoint pictures and (D) endpoint weights of the harvested tumors with 10 days treatment (treatment started 5 days post tumor inoculation). The p values were calculated by One-way ANOVAs analysis of variance.
Figure 3-5 Extracellular PKM2 activates FAK via integrin αvβ3.

Activation of FAK in SK-MEL-28 and SW620 cells was analyzed by immunoblot of the cell extracts using antibody against S-397 phosphorylated FAK (IB: p-FAK). The cells were treated by rPKM2 (PKM2), rPKM1 (PKM1), and buffer saline (NT). Immunoblot analyses of cellular FAK (IB: FAK) is a loading control indicating total cellular levels of FAK.
Activation of ERK1/2 and Akt in SK-Mel-28 and SW620 cells was analyzed by immunoblot of the cell extracts using antibodies against phosphorylated ERK1/2 (IB: p-ERK1/2) and phosphorylated Akt (IB:p-Akt).

The cells were treated by rPKM2 by indicated times. Immunoblot analyses of cellular ERK1/2 (IB: ERK1/2) and Akt (IB: Akt) are loading controls indicating total cellular levels of ERK1/2 and Akt. Immunoblot of GAPDH (IB: GAPDH) is a loading control.
Figure 3-6 AKT inhibitor abolishes PKM2 induced oxaliplatin resistance.

Cell viability of SK-Mel-28 cells under treatments of oxaliplatin (20 µM) and in the presence of Triciribine, rPKM2 or Triciribine+rPKM2. The viabilities were expressed as percent of viability by defining the cells treated with buffer as 100%.
CHAPTER 4  1-[(6, 7-substituted alkoxyquinoxalinyl)aminocarbonyl]-4-(hetero)arylpiperazine Interrupts the Interaction between the Y593 phosphor-p68 and β-catenin

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4.1 Abstract

We previously reported synthesis of 1-[[5 or 6-substituted alkoxyquinoxalinyl]aminocarbonyl]-4-(hetero)arylpiperazine derivatives, and demonstrated that 1-(3,5-dimethoxyphenyl)-4-[[6-fluoro-2-methoxyquinoxalin-3-yl]aminocarbonyl] piperazine (ref to as RX-5902). The synthesized compound exhibited strong activity in inhibition of the growth of human cancer cells with IC50 at around 20 nM [1]. In present study, we show that RX-5902 interacts with p68 RNA helicase leading to disruption of the interaction of the Y593 phosphorylated p68 with β-catenin. Furthermore, treatment of cancer cells with RX-5902 results in downregulation of genes that are known to be regulated by the phosphor-p68 and β-catenin interaction. Thus, our study indicates that interaction with p68 and/or phsopho-p68 may, at least partially, contribute to the anti-cancer activity of our compound RX-5902.
4.2 Introduction

A series of novel quinoxalinyl-piperazine compounds were synthesized and their effects in anti-proliferation and apoptosis were examined [1, 2]. Among them, one representative compound (Ref to as RX-5902) demonstrated very potent activities in inhibition of cancer cell growth and apoptosis induction, with IC50 around 20 nM [1]. Pharmacokinetic analyses of this compound showed also optimal PK properties in rats [2]. These previous studies hold a strong promise for RX-5902 to be an orally available anticancer agent.

A number of heterocyclic N-substituted piperazine derivatives and other piperazine derivatives are long known to have anti-viral activities and for treatment of nervous system disorders. Several anticancer compounds containing a quinoxaline ring have been reported as well [3-14]. Although there is a report suggesting unsubstituted quinoxaline compounds containing piperazine exhibit microtubule-inhibiting activity [15, 16]. The molecular mechanism and cellular targets of anticancer activities are not studied well and should be explored.

In previous studies, we have demonstrated that treatment of cancer cells with our synthesized quinoxalinyl-piperazine derivative RX-5902 led to down-regulation of cellular levels of Bcl-2 and cell cycle arrest at G2/M phases to induce the cell death [1]. However, the molecular target(s) in the cells by which RX-5902 exerts its effects leading to down-regulation of Bcl-2 and cell cycle arresting is not clear yet.

The nuclear p68 RNA helicase belongs to prototypical DEAD box family. A number of studies showed that the protein is involved in cancer progression and cell proliferation/survival. Experiments from our laboratory demonstrate that p68 is phosphorylated at multiple amino acid residues. Phosphorylations of p68 at tyrosine closely correlate with cancer progression. Specifically, phosphorylation of p68 at Y593 mediates the effects of growth factors in promoting epithelial-mysenchymal-transition (EMT). The phosphor-p68 also plays a critical role in up-regulating expression of snail 1, a gene
product plays important role in EMT. These studies suggest that phosphor-p68 may have very important function in tumor development and cancer progression and metastasis. Thus, the phosphor-p68 may constitute an important target for development of anti-cancer therapies.

We report here that our synthesized compound RX-5902 interacts with p68 and/or phspho-p68. The interaction results in abrogation of the interaction between phsopho-p68 and β-catenin and inhibits the β-catenin dependent ATPase activity of phsopho-p68 with no effects on p68 RNA-dependent ATPase activity. Furthermore, cancer cells treated by the compound RX-5902 experienced down-regulation of several proliferation-associated genes that are known to be regulated by p68-β-catenin interaction. Our studies indicate that interaction between RX-5902 and p68 and/or phsopho-p68 may, at least partially, contribute to the anti-tumor activity of the compound.

4.3 Results and discussion

We previously reported the synthesis of a series of quinoxalinyl-piperazine derivatives and evaluation the anti-cancer activities of the synthesized compounds. We asked what would be the cellular target(s) of these compounds for their activities in inhibiting cell proliferation and inducing cell apoptosis. We employed the well established target identification method, called drug affinity responsive target stability (DARTS) method [17], to find target proteins that would interact with the compound RX-5902 in which cells were treated with various concentration of RX-5902 (0, 0.1, 1, and 10 µM) and then cell lysates were collected followed by treatment of thermolysin. The samples were run on SDS/PAGE gel and stained with Coomassie blue staining solution. After comparison with control, the bands only detected from thermolysin treated samples were excised and identified by protein mass spectrometry sequencing. Several candidate proteins were found and they are verified by western blotting. From this experiment, it was found that p68 degradation by thermolysin was protected in the RX-5902 treated sample but not the control sample (Fig. 1), indicating that RX-5902 may interact p68 RNA helicase to ex-
ert its effects in inducing cancer cell apoptosis. P68 RNA helicase is a prototypical member of DEAD box family of RNA helicase. It was well documented that p68 RNA helicase expression and posttranslational modifications play an important role in cancer progression and metastasis [18, 19]. Particularly, phosphorylations of p68 have been shown to associate with cancer progression. Phosphorylation of p68 at Y593 facilitates cancer metastasis via promoting EMT [20]. Furthermore, phosphorylation of p68 at Thr and Tyr residues confer cell apoptosis and survival via interaction with several important apoptosis and proliferation regulators. Thus, we speculated that RX-5902 may target p68 and/or phospho-p68 to exert its activity in inhibiting proliferation and/or inducing apoptosis.

Originally, p68 protein was shown to be an RNA-dependent ATPase and a helicase capable of unwinding RNA [21]. As a helicase, p68 binds to both double- and single- stranded RNA, with greater affinity to double-stranded RNA. Binding to RNA induces ATPase activity of p68 and results in unwinding of RNA in both the 5’ to 3’ and 3’ to 5’ direction. Along with its helicase activity, p68 possesses an RNA annealing activity and can catalyze the rearrangement of secondary structures in RNA. Using RNA as a substrate, p68 possesses a variety of activities including ribosome biogenesis which requires rRNA re-modeling, RNA splicing activity and processing of microRNA [22]. It has been postulated that the main oncogenic functions of p68 are independent of their activity on RNA as a true RNA helicase. To determine the effects of the interaction of RX-5902 with p68 RNA helicase on the ATPase activity, we measured the ATPase activity of recombinant p68 in the presence of RX-5902 with addition of yeast total RNAs. Evidently, RX-5902 was shown not to affect RNA-dependent ATPase activity of p68 (Fig. 2A).

It was demonstrated that interaction with β-catenin and microtubule also stimulates its ATPase activity. We therefore reasoned whether interaction with RX-5902 would affect its β-catenin dependent ATPase activity. To this end, recombinant His-p68 was first treated by RNase cocktail to remove any potential RNA contaminations. The treated His-p68 was phosphorylated by c-Abl kinase. The phosphorylated p68 was then mixed with RX-5902 and a commercially available recombinant β-catenin and then
ATPase activity was measured. It was evident that the β-catenin dependent ATPase activity of phosphor-p68 was largely diminished in the presence of RX-5902 (Fig. 2B) with IC50 61 nM, indicating that RX-5902 potentially disrupted phospho-p68/β-catenin interaction.

Phosphorylation of p68 at Y593 confers cancer cells for proliferation and survival. It was demonstrated that these important functions of the phospho-p68 was mediated by interacting with β-catenin in cancer cells. We therefore sought to detect the effects of RX-5902 on p68/β-catenin interaction. We carried out co-immunoprecipitation experiments with cell extracts prepared from the cells that were treated or untreated by RX-5902 using antibody against p68 or antibody against β-catenin. The immunoprecipitates were analyzed by immunoblot. Clearly, co-precipitation of p68 with β-catenin was largely reduced upon the treatment by the compound RX-5902 (Fig. 3A). The reduction in p68/β-catenin co-immunoprecipitation was confirmed by using both anti-p68 and anti-β-catenin as IP antibody. This result is consistent with the observation that RX-5902 strongly inhibited the β-catenin dependent ATPase activity of phosphor-p68 as shown in Fig 2B. (Please add more explanation on Fig 3, especially 3B).

Since RX-5902 disrupted p68 and/or phosphor-p68 and β-catenin interaction, we therefore questioned whether treatment of cells with the compound RX-5902 would affect the regulatory effects of p68 (and/or phosphor-p68)/β-catenin interaction in regulating several growth associated gene expression. It was reported that phosphor-p68 and β-catenin interaction plays a role in expression of cyclin D1 and c-myc, and also affects activation of c-jun MAP kinase activation. Thus, we analyzed expression of cyclin D1 and c-myc expression, as well as phosphorylation of c-jun in cells that were treated by RX-5902. We used MDA-MB-231 and SK-MEL-28 cells here as we found that the compound was more effective in apoptosis induction these two cell lines. We also used a normal fetal lung fibroblast cell line (WI-38) as control. It was evident that RX-5902 treatment led to decrease in expression of both cyclin D1 and c-myc and decrease in c-jun phosphorylation in MDA-MB-231 and SK-MEL-28 cells, while the treatment
did not resulted in any significant change in cyclin D1/c-myc expressions and c-jun phosphorylation in WI-38 cells (Fig. 4). The results clearly suggested that the effects of the phosphor-p68 and β-catenin interaction on expression of several proliferation and survival related genes were suppressed by the compound RX-5902.

We have developed several novel quinoxalinyl-piperazine derivative compounds and demonstrated high potency of anti-cancer activities in vitro. In present study, we provide evidence that one representative compound RX-5902 interacting with p68 (and/or phosphor-p68) RNA helicase. Our study revealed that RX-5902 interacted with p68 to disrupt the interaction of the phosphor-p68 with β-catenin, which consequently affected the functional role of this critical protein-protein interaction in regulating the expression of several important proliferation and survival associated genes. Although, our study may not pinpoint to the target of the anti-cancer activity of RX-5902 to the phosphor-p68/β-catenin interaction, our results demonstrated that p68/β-catenin interaction, at least, constitute partially

Due to important biological functions of many protein-protein interactions (PPI) in almost all physiological and pathological processes, development of molecules that would disrupt these disease causative PPIs has emerged as an important approach to develop therapeutics for many diseases. One potential advantage of targeting specific disease related PPI is that the targeting can achieve highly disease specific effects. Phosphorylation of p68 at tyrosine residues has been shown to be closely associated with cancer progression and metastasis. Interaction between the phosphorylated p68 and β-catenin plays a role in cancer cell proliferation and survival. This interaction does not exist in normal cells and in normal physiological processes. Thus, this PPI should be an excellent target for develop cancer therapies. Certainly, our study sets an example for further development of molecules that would be effectively in disrupting the phosphor-p68/β-catenin interaction. How does RX-5902 interact with p68 is an open question. Our results showed that the compound did not affect the RNA dependent ATPase activity, but
affected the β-catenin dependent ATPase activity of phosphor-p68, indicating that RX-5902 likely interact with p68 (and/or phosphor-p68) at C-terminal. This is also consistent with previous observations that translational modifications at the C-terminal of p68 mediate pro-growth and anti-apoptosis functions of this RNA helicase. Thus, it would be interested to test whether the compound also interfere with the functional role(s) of other C-terminal modifications of p68 RNA helicase.

