2017

Pyruvate kinase M2 (PKM2), a glycolytic enzyme, is required to maintain vascular barrier function

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

PYRUVATE KINASE M2 (PKM2), A GLYCOLYTIC ENZYME, IS REQUIRED TO MAINTAIN VASCULAR BARRIER FUNCTION

by

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M.S., Boston University, 2017

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2017

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DEDICATION

I would like to dedicate this thesis to my dear friend, Rebecca Robie, who recently was diagnosed with stage 4 Hodgkin's lymphoma. With your wisdom, compassion, and courage you are an inspiration to me and all women. Dear Becca, I know you have a hard fight ahead of you but stay strong. I wish you a speedy recovery and a cancer free happily ever after.

ACKNOWLEDGMENTS

First and foremost, I thank my advisors, Professor Karen Symes and Professor Zoltan Arany. Dr. Karen Symes I would like to thank you for your guidance, patience, encouragement and your motivational conversations. I always walked out of our meeting with renewed confidence. Thank you for being my pillar of support. Dr. Zoltan, I want to thank you for giving me the opportunity to work in your lab and for your valuable guidance in directing me towards completing a successful thesis.

I would like to thank Dr. Boa Kim, for letting me join your project and for sharing your love for PKM2. I would like to also thank all the Arany lab members who helped and supported me in various ways.

I would like to thank my parents, my brother Kiruba, my cousins Sweetha and Mohan, and my friends (Molly, Amber, Rebecca Praneetha and Manasa) for their support, wise counsel and sympathetic ears.

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ABSTRACT

RATIONALE **-** Metabolic enzymes, like pyruvate kinase M2 (PKM2), play an essential role in altering endothelial cell (EC) phenotypes and behavior. Extensive research has elucidated the function of PKM2, a rate-limiting glycolytic enzyme, in the context of cancer cells and in activated immune cells, but its role in EC biology is only newly emerging. Recent findings show PKM2 acts as a key regulator of angiogenesis. Where exogenous circulating PKM2 induces EC cell proliferation leading to increased tumor angiogenesis and growth. Also, PKM2 deficient ECs exhibit decreased proliferation and migration. The relevance of PKM2 in modulating vascular barrier function is yet to be defined.

OBJECTIVE -This study attempts to elucidate the role of PKM2 in regulating vascular barrier function.

METHODS AND RESULTS **-** In vivo, EC specific deletion of PKM2 promotes increased vascular permeability in pulmonary capillary vessels and increased VEGF-induced acute vessel permeability in mouse dermal vessels. Similarly, in vitro, PKM2 deficient ECs exhibit decreased electrical resistance, disrupted VE-cadherin junctions and gap formations (illustrated via florescent VE-cadherin staining and phosphorylation of

VE- cadherin protein at tyrosine residue Y658). Mechanistically, the deletion of PKM2 in ECs leads to increased angiopoietin-2 (Ang-2) expression, a well-known modulator of vascular permeability. Also, deletion of Ang-2 was sufficient to attenuate vascular leakage in PKM2 deficient endothelium, indicating that vascular leaky phenotype observed in PKM2 deficient endothelium is mediated by increased Ang-2 expression.

CONCLUSIONS - PKM2, by modulating Ang-2 expression, plays a vital role in maintaining vascular barrier function.

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LIST OF ABBREVIATIONS

Ang-1 ..... Angiopoietin 1

Ang-2 ..... Angiopoietin 2

EC ..... Endothelial Cell

EBD.....Evans Blue Dye

KO.....Knockout

OX-PHOS .....Oxidative Phosphorylation

PBS .....Phosphate-Buffered Saline

PBS-T.....Phosphate-Buffered Saline/Tween 20

PK ..... Pyruvate Kinase

PKM1.....Pyruvate Kinase M1

PKM2.....Pyruvate Kinase M2

qPCR..... Quantitative Real-Time Polymerase Chain Reaction

TBS .....Tris-Buffered Saline

TBS-T .....Tris-Buffered Saline/Tween 20

VEGF ..... Vascular Endothelial Growth Factor

INTRODUCTION

1. Introduction

An emerging concept is that endothelial metabolism can affect endothelial cell (EC) phenotypes and behavior. Metabolic regulation of angiogenesis is an example of how EC metabolism can induce changes in EC phenotype and behavior (Verdegem et al., 2014). For instance, phosphofructokinase-2/fructose-2,6-bisphosphatase3 (PFKFB3), a rate-limiting glycolytic metabolic enzyme was recently shown to drive sprouting, migration and proliferation of angiogenic ECs (De Bock et al., 2013; Stapor et al., 2014). This brings up the question of what role do other metabolic enzymes, like pyruvate kinase, play in altering EC phenotypes and behavior. This study attempts to investigate the role of pyruvate kinase, another rate-limiting glycolytic enzyme, in endothelial biology. Although extensive research has elucidated the function of PKM2 in context of cancer cells and in activated immune cells, little is known about PKM2 in endothelial cells.

2. Pyruvate Kinase

2.1 Pyruvate Kinase Isoforms

Pyruvate Kinase is a rate-limiting glycolytic enzyme that irreversibly catalyzes phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP) to pyruvate and adenosine triphosphate (ATP). In mammals, the PK family consists of four isozymes: PK liver (PKL), PK red blood cells (PKR), PK muscle 1 (PKM1), and muscle 2 (PKM2). The PK family of enzymes are encoded by two genes PKLR and PKM. (Israelsen and Vander Heiden, 2015, Dong et al., 2016; Alves-Filho and Pålsson-McDermott, 2016).

The four pyruvate kinase isozymes exhibit unique tissue expression and regulatory properties. All the isoenzymes have similar kinetic parameters with respect to ADP, but they differ in their kinetics with respect to PEP (Israelsen and Vander Heiden, 2015).

2.2 PKLR Gene, Expression and Regulation

Under differential promoters, the PKLR gene encodes for: (1) PKL, enzyme found in liver, kidney, and intestine and (2) PKR, enzyme found in red blood cells. PKL has the lowest affinity to PEP and is under hormonal control. PKR is inhibited by glycolytic product ATP and thyroid hormone triiodo-l-thyronine (T3). Both PKL and PKR are activated by fructose-1,6-bisphophate (FBP), an earlier product of glycolysis (Israelsen and Vander Heiden, 2015, Dong et al., 2016; Alves-Filho and Pålsson-McDermott, 2016).

2.3 PKM Gene and Expression

Through alternative splicing, the PKM gene encodes for both the M1 and M2 isozymes (Israelsen and Vander Heiden, 2015). The inclusion of exon 9 generates the PKM1 isoenzyme. Expression of PKM2 isoenzyme requires both repression of exon 9 through splicing factors PTB, hnRNPA1, and hnRNPA2 and inclusion of exon 10 through splicing factor SRSF3 (Israelsen and Vander Heiden, 2015). Although PKM1 and PKM2 have similar 3D structures, the isoform specific exons generate structural differences at the fructose-1,6-bisphophate (FBP) binding site and the dimer-dimer interface (Dong et al., 2016; Alves-Filho and Pålsson-McDermott, 2016).

The PKM1 form is a constitutively active isozyme that predominates in most terminally differentiated cell types and in cells with high catabolic demands (heart, muscle, brain). In contrast the PKM2 is the dominant isoform during development and in cells with more anabolic functions, such as regenerative tissue, cancer cells, and immortal cell lines (MCF10A). PKM2 is regulated by a variety of allosteric effectors (FBP) and post-translational modifications (Figure 1; Alves-Filho and Pålsson-McDermott, 2016).

2.4 Regulation of PKM2 Activity.

The PK enzyme is regulated by stabilizing and destabilizing its oligomeric configurations. The constitutively active PKM1 exists stably as a tetramer, allowing for optimal binding to substrate PEP. The stable tetramer form of PKM1 has higher PK activity than the dimeric or monomer forms. Unlike PKM1, PKM2 requires an activator to have high PK activity. In the presence of activators, PKM2 switches to a tetrameric form which exhibits high PK catalytic activity and is associated with catabolic metabolism. The high PK catalytic activity allows for conversion of PEP to pyruvate, which enter the TCA cycle to generate ATP via oxidative phosphorylation (OX-PHOS). In the absence of activators, PKM2 primarily exist as a dimeric or monomer with low PK activity, facilitating the formation and accumulation of glycolytic intermediates. These glycolytic intermediates are shuttled into the glycolysis branch pathways, such as glycerol synthesis and pentose phosphate pathway, to generate nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) for ROS suppression and nucleotides for anabolic metabolism.

Various endogenous regulators (glycolytic metabolites, non-glycolytic metabolites, amino acids and small molecules) control the switching of PKM2 configuration and activity by affecting the binding affinity of substrate PEP to the active site on PKM2 enzyme (Dong et al., 2016; Alves-Filho and Pålsson-McDermott, 2016). Fructose-1,6-bisphophate (FBP), an upstream glycolytic intermediate, is an activator of PKM2. FBP binds allosterically to PKM2, promoting stabilization of PKM2 tetramer complex and increases its binding affinity to PEP (Christofk et al., 2008). PKM1 and PKM2 bound to FBP exhibit similar kinetic properties. Small molecules, DASA58 and TEPP46 are also allosteric activators of PKM2. Like FBP, these small molecules promote tetramer stabilization and increase PEP binding affinity (Vander Heiden et al., 2010). Additionally, amino acids like serine (Chaneton et al., 2012) and a *de novo* purine synthesis intermediate, succinylaminoimidazolecarboxamide ribose-5′ phosphate (SAICAR), can activate PKM2 activity (Keller et al., 2012; Keller et al., 2014). PKM2 activity can be inhibited by the absence of allosteric activators or the presence of allosteric inhibitors. Some known allosteric inhibitors of PKM2 are amino acids (phenylalanine and alanine), thyroid hormone triiodo-l-thyronine (T3) or via tyrosine phosphorylation (Ashiwaza et al., 1991; (Uebelhoer and Arispe 2016).

2.5 Non-glycolytic PKM2 Activity.

Since the dimeric isoform of PKM2 can translocate to the nucleus, it also exhibits non-glycolytic functions. The nuclear PKM2 interacts with transcription factors (HIF-1α, STAT3, histone H3 and JMJD5) to induce genes required for proliferation and glycolysis (Figure 1; Alves-Filho and Pålsson-McDermott, 2016).

PKM2 can function as a transcriptional coactivator. Under hypoxic conditions, PKM2 translocates to the nucleus and interacts with hypoxia-inducible factor (HIF)-1α to induce glucose metabolism in cancer cells (Luo et al., 2011). PKM2 gene transcription is also activated by HIF-1α, which in turn further promotes HIF-1α transactivation in a positive feedback look. PKM2 binds to Jumonji domain-containing protein 5 (JMJD5) and prevents PKM2 tetramerization. The JMJD5-PKM2 interaction induces PKM2 to be translocated into the nucleus and promotes HIF-1α mediated glucose metabolism in cancer cells (Wang et al., 2014). Nuclear PKM2 can also bind to phosphorylated β-catenin Tyr 333, leading to β-catenin transactivation and epidermal growth factor receptor (EGFR)-promoted tumor proliferation (Yang et al., 2011; Lu, 2012).

PKM2 can act as a protein kinase to phosphorylate substrates involved in proliferation and glycolysis. PKM2, using PEP as a phosphate donor, phosphorylates signal transducer and activator of transcription 3 (STAT3) at tyrosine-705 and histone H3 at threonine-11, promoting cancer progression (Gao et al., 2012).

