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Inhibition of FAK-Actin pathway by indoxyl sulfate suppresses arteriogenesis in a mouse model of chronic kidney disease

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by

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ABSTRACT

Chronic Kidney Disease (CKD) has been implicated in multiple conditions including cardiovascular related diseases and Type 2 Diabetes (T2D). Decreased filtration results in accumulation of urea, electrolytes, and uremic which are implicated in disease progression. Examination of the progression of CKD effect on vascular regeneration, and T2D development are necessary. First, examination of CKD progression and buildup of uremic toxin indoxyl sulfate (IS) was undertaken. *In vivo* and *in vitro* analysis implicated CKD and IS accumulation in impaired vascular regeneration increasing risk of mortality. CKD and the concomitant increase in IS resulted in impaired regeneration through alteration to vascular smooth muscle (VSMC). VSMCs exhibited alteration in actin cytoskeletal structure, and decreased motility. This was driven by loss of focal adhesion protein focal adhesion kinase (FAK). Loss of FAK resulted in decreased signal transduction for neuronal Wiskott-Aldrich syndrome protein (N-WASP) necessary for activation of actin nucleation complex Arp2/3. Secondly, examination was undertaken into T2D progression. High-fat induced T2D resulted in a significant increase in Neuropilin 1 (Nrp1) expression in vascular endothelial cells. Transmembrane Nrp1 receptor is a single-pass glycoprotein with well-established roles in vascular events, including tumor angiogenesis, hypoxia, and growth factor-mediated signal transduction. Increased expression of Nrp1 led to alteration in insulin signaling. Altered insulin signaling by Nrp1 contributed to insulin resistance. Thus, our studies demonstrate that CKD induced accumulation of IS impaired vascular regeneration through loss of VSMC motility and impaired vascular regeneration. Also, it was determined that T2D induced expression of Nrp1 resulted in altered insulin signaling and exacerbating insulin resistance.

INDEX WORDS: Indoxyl Sulfate, Chronic Kidney Disease, Focal Adhesion Kinase, Neuropilin 1, Type 2 Diabetes, Insulin

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DEDICATION

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Abbreviation	Definition
FAK	Focal Adhesion Kinase
Nrp1	Neuropilin 1
N-WASP	Neuronal Wiskott Aldrich Syndrome Protein
IS	Indoxyl Sulfate
PWV	Pulse Wave Velocity
EF	Ejection Fraction
FS	Fraction Shortening
CKD	Chronic Kidney Disease
VSMC	Vascular Smooth Muscle Cells
T2D	Type II Diabetes
IRS1	Insulin Receptor Substrate 1
AAA	Abdominal Aortic Aneurysm
PAD	Peripheral Artery Disease
ECM	Extracellular Matrix

1 INTRODUCTION

Chronic Kidney disease (CKD) is a condition that is defined as any condition that causes reduced kidney function over a period and affects roughly 13.6% of adult Americans (NIH). Currently research is ongoing into uremic toxins that are poorly dialyzable in these patients. These compounds produce a myriad of effects that range from cellular hypertrophy to apoptosis. Compounds that have been shown to cause cardiovascular involvements include, p-cresol, indoxyl sulfate, indole-3-acetic acid, and trimethylamine-N-oxide. Of these toxins indoxyl sulfate has been shown to be the most potent that has been shown to involve the cardiovascular system, renal system, and neural system (1).

Indoxyl sulfate (IS) is a potent uremic toxin that in normally functioning kidneys is easily excreted. However, chronic kidney disease (CKD) prevents renal filtration and directly causes retention of uremic toxins with multiple pathologic consequences. CKD patients have an above average mortality due to cardiovascular complications that are a direct result of uremic toxin accumulation. CKD patients exhibit a multitude of cardiovascular complications including left ventricular hypertrophy, dilated cardiomyopathy, and atherosclerosis (2). IS is produced from the degradation of tryptophan by intestinal microbiota. Within this system indole is used as a potent cellular signal that is used to maintain balance within the intestinal microbiota environment. This prevents the rise of potentially pathogenic bacteria that can lead to dysbiosis within the intestines (3). These complications arise due to the prolonged exposure of IS on various cell types of the cardiovascular system, and renal system.

1.1 Indoxyl Sulfate Generation

IS production is due to a combination of intestinal microbiota degradation of tryptophan, for the purpose of extracellular and intercellular signaling, and hepatic modifications to

potentiate excretion. Indole signaling is necessary for biofilm formation by *Escherichia coli*, and other bacteria (4). Indole is necessary for bacteria to develop drug resistance, spore formation, and virulence. Production of indole is carried out by a variety of bacteria that encompass both Gram-positive and Gram-negative bacteria (5).

IS is produced by metabolism of dietary tryptophan into indole by bacterial derived tryptophanases. These enzymes cleave indole from the amino portion to provide a food source for bacterial metabolism. Once indole enters circulation, through the intestinal lumen, it is transported to the liver. In the liver indole is converted to indoxyl, by the addition of a hydroxyl group to the three-carbon position on indole. Hydroxylation at the three-carbon position allows for the addition of a sulfate to the three-carbon position by a liver sulfotransferase (6). After IS is formed it re-enters circulation and strongly binds to albumin. Albumin then transports IS through the circulation to the kidney's to be excreted. Addition of a sulfate group to indole labels the compound for excretion. In normal patients, the level of circulating IS range around 2mg per liter. This level is variable depending on the study that is reviewed.