4.4 Materials and Methods

4.4.1 Cell Culture and antibodies

SW620, MDA-MB-231, SK-MEL-28, WM266, and WI-38 cells were obtained from ATCC (Manassas, VA, USA) and were cultured according to vendor’s instruction. Antibodies against p68 were raised against bacterially expressed His-tagged C-terminal domain (amino acid 437-614) of human p68 (Invitrogen, Carlsbad, CA, USA, Auburn University Hybridoma Facility). Antibodies against β-actin, phosphotyrosine (p-tyr-100), β-catenin, cyclin D1, p-c-jun, c-myc, were purchased from Santa Cruz, BD Bioscience, and Roche Applied Science respectively. Recombinant β-catenin was purchased from abcam and used without further treatment.

Compound RX-5902 was dissolved in DMSO to prepare a stock solution of 2 mM. The stock solution was stored at -20°C and diluted with medium to prepare working concentrations. The cDNA of p68 ORF was subcloned into pHM6 vector (Roche) at HindIII site to get HA-tagged p68 expression vector. The various p68 single and double threonine mutants (threonine replaced by alanine) were generated by Quick-Change site-directed mutagenesis kit (Stratagene) and the mutations were confirmed by DNA sequencing. P38α cDNA (Origene) was subcloned into p3XFLAG-myc-CMV™-24 Expression Vector (Sigma). A number of mutations to get the constitutively active form of p38 were done using the reference [39]. All DNA transfections were performed using fugene HD (Roche) and lipofectamine 2000 (Invitrogen) while siRNA transfections were done with lipofectamine RNAimax (Invitrogen). The duplex siRNA against
p68 was purchased from Dharmacon and the sequence was as follows: siRNA oligonucleotides against p68 (sense: GCAAGUAGCUGAUAUUU; antisense: 50-PAUAUUGCAGCUAACUUGCUU). Cells were transfected with the indicated plasmids 24 hrs after p68 siRNA knockdown and treated with the drug after further 24 hrs. The cells were then harvested for nuclear extract preparation using a kit from Active motif.

4.4.2 Protein Expression & Purification and in vitro phosphorylation

The procedure used to express and purify p68 is similar to the procedure reported previously. P68 ORF cloned and various mutants cloned into expression vector pET-30a+ using the restriction sites BamHI/HindIII and transformed into E.coli BL21-CodonPlus bacteria (Stratagene) were used to express protein. The bacteria were subcultured in fresh LB broth till OD reached between 0.5 to 0.8 units at 600nm and then subsequently induced with 0.5mM IPTG for 18 hrs at 16°C. The cells were harvested, washed with 1 x PBS buffer, pelleted and stored at -80°C. The cells were then disrupted by one freeze-thaw cycle at -80°C, resuspended in lysis buffer (50mM Tris-HCl pH 8.0, 300mM NaCl, 1mM DTT, 10mM PMSF, 10% glycerol) and subjected to lysozyme (0.5mg/ml) digestion. DTT and PMSF were also added at 1mM final concentration. The cells were further subjected to ultrasonication and pelleted. After centrifugation, the expressed protein was found to be precipitated in the bacterial inclusion bodies (IB). The IB were dissolved in denaturing buffer containing 8M urea, 50mM Tris-HCl pH 8.0, 250mM NaCl and 0.2% Triton-100. The lysate was passed through Ni-NTA column for purification of recombinant protein by affinity separation and the column was washed with the denaturing wash buffer (8M urea, 50mM Tris-HCl pH 8.0, 250mM NaCl, 0.2% Triton-100 and 20mM imidazole pH 8.0). The protein was finally eluted with elution buffer containing 250mM Imidazole, 8M urea, 50mM Tris-HCl pH 8.0, 250mM NaCl, 0.2% Triton-100, 0.5mM DTT and 10% glycerol. The eluted protein solution was refolded using stepwise dialysis procedure (8M→ 6M→ 4M→ 2M→ 0M) to remove urea using refolding buffer (200 mM arginine,
50mM Tris-HCl pH 8.0, 250mM NaCl, 0.2% Triton-100, 0.5mM DTT and 10% glycerol) and preserved in further 15 to 20% glycerol.

Phosphorylation of recombinant His-p68 by v-Abl was carried out by the procedure similar to our previous description. Briefly, the recombinant p68 RNA helicase was first dephosphorylated by protein tyrosine phosphatase 1B (PTP1B, Calbiochem). Approximately 5 µg of protein was incubated with 4 units of the phosphatase in manufacture suggested buffer conditions in total volume of 50 µl at 30°C for 90 minutes. The reactions were either directly used for ATPase assays/western blots or used for re-phosphorylation. Protein phosphatase inhibitor set vanadium was added to the above dephosphorylation reactions. Protein kinase, v-Abl kinase, was added to the reaction mixture. ATP was added to a final concentration of 2 mM. The phosphorylation reactions were further incubated at 30°C for 90 minutes. The re-phosphorylated proteins were re-purified by Ni-NTA column.

4.4.3 Identification of RX-5902 binding proteins by DARTS method

MDA-MB-231 cells were plated onto 6 well plates and treated with RX-5902 at various concentrations (0, 0.1, 1 and 10 µM) for one hour and cells were lysed with p-MER buffer containing protease/phosphatase inhibitors on ice. Cell lysates were treated with thermolysin (1:15 ratio) for 10 min at RT and stopped the reaction with adding 0.5 M EDTA solution. The reaction mixtures were loaded and separated onto 10% SDS-PAGE gel and visualized by Coomassie staining. The bands were picked and sliced out for the Mass spectroscopy analysis. After identifying several candidate proteins from mass spectrometry sequencing analysis, we confirmed the protein which may interact with RX-5902 by western blot analysis.

4.4.4 ATPase Assay

ATPase activities were determined by measuring the released inorganic phosphate during ATP hydrolysis using a direct colorimetric assay. The method is based on the change in absorbance (A623nm)
of malachitegreen-molybdenum complex in the presence and absence of inorganic phosphate. A typical ATPase assay was carried out in 50 µl reaction volumes, containing 20 mM Tris-HCl pH = 7.5, 200 mM NaCl, 1 mM MgCl2, 5 mM DTT, ~1-2 µg of appropriate substrate, 4 mM ATP, and 10 µl of helicase. The ATPase reactions were incubated at 37°C for 30 minutes. After incubation, 1 ml of malachitegreen-molybdenum reagent was added to the reaction mixture, and reactions were further incubated at room temperature for exactly 5 minutes. The absorbance (A) at 630nm was then measured. The concentrations of inorganic phosphate were determined by matching the A630nm in a standard curve of A630nm vs. known phosphate concentrations.

Cell viability of indicated cells was measured using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma). 4000 Cells were seeded per well of 96 well plate 24 hrs before knockdown and transfection of p68 and the mutants. Subsequently, 24 hrs after transfection, the cells were treated with oxaliplatin for 24 hrs. Next day, reconstituted MTT reagent was added in an amount equal to 10% of the culture medium volume and the cells were incubated at 37°C for a further 4 hrs. The formazan crystals were dissolved by adding MTT solubilization solution. Cell viability was measured spectrophotometrically by reading the absorbance at a wavelength of 570 nm.

Cells plated on 6 well plates were treated with indicated drugs. The activity of caspase-3 was measured using caspase-3/CPP32 colorimetric assay kit (Biovision Research products). Briefly, after apoptosis induction, the cells were resuspended in Cell Lysis Buffer for 10 mins and centrifuged. 50 µg of proteins were diluted in Cell Lysis Buffer to which 2 x reaction buffer and 4 mM DEVD-pNA substrate were added and incubated at 37°C. The samples were read at 405 nm using a microtiter plate reader. Alternatively, apoptosis was measured using FITC Annexin V Apoptosis Detection Kit (BD Biosciences). Briefly, the cells were washed twice with cold 1 x PBS after treatment and resuspended in 1 x Annexin Binding Buffer at a final concentration of 1 x 10⁶ cells/ml. To 1 x 10⁵ cells (100 µl) in a 5ml FACS tube, 5 µl of FITC Annexin V and 5 µl of PI were added, gently vortexed and incubated at room temperature for 15
mins in the dark. Finally, 400μl of 1 x Annexin Binding Buffer was added and the samples were analyzed by flow cytometry within 1hr.
4.5 References


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Figure 4-1 RX-5902 interacts with p68 RNA helicase.

MDA-MB-231 cells were treated with RX5902 at various concentrations (0, 0.1, 1, and 10 µM) for an hour and cell lysates were collected followed by treatment of thermolysin for 10 min. The reaction mixtures were loaded and separated onto SDS-PAGE gel and probed with anti-p68 antibody.
ATPase activity of p68 was measured in the presence of 2 µg yeast total RNA, ATP, and recombinant p68 RNA helicase, in the presence or absence of RX-5902 at the indicated concentrations. The ATPase activity is presented as µmol of released inorganic phosphate from ATP hydrolysis. (B) ATPase activity was measured in the presence of 1 µg β-catenin, 2 mM ATP, and 1 µg of recombinant phospho-p68 RNA helicase, in the presence or absence of indicated concentrations of RX-5902. The ATPase activity is presented as percentage of inhibition by defining the ATPase activity of phospho-p68 without RX-5902 as zero percent inhibition and 100% in the presence of 200 µM of RX-5902. The ATPase activity is defined as µM of released inorganic phosphate from ATP hydrolysis.
Figure 4-3 RX-5902 abrogates the p68 and β-catenin interaction.

(A) Interaction of p68 with β-catenin in cell lysate of SW620 (left) and WM266 (right) cells were probed by co-immunoprecipitation using antibody against β-catenin (IP: β-catenin) as pull-down antibody. The co-immunoprecipitates were analyzed by immunoblot using antibody against p68 (IB: p68). Immunoblot of β-catenin in the co-immunoprecipitates (IB: β-catenin) are loading controls indicating the amounts of β-catenin pulled-down by the co-immunoprecipitation. (B) Interaction of p68 with β-catenin in cell lysate of cells that derived from patient with metastatic colorectal adenocarcinoma or malignant melanoma cells were probed by co-immunoprecipitation using antibody against β-catenin (IP: β-catenin) as pull-down antibody. The co-immunoprecipitates were analyzed by immunoblot using antibody against p68 (IB: p68). The cells were treated (+) /untreated (-) by 2 µM of RX-5902 for 24 hours before preparation of cell lysates.
Figure 4-4 Effects of RX-5902 on expression downstream genes of p68 – β-catenin.

Phosphorylation of p68 RNA helicase was analyzed by western blot of cell extracts of indicated cells using an antibody against Y593 phosphor-p68 (IB: P-Tyr-p68). Expression and activation of multiple genes, cyclin D1, C-myc, and c-Jun, that are regulated by p68-β-catein interaction were analyzed by immunoblot using antibodies against indicated proteins (IB: cyclin D1, IB: c-jun, and IB: c-myc). The cells were treated by indicated concentrations of RX-5902. Immunoblots of total p68 (IB: p68) in the cell lysates indicate the cellular levels of p68. Immunoblot of β-actin (IB: actin) is a load control.
CHAPTER 5  CONCLUSION AND DISCUSSION

5.1 PKM2 is a multi-functional protein

Pyruvate kinase has been studied for decades in cell metabolism. Non-proliferative cells or differentiated tissues produce energy to support common cellular functions by oxidative phosphorylation. Glucose is metabolized to pyruvate and further oxidized to CO₂ and generate great amount of ATP. The metabolism is different in highly proliferative cells and cancer cells. Tissues with high proliferation rate produce huge amounts of lactate or ethanol at low oxygen concentration. Cancer cells produce lactate and ethanol even with oxygen supply. This is Warburg effect. It has been reported that altered glycolysis dictates Warburg effect. Pyruvate kinase M2 expression (PKM2) is regulated by HIF1 (Selak, Armour et al. 2005) and c-Myc (David, Chen et al. 2010). PKM2 functions differently to PKM1 due to its low enzymatic activity, which results in the accumulation of metabolic intermediates at the last step of glycolysis. Those intermediates are required for cancer cells rapid cell division. Pyruvate Kinase is one of the key enzymes for cancer cells to manipulate metabolism.

Furthermore, PKM2 has recently been highlighted for its gene transcription activity. Dr. Gao from our lab has reported that PKM2 regulates MEK5 gene transcription by phosphorylating transcription factor STAT3. PKM2 functions as a novel protein kinase and phosphorylates STAT3 at Y705 residue (Gao, Wang et al. 2012; Gao, Wang et al. 2013). Earlier studies also reported that PKM2 interacts with transcription factor Oct-4 and enhances Oct-4 activated gene transcription (Lee, Kim et al. 2008). Dr. Zhimin Lu’s group and our unpublished data both indicated that PKM2 regulates gene transcription by phosphorylating histone and switches on cancer promoting genes.