3. PKM2 and the Warburg Effect

3.1 PKM2 and the Warburg Effect in cancer and immune cells

Typically, most cells utilize oxidative phosphorylation (OX-PHOS) as an energetically more efficient method for generating large amounts of ATP (32-36 ATPs per glucose vs 2 ATPs per glucose during glycolysis). However, many cancer cells primarily are dependent on glycolysis to meet their bioenergetic needs, regardless of oxygen levels (Israelsen and Vander Heiden, 2015, Dong et al., 2016; Alves-Filho and

Pålsson-McDermott, 2016). This increased rates of aerobic glycolysis in cancer cells was termed the Warburg effect and is necessary for tumor growth. The Warburg effect is not exclusive to cancer cells but is also present in normal cells that are rapidly proliferating. (DONG 2016, Uebelhoer and Arispe 2016). Increased aerobic glycolysis has also been observed in proliferating lymphocytes (20-30 fold induction), activated macrophages, proliferating thymocytes, proliferating fibroblasts, and regenerating skeletal muscle cells. (Uebelhoer and Arispe 2016).

In cancer cells, a prominent driver of the Warburg effect is PKM2. In fact, proliferating cancer cells switch from PKM1 to PKM2 to increase glycolysis for biomass production (Verdegem et al., 2014). Also, PKM2 expression is elevated in many cancers and activated immune cells like macrophages (Uebelhoer and Arispe 2016). Thus, PKM2 is a key regulator of the Warburg effect in both cancer and immune cells.

3.1 PKM2 and the Warburg Effect in ECs

The Warburg effect is not limited to only cancer and proliferating cells but is also observed in both proliferating and non-proliferating endothelial cells. Like cancers cells, ECs preferentially use glycolysis for energy production with only 15% of ATP being generated from OX-PHOS (Culic et al., 1997; De Bock et al., 2013). Correspondingly, ECs have decreased mitochondrial usage with only 5% volume fraction consisting of mitochondria versus up to 30% volume fraction in other oxidative promoting cells (e.g. oxidative hepatocytes; Groschner et al., 2012). This preference for aerobic glycolysis is surprising since ECs are in immediate contact with high oxygen and glucose levels in blood, which are favorable for oxidative phosphorylation. However, the preferential use

of aerobic glycolysis in ECs is hypothesized to be advantageous, since glycolysis can: (1) help ECs conserve oxygen for perivascular tissue, (2) protect ECs from oxidative stress unlike oxidative phosphorylation which generates reactive oxygen species (ROS), (3) allow ECs to migrate into hypoxic tissues, (4) generate ATP more rapidly, and (5) facilitate quick adaptations for increased biomass production during angiogenesis (Verdegem et al., 2014; Uebelhoer and Arispe 2016). While all ECs preferential use of aerobic glycolysis, varied amounts of glycolysis is observed in different vascular beds. (Uebelhoer and Arispe 2016). For example, in comparison to quiescent ECs, angiogenic ECs have double rates of glycolysis generating 80% of ATP via glycolysis and less than 1% of ATP via OX-PHOS (Culic et al., 1997; De Bock et al., 2013).

PKM2 also plays a prominent role in ECs. PKM2 is the predominant isoform expressed in ECs and recent findings show PKM2 as a key regulator of angiogenesis. Exogenous circulating PKM2 induces endothelial cell proliferation, migration, and cell-extracellular matrix adhesion leading to increased tumor angiogenesis and growth (Li et al., 2014). In the Arany lab, we have shown that deletion of PKM2 in endothelial cells results in decreased proliferation and migration (data unpublished). Both PKM1 and PKM2 expression are found in the cytosol, nucleus and lamellipodia extensions of ECs (Figure 2A-F, Data generated by Dr. Boa Kim).

4. Mechanisms Regulating Endothelial Permeability

4.1 VE-cadherin junctions

In ECs, pathways involved in mediating changes in permeability affect VE-cadherin expression, function and organization (Gavard et al., 2014). Extracellularly, VE-cadherin mediates cell-cell adhesion by clustering together at cell-cell contacts and adhering to VE-cadherins of adjacent endothelial cells. Intracellularly, VE-cadherin associates with several proteins (p120, β-catenin, and plakoglobin) to form a complex with α-catenin allowing for interaction with the actin-containing cytoskeleton (Dejana et al., 2008; Dejana and Vestweber, 2013). When permeability is induced in ECs, expression and function of VE-cadherin at cell-cell contacts are altered in several ways: (1) phosphorylation of phopho-tyrosine sites on the cytosolic tail of VE-cadherin (2) phosphorylation of other components in the VE-cadherin/catenin complex (3) by cleaving extracellular domains of the VE-cadherin (4) internalization of VE-cadherin/catenin complex (5) or mechanical stresses (Potter et al., 2005; Turoswki et al., 2008; Orsenigo et al., 2012).

4.2 The Angiopoietin -Tie- Signaling Pathway

The secreted, multimeric angiopoietin ligands regulate vascular homeostasis via the endothelial Tie receptor tyrosine kinases, Tie-1 and Tie-2 (Thurston and Daly, 2012). Angiopoietin-1 (Ang-1) functions in a paracrine and agonist manner, promoting vascular stabilizing effects via Tie-2 phosphorylation and activation. Ang-1 is mainly expressed by perivascular cells. Angiopoietin-2 (Ang-2) functions in an autocrine and antagonist manner by inhibiting the constitutively active Ang-1/Tie-2 signaling, promoting pathological angiogenesis, increased vascular permeability and inflammation. Ang-2 inhibition of Tie-2 activity results in Rho-kinase and endothelial myosin light chain

kinase (EC-MLCK) activation, leading to destabilization of the endothelial monolayer (Maisonpierre et al., 1997; Parikh et al., 2006; Hakanpaa et al., 2015). In cells, Ang-2 is stored in intracellular granules called Weibel-Palade bodies (WBP) with a half-life of more than 18 hours (Fiedler et al., 2004). Ang-2 can be rapidly released from WPBs by activated endothelium. In humans and mice, Ang-2 expression is elevated during vessel remodeling of tumor endothelium and in vascular permeability associated pathologies like sepsis and acute lung injury (Parikh et al., 2006; Thurston and Daly, 2012).

5. Preliminary Data

A shotgun approach via RNA sequencing (RNA-Seq*)* of the whole transcriptome reveals reduction of Ang-2 mRNA expression upon overexpression of mouse PKM2 in endothelial cells (Figure 2G, Data generated by Dr. Boa Kim). Correspondingly, PKM2 deletion in the endothelium induces Ang-2 mRNA expression (Figure 2H, Data generated by Dr. Boa Kim). Ang-2 is a well-known modulator of vascular permeability. Thus, based on this preliminary data, we hypothesized that PKM2 deficient endothelium may exhibit a vascular permeability phenotype.

Figure 1

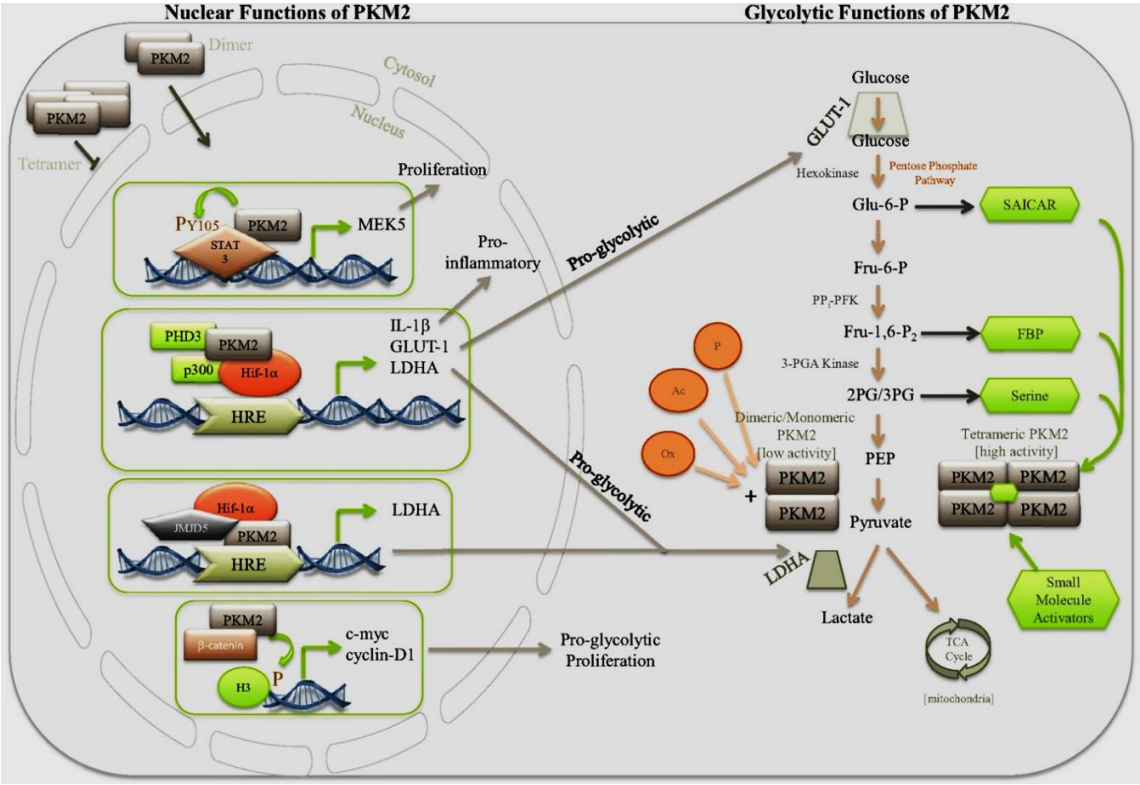


Figure 1: Diagram of PKM2 structure, function (nuclear and glycolytic) and regulation. The tetramer formation of PKM2 promotes glycolysis and the generation of pyruvate, which can enter the TCA cycle for oxidative phosphorylation. The more active tetramer conformation of PKM2 is allosterically regulated by SAICAR, serine, FBP, or small-molecule activators. Without allosteric activators, the less active dimeric form of PKM2 predominates leading to accumulation of glycolytic intermediates that are shuttled into glycolytic branch pathways. Also, the dimeric PKM2 can translocate to the nucleus where it interacts with transcription factors (Hif-1α, STAT3, histone H3 and JMJD5) to induce genes required for proliferation and glycolysis. PKM2 is dominantly expressed in cancer cells, activated or proliferating cells and endothelial cells (modified from Alves-Filho and Pålsson-McDermott, 2016).