When a person is afflicted by chronic and acute kidney disease IS cannot be excreted properly and begins to accumulate within the patient. IS is poorly dialyzed from CKD patient's due to the high binding affinity that it has to serum albumin. The only proper way to remove IS from circulation is for the patient to receive a kidney transplant so that IS can be properly excreted (7).

1.1.1 Indoxyl Sulfate Generation

IS transportation is dependent on Organic anion transporters (OATs) specifically OAT1 and OAT3 in renal tubules, endothelial, skeletal muscle, and vascular smooth muscle cells. OATs play a role in the transport of IS across the renal tubule, however, as OAT3 expression

decreases in renal tubules leading to a possible mechanism for accumulation of IS. OAT3 has been theorized to be necessary for the excretion of IS from the kidneys. However, in other cells outside of renal cells there are shown to be increases in OAT3 expression that plays a major role in the transport of IS into cells (8).

Once transported into the cell IS will bind with the Aryl Hydrocarbon Receptor (AhR) and activates certain genes by binding to Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) transcription factor which will activate multiple transcription factors that increase transcription of genes associated with apoptosis, inflammation, and damage responses (9). These activate genes, and transcription factors are dependent on cell type. AhR activation was determined through the transcription of genes associated with AhR. These genes include *CYP1A1, CYP1B1, UGT1A1, UGT1A6, IL6,* and *SAA1*. Figure 1 graphically represents the OAT to AhR pathway involved in pathogenesis. These genes when measured in cell culture, and tissue samples can act as a marker for AhR induced transcription. It should be stated that IS has a high affinity and high potency when bound to AhR. Based on current research IS appears to be one of the few truly potent activators of endogenous AhR (10).

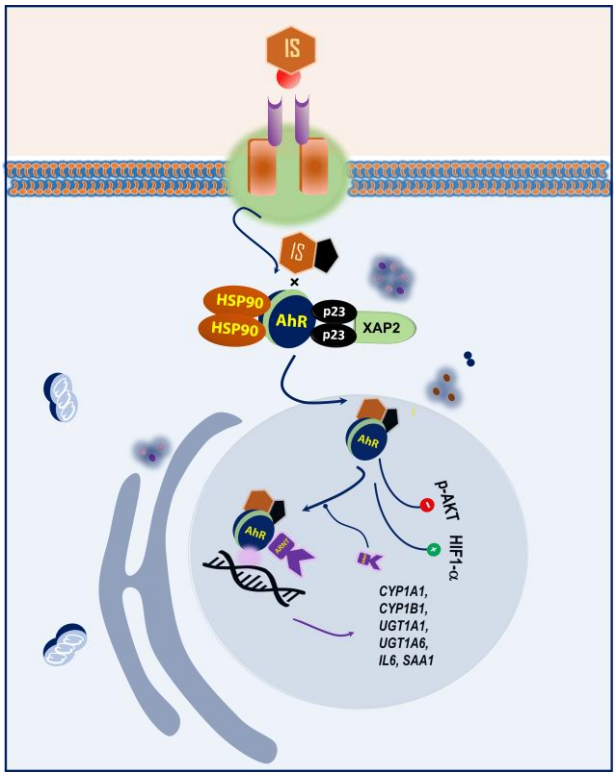

Figure 1: AhR Activation in IS Exposure

When IS is transported into Endothelial cells through Organic Anion Transporter 3 (OAT3) it will encounter Aryl Hydrocarbon Receptor (AhR) it causes it to disassociate from Hsp90 proteins. Once IS is bound to AhR it is transported to into the nucleus and binds to ARNT that acts as a transcription factor that causes expression of *CYP1A1, CYP1B1, UGT1A1, UGT1A6, IL6,* and *SAA1*. This causes the cells to activate inflammatory pathways that effect cellular viability. IS also results in decreased phosphorylation of AKT, and an increase in HIF-1α. HIF-1α activation within the kidney endothelium causes a hypoxic effect within the kidney thus leading to decreased expression of EPO and decreasing endothelial cell proliferation.

1.2 Cardiovascular Disease Exacerbated by Chronic Kidney Disease

Numerous studies have been undertaken to elucidate the effects CKD has on CVD. In a multi-year CKD study that examines the prevalence and mortality of a large cohort of patients in Taiwan was undertaken. They demonstrated an overall 83% increase in mortality in all CKD patients; further co-morbidity with CVD resulted in a 100% increase in mortality over healthy individuals. One surprising factor that was observed in this study was the number of participants who were unaware they had CKD. Age exacerbates the death rate in CKD patients due to comorbidities such as CVD. The rate at which patients are unaware of their underlying conditions which prevents proper treatment and mitigation of the condition (11). It has been determined that younger patients who are aware of their condition and seek medical intervention experience decreased mortality compared with older individuals (12).