In order to regulate gene transcription, PKM2 translocates into nucleus to interact with transcription factor directly. Glycolysis occurs in the cytosol and PKM2 was reported to translocate to nucleus in different studies. PKM2 K399E mutation (K399E mutation promotes PKM2 formation of dimer confor-
mation) increases the nuclear presence of PKM2 to regulate MEK5 gene transcription (Gao, Wang et al. 2012). ERK2 is phosphorylated by EGFR and ERK2 activation phosphorylates PKM2 at Serine 37 and further exposes NLS in PKM2. PKM2 is imported to nucleus by interacting with importin α5 (Yang, Zheng et al. 2012). PKM2 nuclear localization provides the prerequisite for PKM2 in regulating gene transcription.

PKM2 was also reported to localize in extracellular spaces. PKM2 was detected in patients’ blood serum or other body fluids in many types of cancers. Commercially available sandwich ELISA kit for serum or feces PKM2 is currently used in diagnose of colorectal, gastric, lung, breast and pancreatic cancers (Goonetilleke, Mason et al. 2007; Kumar, Tapuria et al. 2007). Fecal PKM2 was also considered as a predictor for bowel inflammation diseases (Day, Judd et al. 2012). PKM2 released from cancer cells was firstly considered to be due to the high turnover rate of cancer cells. However, recent studies indicated that PKM2 might be involved in physiological or pathological processes: PKM2 is actively secreted from cultured cancer cell (Wu, Chen et al. 2008) and secreted PKM2 correlates with 5-FU (5-fluorouracil) resistance (Shin, Yoo et al. 2009). Another study indicated that PKM2 interacts with TEM8 (Tumor endothelial cell marker 8) in vitro (Duan, Hu et al. 2007). All the studies above suggested potential physiological or pathological roles of extracellular PKM2.

5.2 PKM2 regulates tumor growth in vivo by promoting angiogenesis

Since PKM2 is secreted to the cell culture medium, we then studied physiological or pathological relevance of circulating PKM2 in mice model. Serum PKM2 concentration was dramatically increased in mice with SW620 tumors comparing to the mice without tumors. SW620 tumor bearing mice were then administered with either anti-PKM2 antibody, IgGPK or control antibody, IgGCon. Our result indicated that the neutralization of PKM2 by antibody in mice blood led to decreased tumor sizes. It suggested that the existence of PKM2 secreted by cancer cells promotes tumor growth in vivo. Numerous studies have reported that PKM2 serves as a diagnostic biomarker for various types of cancers. Our study sug-
gested that neutralization of PKM2 in circulation by anti-PKM2 antibody could also be a promising therapeutic strategy for cancer.

In order to further confirm that PKM2 is involved in tumor growth in vivo, I administered recombinant human PKM2 (rhPKM2) to mice with SW620 tumor. Mice tumor size and weight measurements have shown that rhPKM2 promoted SW620 tumor growth compared with recombinant human PKM1 (rhPKM1) and saline control. Therefore, I have confirmed that PKM2 concentration is highly related to SW620 tumorigenesis in nude mice model. PKM2, but not PKM1 promoted tumor growth. This is either due to the 23 amino acids difference in amino acid sequences or due to the structural difference regarding to dimer and tetramer conformations. I am going to discuss this issue in the late section.

Is the effect of PKM2 on tumor growth a universal mechanism or is it tumor type specific? To address this question, mice model with s.c. prostate cancer PC-3 tumor was employed. This result indicated that PKM2 also promoted PC-3 tumor growth. However it seems that PKM2 has a stronger effect in promoting tumor growth in SW620 tumor than that in PC-3 tumor. This is likely due to that VEGF (vascular endothelial growth factor) is a dominant angiogenesis regulator in promoting PC3 tumor growth. Though SW620 and PC3 tumors are both highly vascularized (Gardner, Kelly et al. 2012), VEGF singling is essential in PC-3 tumor angiogenesis (Schulman, Davis et al. 1987) and inhibition of VEGFR kinase activity completely eliminated PC-3 tumor angiogenesis but only partially inhibited SW620 tumor angiogenesis. This implies that PKM2 might play more crucial role in less VEGF dependent tumor such as SW620 (Dev, Dornsife et al. 2004). This data also suggested that PKM2 could also serve as a complementary target for treating VEGF-dependent cancers. We are going to test it by administering either Avastin along or Avastin with anti-PKM2 antibody in PC3 tumor bearing mice. Avastin (Bevacizumab) is a FDA approved anti-cancer therapeutic drug for blocking VEGF.

By finishing animal experiments, we drew a conclusion that PKM2 regulates tumor growth in vivo and this regulation is involved in a common mechanism in different types of cancers. We then stud-
ied how PKM2 regulates tumor growth. Our original hypothesis is that PKM2 promotes tumor growth directly. We firstly analyzed SW620 frozen tumor sections. Ki67 staining has shown a relatively higher proliferation rate of SW620 cancer treated with rhPKM2 compared to saline group. Then, we applied rhPKM2 and anti-PKM2 antibody to cultured SW620 cells and tested cell proliferation. However, our results indicated that rhPKM2 did not significantly promote SW620 and PC-3 cell proliferation in vitro. Therefore, we concluded that PKM2 does not promote cancer cells growth directly. We then modified our hypothesis to test whether rhPKM2 affects tumor angiogenesis and further facilitates tumor growth. CD31 immunofluorescence staining result suggested a potential role of PKM2 in angiogenesis. Mice administered with rhPKM2 have substantially higher MVD (Microvessel Density) and vessel length. Mice contain less MVD and vessel length in IgGPK treated group than those in IgGCon treated group. CD31 staining has also shown that PKM2 promoted tumor angiogenesis in PC-3 tumor model. In conclusion, our data implied that PKM2 facilitates tumor growth by promoting tumor angiogenesis in vivo.

5.3 PKM2 promotes endothelial tube formation

We have discussed that PKM2 is involved in tumor angiogenesis in nude mice model. In order to establish a direct relationship of PKM2 on angiogenesis, we performed in vitro angiogenesis assay. The endothelial cell tube formation assay result indicated that PKM2 does promote human umbilical vein endothelial cell angiogenesis in vitro. We also tested tube formation assay on another endothelial cell line: HMEC, Human Microvascular Endothelial Cell, generously gifted by Dr. Yuan Liu from our department. RhPKM2 significantly increases numbers of branch points and stabilizes tube structure of HMEC at both high (2µM) and low (0.5µM) concentration. Then we also demonstrated that PKM2 secreted by cancer cells promotes endothelial cell tube formation. In conclusion, PKM2 promotes angiogenesis in vivo and in vitro. However, angiogenesis is a complicated process which is involved in endothelial cell proliferation, migration and survival.
5.4 PKM2 promotes endothelial cell proliferation, migration and survival

Angiogenesis is a complicated process, which includes endothelial cell migration, survival and proliferation. Therefore, we first tested HUVEC cell proliferation and migration of HUVEC cells in vitro. Our result indicated that rhPKM2 increased HUVECs cell proliferation up to 40% comparing to saline control and rhPKM1. We also tested whether PKM2 has effects on endothelial cell adhesion on extracellular matrix proteins. Endothelial cells attach to ECM such as collagen, laminin and fibronectin in physiological conditions. Therefore, we are interested in whether PKM2 promotes or inhibits endothelial cells binding to ECM proteins. The data have shown that rhPKM2 facilitates HUVECs attach to ECM proteins, such as fibronectin and vitronectin. However, if HUVECs were incubated for 2 hour or longer after pre-incubation with rhPKM2, no significant difference was observed in this situation. This suggests an early-phase angiogenic role of PKM2. We think that rhPKM2 either shortens the time period for HUVECs attachment or rhPKM2 promotes HUVECs spreading and facilitating endothelial cells attach to ECM strongly. After all, this phenomenon illustrated that rhPKM2 activates and stabilizes focal adhesion complexes. This can also be interpreted as that PKM2 physiological or pathological role might be promoting endothelial cell migration and proliferation, not adhesion. We thereafter tested whether HUVECs spreads on the surface of coated collagen upon treatment of PKM2. The data have shown that HUVECs treated with rhPKM2 possessed higher percentage of spreading cells than those treated with either rhPKM1 or BSA.

Endothelial cell migration is one of the most important contributors to angiogenesis. In order to demonstrate whether PKM2 regulates HUVEC migration, we employed Boyden Chamber assay to test HUVEC cell migration. We tested endothelial cell migration with two different methods. Firstly, rhPKM2, rhPKM1 or saline were incorporated to the cell culture medium in the upper chambers along with HUVECs while cells were seeded. This result verified that existence of rhPKM2 in the culture medium significantly promoted HUVECs migration. Interestingly, we also observed that PKM2 possesses some
characteristic of ‘chemoattractants’, inducing endothelial cell to migrate towards PKM2. In order to test this specific aim, rhPKM2, rhPKM1 or saline was mixed with medium in the bottom chambers of Boyden Chamber. Migrated HUVECs are greater in numbers in PKM2 loaded chamber than PKM1 or BSA loaded chambers. This strongly implied a novel concept that PKM2 secreted by the cancer cells could induce endothelial cell to migrate towards cancer cells. This is consider to be crucial that cancer cells communicate with surrounding cells such as vascular endothelial cells or fibroblast cells in pathological cancer scenarios. Further studies are needed to test this hypothesis.

Previously, I have discussed my result that PKM2 promotes endothelial cells to form more vascular tubes (branch points). I also observed that endothelial vascular tubes only last 24-48 hours on Matrigel in saline treated group, but the tubes sustain to 72 hours or even longer when treated with PKM2. This suggests that PKM2 might enhance endothelial cell survival. Endothelial cell survival ability is an essential factor in early angiogenesis (Chavakis and Dimmeler 2002). At the early stage of angiogenesis, endothelial cells is devoid of the protection from extracellular matrix and surrounding smooth muscles as the matured blood vessels have. PKM2 could activate endothelial cell survival signaling pathway and protect endothelial cell from apoptosis.

Based on our results from the BrdU incorporation, ECM attachment assay and Boyden chamber assays, we have successfully demonstrated that PKM2 is sufficient to facilitate endothelial cell proliferation, migration and survival.

5.5 PKM2 interacts with Integrin family proteins

As we had found that PKM2 promotes endothelial cell proliferation, migration and survival, the next question we asked is how PKM2 regulates proliferation, migration and survival. In order to address this question, we tested whether PKM2 directly interacts with endothelial cells and what is the binding partner for PKM2 on the endothelial cell membrane.
Our direct binding assay revealed that endothelial cells bind to rhPKM2, but not to rhPKM1 and BSA. The binding activity of endothelial cells to PKM2 was able to be blocked by anti-integrin αvβ3 antibody LM609, antibody 23C6 and anti-PKM2 antibody IgGPK, but not by other two integrin H-2 and Q20 antibodies. It is known that LM609 and 23C6 bind to ligand binding region of integrin αvβ3, but H-2 and Q20 do not. Thus, the blocking assay suggested that PKM2 binds to the region close to the active site of integrin αvβ3. Our speculation was also confirmed by competitive binding assay with RGD peptide, Cilengitide. Cilengitide is small cyclic peptide with a sequence of RGDFv and it specifically inhibits RGD dependent integrin binding to their ligands. HUVECs binding to PKM2 can be strongly inhibited by Cilengitide. Therefore, we concluded that PKM2 interacts with endothelial cells through binding to integrin and this interaction is involved in integrin ligand binding site.

In order to further investigate the binding receptors for PKM2, we utilized SPR (Surface Plasmon Resonance or Biacore) assay. CM5 chip was coated with Integrin αvβ3, α5β1 and α1β1 to three different channels. Purified recombinant protein rhPKM1, rhPKM2 and BSA were flowed though the chip. The normalized curves indicated that PKM2 binds to integrin αvβ3 and α5β1, but not integrin α1β1 (binding to αvβ3 is stronger than that to α5β1). This is consistent with our antibody and peptide blocking assay which I have described above. Biacore results also implied that rhPKM2 has higher affinity to integrin than rhPKM1 or BSA does. This data provided strong evidence that PKM2 binds to integrin in vitro. Integrin family is a well-studied transmembrane receptor family which regulates cell adhesion and migration. After integrins bind to their ligands in extracellular matrix, integrins are activated by either conformational change (affinity) or integrin clustering (avidity).