Figure 2

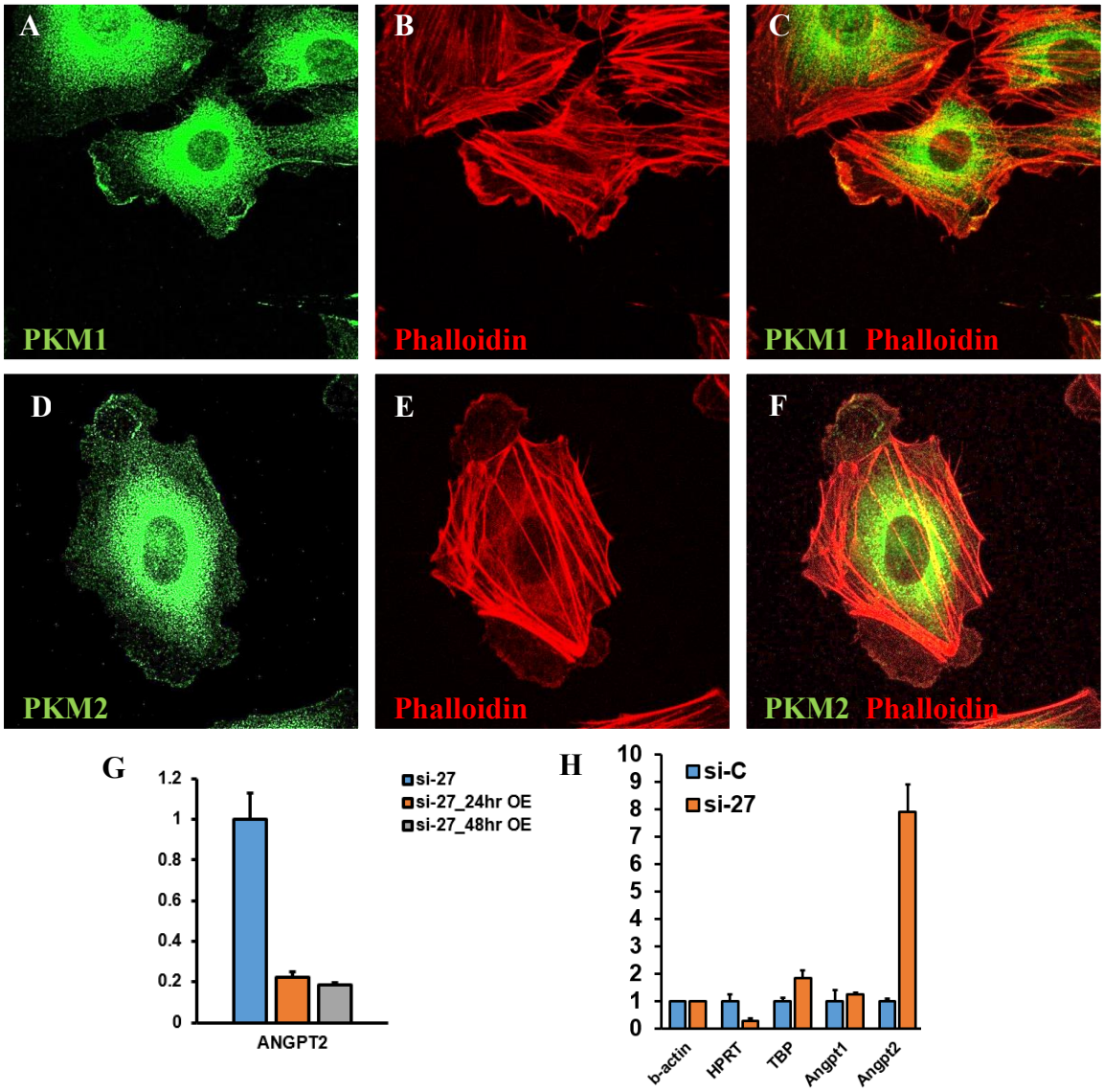


Figure 2: PKM1 and PKM2 subcellular localization in endothelial cells and Ang-2 mRNA expression in PKM2 deficient endothelium. Immunofluorescence staining of PKM1 (green, **A-C**) and PKM2 (green, **D-F**) reveals expression of both isoforms in the cytosol, nucleus and lamellipodia extensions. **G,** A shotgun approach via RNA sequencing (RNA-Seq*)* of the whole transcriptome in reveals reduction of Ang-2 mRNA expression levels in human PKM2 deficient endothelium (si-27) that are overexpressing mouse PKM2 for 24hr and 48hrs (OE). **H,** Quantitative reverse transcription polymerase chain reaction analysis reveals induction of Ang-2 mRNA expression in PKM2 deficient endothelium.

METHODS

Miles Assay

Hair was shaved from the entire back of the mice one day prior to experiment. Mice were anesthetized with isoflurane and retro-orbitally injected with Evans blue dye (EBD, Sigma-Aldrich) at 50 mg/kg. After 10 minutes, recombinant human VEGF-A121 or phosphate buffered saline (PBS) as a vehicle control was injected intradermally. After 20 minutes, organs and skin were harvested and photographed. Tissues were incubated in 1ml formamide at 56°C for 48 hours to extract extravasated dye. Absorbance of extravasated dye was measured at 620 nm with a standard curve of known dye concentrations and normalized to tissue weight.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) and endothelial colony forming cells (ECFCs) were grown in endothelial basal medium (Lonza), supplemented with 10% FBS, 1% Pen/Strep and growth supplements (EGM-MV BulletKit, Lonza). All experiments with HUVECs were carried out between passage three and nine. All experiments with ECFCs were carried out between passage four and twenty.

siRNA transfection

HUVEC or ECFCs were seeded onto 0.1% gelatin-pretreated 10-cm or 6-cm plates. Cells were transfected with 10uM of siCTL (Sigma, SIC001), si-27 (Sigma, WDO5110433), si-87 (Sigma, WDO4615699), si-155 (Sigma, WDO4615697), si-Ang-2

(Sigma, WDO5975627), siPKM (Sigma, WDO5742340) in Opti-MEM (Fisher, cat no: 31985-070) with Lipofectamine RNA iMAX (Invitrogen) for 6 hours. After 30-72 hours of transfection, cells were either fixed with 2% formalin for morphological examination or collected for mRNA, protein, or transendothelial electrical resistance (TER) analysis.

Transendothelial Electrical Resistance (TER) Measurements

Changes in transendothelial electrical resistance (TER) was measured in an endothelial monolayer using a 8-well gold microelectrode (8W10E+, 40 micro-electrodes per well, Applied Biosciences) pretreated with 10 mM L-cysteine and 1% gelatin. The 8W1E+ electrode was connected to an electric cell-substrate impedance sensor (ECIS) system (Applied BioPhysics) in a chamber at 37°C with 5% CO_2. Cells were reseeded from a 10cm plate to 8W1E+ electrode, 30 hours after transfection at a super confluent density of 250,000 per well. To measure endothelial permeability, resistance changes were measured for 14 hours at 4000Hz. Values from three to four wells were pooled and plotted as means ± SD.

Quantitative real-time polymerase chain reaction (qPCR) assays

Total RNA was isolated from cells using the TurboCapture 384 mRNA Kit (Qiagen, CA). RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA). qPCR reactions were performed using the SYBR Green Master Mix and the CFX384 Real-Time System (Bio-Rad). All protocols were performed according to the manufacturers' instructions. The qPCR data was

normalized to actin and calculated per the $2^{-\Delta Ct}$ method. The list of genes investigated by qPCR and their respective primers are listed in Table 1.

Table 1: Forward and reverse primer sequences utilized for quantitative real-time polymerase chain reaction (qPCR) assay.

	Species	Name	Sequence
1	Human	36B4- Forward	AGATGCAGCAGATCCGCAT
2	Human	36B4- Reverse	GTTCTTGCCCATCAGCACC
3	Human	Angiopoetin 1 (Ang-1)- Forward	CTCGCTGCCATTCTGACTCAC
4	Human	Angiopoetin 1 (Ang-1)- Reverse	GACAGTTGCCATCGTGTTCTG
5	Human	Angiopoetin 2 (Ang-2)- Forward	AATGCAGTACAGAACCAGACG
6	Human	Angiopoetin 2 (Ang-2)- Reverse	TTAACTTCCGCGTTTGCTCAG
7	Human	Beta-Actin - Forward	GAGCGCGGCTACAGCTT
8	Human	Beta-Actin - Forward	TCCTTAATGTCACGCACGATTT
9	Human	Pyruvate kinase M1 (PKM1)- Forward	ATAGCTCGTGAGGCTGAGGCAG CCATGTT
10	Human	Pyruvate kinase M1 (PKM1)- Reverse	ACTCCGTCAGAACTATCAAAGCT GCTGCTA
11	Human	Pyruvate kinase M2 (PKM2)- Forward	TGAGGCAGAGGCTGCCATCTAC CACTT
12	Human	Pyruvate kinase M2 (PKM2)- Reverse	TGCCAGACTTGGTGAGGACGAT TATGGC
13	Human	Tata Binding Protein (TBP)- Forward	GAGCCAAGAGTGAAGAACAGTC
14	Human	Tata Binding Protein (TBP)- Reverse	GCTCCCCACCATATTCTGAATCT
15	Human	VE-cadherin/Cadherin 5- Forward	AAACACCTCACTTCCCCATC
16	Human	VE-cadherin/Cadherin 5- Reverse	ACCTTGCCCACATATTCTCC

Immunostaining

Cells were fixed in 2% neutral buffered formalin for 10 minutes and briefly permeabilized with 0.5% PBS-T for 5 minutes. Cells were rinsed three times in PBS for 1

minute and blocked in Protein Block Serum-Free (Dako) for 1 hour. Cells were incubated with anti-goat VE-cadherin antibody (1:50, Santa Cruz, sc-8439) in a humidified chamber overnight at 4˚C. Cells were rinsed three times in PBS for 1 minute, and incubated with anti-phallodin 488 (1:200, Fisher) and donkey anti-goat Alexa fluor 594 antibody (1:200, LifeTech, A11056) for 2 hours. Cells were rinsed three times in PBS for 1 minute and mounted using Vectashield mounting medium (Vector Laboratories). Cells were examined using a Nikon Eclipse 80i microscope or an Olympus IX81 confocal microscope. All incubations were performed at room temperature unless otherwise noted.

Westerns blots

Cell lysates were collected using RIPA lysis buffer with protease and phosphatase inhibitors (Roche). Lysates was mixed with 6X SDS sample loading buffer (Bioworld), boiled for 5 minutes at 95 °C, and loaded onto 4–20% gradient Tris Glycine X-PAGE gels (Bio Rad). Separated proteins were wet transferred to a methanol activated PVDF membrane (Millipore). The membrane was blocked with 5% milk in TBS-T buffer for 1 hour at room temperature and incubated with a primary antibody (1:5000) in 5% BSA in TBS-T buffer overnight at 4°C. The membrane was then washed and incubated with secondary HRP-linked antibody (1:10,000) in 5% milk in TBST buffer for 1 hour at room temperature. All washes were performed with TBS-T. Membranes were probed with the following antibodies: anti- Ang-2 (R&D systems, MAB0983-100), anti-phospho Y658-VE-Cadherin (Thermo fisher, 44-1144G), anti-phospho Y685-VE-Cadherin (ECM Biosciences, CP1981), VE-CAD (Santa Cruz, sc-6458), pyruvate kinase M1 (Cell

signaling, 7067s), pyruvate kinase M2 (Cell signaling, 4053s), and actin (Cell signaling, 4097s). The following HRP-line secondary antibodies were used: anti-mouse-HRP linked (Cell signaling, 7076s), anti-rabbit-HRP linked (Cell signaling, 7074s), or anti-goat-HRP linked (Thermo, 31400). The bands were visualized with a chemiluminescence detection kit (ECL, Amersham Biosciences) and imaged using (ImageQuant LAS 4000 series, GE Healthcare).

Statistical Analysis. Data were expressed as means ± standard error of mean unless otherwise indicated. *P* values were calculated using the two-tailed Student's *t*-test *or* one-way analysis of variance, followed by Bonferroni *post hoc* testing with $p<0.05$ as statistically significant. Data analysis and generation of all graphs were performed in PRISM5 software (Graphpad).

RESULTS

Endothelial-specific PKM2 deletion in mice induces vessel permeability.

To examine basal vascular permeability, Evans blue dye (EBD) was injected in control and PKM2 EC-specific KO mice, and various organs were assessed 30 minutes post injection. Compared to control mice, PKM2 EC-specific knockout (KO) mice exhibited increased extravasation of EBD into lung tissue (Figure 3A). No significant differences in EBD leakage were observed in kidney, liver and heart (Figure 3B-D) indicating that PKM2 deletion affects vessel permeability in an organ-specific manner.