Most studies it should be stated that most of these studies have been done on the adult population however recent studies have focused on the correlation of CKD and pediatric mortality. Children who suffer from late-stage CKD exhibit multiple pathologies indicative of CVD including left ventricular hypertrophy, carotid artery intima-media thickness, carotid arterial wall stiffness, and coronary artery calcification.

1.2.1 Cardiac Cell Dysfunction

Early research into the effects of uremic toxins effect on cardiovascular disease centered on cardiac complications. First it was necessary to determine the effect IS has on markers of cardiac fibrosis. Utilizing IS-exposure in Dahl Salt normotensive versus hypertensive rats demonstrated there was a significant increase in fibrotic markers, and cell size. These factors include α-smooth muscle actin, transforming growth factor 1β (TGF-1β), and type 1 collagen.

Oxidative marker NADPH oxidase 4 (Nox4) is increased, and inhibition of anti-oxidative markers Heme Oxygenase 1 (HO-1) and Nuclear like-factor 2 (Nrf2). The increase of the fibrotic markers leads to heart stiffness and eventual failure. These data demonstrate that IS has a direct effect on cardiac dysfunction (13).

After it was determined that cardiomyocytes exhibit increased fibrosis, cell size, and increased oxidative stress it became necessary to delve into the mechanistic pathways involved in cardiac dysfunction. Firstly, it was again demonstrated that cardiomyocytes exhibit increased ROS production, decreased cell viability in a dose and time dependent manner, and cell size. Next, mechanistic analysis was utilized to elucidate the pathway by which cardiomyocyte dysfunction is potentiated via IS. Uncoupling protein 2 (UCP2) has been shown to be protect cardiomyocytes by inhibiting ROS production; however, IS-exposure results in UCP2 is downregulation thus leading to increased levels of ROS. AMP-activated protein kinase (AMPK) was also examined for its potential role in activating UCP2. As cardiomyocytes are exposed to IS; phosphorylation of AMPK decreases demonstrating that IS inhibits the activation of AMPK. Consequently, when cardiomyocytes were exposed to an AMPK activator there was a recovery in UCP2 expression, and conversely, inhibition of AMPK reduced UCP2 expression. Thus, demonstrating that AMPK activation or deactivation directly effects UCP2. Further demonstrating that IS-induced deactivation of AMPK will results in decreased UCP2 and an increase in ROS production. Activation of AMPK or overexpression of UCP2 was further utilized to demonstrate that recovery of this pathway inhibits hypertrophic markers and reduces cell size (14).

1.2.2 Indoxyl Sulfate Effect on Vascular Endothelial Dysfunctions

IS causes activation of inflammatory cytokines that cause arrest of cellular proliferation. This damage will lead to weakening of ventricles and weakened arteries that could rupture due to increased stress due to hypertension. As mentioned above AhR is the intracellular receptor responsible for binding IS and activating the production of pro-inflammatory cytokines. Endothelial cells when exposed to IS exhibit dysfunction due to increasing levels of ROS. IS causes the expression of monocyte chemotactic protein-1 (MCP-1) and decrease in the production of nitric oxide. This is caused increased production of ROS, and activation of AhR. ROS production is induced through the activation of Nox4. The increase in MCP-1 causes increased inflammation of the vascular endothelium (15).

Erythropoietin (EPO) is an erythropoiesis-stimulating cytokine that initiates endothelial proliferation of vascular endothelial cells through activation of the eNOS pathway. CKD patients have been shown to exhibit anemia due to reduced expression of erythropoietin. Research was undertaken to demonstrated IS also prevents the expression of EPO through suppression of nuclear localization of hypoxia-inducible transcription factor (HIF). Loss of nuclear localization of HIF results in a desensitization of the kidney oxygen-sensing pathway preventing the release of EPO, thus possibly effecting other cellular systems (16). The next question that had to examined was does IS affect EPO pathways and through which mechanism. Successive research done by Adelibieke *et al.* demonstrated that IS inhibits EPO signaling through inhibition of AKT phosphorylation. Decreased AKT phosphorylation was traced back to erythropoietin receptor (EPOR) inactivation in the presence of EPO. EPOR induced loss of AKT phosphorylation then directly interfered in EPO activation of eNOS. These aberrations in the EPO pathway directly led to endothelial dysfunction and apoptosis. Caspases 3 and 7 are

directly increased after IS stimulation in endothelial cells providing direct evidence of elevated apoptosis rates. From this it can theorized that aberrations in the EPO pathway may be due to IS induced resistance to EPO (17).

Paradoxically, there has been research that demonstrates IS can induce angiogenesis however, contradictory experimentation demonstrated a decrease in neovascularization. Firstly, it was demonstrated that IS could induce angiogenesis through ROS production and *CYP1B1*-dependent manner. *CYP1B1* as previously stated is a target of the AhR response. However, contradicting research has demonstrated that IS affected the recruitment of endothelial progenitor cells (EPC) and neovascularization in CKD. IS-exposure resulted in impaired recruitment of EPCs that was directly linked to reduced neovascularization which potentiates blood flow disruption. Taking those two contradictory experiments it can be stated that further work must be done to fully investigate the role IS plays in endothelial dysfunction.