Since PKM2 and integrin interaction is blocked by RGD peptide and αvβ3 integrin is a RGD dependent integrin, we speculated that PKM2 binds integrins through RGD sequence. RGD sequence is an amino acid sequence, Arg-Gly-Asp, which binds von Willebrand factor A domain on integrin β3 subunit. Therefore, we started to search putative RGD on PKM2 protein sequence. We found one RGD sequence
in PKM2, but this RGD sequence (294-296) is partially buried inside from analysis of PKM2 crystal structure. However, this RGD sequence still could be the binding region for integrins even it is not completely assessable. It has been reported that proteins such as collagen and thrombin expose their RGD binding sites after the cleavage by extracellular matrix proteases, MMP-2 or MMP-9 (Papaconstantinou, Carrell et al. 2005). More importantly, PKM2 might undergo conformational change while converting from tetramers to dimers. RGD site is likely to be exposed during this conformational change. It is still possible that PKM2 binds integrins through other sequences than RGD. Numerous studies have reported that proteins containing no canonical RGD sequence bind to RGD dependent integrins. CCN1 (CYR1) is one example. It binds integrins via a sequence NCKHQCTCIDGAVGCIPLCP (Chen, Leu et al. 2004), which does not contain any RGD. It is also reported that RGD synergy binding site PHSRN sequence is able to activate fibronectin binding to RGD-dependent integrin αvβ3 and stimulate angiogenesis (Zeng, Yao et al. 2009). Within these 5 residues, Serine and Arginine are the most conservative residues and both mutations completely abolished the binding affinity of PHSRN peptide to integrins. Meanwhile, Serine mutated to Threonine or Tyrosine residue maintained the binding activity over 80% (Hattori, Hozumi et al. 2009). Interestingly, PKM2 contains a sequence similar to PHSRN: AVTRN, which could be another potential binding sequence to integrins. We are currently testing the integrin binding site on PKM2.

Duan et al has reported that PKM2 might interact with TEM8 (Tumor Endothelial Marker 8). To test whether PKM2 interacts with TEM8, we expressed recombinant GST-TEM8 extracellular domain (ECD) and performed GST pull-down assay. We did observe weak binding of PKM2 to TEM8 ECD. We also overexpressed full length TEM8 in HEK 293 cells, which is reported to have basal expression level of TEM8, but overexpression of TEM8 in HEK did not significantly enhance HEK cells binding to PKM2. However it is noticed that TEM8 has structural similarity with integrins on their ligand binding site. The MIDAS (metal ion-dependent adhesion site) motif in vWA domain in TEM8 is very similar to the I domain in integrin β subunit. Magnesium is actively involved in the binding which is also supported by our exper-
iment result: EDTA completely inhibits endothelial cell to attach to immobilized PKM2. This evidence suggests the possibility that PKM2 could bind to vWA (I domain) located in integrins and TEM8. We did not exclude that possibility that PKM2 could also interact with TEM8 in certain cells or circumstances. We are going to isolate tumor endothelial cells to test whether PKM2 could regulate tumor endothelium through TEM8 or not. We will further investigate PKM2 interacting proteins on the cell membrane in our future studies by co-immunoprecipitating membrane proteins abstract and identifying candidate proteins by 2-D electrophoresis and mass spectrum.

5.6 How does PKM2 bind to integrins and activate integrins

Evidently, we speculated that PKM2 binds to the ligand binding site in integrin αvβ3. This is supported by the evidence that the interaction of integrin and PKM2 can be inhibited by RGD peptide or LM609, an antibody binding to integrin αvβ3 ligand binding region. Further studies need to be done on the detailed binding sequences. Several strategies can be employed: 1. Crystallography. Co-crystallization of PKM2 and integrin complex is the most straightforward method. If PKM2 and integrins are co-crystallized, we are able to identify the binding site locating on both PKM2 and integrins by structure analysis. However, certain technical difficulties have to be considered. Integrins are too large in size to be expressed in bacteria. Mammalian expression of integrins is also hampered by low expression level and inhomogeneous glycosylation. Crystallization condition needs to be explored as well. 2. Protein cross-linking assay. After PKM2 forms complex with integrin αvβ3, chemical reagents can be employed to covalently cross-link the protein complex. Cross-linked proteins are further digested with trypsin and analyzed by LC-MS. Selection of cross-linker and optimizations of cross-linking parameters are most important for this method. 3. Electron microscopy. Conformational changes of integrins (close or open form) can be visualized and differentiated by electron microscopy. Therefore, we are able to identify
binding regions approximately and then carry out mutagenesis assay to further locate the binding sequences.

If it mimics the ligand binding, then PKM2 might induce the separation of c-terminus of α and β subunits. If PKM2 does not bind to the ligand binding site, we need to further test how integrins respond to PKM2 binding.

5.7 PKM2 binds integrins and activates integrin signaling pathways and cellular responses

I have described above that PKM2 binds to recombinant integrins and endogenous integrins on the cytoplasm membrane of endothelial cells. We also demonstrated that PKM2 facilitates endothelial cell adhesion, proliferation and migration. Therefore, we next tried to establish the linkage between protein interaction and cellular responses. It is well known that immobilized extracellular matrix proteins bind and activate integrins. Activated integrins initiate downstream pathways via FAK phosphorylation or ILK phosphorylation. In my study, I am interested in whether PKM2 also activates integrin signaling. Western blot result showed that PKM2 induces FAK tyrosine 397 phosphorylation, which is an early event of FAK activation. FAK activation is reversed by PKM2 antibody and LM609 the αvβ3 integrin specific antibody. This indicated that PKM2 and integrin interaction is required for FAK phosphorylation. FAK inhibitor I14 inhibited PKM2 induced FAK activation and blocked PKM2 induced cell adhesion. All the evidences suggested that integrins are the main receptors for PKM2 on endothelial cell membrane. We further tested whether PKM2 is able to facilitate focal adhesion complex assembly. The data have shown that integrin αvβ3 started clustering at the leading edge of HUVEC upon the treatment of PKM2 compared with BSA and PKM1. We also demonstrated that PKM2 promotes vinculin recruiting to focal adhesion complex and enhances actin stress fiber formation. Actin stress fiber formation and vinculin recruitment both are the downstream events of FAK activation during cell migration and adhesion. In conclusion, we have shown that PKM2 activates integrin αvβ3 and its intracellular signaling pathways.
The classical role of integrins is to mediate attachment to ECM (extracellular matrix, immobilized lig- and) or migration on ECM. Many studies have revealed that soluble ligands binding to the active site of integrins inhibit cell adhesion and migration. In my dissertation, PKM2 is a soluble protein in culture me- dium or cancer patient blood. It is interesting why PKM2 promotes endothelial cells adhesion and migra- tion instead of inhibition. A number of other studies have shown that soluble proteins are also able to activate integrin and its downstream signaling pathway, such as TAT protein in HIV virus, CD40L, CD97 (Wang, Ward et al. 2005) and fibronectin. Cilengitide, a designed integrin inhibitor was originally consid- ered to repress angiogenesis by inhibiting integrin, but was proved to activate integrins and potentially promote angiogenesis instead (Alghisi, Ponsonnet et al. 2009).

How do soluble proteins induce endothelial cell adhesion or migration and further angiogenesis? We are trying to understand how soluble proteins induce endothelial cell adhesion and migration. Firstly, cell adhesion and migration is a complicated process in human body during physiological and pathologi- cal situation. Plenty of studies have reported that certain types of integrin overexpression results in de- regulation of cell adhesion and migration (Margadant, Raymond et al. 2009). Integrin α1β1, α2β1 and α3β1 mainly mediate cell adhesion to extracellular matrix; while αvβ3 is responsible for cell migration. In the adult tissues, almost all cell types except the cells in blood vessels or lymphatic vessels attach to extracellular matrix. Therefore, blocking of integrin α1β1 and α2β1 to their ligands causes cell adhesion defects. However, inhibition of αvβ3 does not necessarily inhibit endothelial cell adhesion (Rainero, Caswell et al. 2012). Secondly, cell migration is a dynamic process. One of the explanations could be that soluble PKM2 from cancer cells drives endothelial cells to migrate towards cancer cell through integrin recycling. This mechanism was studied on osteopontin and Cilengitide cases. Both proteins in soluble forms induce cell migration through activating Rab dependent pathway (Caswell, Chan et al. 2008). At the leading edge of endothelial cells, integrins are activated; while integrins dissociate at the rear of the migrating cells. Soluble proteins might promote integrin recycling process to facilitate cell migration
(Huveneers, Truong et al. 2008). Thirdly, whether soluble proteins or ligands are able to inhibit αvβ3 is dependent on the binding affinity and soluble protein concentration. Cyclic RGD peptide, Cilengitide is able to inhibit angiogenesis at high concentration. At low concentration, Cilengitide tends to promote endothelial cell migration and angiogenesis (Reynolds, Hart et al. 2009). In summary, soluble PKM2 activates FAK in endothelial cells and induces integrin αvβ3 redistribution to the leading edges in endothelial cells.

5.8 PKM2 dimeric and tetrameric forms

PKM2 has two conformational structures in the cells: dimer and tetramer formations. Tetramers have higher affinity to PKM2 substrate PEP and higher enzymatic activity, however, dimeric PKM2 has relatively lower enzymatic activity. We found a very interesting fact which had not been addressed by other studies before. We revealed that recombinant PKM2 mainly forms tetrameric conformation in solution at a concentration of 8-15µM. However, upon the dilution, PKM2 converts from tetramers to dimers at lower concentration, 1 µM. Unlike rhPKM2, rhPKM1 has tetrameric formation at both low and high concentration. We also observed FBP induces PKM2 to form tetrameric forms, which was consistent with many other studies (Merrins, Van Dyke et al. 2013). This implicates that PKM2, PKL and PKR have a relatively loose interaction between their dimer-dimer association interfaces. This conformation might be evolutionally apt to functional modulation in the cells for rapid cell proliferation. We have verified that recombinant PKM2 is dimer at low concentration and we are also interested in how PKM2 is converted to dimer in the cell. Many groups have reported that tumor PKM2 in patient blood serum exists as dimers. Dr. Jing Chen’s group at Emory University and Dr. Zhimin Lu’s group at UT Anderson both reported that modification (such as phosphorylation) to PKM2 stabilizes dimer PKM2. Tetrameric PKM2 is assembled in the glycolytic enzyme complex and vulnerable to dissociation to dimers. After converting into dimeric PKM2, dimeric PKM2 dissociates from enzymatic complex and phosphorylated by FGFR to
stabilize its dimer formation. In our study, we speculated that it is the dimer PKM2 that interacts with integrin and exerts angiogenesis functions.

The next question we answered in our study is which PKM2 conformation interacts with integrins, dimer or tetramer. In order to test this, we employed Pyruvate kinase synergetic co-activator FBP. FBP association decreased the binding activity of PKM2 to endothelial cells. Biacore result also confirmed that FBP weakened PKM2 binding to integrins in vitro. Since FBP represses the interaction of PKM2 and integrin, we reasonably think that FBP might affect endothelial cell angiogenic responses induced by PKM2. Therefore we further demonstrated that FBP impaired endothelial cell adhesion and migration induced by PKM2. In vivo, tumor sizes were significantly smaller in the group treated with rhPKM2 than the one treated with Saline or rhPKM1. As we expected, FBP decreased tumor size (comparing the p value of rhPKM2+FBP with saline group). However, p value is not significantly different between groups treated with rhPKM2 alone and rhPKM2 with FBP. It could be explained by two reasons: 1) FBP easily dissociates from PKM2/FBP complex and is excreted by kidney or absorbed by tissues in vivo; 2) PKM2 could be modified or bind to other proteins after injected to mice and cannot efficiently bind FBP anymore. We analyzed mice blood serum by western blot on mice blood serum after gel filtration chromatography. RhPKM1 and rhPKM2 in mice blood circulation maintain as tetrameric and dimeric formation respectively. RhPKM2 with FBP partially converts dimeric PKM2 to tetramers, which indicated that FBP induces PKM2 tetrameric conformation but is not able to completely convert PKM2 to tetramers.

5.9 PKM2 secretion mechanism

First of all, I have examined the secretion of PKM2 in vitro and in vivo. PKM2 was detected by western blot in the culture medium from eight types of cancer cell lines. An interesting observation is that primary cancer cell lines release higher levels of PKM2 than their metastatic counterparts do respectively within each cancer cell line pair. This result is consistent with the founding on the relationship
between metastasis and angiogenesis which has been acknowledged for decades. Angiogenesis of metastatic tumor is repressed in vivo. Metastatic cells secrete less PKM2 and assist metastatic tumor to keep the minimum angiogenesis level (Kirsch, Schackert et al. 2004). But primary solid tumors, on the contrary, require more angiogenesis for nutrition transportation. Meanwhile, we also realized that Melanoma cell pairs had the highest level of PKM2 in the culture medium, followed by colon and lung cancer cell pairs, while breast cancer pairs had a comparatively low level of PKM2. These results are entirely supported by early reports. In early 1980s, Chaudhury et al. reported that melanoma cells produce higher angiogenic activity than breast cancer cells in rabbit cornea model (Chaudhury, Lerner et al. 1980).