To examine acute vascular permeability, EBD was injected in control and PKM2 KO mice and were subsequently injected intradermally with PBS and VEGF-A121. When compared with PBS, both control and PKM2 EC-specific KO mice exhibited increased extravasation of EBD vascular in response to VEGF-A121 (Figure 4A-B). But PKM2 EC-specific KO mice exhibited significantly more EBD extravasation than control mice in response to VEGF-A121 (Figure 4A-B), indicating that PKM2 EC-specific KO mice are more sensitive to VEGF-induced vascular permeability

Endothelial-specific PKM2 deletion reduces endothelial monolayer integrity

To evaluate whether PKM2 deletion in ECs alters endothelial barrier integrity, we measured transendothelial electrical resistance (TEER) using electrical cell impedance sensing (ECIS). Statistical analysis at a 14-hour end time point showed PKM2 deletion (si-27 and si-87) in human umbilical vein endothelial cells (HUVECs) significantly reduced TEER levels in the endothelial monolayers compared to control (Figure 5A-B). Interestingly, deletion of both PKM1 and PKM2 (si-PKM) did not alter TEER levels in

the endothelial monolayers compared to control (Figure 5A-B). Significant knockdown of both PKM1 (si-PKM) and PKM2 (si-27, si-87 and si-PKM) was verified via mRNA expression (Figure 5C-D). Upon knockdown of PKM2 with si-27, there is an induction of both mRNA (Figure 5D) and protein (Figure 7B) PKM1expression. This was not observed with the other PKM2 si-87 (Figure 5D).

Endothelial-specific PKM2 deletion reduces VE-cadherin localization at cell-cell contacts

To study the molecular organization of the adherent junctions that play an important role in maintaining endothelial barrier integrity, VE-cadherin localization was visualized by immunofluorescence. Endothelial colonizing forming cells (ECFCs) transfected with sRNA against PKM2 (si-27, si-155 and si-87) and a negative control scrambled sequence (si-CTL), were assessed at 1 hour, 4 hours, and 24 hours after reseeding and 48-72 hours after transfection. Knockdown of PKM1 (si-PKM) and PKM2 (si-27, si-87 and si-PKM) was verified via mRNA expression (Figure 5C-D). Both control and PKM2 deficient endothelial cells show endogenous VE-cadherin expression in the perinuclear region 1 hour after of reseeding (Appendix A1). 4 hours after of reseeding, both control and PKM2 deficient endothelial cells began to exhibit VE-cadherin localization at plasma membrane in regions where cell-to-cell contacts occur (Appendix A2). Highest expression of VE-cadherin was observed 24 hours after of reseeding in a confluent monolayer (Figure 6 and Appendix A3). VE-cadherin staining in confluent monolayer (Si-CTL) show continuous and narrow lining at cell-cell borders reflecting stable junctions (Figure 6A-C and Appendix A3, A-D). In PKM2 deficient

endothelial cells, VE-cadherin staining was discontinuous, a pattern typical for unstable junctions and the generation of intercellular gaps (Figure 6D-L and Appendix A3, E-P). Quantification of percent of cell membrane attached to adjacent cells revealed PKM2 deficient endothelial cells have significantly less attachment to adjacent cells compared to control (Figure 6M).

Interestingly, when PKM2 deficient endothelial cells are assayed without reseeding, VE-cadherin junctions are not disrupted (Appendix A4). Control cells show the characteristic differential pattern in VE-cadherin staining typically found in an endothelia monolayer (Appendix A4, A-C). In contrast, PKM2 deficient endothelial cells show a robust and uniform expression pattern of VE-cadherin (Appendix A4, D-I) indicating a loss in differential VE-cadherin expression pattern.

Endothelial-specific PKM2 deletion induces phosphorylation of VE-cadherin at Y658

Vessel permeability is accompanied by phosphorylation, disruption and internalization of VE-cadherin/catenin complex leading to weak cell–cell junctions. Thus, we evaluated both total VE-cadherin levels and phosphorylation of VE-cadherin at tyrosine residues 685 and 658. Deletion of PKM2 in ECs do not alter either total VE-cadherin mRNA expression (Figure 7A) or total VE-cadherin protein levels (Figure 7B). But deletion of PKM2 in ECs increased VE-cadherin phosphorylation at tyrosine residues 658 but not 685 compared to control (Figure 7B). Deletion of both PKM1 and PKM2 deletion (si-PKM) decreased VE-cadherin phosphorylation at tyrosine residues 658 but not 685 compared to control (Figure 7B).

Endothelial-specific PKM2 deletion induces Ang-2 expression.

The Ang-2 is a well-known modulator of vascular permeability. Based on our preliminary data of Ang-2 induction from qPCR and RNA-seq analysis, we further assessed Ang-2 expression in ECs. Compared to control, PKM2 deficient endothelium significantly induces Ang-2 mRNA expression with one of the PKM2 siRNAs (si-27 but not in si-87; Figure 8A). However, PKM2 deficient endothelium significantly induces Ang-2 protein expression with the both the PKM2 siRNAs (si-27 and si-87) compared to control (Figure 8B). Deletion of both PKM1 and PKM2 (si-PKM) reduced Ang-2 protein expression compared to control (Figure 8B).

Loss of Ang-2 is sufficient to attenuate vascular leakage in PKM2 deficient endothelium

We knocked down Ang-2 along with PKM2 to evaluate whether removal of Ang-2 attenuated the effect of PKM2 deletion on leaky barrier function. Statistical analysis at 14-hour end time point showed PKM2 deletion (si-87) in human umbilical vein endothelial cells (HUVECs) significantly reduced TEER of the endothelial monolayers compared to control (Figure 9A-B). Knockdown of Ang-2 (si-Ang-2) along with PKM2 rescued TEER of the endothelial monolayers compared to control (Figure 9A-B). Thus, indicating that Ang-2 deletion is sufficient to attenuate vascular leakage in PKM2 deficient endothelium. Knockdown of Ang-2 and PKM2 was verified via mRNA expression (Figure 9C-D). Similar to the preliminary data, PKM2 deficient endothelium induced Ang-2 mRNA expression (Figure 9D). Interestingly Ang-2 knockdown also

induces PKM2 expression (Figure 9C) suggesting that PKM2 and Ang-2 may reciprocally regulate each other.

Figure 3

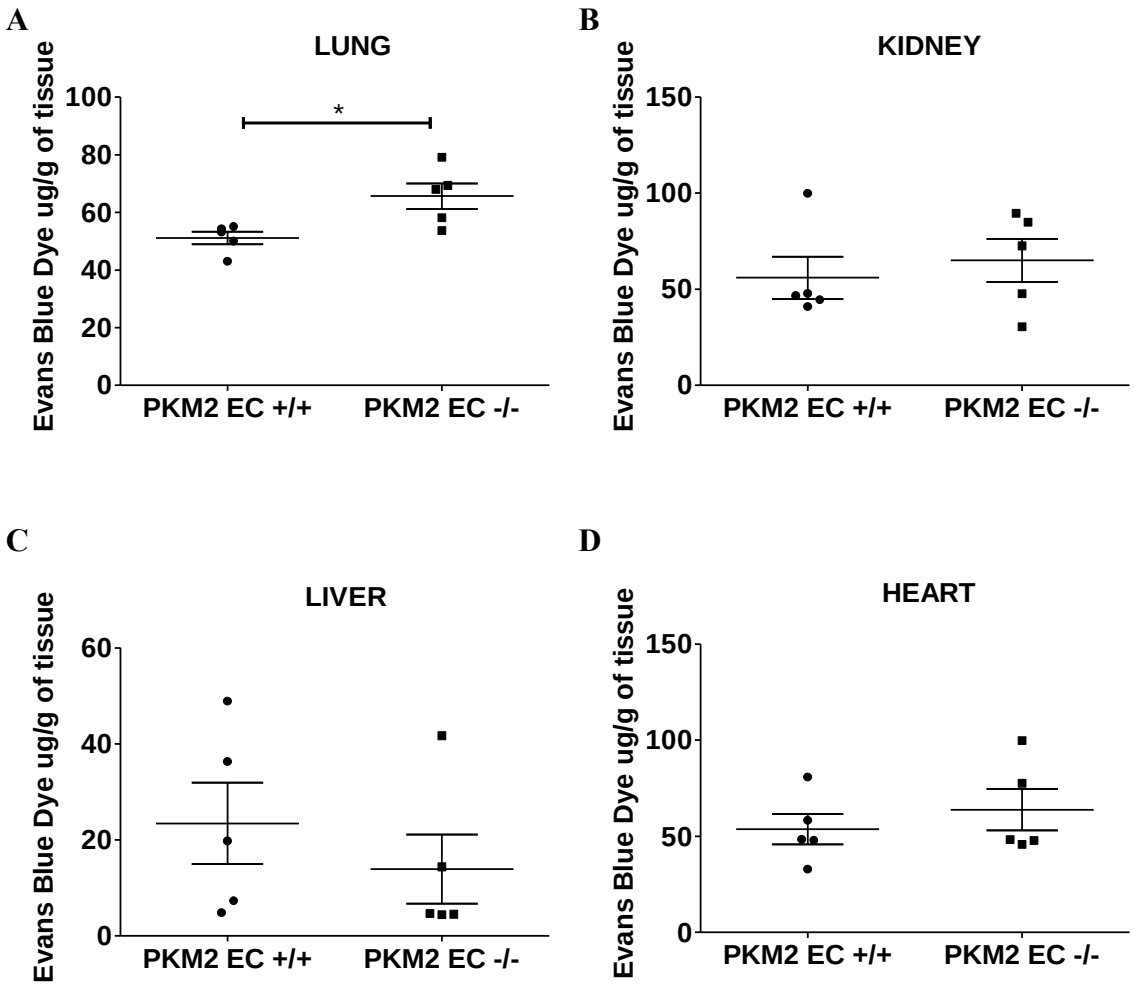


Figure 3: Endothelial-specific PKM2 knockout mice show increased basal pulmonary microvascular permeability. Basal vascular permeability was assessed in various organs 30 minutes after retroorbital injection of EBD. **A-D,** Quantification of extracted EBD from lung **(A),** Kidney **(B),** liver **(C),** and heart **(D).** EBD values were normalized to tissue weight (μg of EBD/g of tissue). Only the lung from EC-specific PKM2 KO mice showed significantly increased EBD leakage compared with control mice. Data are presented as mean± SEM. **P*<0.05. *n* = 5 per experimental group.

Figure 4

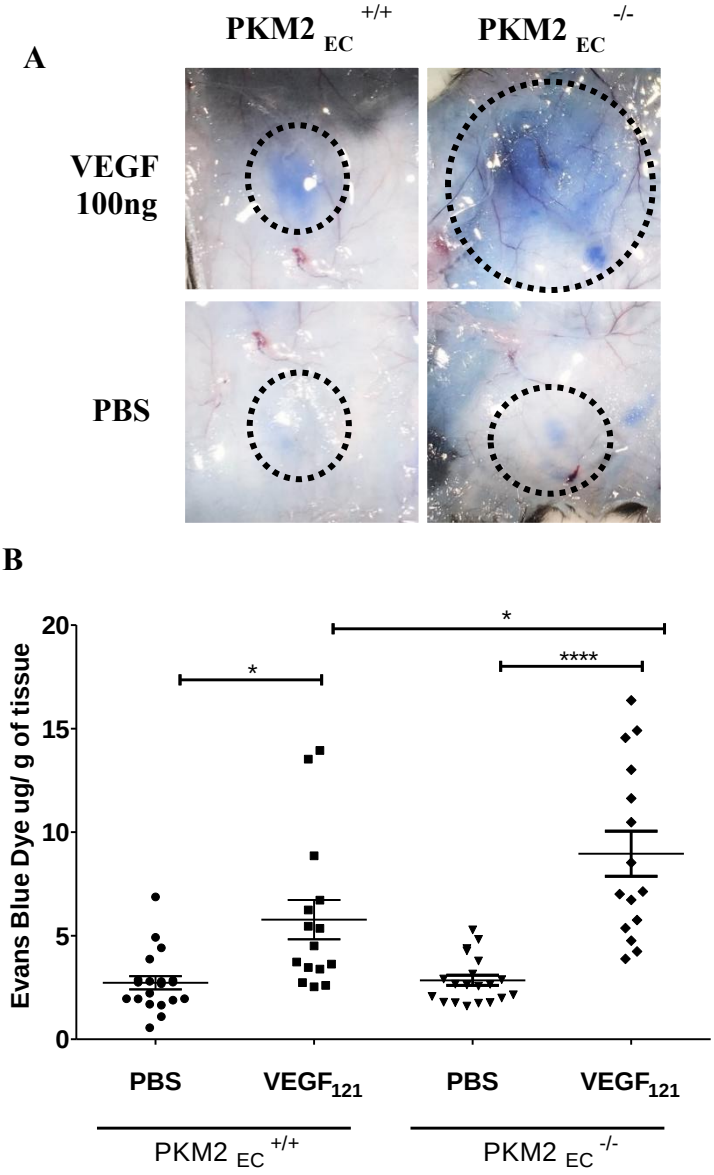


Figure 4: Endothelial-specific PKM2 knockout mice are more sensitive to VEGF-induced vascular permeability. Acute vascular hyperpermeability was assessed through miles assay. Mice were injected retro-orbitally with EBD and were subsequently injected intradermally with PBS and VEGF-121 (100ng). **A,** Representative skin images in response to PBS or VEGF-A121 intradermal injections. **B,** Quantification of extracted EBD normalized to tissue weight (µg of EBD/g of tissue). EC-specific PKM2 KO mice showed significantly increased leakage compared with control mice when treated with VEGF-A121. Data are representative of two independent experiments and are presented as mean± SEM. **P*<0.05, ****P*<0.001. *n* = 10 per experimental group.