1.2.3 Indoxyl Sulfate Induced Complications in Vascular Smooth Muscle

As vascular smooth muscle is a prominent part of the vascular system it is only reasonable that this tissue will be affected by IS exposure. Vascular smooth muscle is shown to undergo increased proliferation due to the activation of the (Pro)renin receptor. This is achieved through IS stimulating the expression of NF-kB due increased activity by transporter OAT3, and transcriptional activation by AhR. This effect in conjunction with oxidative stress causes the increased proliferation of smooth muscles cells. Increased smooth muscle proliferation will produce thickening of vascular walls and promotes calcification in hypertensive models. (Pro)renin receptor activation causes increased cell proliferation during IS exposure; it has also been shown that platelet derived growth factor beta (PDGF-β) and ROS are involved in the

proliferation and migration of smooth muscles cell (18-19). Activation of p38 MAPK, and NADPH oxidase in the presence of IS-induced ROS production increased proliferation, and migration due to the increased sensitivity to PDGF-β due to IS exposure (20).

Angiotensin II activation axis is a model of study into the progression of atherosclerosis. When vascular smooth muscles were exposed to IS, and angiotensin II it was observed that ERK activation was significantly upregulated, but ERK expression levels did not increase. This increase in ERK activation leads to increased VSMC migration. It was also determined that when smooth muscle cells were exposed IS epidermal growth factor receptor (EGFR) had increased expression. EGFR has previously been reported to be upregulated during angiotensin II induction (21). Previously stated it has been shown that IS causes the expression of PDGFRβ due to ROS production. When ROS inhibitors were employed to determine if EGFR responded in the same manner as PDGFRβ it was determined that EGFR levels decreased in antioxidant treated cells. EGFR phosphorylation was also increased when treated with both angiotensin II and IS; angiotensin II induction alone showed increased phosphorylation but not at the levels experienced in the dual treatment. Thus, showing that IS can exacerbate the effects of angiotensin II on vascular smooth muscle cells (22). IS has a mode of action that increases apoptosis leading to increased pathogenesis. This is achieved through activation of certain pro-senescent proteins increase pathogenesis. Within renal proximal tubular cells NF-kB p65 has increased expression by IS induction, which causes the activation of genes such as p53, α-SMA, and TGF-1β. Through activation of senescence pathways there is a concomitant decrease in cell division and increased fibrotic gene expression in the proximal tubules. Senescence of the proximal tubular cells thus exacerbates the renal dysfunction causing further progression of CKD (23).

NF-kB activation of senescence has also been reported in the progression of vascular smooth muscle dysfunction. Oxidative stress is induced by IS exposure leading to upregulation of senescence markers p53, p21, and prelamin A. Even though it has been shown that vascular smooth muscles undergo increased proliferation, paradoxically senescence is also upregulated in VSMC. Senescence marker increase causes the cell to downregulate the expression of FACE1 which is a metalloproteinase responsible from cleaving prelamin A to lamin A. Lamin A is an integral part of the nuclear membrane that requires FACE1 processing to be functional. Build-up of prelamin A will then result in DNA damage and mitotic dysfunction that can directly related to cell death and senescence (24).

1.2.4 Indoxyl Sulfate-induced Skeletal Muscle Atrophy

Skeletal muscle atrophy is a pathologic hallmark of CKD which leads to loss of protein, and a decrease in muscle mass (Wang *et al.* 2014)25. One recently elucidated pathway is related to the release of cytokines and increase in ROS. Skeletal muscle exposure to IS results in release of inflammatory cytokines such as TNF-α, IL-6, and TGF-1β, all these cytokines have been shown to increase in multiple tissues. Following upregulation of inflammatory cytokines there's a resultant increase in myokine Myostatin. Myostatin has previously been linked to skeletal muscle atrophy, but until this study it was not sure by which possible pathway it was interacting with (26-27). Due to the increase of ROS in skeletal muscle cells caused by IS-exposure; myostatin expression increased through NF-kB induction in response to inflammatory cytokines. Previously it had been shown that myostatin will cause increased ROS production through stimulation of TNF-α and NF-kB (28-29). This increased expression of ROS thus in turn causes increased myostatin leading to increased muscle atrophy. Until this point the control mechanisms

for myostatin was unknown, but pharmacologically inhibition through anti-inflammatories prevented muscle atrophy. Atrogin-1 is an E3-ligase linked to skeletal muscle proteolysis has been directly linked to muscle wasting. When myostatin or TGF-1β are upregulated, there is a concomitant activation of Atrogin-1. Then the question becomes how these components control atrogin-1 upregulations. Atrogin-1 expression has been directly linked to upregulation of Smad2/3 (30). It has previously been shown that TGF-1β and myostatin control expression of Smad2/3. This directly links expression of myostatin and TGF-1β to IS-induced atrogin-1 skeletal muscle atrophy (Figure 2) (31).