It has been well-studied that the hypoxia condition in solid tumors promotes tumor angiogenesis (Pugh and Ratcliffe 2003). To elucidate this question, I measured PKM2 concentration in culture medium during hypoxia comparing with normoxia. The data indicated that CoCl2 up-regulated PKM2 secretion to medium. CoCl2 is reported to induce the expression of HIF1α and to mimic hypoxia condition (Lum, Bui et al. 2007). This result suggested that hypoxia plays an important role in PKM secretion. We also examined serum PKM2 level in mice blood with or without tumor inoculation. Mice with subcutaneous SW620 tumor contain more PKM2 in blood than mice without tumors. This is consistent with many clinical reports that PKM2 was detected in patients’ blood serum with various types of cancers.

It remains mysterious how PKM2 is secreted to the extracellular spaces. PKM2 were reported to be present in exosomes or microvesicles and tumor cells have elevated vesicles secretion (Wubbolts, Leckie et al. 2003; Park, Tan et al. 2010). Exosome and microvesicles are small extracellular vesicles released by many cell types including cancer cells. (Steiner, Angot et al. 2011). We speculated that PKM2 secretion is related to those vesicles from cancer cells. Our data supported that DMA dramatically inhibits PKM2 secretion into the cell culture medium of WM115 cells. DMA is an amiloride homologue which has been reported to specifically inhibit exosome and microvesicles release. But classic exocytosis pathway inhibitors such as BFA (Brefeldin A) and Exo1 do not inhibit PKM2 secretion. This indicated that
PKM2 secretion is related to exosome and microvesicle release or PKM2 is included in those vesicles. The detailed mechanism needs to be further studied.

5.10 PKM2 is involved in cancer cell drug resistance

We have tested proliferation and migration of SW620 and PC3 cells upon PKM2 treatment. The data have shown that PKM2 did not promote SW620 and PC3 cell proliferation and migration significantly compared to HUVEC cells. It seems that PKM2 only recognizes endothelial cells. Therefore, we tested whether PKM2 functions on endothelial cells exclusively. Since we have demonstrated that PKM2 promoted endothelial cells through integrins, we examined integrin αv and β3 subunits expression in SW620, SW480, PC3 and SK-MEL-28 cell lines. It has shown that expression level of integrin αv and β3 is dramatically higher in SK-MEL-28 than in SW620 and PC3. We also tested the downstream pathways of integrin and the data have shown that PKM2 phosphorylated Akt. This result led us to speculate that PKM2 enhances SK-MEL-28 cell against apoptosis. Integrins are known to protect cancer cell from apoptosis. Integrin binding to ECM activates FAK signaling pathways leading to the activation of Erk or Akt pathway. Akt is the key factor in survival pathways to protect cells from apoptosis. Moreover, we treated SK-MEL-28 and SW620 cells with anti-cancer drug, oxaliplatin. We also applied recombinant PKM2 or PKM2 antibody (IgGPK) along with oxaliplatin. MTT assay and TUNEL assay both have indicated that PKM2 protected SK-MEL-28 cells from apoptosis induced by oxaliplatin and PKM2 antibody, on the contrary, enhanced SK-MEL-28 apoptosis. However, either PKM2 or PKM2 antibody does not have significant effects on SW620 cell. Based on this result, we tested whether integrins play a crucial role on the anti-apoptosis effect of PKM2. We overexpressed integrin αv and β3 subunits in SW620 cell and then tested PKM2 anti-apoptosis activity in SW620 wild type (SW620WT) and SW620 αv and β3 transfected (SW620AB) cells. The data has shown that PKM2 protected SW620AB cells from oxaliplatin induced apoptosis, but not SW620WT cells. PKM2 was also reported be involved in another cancer drug 5-FU
induced resistance. 5-FU resistant cell line has more PKM2 secretion in the culture medium comparing with 5-FU non-resistant cell line (Shin, Yoo et al. 2009). In summary, PKM2 also binds cancer cells through integrins and enhances cancer cell drug resistance.

In my dissertation, I have successfully demonstrated that PKM2 secreted by cancer cell promotes endothelial proliferation, migration and survival. PKM2 has been proved to facilitate angiogenesis in vitro and in vivo. The mechanism is that PKM2 recognizes and binds to integrin αvβ3 on endothelial cells and this interaction of PKM2 with integrin phosphorylates Focal Adhesion Kinase and induces integrin αvβ3 to redistribute to the leading edge of endothelial cells. Endothelial cell activation and endothelial angiogenesis is able to be blocked by PKM2 antibody. Therefore, this indicates that PKM2 can be exploited as a potential therapeutic target for cancers. Anti-PKM2 antibody could serve as a combination treatment with existing drugs to certain types of cancers.
CHAPTER 6  METHODOLOGY

6.1 Molecular techniques

6.1.1 Polymerase Chain Reaction (PCR)

PCR technique was used to amplify PKM2 and other mutant DNA plasmids. The reaction system includes the following materials: 1ul of template DNA (50ng/µl), 5ul dNTPs (final concentration 0.2mM), 5ul 10X KOD HOT START DNA polymerase buffer, 2ul MgSO4 (final concentration 1mM), 5µl primer mixture (forward and reverse primers at final concentration 1uM), 1µl KOD HOTSTART DNA polymerase (1U/µl) and H2O. After all the components were mixed, a brief spin-down was performed and PCR tubes are placed into eppendorf Mastercycler. The PCR procedure for amplifying the whole plasmid was: 2min 95°C; 30 sec 95°C, 30 sec 56°C, 10min 72°C, 30 cycles; 15min 72°C; the PCR procedure for amplifying gene fragment is: 2min 95°C; 30sec 95°C, 30sec 56°C, 2min 72°C, 30 cycles; 8min 72°C The PCR results were analyzed by 0.8 %-1.5 % agarose gel electrophoresis.

6.1.2 Agarose gel electrophoresis

PCR fragments were analyzed by Agarose gel electrophoresis. 0.8 % agarose gel was made by adding 0.4g agarose to 50mL 1XTBE buffer. Gel mixture was boiling 1.5min by microwave to completely dissolve agarose. After cooling down, 2µl Ethidium Bromide was added into gel mixture and gel mixture was poured to gel casting tray to solidify the gel. PCR samples were mixed with 1 X Loading dye and loaded to the wells. 6µl of 1K plus DNA ladder was loaded to a separate well. Agarose gel was run at the voltage of 100V for 1hour. Agarose gel was visualized by UVP biospectrum 410 imaging system.

6.1.3 PCR gel extraction

Qiagen gel extraction kit was utilized to purify PCR product. Buffer Q was mixed with PCR product with the ratio of 3:1. The mixture applied to the columns provided in the kit. Columns were centri-
fuged for 1min at 13,000 rpm in the bench-top eppendorf centrifuge. The mixture centrifuged down was returned to the column and another centrifuge was performed. The flow-through was discarded and 750 µl Buffer PE was added to the column. After centrifuge 1min at 13,000 rpm, the flow-through was discarded. 40-50 µl de-ionized water was added to the column and the elution was collected to a new 1.5ml eppendorf tube. De-ionized water can be heated to 60-70°C if necessary.

Gel extraction was carried out with the similar protocol. PCR product was separated by agarose gel. Targeted DNA fragments were cut off by blades and the gel is weighted on the scale. Buffer Q was mixed with gel fragments with the ratio of 3:1 to 5:1. If the PCR fragments are larger than 5K bp, Isopropanol was added into the gel/Buffer Q mixture. The mixture was placed in the hotplate at 60-70°C for 10 minutes and vortexed twice while heating. The rest procedure is the same as mentioned above in PCR fragments extraction procedure.

6.1.4 Restriction Digestion

In order to construct foreign DNA expression plasmid, PCR fragments and empty vectors need to be digested with the same restriction endonucleases. Those restriction endonucleases are generated by bacteria originally to protect bacteria from attacking of phage. Restriction endonucleases specifically recognize unique sequences in PCR products and vectors as well, and cleave DNA sequence. Briefly, DNA substrates, enzyme and reaction buffer were mixed together and the mixture was incubated in 37°C for 1 hour to 2 hours. The restriction digestion product can be analyzed by agarose gel electrophoresis or purified by gel extraction kit.

6.1.5 PCR products phosphorylation

Some PCR products require phosphate group in 5’ end in order to be ligated. T4 polynucleotide kinase (T4 PNK) was used transfer γ-phosphate from ATP to 5’-OH group of double stranded DNA. Phosphorylated PCR products can be used for DNA ligation to form circular plasmids. The reaction mixture
contained: PCR product 5 µl (100ng/µl), 10 X T4 PNK reaction buffer A 2µl, ATP (10mM) 2µl, T4 PNK 1µl (1Unit) and 10µl nuclease-free water. After mixed and spin down, the reaction was incubated at 37°C for 1 hour. The reaction was quenched at 75°C for 10min.

6.1.6  DNA ligation

After PNK treatment, PCR products were treated T4 DNA ligase to form a phosphodiester bond between 5’ phosphate and 3’ hydroxyl termini. DNA ligation reaction can be used to circularize linear RCP products or insert a gene into a vector. T4 DNA ligase can be used for both blunt end and sticky end. Reaction mixture contains: linear DNA (after PNK treatment) 10µl, T4 DNA ligase buffer 2µl, T4 DNA ligase 1µl, nuclease-free water 7µl. The reaction was incubated at room temperature for 1 hour.

6.1.7  Transformation

Self-circularized by DNA T4 DNA ligase was transformed to E. coli competent cells. The strains used in this study are BL21 (DE3), BL21 (DE3) plys and Tuner (DE3). Competent cells were originally stored in -80°C. Prior to transformation, competent cells were incubated on ice for 5min. Then ligation products were added into 50µl competent cell and mixed by gentle finger tapping. After incubation for 10min on ice, competent cells were placed at 42°C on heat block for 90 sec. The competent cells were then kept on ice again for another 2min and 950µl warm LB medium was added to competent cells. After 60mins, 100µl LB containing competent cells was spread onto LB agar plates by bacteria spreader. LB agar plates were placed in the 37°C incubator for overnight.

6.1.8  Plasmid miniprep

After transformation, single colony was picked up to fresh LB medium with Kanamycin at the concentration of 1µl/ml and cultured at 37°C for overnight 250rpm in bacteria shaker. QIAprep spin miniprep kit was applied to extract plasmids from E. coli. 3ml bacteria overnight culture was centrifuged
for 2min at 12,000g and supernatant was removed by vacuum. The bacteria pellets were re-suspended by 250 μl Buffer A containing RNase A. After completely vortexed, bacteria cells were broken down by 250 μl Buffer B (containing SDS and NaOH). Then 250μl of neutralization buffer or Buffer N was added. The bacterial proteins were denatured and precipitated out by centrifuge at 12,000g for 10min. The supernatant containing DNA was applied to Qiagen miniprep column. The miniprep column was washed twice with 75-80% ethanol buffer, Buffer PE and was dried in room temperature for 2min. Finally, DNA on the column was eluted out by nuclease-free deionized water. DNA extracted can be applied to agarose gel electrophoresis for analysis.

6.1.9 **Recombinant protein expression**

After plasmid verification by DNA sequencing, PKM2 or other mutants were cultured in 10mL LB medium at 37°C, 250rpm for overnight. The second day, 10 ml bacterial culture was transferred to a 1L fresh LB medium containing 1mM Kanamycin. And the 1L LB Broth was subjected to culture at 37°C 250rpm until OD600 reaches 0.3. 1L bacterial culture was cooled down in 4°C and then 0.2mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture. The bacteria were continuously cultured for 16°C 150rpm for 18 hour for recombinant protein expression. The bacteria were collected by centrifuge at 4°C, 6000rpm for 15min. A portion of bacterial pellets were suspended with DI water and mixed with 2 x SDS loading buffer.

6.1.10 **Recombinant protein purification**

Bacteria pellets were suspended and lysed by 20mL lysis buffer containing 50mM Tris-HCl pH 7.5, 50mM NaCl, 10mM Imidazole and protease inhibitor cocktail. The bacteria suspension was kept on ice for 10min and bacteria were broken down by French Press. Then sonication was applied to the bacteria lysate for 1min with 10sec pause in every 10sec. The bacteria lysate was centrifuged at 4°C, 15,000g for 30min. Since recombinant proteins contain 6 X histag, the supernatant was applied to Ni-
NTA on (Fast Protein Liquid Chromatography) FPLC. 6X histag proteins bind to nickel-chelated column and high concentration of imidazole. Briefly, recombinant PKM2 was loaded to the Ni-NTA column at the concentration of 20mM imidazole. Then imidazole concentration was increased to 250-300mM to elute recombinant PKM2. Elution was dialyzed against the buffer containing 50mM Tris HCl pH 7.5, 50mM KCl, 15% glycerol and 1μM β-mercaptoethanol (2ME) at 4°C for overnight. Recombinant protein solution concentration was measured by Bio-rad Bradford assay.