Figure 5

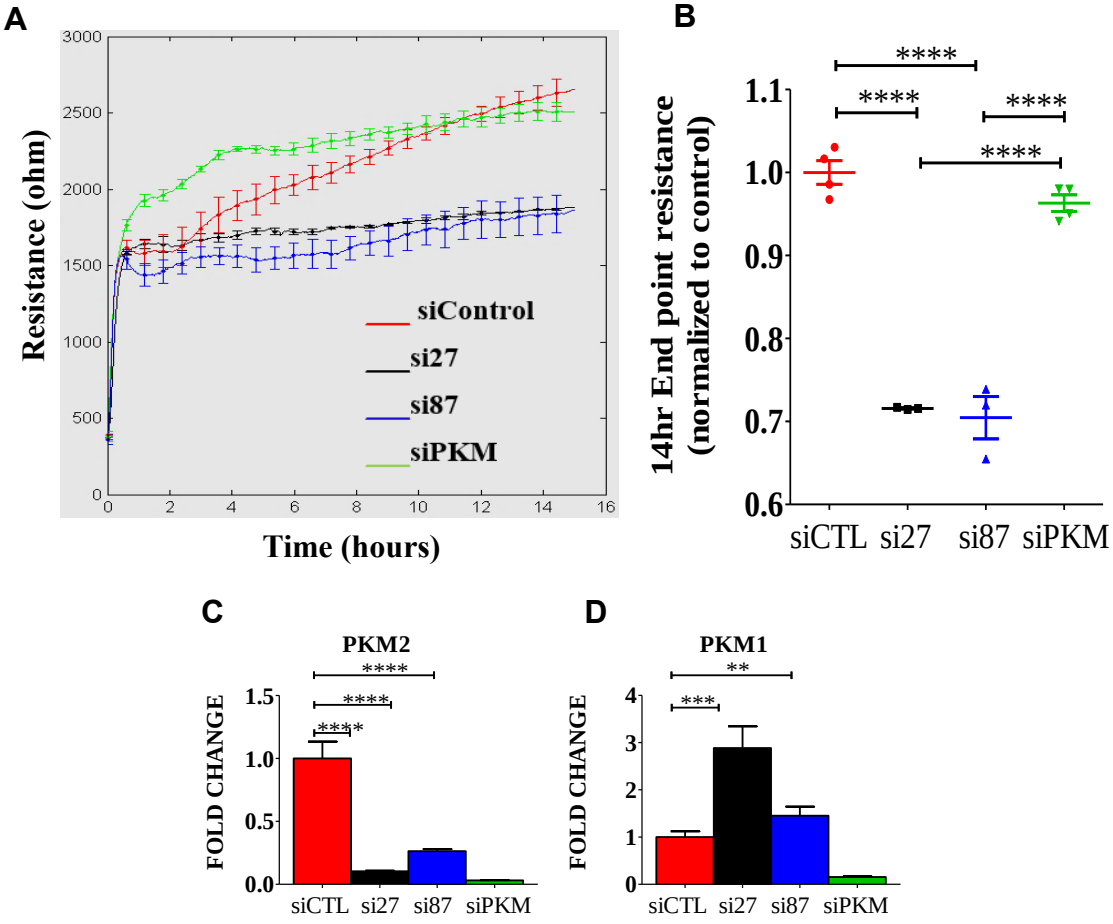


Figure 5: Loss of PKM2 expression results in decreased transendothelial electrical resistance (TEER). Human umbilical vein endothelial cells (HUVECs) were transfected with small interfering RNA (siRNA) targeted to PKM2 alone (si-27 and si-87), to both PKM1+PKM2 (si-PKM) and to a scrambled sequence (si-CTL) as a negative control. **A,** Changes in electrical resistance in HUVEC monolayer were measured for 14 hours on an electric cell-substrate impedance sensor (ECIS) in 8W1E+ plates at 4000Hz. Cells were reseeded from a 10cm plate to 8W1E+ plate 30 hours after transfection. Data are presented as means ± SD (n = 3-4). **B**, End point electrical resistance was normalized to negative control group at 14 hours. EC-specific deletion of PKM2 (si-27 and si-87) results in decreased TEER. While EC-specific deletion of both PKM1 and PKM2 did not alter TEER. Data are presented as mean ± SEM (n = 3-4). ****P<0.0001. **C and D,** mRNA expression was assessed using quantitative reverse transcription polymerase chain reaction. There was significant knockdown of PKM1 (si-PKM) and PKM2 (si-27, si-87 and si-PKM) mRNA expression compared to control. mRNA expression was normalized to actin expression using the $2^{-\Delta Ct}$ method. Data are presented as mean ± SEM (n = 3). **P<0.01, ***P<0.001 ****P<0.0001.

Figure 6

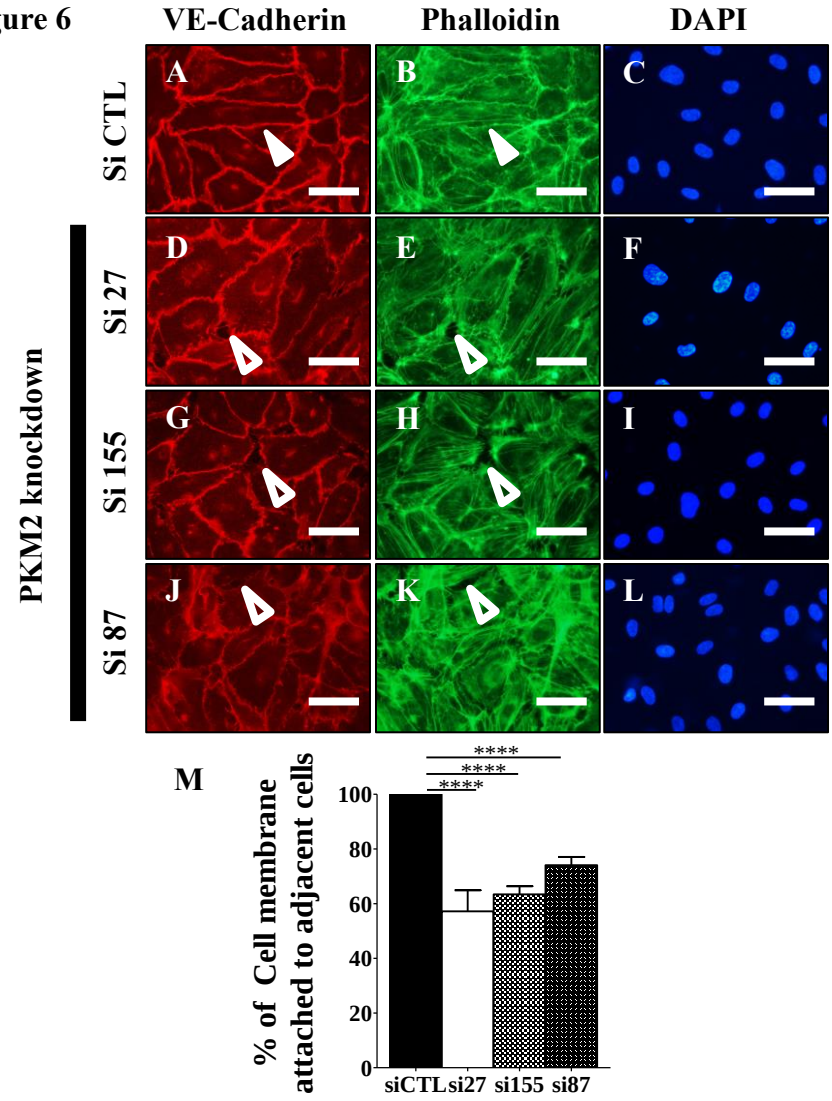


Figure 6: Loss of PKM2 expression results in disrupted VE-cadherin adherent junctions and gap formations. Endothelial colonizing forming cells (ECFCs) were transfected with small interfering RNA (siRNA) targeted to PKM2 (si-27, si-155, si-87) and to a scrambled sequence (si-CTL) as a negative control. Cells were assessed 24 hours after reseeding and 72 hours after transfection **A-L,** Representative images of a ECFC monolayer immunolabeled for VE-cadherin (Red), phalloidin (Green) and DAPI (Blue). In confluent endothelial cells, VE-cadherin staining was continuous, linear and distributed around the entire periphery of the cells reflecting stable junctions (**A-C,** arrow). In PKM2 deficient endothelial cells, VE-cadherin staining was discontinuous reflecting unstable junctions and formation of intercellular gaps (**D-L,** open arrow head = discontinuous junctions and gap formations). **M,** Quantification of cell membrane (%) attached to adjacent cells. PKM2 deficient endothelial cells showed significantly less attachment to adjacent cells compared to control. Scale bars: 50 µm. Data are presented as mean ± SEM (n = 3 random fields). ****P<0.0001.

Figure 7

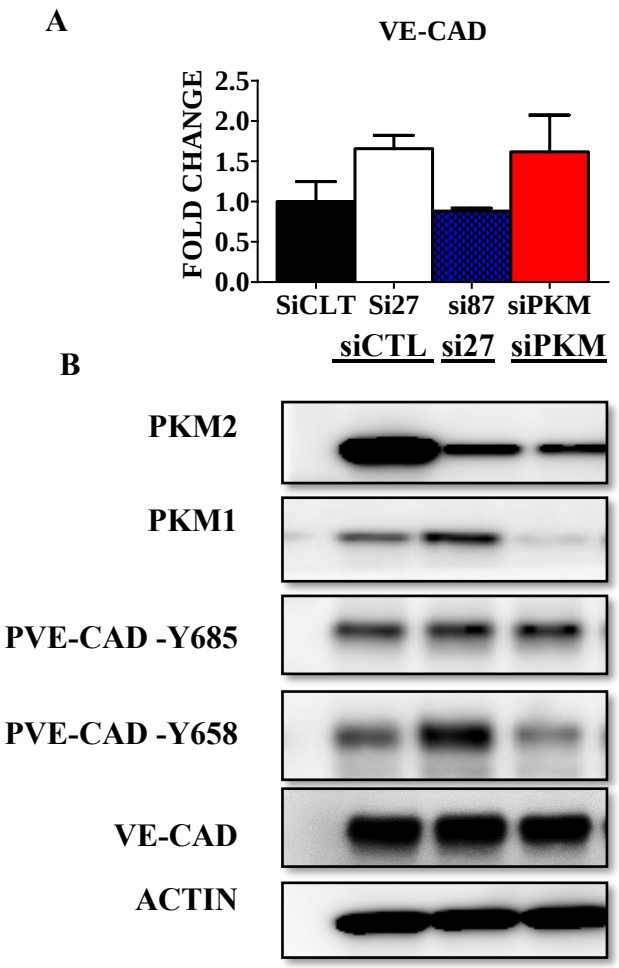


Figure 7: PKM2 deficient endothelium induces VE-cadherin tyrosine phosphorylation at residues Y658. Human umbilical vein endothelial cells (HUVECs) were transfected with small interfering RNA (siRNA) targeted to PKM2 alone (si-27 or si-87), both PKM1+PKM2 (si-PKM) and a scrambled sequence (si-CTL) as a negative control. **A**, Quantitative analysis of total VE-Cadherin (VE-CAD) mRNA expression normalized to actin. Deletion of PKM2 in ECs do not alter VE-cadherin mRNA expression. Data are presented as mean ± SEM (n = 3). **B**, Western blot analysis of phospho Y658-VE-Cadherin (p-VE-CAD-Y658), phospho Y685-VE-Cadherin (p-VE-CAD-Y685), VE-CAD, pyruvate kinase M1 (PKM1), pyruvate kinase M2 (PKM2), and actin (as loading control). PKM2 deficient endothelium induces VE-cadherin tyrosine phosphorylation at Y658 without altering total VE-cadherin protein levels.