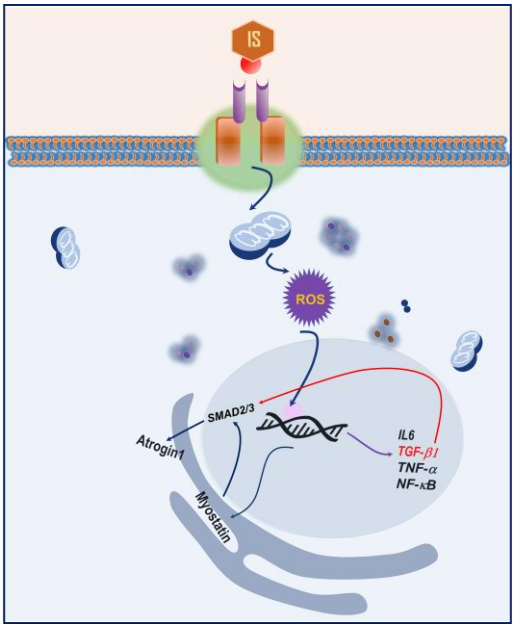

Figure 2: IS Induced Skeletal Muscle Atrophy

When IS encounters OATs on the surface of skeletal muscle cells IS is transported into the cell and binds to AhR. Once activated AhR is translocated to the nucleus through the action of ARNT and causes the transcription of inflammatory cytokines such as, IL-6, and TNF-α. This in turn causes increased expression of Myostatin leading to muscle atrophy. Once myostatin is upregulated there is increased activity of Atrogin-1, an E3-ligase, that causes protein degradation

leading to muscle wasting. Muscle atrophy is achieved through the upregulation of myostatin through increased ROS production and activation of NF-kB.

1.3 Type II Diabetes and Chronic Kidney Disease

Type II diabetes (T2D) can be characterized by insulin insensitivity and inhibited glucose response. Examination of multiple facets of associated with diabetes open alternate pathways of examination. Vascular systems are made up of multiple cell types that react in various ways to stimuli. It has been observed that endothelial cells are particularly affected by uremic toxin exposure. The insulin signaling pathway is a complex signaling pathway necessary for glucose uptake and utilization. Insulin signaling starts by the interaction insulin with insulin receptor alpha (IR-α) which in turn phosphorylates the insulin receptor beta (IR-β). IR-β will then potentiate this signal by activating insulin receptor substrate 1 (IRS-1) which allows for signal propagation and activation (32). Endothelial cells line the entirety of blood vessels and are the first line of exposure to toxins and elevate insulin levels.

T2D has been shown to exacerbate multiple disease conditions, including T2D, leading to increased mortality. In the case of T2D multiple studies have demonstrated a relationship to CKD and subsequent disease progression (33-34). Patients who suffer from both T2D and CKD are at increased rates of CVD that directly contributes to mortality. By understanding the disease progression into T2D will shed light onto how CKD is potentiated and open new treatment directions.

1.4 Treatment to Combat Indoxyl Sulfate Accumulation

Currently there are few treatments to reduce production of IS from indole produced by the intestinal microbiota. One way that indoxyl levels are reduced in CKD patients is through the treatment with AST-120. AST-120 is an oral sorbent compound that will bind to indole within the lumen of the intestines which allows for it to be excreted in feces. This method is used to help reduce the levels of IS and decrease the cardiovascular complications that arise from the increased levels of IS (35).

Another treatment that has shown so effect in counteracting the effect of IS is treatment with Vitamin C and N-Acetylcysteine. As stated previously IS acts to increase the level of antioxidants that cause many of the cellular dysfunctions that are experienced by CKD patients. Both compounds act as ROS scavengers to reduce the oxidative stress that occurs due to the increasing levels of IS (36). Another way to control the levels of IS in CKD patients is for them to ingest very low protein diet to reduce the intake of tryptophan so that indole is produced in smaller quantities within the bacterial community (37). CVD disease progression could be blunted in CKD patients by inhibiting IS production. Thus, preventing CKD patients from increased mortality.

Recently, work has been done to develop a new method of dialysis to remove IS from CKD patient serum. Until this point there is not a commercially available method to remove IS from its albumin carrier. In the development of a new dialysis method; poly-cyclodextrans were chosen for their binding ability to multiple molecules. It was demonstrated that addition of poly-cyclodextrans to dialysis setups increased removal of IS compared to conventional dialysis. This new method provides new hope in the future for better dialysis setups to reduce IS concentrations and reduce cardiovascular complications (38).

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2 MATERIAL AND METHODS

2.1 *Cell culture*

VSMCs were purchased from American Type Culture Collection (ATCC) and maintained in SmGM (Lonza) with 10% FBS. All cells were maintained at 37°C in 5% CO_2 and 95% air. All 3T3-L1 fibroblasts and all cancer cell lines including H1792, HepG2, H1299, and H1650 were purchased from American Type Culture Collection (ATCC) and maintained in either DMEM F-12 50/50 or RPMI-1640 medium (Lonza) with 10% FBS. HUVECs (Thermo Scientific, USA) were cultured in EGM-2 basal medium supplemented with EGM-2 BulletKit (Lonza). HASMCs (Thermo Scientific, USA) were cultured in Medium 231 (Catalog #M231500; Thermo Fisher, USA) supplemented with smooth muscle cell growth supplement (Catalog #1101; Sciencell, USA). Adipocyte differentiation studies were carried out by using DMEM F-12 50/50 added with 3T3-L1 differentiation kit (Catalog #: K579; BioVision, USA). All cells were maintained at 37°C in 5% CO_2 and 95% air.