6.1.11 Protein concentration measurement

Bio-rad Bradford assay was used to measure protein concentration. Bio-rad Bradford solution was mixed with water with a ratio 1:4. 1ml was added into cuvettes to measure OD595 by spectrophotometer. To establish the standard curve, 0μg, 2μg, 4μg, 6μg, 8μg and 10μg BSA was added to separate cuvettes and OD595 was measured. Recombinant protein and control buffer were added to cuvettes to measure OD595.

6.1.12 Gel filtration

Gel filtration was utilized to analyze PKM2 dimer/tetramer ratio. Firstly, we established the standard curve by loading high molecular weight gel filtration calibration kit. This calibration kit contains five proteins with molecular weight from 44,000 Dalton to 669,000 Dalton: Ovalbumin, Conalbumin, Aldolase, Ferritin and Thyroglobin. The kit also includes Blue Dextran 2000. Standard curve is set up by taking log Molecular weight as X-axis and $K_{av}$ as Y-axis. $K_{av}$ is calculated by equation \( \left( V_e - V_o \right) / \left( V_c - V_o \right) \). \( V_o \) is column void volume. The elution volume for Blue Dextran 2000, a protein with molecular weight 2000Kd, is taken as $V_o$. $V_e$ is determined by the elution volume of all the proteins in the calibration kit respectively. $V_e = r^2 \pi x l$, \( r \) is the radius of the column and \( l \) is the length of the column. After linear fitting, excel generated an equation to represent the standard curve. Superdex 200 was used for analysis in this experiment. Briefly, Blue Dextran 2000 was firstly applied to the column to determine $V_o$. Then
proteins in the calibration kit were dissolved and mixed as the concentration of 3-5 mg/ml of each protein (with the exception of Ferritin, 0.3 mg/ml). The mixture was applied to superdex 200. Then recombinant PKM1 and PKM2 were diluted to 1μM and 8μM respectively. 200μL of each protein sample was loaded to the column. The equilibration buffer used in this experiment is 50mM Tris pH 7.5 and 50mM KCl.

6.1.13 Pyruvate kinase enzymatic activity assay

Pyruvate kinase enzymatic activity was measure by pyruvate kinase activity assay kit from Biovision. Pyruvate kinase catalyzes PEP and ADP to produce ATP and pyruvate. This reaction is coupling with pyruvate oxidase reaction. Pyruvate generated by pyruvate kinase is oxidized by pyruvate oxidase to produce color at wavelength 570nm. Standard curve was prepared before measuring samples. Firstly, pyruvate standard in the kit was diluted 100 folds with Assay Buffer. 0, 2, 4, 6, 8, 10μl was added to each with each well in 96 well plate and Assay buffer was added to make the total sample volume to be 50 μl. Then another 50μl of reaction mixture was added to each well. Reaction mixture contains: Assay buffer, substrate mix, enzyme mix and OxiRed Probe. Secondly, the samples were diluted and added to 96 well plate (50 μl each well) separately. 50μl reaction mixture was mixed with the samples. The plate was read by Enspire plate reader in GSU core facility at wavelength 570nm every 5min for 20min at room temperature. Every standard point and sample was measured in triplet.

6.1.14 SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is for analyzing or separating proteins based on molecular weight. Polyacrylamide gel generally has two layers: bottom layer is separating gel and upper layer is stacking gel. Separating gel is made by mixing the following materials: for 10% gel, 5.95ml DI water, 3.75ml 1.5M Tris-HCl pH 8.8, 150μl 10% SDS, 5ml Acrylamide/Bis-acrylamide (29:1), 150μl 10% Ammonium persulfate and 6μl TEMED. Total volume is 15ml. Stacking gel
is made by: 6.8ml DI water, 1.25ml 1M Tris-HCl pH 6.8, 100µl 10% SDS, 100 µl 10% Ammonium persulfate, 1.7ml Acrylamide/Bis-acrylamide (29:1) and 10µl TEMED. Total volume is 10ml. The comb was inserted to the upper stacking gel to generate loading wells. After becoming solidified, gels were placed in the gel running cassettes, which was filled with gel running buffer (containing 25mM Tris, 192mM Glycine and 0.1% SDS) on both positive and negative ends. All the samples were mixed with 5X SDS loading buffer (50 mM Tris-HCl pH6.8, 2% SDS, 10% Glycerol, 1% β-Mercaptoethanol, 0.02% Bromophenol Blue and 12.5 mM EDTA). Then 20-30µl samples were loaded to each well and gels were ran at constant voltage of 50-120V for 2 hours.

6.1.15 Coomassie brilliant blue staining

After SDS-PAGE, protein bands can be visualized by Coomassie Brilliant Blue staining. The gel was removed from gel running plates by gel lifter and washed with DI water twice. Then the gel was stained in Coomassie blue staining buffer for 30min-60min. After washed twice with DI water, the gel was destained with destaining buffer till the target protein bands can be visualized clearly. Coomassie blue staining buffer contains 0.1% Coomassie Brilliant Blue, 20% Methanol, and 10% Acetic acid, and dissolved in DI water. Destaining buffer contains: 20% Methanol and 10% Acetic acid, and dissolved in DI water.

6.1.16 Gelcode gel staining

This staining method is to stain proteins with low concentration or for proteins subjected to Mass Spectrum. The gel is removed by the gel lifter mentioned above and washed twice with DI water. The gel was then stained with 20ml Gelcode staining buffer for minimum 60min at room temperature. The gel was destained by DI water instead of destaining buffer. The bands were able to be visualized under UV 256nm by UVP biospectrum 410 imaging system in our lab.
6.1.17 **Protein phosphorylation assay of pyruvate kinase**

To test the protein kinase activity, western blot assay was utilized. Reaction mixture contains: PKM2 (BSA or PKM1), 5mM PEP, 2mM DTT, and substrates such as STATs or Vitronectin. The reaction was kept in room temperature for 2 hours or 4°C for overnight. The reaction was quenched by adding SDS loading buffer. Reaction products were applied to SDS-PAGE and western blot. Anti-phosphoserine, phosphotheonine and phosphotyrosine primary antibodies were used to blot the membrane.

6.1.18 **Western blot**

Western blot was used to specifically detect the existence of contain proteins. Protein sample or cell lysates were separated by SDS-PAGE. After SDS gel was lifted, the gel, filter paper, the sponges and nitrocellular membrane were soaked in the transfer buffer for 10min. Transfer buffer was made by 25mM Tris, 192mM Glycine, 10% methanol and DI water. Then the gel, filter paper, the sponges and nitrocellulose membrane were placed together to form a sandwich structure. The gel has to face the negative side and membrane faced to positive side. The sandwich structure was inserted to the transfer cassette and the cassette was placed into the transfer chamber filled with transfer buffer. The transferred membrane was staining with Ponceau S. Prestained protein ladder on the membrane was labeled. The membrane was washed twice with TBST (1xTBS containing 0.05% Tween) to wash off the Ponceau. Then the membrane was incubated with blocking buffer (5% BSA dissolved in 1XTBS) for 1 hour at room temperature. After brief wash, the membrane was incubated with primary antibody diluted with blocking buffer for overnight at 4°C or 2 hours at room temperature. Then the membrane washed three times with 1xTBS (each time 5min) and incubated with HRP-conjugated goat-anti-mouse or goat-anti-rabbit for 1 hour at room temperature. The membrane was washed three times with 1xTBS (each time 5-10min). Pierce chemiluminescent substrates (supersignal western pico substrate) was add-
ed to the membrane. After two minutes, the membrane was exposed to X-ray film for indicated time and the film was development by X-ray film developer in GSU core facility.

6.1.19 Antibody generation

Recombinant human PKM2 (rhPKM2) was purified after Ni-NTA and gel filtration describe above. rhPKM2 (1mg/ml) was mixed with either complete Freund’s adjuvant or incomplete Freund’s adjuvant at a ratio of 1:1. Complete and incomplete Freund’s adjuvants were used to boost the rabbit immune system. PKM2 and adjuvants were mixed completely to form a stable emulsion and 1ml of the mixture was injected under the skin of New Zealand Rabbits. After the inoculation injection and three follow-up boost injections (6-8 weeks in total), rabbit blood was collected and serum was separated by centrifugation at 2500 rpm for 20min. The anti-PKM2 serum can be applied to western blot to analysis the antibody titer and efficacy.

6.1.20 Antibody purification

Polyclonal antibody was purified from rabbit serum by Protein A sepharose 4B fast flow (GE life science). In general, anti-PKM2 rabbit serum was centrifuged or filtered to remove the junks. Antiserum was mixed with equilibrium buffer (20mM sodium phosphate pH 7.5) with a ratio of 1:1 and then mixed with Protein A sepharose 4B resin beads for 1 -2 hours at room temperature. The beads were washed twice with equilibrium buffer. Then IgG was eluted out from beads by elution buffer containing 0.1 M glycine-HCl pH 2.7. The elution fractions were neutralized by 1.0 M Tris-HCl pH 9.0. SDS-PAGE was applied to analyze the purification results. The fractions containing IgG were combined and desalted by desalt column. In the meantime, pre-bleeding rabbit serum was also used to purify control antibody with the same procedure described above.
6.1.21 PKM2 specific antibody purification

The method mentioned above is to purify total rabbit IgG from rabbit serum. PKM2 specific antibody only takes 10-20% of total IgG. We wanted to exclude the possibility that those non-specific antibodies do not affect PKM2 function. Therefore we carried out this assay to specifically purify PKM2-specific antibodies from total rabbit IgG. The method we used to conjugated rhPKM2 to CNBR-activated Sepharose 4B. Briefly, CNBR-activated Sepharose 4B was washed at low pH (pH 3) to remove additives. 1g CNBR lyophilized powder gives roughly 3.5 ml final volume. RhPKM2 was dissolved in the coupling buffer (0.1 M NaCO3 pH 8.3 and 0.5 M NaCl). Mix rhPKM2 with CNBR-activated Sepharose 4B at room temperature for 1 hour or at 4°C for overnight. Sepharose 4B beads were washed with coupling buffer for 3 times and blocked with blocking buffer containing 0.1 M Tris-HCl pH 8.0 for 2 hours. Then Sepharose 4B beads were washed for 3 times with each buffer. Buffer1 is made by 0.1 M acetic acid / sodium acetate, pH 4.0 containing 0.5 M NaCl; Buffer 2: 0.1 M Tris-HCl, pH 8 containing 0.5 M NaCl. After rhPKM2 was successfully conjugated to Sepharose beads, purified rabbit IgG from rabbit serum were incubated with rhPKM2-Sepharose at 4°C for 2 hours. Unbound proteins were washed away with PBS. Then PKM2 specific antibodies were eluted out from beads by elution buffer containing 0.1 M glycine-HCl pH 2.7. The elution fractions were neutralized by 1.0 M Tris-HCl pH 9.0. SDS-PAGE and western blot were applied to analyze the purification results.

6.1.22 PKM2 purification from cell culture medium

Polyclonal anti-PKM2 antibody was incubated with Protein A sepharose (GE lifesciences) beads for 1-2 hours at room temperature (Protein A sepharose binding capacity is 5mg/ml). The sepharose beads were washed with PBS to remove unbound protein and then were washed twice with 0.1 M sodium borate, pH 9. The beading binding antibody was incubated in sodium borate buffer containing 20mM DMP (dimethyl pimelimidate, from Sigma) for 30min in room temperature. Repeat this step once. The
beads were incubated with buffer containing 50mM glycine pH 2.5 to quench the DMP activity for 30min. Finally, the beads were washed twice with PBS. After Protein A sepharose beads were successfully conjugated with polyclonal anti-PKM2 antibody, conditional culture medium from SW620 cancer cell line was subjected to be incubated with conjugated Protein A sepharose. The beads then were washed twice with PBS and eluted by 50mM glycine pH 2.5. 1M Tris-HCl pH 9.0 was added to the elution to neutralize pH. Purified exogenous PKM2 underwent dialysis to reduce salt concentration.

By using the same method, normal rabbit IgG was also purified by Protein A sepharose beads. The elution was collected and neutralized by future experiment.