Figure 8

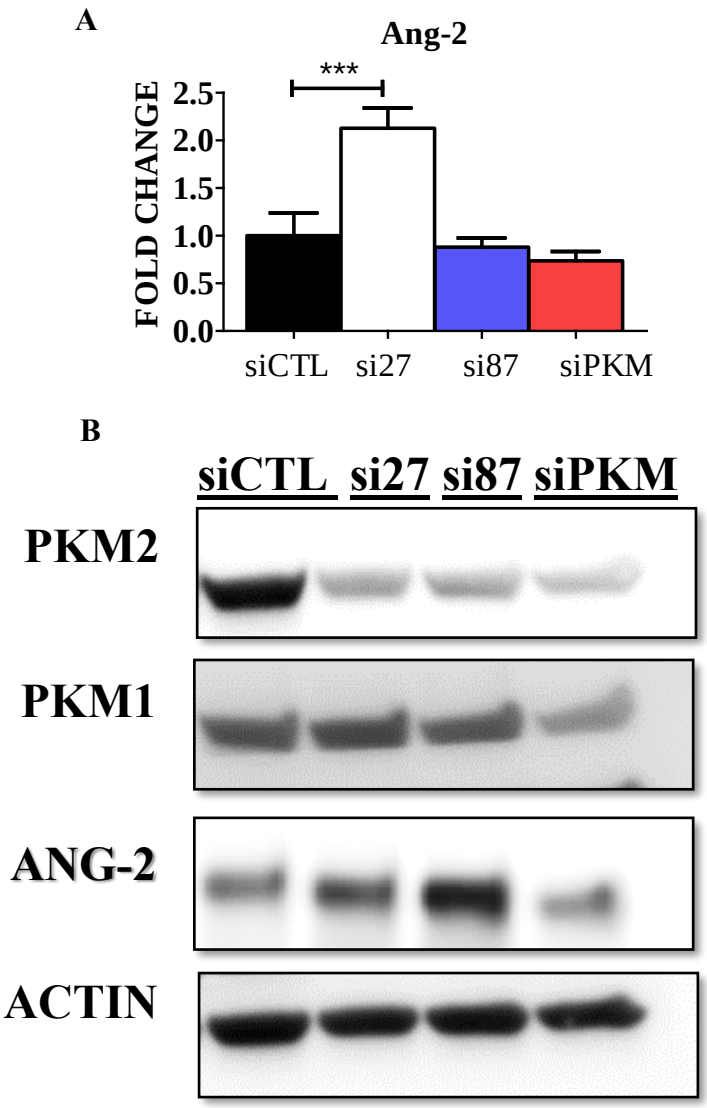


Figure 8: PKM2 deficient endothelium induces Ang-2 expression. Human umbilical vein endothelial cells (HUVECs) were transfected with small interfering RNA (siRNA) targeted to PKM2 alone (si-27 and si-87), both PKM1+PKM2 (si-PKM) and a scrambled sequence (si-CTL) as a negative control. **A,** Quantitative analysis of angiopoietin-2 (Ang-2) mRNA expression normalized to actin. Deletion of PKM2 in ECs induces Ang-2 mRNA expression. Data are presented as mean ± SEM (n = 3). ***P<0.001. **B,** Western blot analysis of Ang-2, pyruvate kinase M1 (PKM1), pyruvate kinase M2 (PKM2), and actin (as loading control). Deletion of PKM2 in ECs induces Ang-2 protein expression.

Figure 9

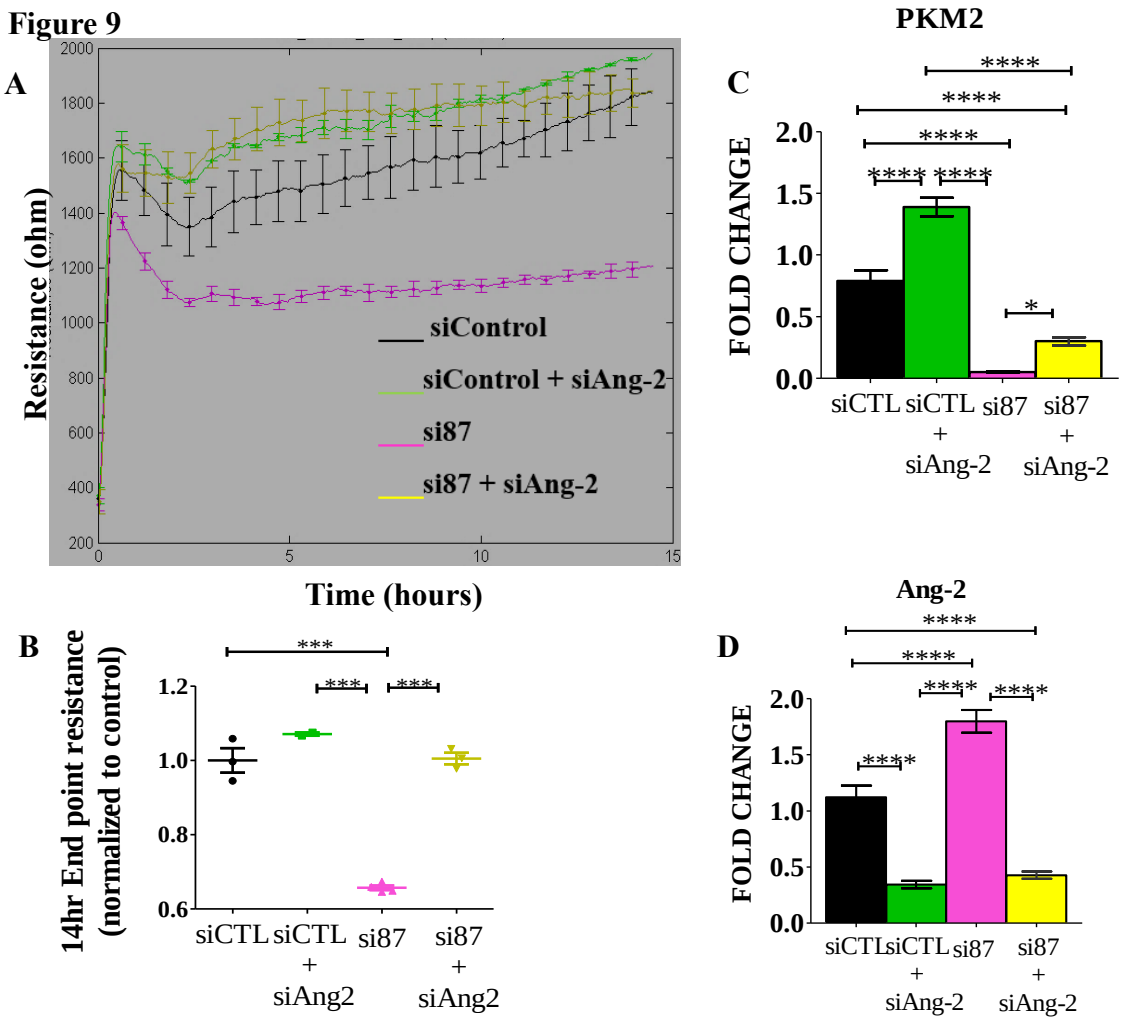


Figure 9: Knockdown of Ang-2 in PKM2-deficient cells is sufficient to rescue decreased transendothelial electrical resistance (TEER). Human umbilical vein endothelial cells (HUVECs) were transfected with small interfering RNA (siRNA) targeted to PKM2 (si-87), angiopoietin 2 (si-Ang-2) or a scrambled sequence (si-CTL) as a negative control. **A,** Changes in electrical resistance in HUVEC monolayer were measured for 14 hours on an electric cell-substrate impedance sensor (ECIS) in 8W1E+ plates at 4000Hz. Cells were reseeded from a 10cm plate to 8W1E+ plate 30 hours after transfection. Data are presented as means ± SD (n = 3-4). **B,** End point electrical resistance was normalized to negative control group at 14 hours. Loss of Ang-2 is sufficient to attenuate vascular leakage in PKM2 deficient endothelium. Data are presented as mean ± SEM (n = 3-4). ****P<0.0001. **C-D,** mRNA expression of PKM2 and angiopoietin 2 were assessed using RT-PCR. PKM2 deficient endothelium induces Ang-2 mRNA expression and interestingly Ang-2 knockdown also induces PKM2 expression. mRNA expression was normalized to actin expression using the $2^{-\Delta Ct}$ method. Data are presented as mean ± SEM (n = 3). ***P<0.001, ****P<0.0001.

DISCUSSION

Impairment in vessel stability and subsequent vascular permeability is the hallmark of many pathological diseases like cancer and sepsis. Thus, the control of the endothelial barrier function is essential for maintaining vascular homeostasis. Here we demonstrate using both in-vivo and in-vitro models, that pyruvate kinase M2 is required to preserve endothelial barrier function.

Endothelial PKM2 deficient mice display increased basal vessel permeability in pulmonary capillary vessels, demonstrating that lung vascular permeability is regulated by PKM2. Due to its enormous surface area, the pulmonary vasculature is known to be particularly sensitive to barrier dysregulation (Xing and Birukova, et al. 2009). However, the kidney, heart and liver vasculature remained intact in EC-specific PKM2 deficient mice. Thus, PKM2 deletion affects vessel permeability in an organ-specific manner. This is not surprising as permeability of capillaries varies considerably from organ to organ due to their heterogenous morphologies and function (Aird et al., 2015). Also, endothelial PKM2 deficient mice display increased VEGF-induced acute vessel permeability in dermal vessels, suggesting that VEGF-induced vascular permeability is mediated by PKM2.

Similar to in-vivo results, PKM2 deficient endothelium exhibited decreased transendothelial electrical resistance (TEER), indicative of reduced tightness of junctions in endothelial cell monolayer. PKM2 deficient endothelial cells have disrupted localization of VE-cadherin at the intercellular junctions and significantly less attachment to adjacent cells, but total VE-cadherin protein expression was conserved. This altered

VE-cadherin localization is consistent with the observed leaky phenotype given that previous studies have shown VE-cadherin adherent junctions play a crucial role in maintaining barrier integrity in ECs. Where loss of VE-cadherin at cell–cell contacts produces unstable junctions, intercellular gaps (Lampugnani et al., 2012) resulting in increased permeability.