2.2 Hindlimb Ligation

Mice were fed 0.25% Adenine diet for 1.5 weeks followed by 0.5 weeks recovery before surgery (21). Hindlimb ligation was performed as previously described (22). Blood flow restoration was examined at days 0, 1, 3, 7, 14, 21, and 28 days using laser doppler. Mice were sacrificed at the end of the experiment and tissues were collected for WB and IF examination. Tissue was either stored in formaline fixative solution or -80C.

2.3 HPLC determination of IS

Sample preparation for plasma was done by mixing 50µl of sample by vortexing in a 1.5ml eppendorf tube with 1/10 volume of ice-cold 2.4 mol/L perchloric acid. The cloudy suspension

was chilled on ice for 15' and then centrifuged at 8,000g for 3 minutes. The clear protein-free supernatant was analyzed directly. IS content was estimated along with standards ranging from 0.250-25μM IS (Sigma, I3875), using a C18 column (Symmetry C18 Column, 100Å, 5 μm, 4.6 mm X 250 mm; Waters, USA). The mobile phase was ACN/H2O: 66/34 and the injected sample volume was 20 μl. The flow rate was 1 ml/min at 38 ^{O}C. The column effluent was monitored at 278nm by a UV detector.

2.4 Cell Attachment and Size Assay

ECM Laminin (20ug/mL), Fibronectin (10ug/mL), and liquid gelatin were diluted in PBS and incubated on plates for 1 hour. Plates were blocked with 5% BSA for 1 hour to prevent nonspecific binding. Cells were plated on ECM for 6 hours in 2% FBS media then fixed with 4% paraformaldehyde. Stained with Phalloidin (1:1000) for 20minutes and mounted with DAPi gold mounting media. Size was determined as previously described.

2.5 ***siRNA Transfection***

VSMCs were routinely passaged 24 hours before transfection and transfected at approximately 70% confluency. Cells were transfected with either siRNA for FAK, Paxillin, or N-WASP (Santa Cruz Biotechnology) for 6 hours in Optimem (Gibco) using the RNAImax (Invitrogen) then incubated for 24 hours in 2% FBS media before incubation under normoxic or hypoxic conditions (1% O_2; 12hrs) or further manipulation. Lung cancer cell lines were routinely passaged 24 hours before transfection and transfected at approximately 70% confluency. Cells were transfected overnight in 1% FBS media using the RNA IMAX protocol (Invitrogen) before incubation under hypoxic conditions or further manipulation. Stable Nrp1-deficient cell lines were achieved using lentiviral shRNAs under puromycin selection (Santa Cruz Biotechnology).

2.6 *Reagents and Cell Treatments*

To inhibit the lysosomal activity, Bafilomycin A1 (BafA; 8hrs), Chloroquine (CQ; 8 hours), and to inhibit proteasome degradation Mg132 (8hrs) (Invitrogen, USA) was used. Control cells were treated with vehicle only (PBS). IS stimulation experiments were done with various insulin concentrations (nM) for 2 days in 2% serum media. To inhibit the lysosomal activity, Chloroquine (CQ; 3 hours) (Invitrogen, USA) was used. Control cells were treated with vehicle only (PBS). Insulin stimulation experiments were done with various insulin concentrations (nM) at varying time duration (5 to 120 minutes) in serum-starved cells.

2.7 *Western Blotting and Immunoprecipitation*

As previously described, Western blots were done, and blots were probed with specific antibodies (23). Primary antibodies used as follows: β-actin and GAPDH anti-mouse monoclonal antibody (Santa Cruz Biotechnology); anti-rabbit polyclonal FAK, FAK (Y397), Paxillin, Paxillin (Y118), Hif1α, Arp2, and Arp3(Cell Signaling); anti-rabbit polyclonal N-WASP (Sigma). After background subtraction from the calculated area, band intensities were measured and quantified by densitometry (GS-700 Imaging Densitometer; Bio-Rad). Quantification of Western blot was based on the ratio of the target protein to GAPDH or β-actin housekeeping protein.

As previously described, Western blots were done, and blots were probed with specific antibodies (23). Primary antibodies used as follows: β-actin and GAPDH anti-mouse monoclonal antibody (Santa Cruz Biotechnology); anti-rabbit polyclonal RAB5, RAB7, RAB11, pAkt (Ser473), tAkt, pIRS1 (Ser612), tIRS1, PPARγ and C/EBPα (Cell Signaling); anti-rabbit polyclonal Nrp1 and Lamp2 (Abcam, USA). After background subtraction from the calculated area, band intensities were measured and quantified by densitometry (GS-700 Imaging

Densitometer; Bio-Rad). Quantification of Western blot was based on the ratio of the target protein to GAPDH or β-actin housekeeping protein.