6.2 Cellular techniques

6.2.1 Mammalian Cell culture

Human umbilical vascular endothelial cell (HUVEC) and cancer cell lines were cultured in 75cm² culture flasks containing 20-30 ml designated culture medium. The flasks were placed in the culture incubator in a condition of 37°C, 5% CO₂/95% air and 95% humidity. Culture medium for cancer cell lines was commonly supplemented with 5-10% Fetal Bovine Serum (FBS) to provide growth factors needed. Culture medium for HUVEC cell is supplemented with low serum growth supplement (LSGS). While cultured cells reached 95% confluence, cells were digested and split into new flasks.

6.2.2 Subculture

Firstly, the culture medium was discarded by pipetting or vacuuming. Cells were then briefly washed with 0.05% (w/v) Trypsin and 0.53 mM EDTA solution to remove all traces of serum in the flasks. 2mL of this Trypsin-EDTA solution was added to the flask for 5-10min. Cell culture flasks can be observed under microscope to determine the digestion time. After cells detached from the flasks, 8mL culture medium containing 10% FBS was added to the quench Trypsin. Trypsin-EDTA can be removed by centrif-
ugation at 800 rpm for 5 min at room temperature. Cells were suspended with culture medium containing 10% FBS following centrifugation and cells were split to new flasks with a ratio of 1:3 to 1:5.

6.2.3 **Cell storage**

Cultured cells need to be stored for future use. Cells were trypsinized following the method mentioned above. After neutralization and centrifugation, cells were suspended with culture medium supplemented with 10% DMSO and 10% FBS. The cell suspension was transferred to Cryovials and Cryovials were placed into freezing container containing filled with isopropyl alcohol. Freezing container was stored at -80°C overnight and then Cryovials were transferred to liquid nitrogen for long term storage.

6.2.4 **Cell lysis preparation**

Culture medium was evacuated by vacuum and cells were washed twice with PBS containing sodium orthovanadate. After removing PBS, 1mL fresh PBS was added to culture flasks and cells were detached by cell lifters. Cells were transferred to eppendorf tube and centrifuged at 3000 rpm for 5 min at 4°C. Cell pellets were suspended with 500-1000µl cold 1X RIPA buffer containing Protease inhibitor cocktail 1 and Protease inhibitor cocktail 2. The cell suspension was incubated for 1 hour at 4°C on a rotator and centrifuged at 13,200 rpm for 10 min at 4°C. The supernatant was collected and stored at -80°C for further immunoprecipitation or western blot analysis.

6.2.5 **Immunoprecipitation**

Wash protein A/G beads twice with PBS. Mix polyclonal rabbit serum (purified antibody) or normal rabbit IgG with protein A/G beads at 4°C for 1 hour. Protein A/G beads were washed three times with PBS to remove unbound antibody and other unspecific proteins. Cell lysates were thawed and mixed with antibody bound protein A/G beads. The mixture was incubated at 4°C on a rotator for 2-4 hours.
The supernatant was discarded and the beads were washed for three times with PBS. Finally, discard all supernatant and add 60µl of SDS-loading dye to the beads. The beads were boiled for 5mins and then the sample was ready for SDS-PAGE.

6.2.6 DNA precipitation

After mini- or midi- preparation, add 2 volume of cold 100% ethanol and 1/10 volume of 3M sodium acetate (NaAc), pH 5.2 to one volume of DNA solution. The whole mixture was placed at -20°C for from 1 hour to overnight. Thereafter, the solution was centrifuged at 13,000 rpm for 10min at 4°C in a bench-top eppendorf centrifuge. The supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was centrifuged again at 13,000 rpm for 10min at 4°C. Remove the ethanol and air-dry the pellet or by vacuum. DNA pellet was re-suspended with Tris-HCl buffer pH 8.0.

6.2.7 Cell transfection

Cells were seeded in 6-well culture plates to 60-80% confluence. 2µg DNA plasmids and 3µl Lipofectamine 2000 were diluted into 50µl Opti-MEM respectively for 5 min. Mix Opti-MEM containing plasmids and Lipofectamine together and incubate at room temperature for 10 min. Total 100µl Opti-MEM was added to cell culture medium in 6-well plates. Cells were continuously cultured for 48-72 hours for further testing.

6.2.8 Immunofluorescence

When the cells grew to 80-90% confluence, the cells were trypsinized and seeded in a 4 well chamber slides (1x10^4 per well). After cultured overnight, cells were washed with non-FBS medium and treated with rPKM2 and BSA respectively for 1-2 hours at 37°C. Thereafter, culture medium was discarded and the cells were fixed with 200mL 4% formaldehyde in PBS for 5-10 min at room temperature. After fixation, cells were washed twice with PBS. Cells were incubated with 0.5% Triton X-100 in PBS for 10
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min to penetrate cell membrane, followed by blocking with 5% BSA in PBST (0.05% Tween 20 in PBS). Cells were treated with primary antibody diluted in blocking buffer at 4°C overnight. On the second day, cells were washed 3 times with PBST and stained with fluorescence-conjugated secondary antibody at room temperature for one hour. This and following steps should be conducted in a humid light-tight box. After washed 3 times, Prolong Gold anti-fade reagent with DAPI was added to slides, followed by covering the slides with coverslips. The stained slides were dried for overnight and sealed with nail polish for long time storage.

6.2.9 Cell attachment assay

Extracellular matrix proteins such as vitronectin or fibronectin were coated to 96 well ELISA plates at 4 °C for overnight or recombinant pyruvate kinase proteins were coated at room temperature for 2 hours. The plates were washed twice with PBS and blocked with 5% BSA diluted in PBS at room temperature for 1 hour. Endothelial cells were cultured in a 75 cm² flasks to 80% confluence and were detached by 10mM HEPES buffer pH 7.4 containing 20mM EDTA and seeded to the ECM or recombinant proteins coated ELISA plates. 2 x 10⁴ endothelial cells were added to each well and plates were incubated at 37 °C incubator for 30-60 min. After removing the unbound cells with PBS by wash, attaching cells were fixed with 3.7% formaldehyde solution at room temperature for 10 min and then stained with 0.1% crystal violet for 15min. Crystal violet out of the cells were washed away by PBS for 3 times. After complete air dry, crystal violet was dissolved with 50-100 μL extraction buffer solution. The extraction buffer is made of 0.1 M sodium citrate, 50% ethanol and 50% H₂O. Transfer 50 μl extraction buffer solution to a new well and measure OD590. This experiment has been done three times independently.

6.2.10 Cell migration assay

Endothelial cells were seeded in 75 cm² flask and cultured at 37 °C to 70-80% confluence. Cells were washed with serum-free medium and detached with 10 mM HEPES buffer pH 7.4 containing 20mM
EDTA, and neutralized by 3.5mM CaCl$_2$ and 3.5mM MgCl$_2$. Cell suspension was centrifuged at 800g for 5min and washed twice with serum-free medium or HBSS. Endothelial cells were then seeded onto the upper chamber of Boyden chamber. 5x10$^4$ cells per well in 100 µl serum-free medium. Add 400 µl medium with FBS or with recombinant PKM2 proteins or control proteins to the lower chamber. Boyden chambers were incubated at 37 °C for 12-24 hours with 5% CO2. The cells was fixed with 3.7% formaldehyde at room temperature for 10 min and washed twice with PBS, followed by staining the cells with 0.1% crystal violet for 15min. The cells on the membrane of the lower chamber were removed by cotton swabs. After air dry, crystal violet was dissolved by extraction buffer for 5min (100 µl each well). Transfer 50µl of the extracted solution and measure OD at 590 nm. Each sample was done by 3 parallel transwells and each experiment was repeated for 3 times independently.

### 6.2.11 Cell proliferation assay

Cell proliferation is assessed by BrdU incorporation assay. Briefly, cells were seeded into 96 well plates (2500-100000 cells per well) and were incubated with recombinant proteins respectively at 37°C for certain period (from 24-72 hours). Culture medium was changed to fresh medium containing 1:1000 diluted BrdU (100µL each well). Place the plates back to the incubator for another 4-12 hours based on the cell growth rate. After removal of the culture medium, cells were fixed at room temperature for 30 min with fixing/denaturing solution, followed by 3 times wash; cells were incubated with primary antibody for 1 hour. Cells were incubated with HRP-conjugated secondary antibody for 30 min after another 3 times wash. Remove secondary antibody, wash plates 3 times with washing buffer and add TMB substrates solution. Add stop solution after incubating with substrates for 30 min and measure absorbance at 450nm. Each sample was done by 5 parallel wells and each experiment was repeated for 3 times independently.
6.2.12 Culture medium collection

Cancer cell lines were cultured in full FBS medium to 50-60% confluence in 75 cm² flasks. The cells were washed twice with non-FBS medium and fresh non-FBS medium was added. After 12 hour culturing, cell culture medium was collected and suspended cells were removed by filtering. The culture medium can be used for western and immunoprecipitation.

6.2.13 Tumor cell preparation for animal experiment

Prostate cancer cell line PC-3 and colon cancer cell line SW620 were cultured in 37 °C incubator. Upon the injection day, cells were trypsinized by trypsin-EDTA and neutralized by Full FBS medium. After centrifuge at 800g, cells were washed with non-FBS medium. Then cells were washed 2-3 times with HBSS. The cell pellet was responded with HBSS to a concentration of 2x10^7 cells per 1mL and places on ice. Equal volume of Matrigel from BD Bioscience was mixed with cell suspension. The cell suspension was left on ice for mice subcutaneous injection.

6.3 Animal experiments

6.3.1 Nude mice tumor model

The mice using is 4-6 week old Nu/Nu male mice from Harlan. Nude mice were housed in animal facility at Georgia State University. One week later, 50-100 µl tumor cells were mixed with BD Matrigel and injected on the back flank of nude mice under anesthesia. After tumor inoculation, mice were administered with recombinant proteins intraperitoneally three doses every week. Three days after tumor implantation, tumor sized was by measured by length and wild. The tumor volume was calculated by formula: \( V=\frac{\pi}{6} \times L \times W^2 \) (width). 15-20 days after inoculation, nude mice were euthanized by CO₂ and tumors or tissues were collected. The tumors were measured and weighted, followed by being embedded in OCT reagent and frozen at -80°C for further sectioning.
6.3.2 Breast Cancer Orthotopic model

Mouse breast cancer cell line 4T1 was maintained in DMEM at 37°C incubator. Cells were trypsinized and collected by centrifuge. 4T1 cells were then washed three times with HBSS and concentrate to 1 million cells per 1ml. Female Bal b/c mice were purchased from Harlan and separated randomly to each cage. Mice were shaved and injected 20-30 µl to the fat pat on the left under anesthesia. IP injection, tumor measurement and euthanasia were conducted by following the same protocol as the nude mice model. The only difference is the tumor volume calculation. Tumor area volume is calculated by Width x Length. This reason we were calculating tumor area instead of tumor volume is because the fat pat tumor is much flatter.

6.3.3 Frozen tissue sectioning

The mice tissues and tumors were embedded in OCT and stored at -80°C. Before sectioning, tissues and tumors were placed in -20°C for 10min. This may prevent sample cracking while sectioning. The section machine is Cyrostat. Tissues were sectioned at a range 8-12µm and placed on the positively charged slides. Allow sections to air dry on the slides for 1-2hour. Before fixation and staining, OTC was washed away by Ethanol. The tissue area was circulated by Dako pen to maintain the solutions or antibodies at the tissue area.

6.3.4 Matrigel plug assay

Prepare the samples: rPKM2 or saline solution was mixed with high concentration Matrigel with growth factor reduced. The mixture was kept on ice. Transfer the Matrigel/protein mixture to a syringe. Nude mice were anesthetized and injected with 300µl of the Matrigel with protein on the back of the mice. Ten days after Matrigel implantation, mice were euthanized and Matrigel plugs were harvested. Take pictures on the Matrigel plug to record the peripheral angiogenesis. 10µg of the plug was cut and
submitted for hemoglobin measurement. And the rest of the gel plug was frozen in OCT for tissue sectioning.

6.4 Statistics

All the in vitro data were analyzed by one-tailed, unpaired student t test. All the in vivo data were performed by pairing nude mice and Balb/c mice after tumor implantation and before treatment. Mice were paired based on the tumor size. If the tumor size were similar, mice were further paired by mice weight. Mice tumor sizes were compared in the pairs before and after treatment. Nude mice experiment results have shown significance with one-tailed, unpaired student t test (p value is lower than 0.05); while orthotopic 4T-1 model didn’t show significance with one-tailed, unpaired student t test. Therefore, we tried one-tailed, paired student t test. P values is lower than 0.05. Since we paired the animals while treated, it is reasonable to analyze the data by paired t test.