Modulation of VE-cadherin is mediated by several mechanisms one of which is by phosphorylation of VE-cadherin (Turowski et al., 2008). The cytoplasmic domain of VE-Cadherin has several phospho-tyrosine sites, but only three (Y658, Y685 and Y731) are involved in the regulation of barrier function (Adam, 2015). Thus, we evaluated phosphorylation of VE-cadherin at two of three tyrosine residues (Y685 and Y658). Loss of PKM2 in endothelial cells induces phosphorylation of VE-cadherin at residue Y658 but not Y685. Studies have shown phosphorylation at Y658, mediated by Src, has been implicated as a mechanism for loss of barrier function (Garrett et al., 2017). Specifically, phosphorylation at Y658 disrupts p120 binding to VE-cadherin, leading to internalization of VE-Cadherin. VEGF also is known to alter endothelial barrier function via phosphorylation of VE-Cadherin at residue Y658 (Potter et al., 2005; Gavard and Gutkind, 2006). Taken together, phosphorylation of VE-cadherin and the subsequent loss of VE-cadherin at cell-cell contacts suggests a mechanism by which PKM2 alters intercellular junctions leading to a leaky endothelium.

Surprisingly, PKM2 deletion in an established endothelial monolayer (without reseeding) did not affect VE-cadherin localization at the cell-cell contacts. Instead, PKM2 deletion in an established endothelial monolayer led to a loss of differential VE-

cadherin expression. Recent studies have linked loss of differential VE-cadherin junctional regulation to abnormal vascular phenotypes (Bentley et al., 2014).

In this study, we observed a significant increase in the expression of Ang-2 mRNA and protein in PKM2 deficient endothelium. The upregulation of Ang-2 mRNA and protein levels is consistent with the observed leaky phenotype given that previous studies have shown overexpression of Ang-2 leads to destabilization of vessels. Ang-2 is as an antagonist of the constitutively active Ang-1/Tie-2 signaling which participates in vessel stabilization (Maisonpierre et al., 1997; Fiedler et al., 2004). Specifically, Ang-2 inhibits Tie-2 phosphorylation, resulting in Rho-kinase and endothelial myosin light chain kinase (EC-MLCK) activation and destabilization of the endothelial monolayer (Parikh et al., 2006). We also performed rescue experiments using TEER measurements to verify if leaky barrier function observed upon PKM2 deletion was specifically due to Ang-2 induction. Indeed, removal of Ang-2 was sufficient to attenuate the effect of PKM2 deletion on leaky barrier function. Therefore, in the endothelium PKM2 effects are mediated in part by altering Ang-2 expression. Furthermore, PKM2 and Ang-2 seem to reciprocally regulate each other, since knockdown of either PKM2 or Ang-2 induces mRNA expression of Ang-2 or PKM2, respectively.

In PKM2 deficient endothelium, there is greater induction of PKM1 than in basal conditions. Thus, it is possible that PKM1 induction, rather than PKM2 loss, is mediating the leaky phenotype through VE-cadherin phosphorylation and Ang-2 induction. This matches our observation that upon double knockdown of PKM2 and PKM1, we see an intact vascular barrier function and concurrent reduction of VE-cadherin phosphorylation

and Ang-2 protein expression. In tumor cells a similar mechanism is observed, where PKM1 expression, rather than PKM2 loss, is responsible for proliferation arrest (Lunt et al., 2015). However, it is noted that we do not see a consistent induction of PKM1 among the different PKM2 siRNAs, but we do see a consistent induction in Ang-2 expression. Thus, it is not clear if PKM1 is indeed mediating the leaky phenotype. To resolve this question, looking at barrier function in PKM1 overexpressing ECs would elucidate if PKM1 expression, rather than PKM2 loss, is responsible for leaky vascular barrier.

Conclusions

Collectively, the data reported here suggests that pyruvate kinase M2 is required for maintaining vessel integrity and barrier function in endothelial cells. Specifically, the observed vascular leakiness of PKM2 deficient endothelium is mediated by increased Ang-2 expression and disrupted VE-cadherin junctions at cell-cell contacts. Recent studies have described the PKM2's role in tumorigenesis and angiogenesis (Christofk et al., 2008; Azoitei et al., 2016), but this is the first study to describe PKM2's role in barrier function.

Future Directions

Our results suggest that PKM2 regulates Ang-2 expression. However, it is unclear how and through which mechanism PKM2 mediates the leaky phenotypes via ANG-2 modulation.

PKM2 may alter other components of the angiopoietin-Tie-2 signaling axis and the downstream pathways. Ang-1/Tie-2 phosphorylation and activation leads to Rac1 activation and RhoA inactivation, mediated by PI3K/Akt. Ultimately, increasing

accumulation VE-cadherin at the adherent junctions and preserving vascular barrier function (Milam and Parikh et al., 2015). By looking at protein activity of Tie-2, Rac1 and RhoA, we clarify where PKM2 loss leads to changes in the angiopoietin-Tie-2 signaling axis. Also, since Ang-1 is an agonist of the Tie-2 receptor and opposes the antagonistic action of Ang-2 (Maisonpierre et al., 1997; Fiedler et al., 2004), Ang-1 treatment may possibly rescue the leaky phenotype of PKM2 deficient endothelium. Together this would further validate if and how PKM2 acts on the angiopoietin-Tie-2 signaling axis.

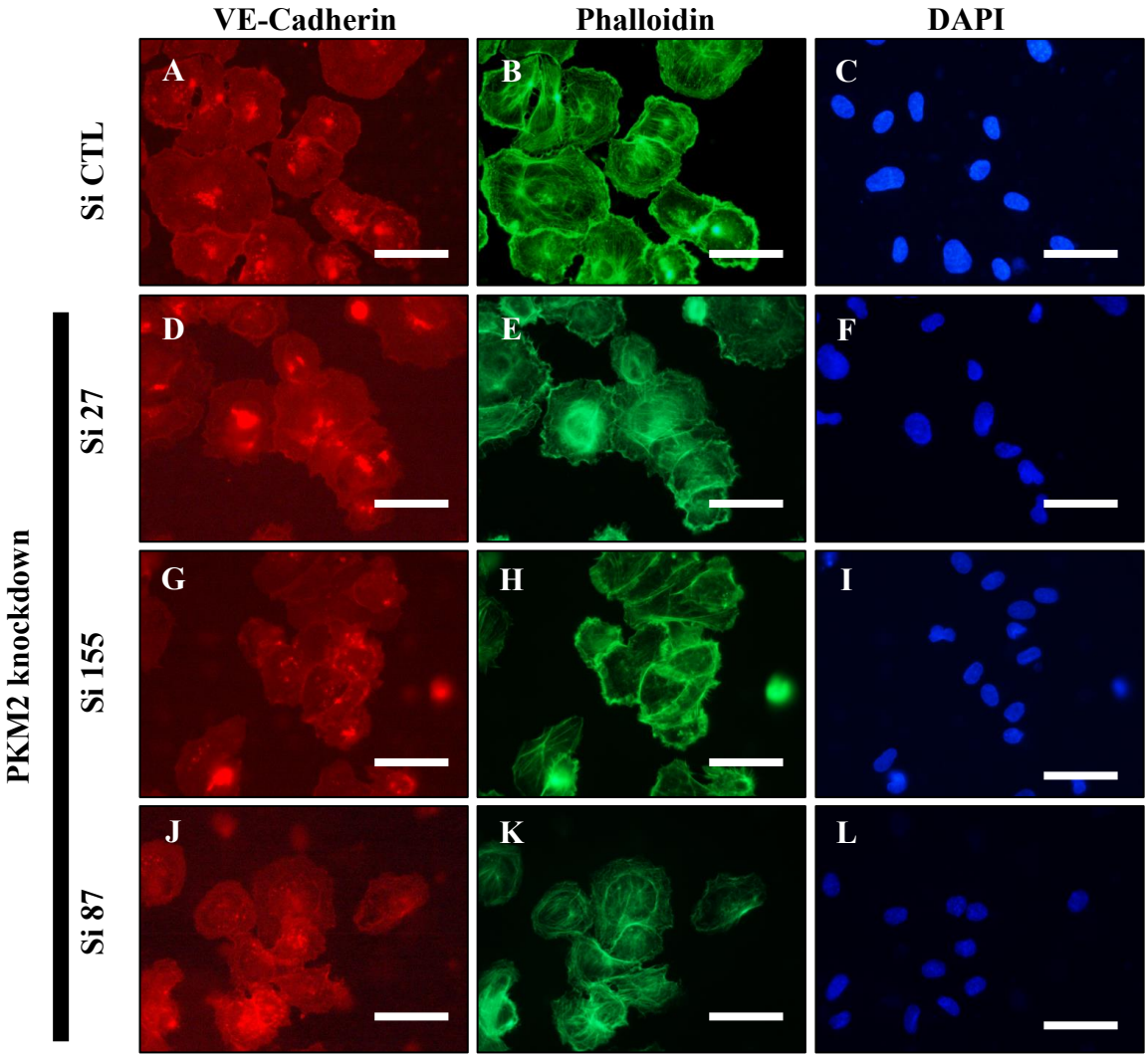
PKM2 may act more upstream by regulating both packaging and release of Ang-2 from Weibel-Palade bodies (WBP). In lung microvascular endothelial cells, Ang-2 release is regulated by the PTEN/PI3-kinase/Akt pathway. PTEN is a positive modulator of Ang-2 release, while activation of PI3-K/Akt pathway downregulates Ang-2 (Tsigkos et al., 2006; Parikh et al., 2006). By looking at protein activity of PTEN, PI3K and AKT, we can clarify if PKM2 loss alters both packaging and release of Ang-2. Studies have shown elevated serum Ang-2 levels elicit leaks in pulmonary endothelium of septic patients and mice (Hashimoto and Pittet, 2006, Parikh et al., 2006). Since we observe a similar leaky lung phenotype upon PKM2 deletion in mice, it would be valuable to assess if PKM2 deficient mice have a corresponding increase in circulating Ang-2. Thus, revealing if PKM2 deletion stimulates release of Ang-2 from WBP.

Also, it is important to delineate the role of PKM2 in the context of a pathological disease like sepsis, which is associated with abnormally increased vascular permeability. Will PKM2 deletion in the endothelium sensitize the mice to septic shock? Or will

overexpression of PKM1 or PKM2 mitigate and rescue the effects of septic shock? Ultimately, if PKM2 is relevant in sepsis or other permeability associated pathological diseases, regulation of PKM2 could open up possible new therapeutic targets.