2.8 Light and Confocal Microscopy

Cells were plated on glass coverslips and fixed in 4% paraformaldehyde. Cells were permeabilized with either 0.1% Triton X-100 and blocked with 0.1% BSA before antibody addition. Indirect immunofluorescence experiments were performed using the following primary antibodies: anti-rabbit FAK (1:200; Cell Signal), anti-mouse N-WASP (1:50; Santa Cruz), anti-mouse Paxillin (1:50; Santa Cruz), anti-rabbit N-WASP (1:50; Sigma), anti-mouse Arp2 (1:50; Santa Cruz). Secondary antibodies consisted of Alexa Fluor 488, 555, and 647 (donkey anti-goat, -rabbit, or -mouse, respectively) used at 1:400 dilution.

Cells were plated on glass coverslips and fixed in 4% paraformaldehyde. Cells were permeabilized with either 0.1% Triton X-100 or 0.2% saponin and blocked with 0.1% BSA before antibody addition. Indirect immunofluorescence experiments were performed using the following primary antibodies: anti-goat Nrp1 (1:100; Santa Cruz), anti-rabbit Nrp1 (1:100; Santa Cruz), anti-rabbit RAB7 and PPARγ (1:100; Cell Signaling), anti-rabbit Lamp2 (1:750; Abcam). Secondary antibodies consisted of Alexa Fluor 488, 555, and 647 (donkey anti-goat, -rabbit, or -mouse, respectively) used at 1:400 dilution.

2.9 IHC

Tissues were fixed in 10% ice-cold formalin overnight, then embedded in paraffin. Briefly, slides were deparaffinized and dehydrated for 30 minutes at room temperature. Sections were incubated for 30 minutes in diluted normal blocking serum, and primary antibodies were added to

slides after excess serum had been removed. Respective antibodies against vWF and α-SMA (Santa Cruz Biotechnology) were diluted according to the manufacturer's instructions.

Tissues were fixed in 10% ice-cold formalin overnight, then embedded in paraffin. Briefly, slides were deparaffinized and dehydrated for 30 minutes at room temperature. Sections were incubated for 30 minutes in diluted normal blocking serum, and primary antibodies were added to slides after excess serum had been removed. Respective antibodies against Nrp1 and Rab7 (Lifespan Biosciences) were diluted according to the manufacturer's instructions.

2.10 Nrp1 Plasmid Transfection

Nrp1 plasmid was a gift from Christiana Ruhrberg (University College London, UK), and was transfected in H1299 cells using optiMEM medium and Lipofectamine 2000 transfection kit (Thermo Fisher, USA).

2.11 *Quantitative RT-PCR*

Oligos used to quantify as follows: *Nrp1* (forward, 5′-GTTCATCCACCGCATCGAC-3′; reverse, 5′-CACATCCACCAGCTGGATG-3; 277bp′), *Nrp1* (forward, 5′-AAATGCGAATGGCTGATTCAG-3′; reverse, 5′-CTCCATCGAAGACTTCCACGTAGT-3′; 121bp), and 1*8s* (forward, 5′-GTCTGTGATGCCCTTAGATG-3′; reverse, 5′-AGCTTATGACCCGCACTTAC-3′). Real time quantification was performed by SYBR Green (Bio-Rad, USA) with the C1000 thermal cycler, CFX96 detection system (Bio-Rad, USA). Relative gene expression was normalized to *18s* and compared using the ΔΔCt method.

2.12 FACS Assay

Insulin internalization into the different cell types was measured using FITC-labeled insulin (Ins. FITC, Sigma-Aldrich, USA). A monolayer of serum-starved siNrp/si-Scr or

LacZ/Nrp1-transfected cells were treated with insulin (50nM) for 15 min. The excess insulin was removed and washed with cold PBS. Fresh serum-free RPMI medium was added to the cells on ice. Cells were placed back at 37 °C for 10 min. Subsequently Ins.FITC (100nM) in PBS was added and incubated for 30 min. The reactions were stopped by the removal of Ins.FITC and cold PBS washes (2×). Cells were fixed in 1% paraformaldehyde, and FITC staining was detected by flow cytometry (BDLSRFortessa), and data was analyzed using FlowJo software.

2.13 Glucose and Insulin Tolerance Tests

Wild type, $Nrp1^{endo-/-}$ mice and littermates ($Nrp1^{endo+/+}$) mice were fed with either chow or high fat diet. Glucose and insulin tolerance tests were performed. Briefly, for the glucose tolerance test (GTT), the mice were fasted for 16 h. After determining fasted blood glucose levels, each animal received an intraperitoneal glucose injection of 1 g/kg body weight. Blood glucose levels were determined at 0, 15, 30, 60, and 120 min. For insulin tolerance tests (ITTs) 4 h fasted mice received an intraperitoneal injection of 1.5 units/kg body weight of insulin. Blood glucose levels were measured at 0, 15, 30, 60, and 120 min.

2.14 ***Statistics***

Data are expressed as mean ± SEM or mean ± SD as indicated in the figure legends. Statistical comparisons were performed using unpaired 2-tailed Student's *t*-test or 1-way ANOVA with Bonferroni multiple-comparison test. A *P* value less than 0.05 was considered significant.