6.5 Materials

Chemicals

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<th>Chemical</th>
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<tr>
<td>(IPTG) Isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>HEPES</td>
<td>Sigma Aldrich</td>
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<td>Item</td>
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<td>High Concentration Matrigel, phenol red-free</td>
<td>BD Biosciences</td>
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<tr>
<td>HiTrap Q column</td>
<td>GE Healthcare Lifesciences</td>
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<tr>
<td>Hydrocholoric Acid</td>
<td>VWR international</td>
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<tr>
<td>Hydrocortisone</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Igepal (NP-40)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Imidazole</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Incomplete Freud's Adjuvant</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Isopropyl Alcohol</td>
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<td>Kanamycin</td>
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<tr>
<td>Lipofectamine 2000 Transfection Reagent</td>
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<td>Low Melting point Agarose</td>
<td>Sigma Aldrich</td>
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<td>LSGS (Low serum growth supplement)</td>
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<tr>
<td>Magnesium Chloride</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Magnesium Chloride 6H(\text{H}_2\text{O})</td>
<td>Sigma Aldrich</td>
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<td>Matrigel Basement Membrane Matrix</td>
<td>BD Biosciences</td>
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<td>Matrigel, phenol red-free, growth factor reduced</td>
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<td>Methanol</td>
<td>VWR international</td>
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<tr>
<td>Nail polish</td>
<td>Fisher Scientific</td>
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<td>Ni-NTA agarose</td>
<td>Qiagen</td>
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<td>OCT</td>
<td>Fisher Scientific</td>
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<td>Oxalate</td>
<td>Sigma Aldrich</td>
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<td>PCR Master Mix</td>
<td>Promega</td>
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<tr>
<td>Penicillin-Streptomycin solution</td>
<td>Cellgro</td>
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<td>PEP(Phosphoenolpyruvic acid monopotassium salt)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Phosphatase inhibitor cocktail I</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Phosphatase inhibitor cocktail II</td>
<td>Sigma Aldrich</td>
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<tr>
<td>PMSF (Phenylmethylsulfonyl FLuoride)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Ponceau</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Potassium Chloride</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Potassium phosphate monobasic</td>
<td>Sigma Aldrich</td>
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<td>Product Name</td>
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<tr>
<td>Prolong gold anti-fade reagent with DAPI</td>
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<tr>
<td>Protease inhibitor cocktail</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Protein A agarose</td>
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<td>Protein G agarose</td>
<td>Millipore</td>
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<tr>
<td>Rhodamin Phalloidin</td>
<td>Cytoskeleton</td>
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<tr>
<td>RIPA buffer 10X</td>
<td>Millipore</td>
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<td>Sodium Acetate</td>
<td>Sigma Aldrich</td>
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<td>Sodium Azide</td>
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<td>Sodium Chloride</td>
<td>VWR International</td>
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<tr>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>Sodium Hydroxide</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Sodium Orthovanadate</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Sodium phosphate dibasic</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Sodium Pyruvate</td>
<td>Cellgro</td>
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<tr>
<td>Supdex 200</td>
<td>GE Healthcare Lifesciences</td>
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<tr>
<td>TEMED</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>TEMED (N,N,N',N'-TetramethylEthlenediamine)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Tris base</td>
<td>Fisher Scientific</td>
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<tr>
<td>Triton X-100</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Tryptone</td>
<td>BD Bioscience</td>
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<tr>
<td>Tween-20</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Urea</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Western Blot Stripping Buffer</td>
<td>Thermo Scientific</td>
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<tr>
<td>Yeast Extract</td>
<td>BD Bioscience</td>
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<tr>
<td>Nitrocellulose Membranes</td>
<td>Thermo Scientific</td>
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<tr>
<td>0.25% Trypsin-EDTA</td>
<td>Cellgro</td>
</tr>
<tr>
<td>FBP (Fructose 1,6-bisphosphate)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Acrylamide-Bisacrylamide 40% (w/v 37.5:1)</td>
<td>VWR international</td>
</tr>
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</table>

Kits and disposables
<table>
<thead>
<tr>
<th>Product Description</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5ml eppendorf tube, 15ml, 50ml conical tube</td>
<td>VWR International</td>
</tr>
<tr>
<td>10ml Bacteria cell tube</td>
<td>VWR International</td>
</tr>
<tr>
<td>20µl, 200µl and 1000µl pipette tips</td>
<td>VWR International</td>
</tr>
<tr>
<td>24-well transwell insert, 8.0 µm</td>
<td>BD Falcon</td>
</tr>
<tr>
<td>96-well ELISA plates</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Bio-Rad Protein Assay</td>
<td>Bio-Rad Laboratories</td>
</tr>
<tr>
<td>C18 ZipTip Pipette Tips</td>
<td>Millipore</td>
</tr>
<tr>
<td>Cell culture flasks and plates</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>FuGENE HD transfection kit</td>
<td>Promega</td>
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<tr>
<td>Nuclear Extraction Kit</td>
<td>Active Motif</td>
</tr>
<tr>
<td>PCR tubes</td>
<td>VWR International</td>
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<tr>
<td>QIAGEN Plasmid Midi kit</td>
<td>Qiagen Inc.</td>
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<tr>
<td>QIAdapta Spin Miniprep Kit</td>
<td>Qiagen Inc.</td>
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<tr>
<td>QIAquick Gel Extraction Kit</td>
<td>Qiagen Inc.</td>
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<tr>
<td>Virapower Lentiviral Expression System</td>
<td>Invitrogen Life Technologies</td>
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<tr>
<td>BiaCore CM5 chip</td>
<td>BIACORE</td>
</tr>
<tr>
<td>DAKO Delimiting pen</td>
<td>DAKO</td>
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</table>

**Laboratory Equipment**

<table>
<thead>
<tr>
<th>Equipment Description</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>Hemocytometer</td>
<td>Hausser Scientific</td>
</tr>
<tr>
<td>Allegra 6R Benchtop Centrifuge</td>
<td>Beckman Coulter</td>
</tr>
<tr>
<td>C25 Incubated Floor Shaker</td>
<td>New Brunswick Scientific</td>
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<tr>
<td>Class II Biosafety Cabinet</td>
<td>Labconco</td>
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<tr>
<td>EC3 BioImaging System</td>
<td>UVP</td>
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<tr>
<td>UV-1700 Spectrophotometer</td>
<td>Shimadzu North America</td>
</tr>
<tr>
<td>AKTA fplc</td>
<td>GE Healthcare Lifesciences</td>
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<tr>
<td>BiaCore 3000</td>
<td>BIACore</td>
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<tr>
<td>MALDI TOF 4800 PLUS</td>
<td>Applied Biosystems</td>
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<tr>
<td>Enspire Plate Reader</td>
<td>Perkin Elmer</td>
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<tr>
<td>Film Processor</td>
<td>Kodak</td>
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<tr>
<td>Equipment/Reagent</td>
<td>Supplier</td>
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<tr>
<td>French Pressure Cell Press</td>
<td>SLM/Aminco</td>
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<tr>
<td>Sonifier 450</td>
<td>Branson</td>
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<tr>
<td>Zeiss 510 Laser Scanning Microscope</td>
<td>Zeiss</td>
</tr>
<tr>
<td>CO₂ incubator</td>
<td>Thermo Scientific</td>
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<tr>
<td>-80°C freezer</td>
<td>Fisher Scientifics</td>
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**Proteins, peptides and antibodies**

<table>
<thead>
<tr>
<th>Protein/Peptide/Antibody</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>22% v/v Bovine Serum Albumin</td>
<td>Sera Care Lifesciences</td>
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<tr>
<td>Anti-Actin antibody</td>
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<tr>
<td>Anti-FAK antibody</td>
<td>Millipore</td>
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<tr>
<td>Anti-Histag antibody</td>
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<tr>
<td>Anti-human GAPDH antibody</td>
<td>Millipore</td>
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<tr>
<td>Anti-human Integrin α5/β1 antibody (JBS5)</td>
<td>Santa Cruz biotechnology, inc.</td>
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<tr>
<td>Anti-human Integrin αV antibody (H-2)</td>
<td>Santa Cruz biotechnology, inc.</td>
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<tr>
<td>Anti-human Integrin αV antibody (Q-20)</td>
<td>Santa Cruz biotechnology, inc.</td>
</tr>
<tr>
<td>Anti-human Integrin αV/β3 antibody (23C6)</td>
<td>Santa Cruz biotechnology, inc.</td>
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<tr>
<td>Anti-human Integrin αV/β3 antibody (LM609)</td>
<td>Millipore</td>
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<tr>
<td>Anti-human TEM8 antibody</td>
<td>IMGENEX</td>
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<tr>
<td>Anti-Ki67 antibody</td>
<td>Millipore</td>
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<tr>
<td>Anti-mouse CD31 antibody FITC-labeled</td>
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<tr>
<td>Anti-phospho-FAK (Tyr397) antibody</td>
<td>Millipore</td>
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<tr>
<td>Anti-Vinculin antibody</td>
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<tr>
<td>Bovine Serum Albumin standard</td>
<td>Sigma Aldrich</td>
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<td>Collagen I from Rat tail</td>
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<td>DNase</td>
<td>Promega</td>
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<td>Gel Filtration Calibration Kit (Low molecular weight)</td>
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<td>HA antibody (mouse monoclonal)</td>
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<tr>
<td>HRP-conjugated goat-anti-mouse antibody</td>
<td>Thermo Scientific</td>
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<tr>
<td>HRP-conjugated goat-anti-mouse antibody</td>
<td>Thermo Scientific</td>
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<tr>
<td>Normal rabbit IgG</td>
<td>Cell Signaling Technology</td>
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<tr>
<td>PDGF-BB</td>
<td>Pepro Tech</td>
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<td>Pfu DNA polymerase</td>
<td>Promega</td>
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<tr>
<td>PKM2 (rabbit polyclonal)</td>
<td>Lab generated</td>
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<tr>
<td>p-Tyr-100 antibody (mouse monoclonal)</td>
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<tr>
<td>p-Tyr-20 antibody (mouse monoclonal)</td>
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<tr>
<td>Pyruvate kinase antibody (both M1 and M2)</td>
<td>Lab generated</td>
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<tr>
<td>Rabbit muscle Pyruvate Kinase (M1 type)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Recombinant human Fibronectin</td>
<td>R&amp;D systems</td>
</tr>
<tr>
<td>Recombinant human Integrin α1β1</td>
<td>R&amp;D systems</td>
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<td>Recombinant human Integrin α5β1</td>
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<tr>
<td>Recombinant human Integrin αvβ3</td>
<td>R&amp;D systems</td>
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<td>Recombinant human vitronectin</td>
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<td>Restriction enzymes</td>
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<td>RNase A</td>
<td>Thermo Scientific</td>
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<td>T4 DNA ligase</td>
<td>Fermentas</td>
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<tr>
<td>Thrombin from bovine plasma</td>
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<tr>
<td>TNK (T4 Polynucleoide Kinase)</td>
<td>Fermentas</td>
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<tr>
<td>Trypsin proteomics grade</td>
<td>Sigma Aldrich</td>
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**Plasmids, bacteria stains, cell lines and animals**

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<table>
<thead>
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<tr>
<td>pHM6 mammalian expression vector</td>
<td>Roche Applied Science</td>
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<td>pLenti6/V5-D-TOPO</td>
<td>Invitrogen Life Technologies</td>
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<td>pLenti6/V5-DEST</td>
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<td>PET30a</td>
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<td>PGEX 2TK</td>
<td>GE Lifesciences</td>
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<tr>
<td>Human Pyruvate kinase M2 gene</td>
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<tr>
<td>Human Pyruvate kinase M1 gene</td>
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<tr>
<td>Tumor endothelial marker 8 gene</td>
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<td>Colon cancer cell line SW620</td>
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<td>Cell Line</td>
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<tr>
<td>Human Umbilical Vein Endothelial cell line</td>
<td>Invitrogen Life Technologies</td>
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<tr>
<td>Microvascular endothelial cell line</td>
<td>Provided by Dr. Yuan Liu, Georgia State University</td>
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<td>HEK 293 human embryonic kidney cell line</td>
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<td>H460 human lung cancer cell line</td>
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<td>H146 human lung cancer cell line</td>
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<td>WM115 human melanoma cancer cell line</td>
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<td>WM266 human melanoma cancer cell line</td>
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<td>PC3 human prostate cancer cell line</td>
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<td>4T1 mouse breast cancer cell line</td>
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<td>NM2C5 human breast cancer cell line</td>
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<td>M4A4 human breast cancer cell line</td>
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<td>Nuce mice</td>
<td>Harlan</td>
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<td>Bal b/c mice</td>
<td>Harlan</td>
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<td>Tuner (DE3)</td>
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<td>JM109</td>
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<td>pcNDA3.1+</td>
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