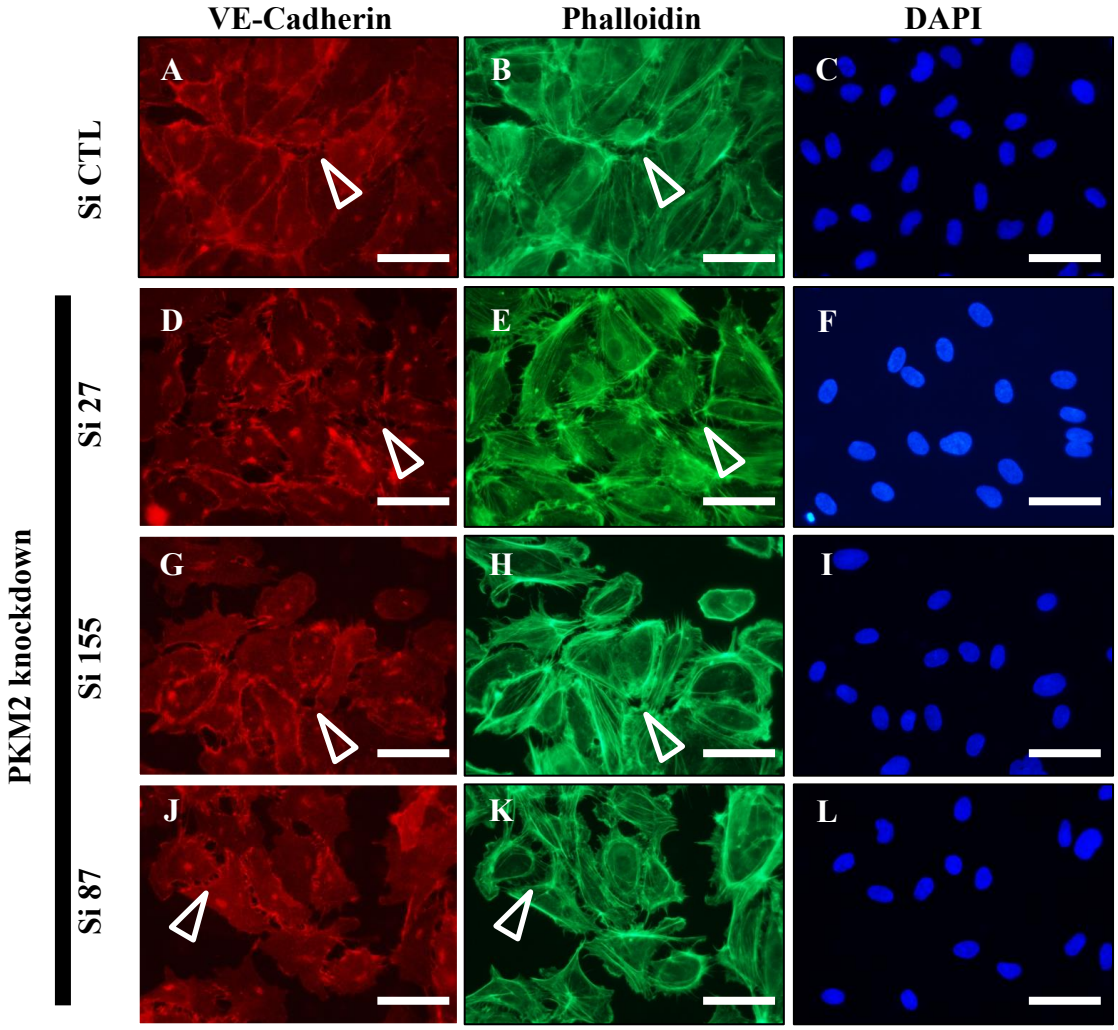
APPENDIX

Appendix A1



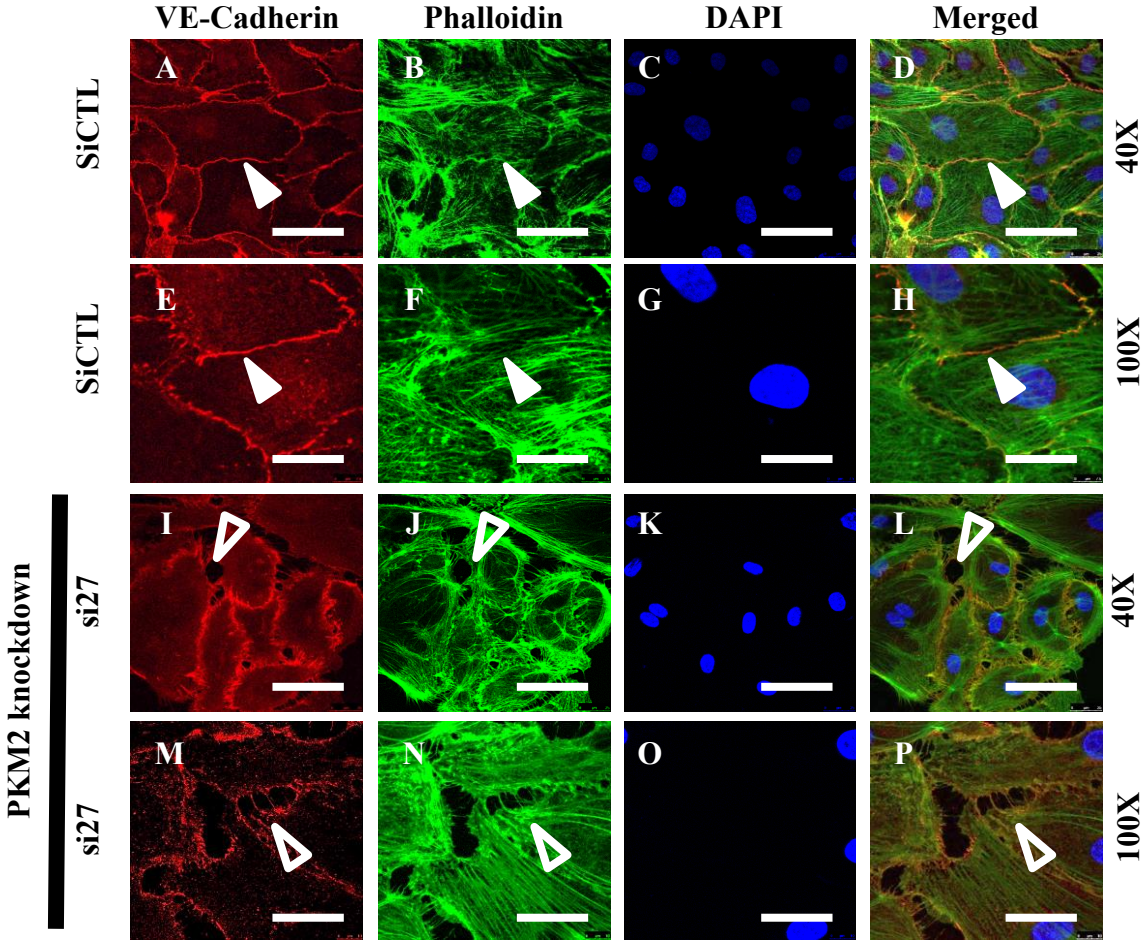
Appendix A1: 1 hour after of reseeding, endogenous VE-cadherin expression localizes to the perinuclear region. Endothelial colonizing forming cells (ECFCs) were transfected with small interfering RNA (siRNA) targeted to PKM2 (si-27, si-155, si-87) and to a scrambled sequence (si-CTL) as a negative control. Cells were assessed 1 hour after reseeding and 24 hours after transfection **A-L,** Representative images of a ECFC monolayer immunolabeled for VE-cadherin (Red), phalloidin (Green) and DAPI (Blue). Scale bars: 50 µm.

Appendix A2:



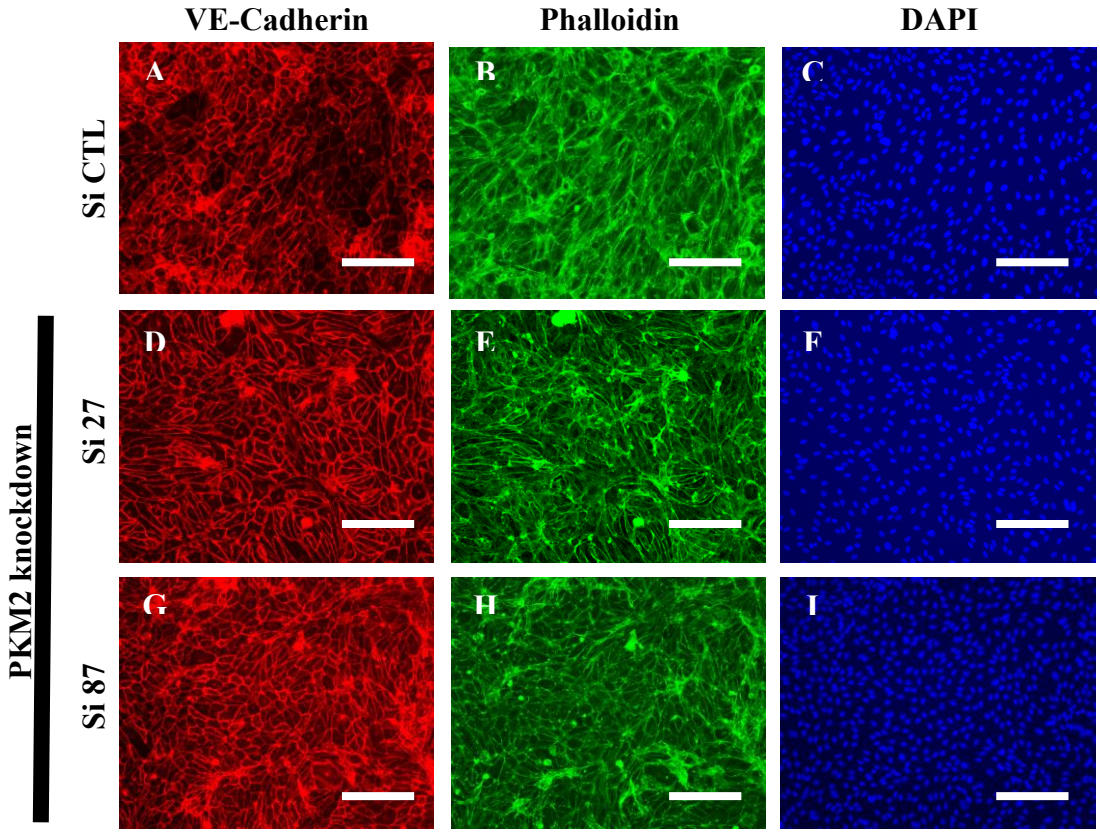
Appendix A2: 4 hours after of reseeding, VE-cadherin localizes at plasma membranes where cell-to-cell contacts occur. Endothelial colonizing forming cells (ECFCs) were transfected with small interfering RNA (siRNA) targeted to PKM2 (si-27, si-155, si-87) and a scrambled sequence (si-CTL) as a negative control. Cells were assessed 4 hours after reseeding and 24 hours after transfection. **A-L**, Representative images of a ECFC monolayer immunolabeled for VE-cadherin (Red), phalloidin (Green) and DAPI (Blue). Arrow heads indicate VE-cadherin localization at plasma membranes. Scale bars: 50 µm.

Appendix A3



Appendix A3: 24 hours after reseeding, loss of PKM2 expression results in disrupted VE-cadherin adherent junctions and gap formations. Endothelial colonizing forming cells (ECFCs) were transfected with small interfering RNA (siRNA) targeted to PKM2 (si-27) and a scrambled sequence (si-CTL) as a negative control. Cells were assessed 24 hours after reseeding and 72 hours after transfection **A-P,** Representative images of a ECFC monolayer immunolabeled for VE-cadherin (Red), phalloidin (Green) and DAPI (Blue). Confocal imaging of confluent endothelial cells show VE-cadherin staining as continuous, linear and distributed around the entire periphery of the cells reflecting stable junctions (**A-H,** arrow). In PKM2 deficient endothelial cells, VE-cadherin staining was discontinuous reflecting unstable junctions and formation of intercellular gaps (**I-P,** open arrow head = discontinuous junctions and gap formations). Scale bars: 40x = 50µm, 100x = 20µm.

Appendix A4:



Appendix A4: PKM2 deficient endothelium lack differential VE-cadherin expression. Endothelial colonizing forming cells (ECFCs) were transfected with small interfering RNA (siRNA) targeted to PKM2 (si-27 and si-87) and a scrambled sequence (si-CTL) as a negative control. Cells were assessed 48 hours after transfection with no reseeding. **A-I,** Representative images of a ECFC monolayer immunolabeled for VE-cadherin (Red), phalloidin (Green) and DAPI (Blue). Cells transfected with a si-CTL show the characteristic differential pattern in VE-cadherin staining in an endothelia monolayer **(A-C)**. In contrast, PKM2 deficient endothelial cells show a robust and uniform expression pattern for VE-cadherin **(D-I)**. Scale bars: 50 μm.

LIST OF JOURNAL ABBREVIATIONS

J Cell. Physiol	Journal of Cell Physiology
J. Cell Sci	Journal of Cell Science
Nat Cell Biol	Nature Cell Biology

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