3 INHIBITION OF FAK-ACTIN PATHWAY BY INDOXYL SULFATE SUPPRESSES ARTERIOGENESIS IN A MOUSE MODEL OF CHRONIC KIDNEY DISEASE

3.1 Abstract

Chronic Kidney Disease (CKD) and the concomitant accumulation of uremic toxins has been implicated in impaired vascular regeneration and arteriogenesis. The objective of this study is to examine the role uremic toxin indoxyl sulfate (IS) has on arteriogenesis, vascular regeneration, and the underlying molecular mechanisms. C57BL6/J mice were fed 0.25% adenine diet for 1.5 weeks to induce CKD. CKD mice exhibited increased serum levels of uremic toxin IS, nitrogen, and creatine. Mice underwent hindlimb ligation and CKD mice exhibited decreased blood flow restoration (44.7% relative blood flow) compared to control (82.6% relative blood flow) and presented with decreased arteriole formation as evidenced by reduced VSMC content (decreased α-SMA) in vessels of ligated limb. Wound healing and transwell migrations assays demonstrated IS-exposed VSMCs had significantly reduced motility. IS-exposure also resulted in disorganized actin cytoskeleton, alterations in actin structure, attachment, and cell size in VSMCs. Focal adhesions, actin attachment points, were decreased due to IS-exposure. Specifically, focal adhesion kinase (FAK), a vital component of cell attachment and attachment, was significantly decreased due to CKD progression and IS-exposure. Loss of FAK directly decreased motility and impacted N-WASP expression necessary for activation of actin polymerization complex Arp2/3. Arp2/3 complex was further affected by IS-exposure which resulted in decreased expression. Loss of Arp2/3 would thus inhibit motility through the loss of actin polymerization. Thus, our studies demonstrate that CKD accumulation

of uremic toxin IS inhibits VSMC motility and impairs vascular regeneration and arteriogenesis through inhibition of FAK-actin pathway.

3.2 INTRODUCTION

Chronic Kidney Disease (CKD) a serious medical condition that causes not only medical burden but socioeconomic pressures that are only exacerbated by the ever-increasing disease prevalence. Figures from 1998 demonstrated, approximately 20 in every 1000 individuals over the age of 60 were affected by CKD, however by 2012 that figure had more than doubled to 50 in every 1000 individuals in the United States (1-3).

CKD patients present with decreased filtration of the blood, accumulation of uremic toxins, and other solutes normally excreted by healthy kidneys. Uremic toxins have been implicated in the progression of multiple diseases or the exacerbation of already existing conditions. These compounds are derived from amino acid degradation by gut microbiota. Currently, the most potent uremic toxin elucidated is that of IS. IS is generated by the degradation of tryptophan by *E. coli*. Specifically, *E. coli* will cleave the function and amino groups and utilize each component. Gut microbiota will utilize the functional group, indole, as an intracellular signaling molecule (4). Once indole is released by the microbiota it is cross the intestinal lumen and enters the circulation. Normally, excess indole will be transported to the liver and undergo sulfonated conversion into IS. Upon conversion IS will be released into the circulation and quickly binds albumin for transport to the kidneys for excretion. However, CKD impairs kidney filtration resulting in the accumulation of IS. As the levels rise cardiovascular complications increase ultimately leading to mortality (5-6).

Cardiovascular disease (CVD) is one of the main routes of mortality in CKD patients especially those in late stage of disease progression. CVD progression has been tied to the accumulation of said uremic toxins. Uremic toxin accumulation has been shown to cause or exacerbate dilated cardiomyopathy, coronary artery disease, atherosclerosis, and peripheral artery disease (PAD). Studies have demonstrated CKD patients are susceptible to PAD which leads to ischemia, extremity ulcerations, and ultimately infections (38&39). Examination of CKD patients has demonstrated ~25.2% of patients also suffer from PAD which impairs blood flow and wound healing. PAD results in narrowed blood vessels leading to impaired collateral formation necessary for blood flow restoration. CKD patients are known to suffer from leakage at canulation sites which interferes with hemodialysis preventing proper blood filtration (43). Also, CKD patients are at increased risk of adverse outcomes from AAA repair and lower extremity amputations. They are more likely to have failed surgical repairs and or impaired blood flow that will prevent repair and increase mortality. These reports demonstrate that vascular structure is disrupted, and these disruptions will directly contribute to impaired vascular regeneration and arteriogenesis (7-9).

Vascular regeneration and arteriogenesis rely on a complex signaling pathway necessary to activate with expansion of new vessels necessary to alleviate hallmarks, including ischemia, necrosis, and infections, of impaired blood flow (10&11). Vasculature regeneration first relies on the activation of endothelial cells to construct new capillaries to begin blood flow blood flow restoration. These capillaries are essential for nutrients and oxygen to reach the damaged tissue. However, these capillaries are incapable of high-capacity blood flow limiting the amount of nutrients and oxygen reaching said wounds. Capillaries experiencing elevated levels of endothelial shear stress, radial wall stress, and high blood pressure must undergo arteriogenesis to allow for blood vessel expansion. Arteriogenesis allows for blood vessel expansion through the recruitment

vascular smooth muscle cells (VSMCs) that will provide support necessary for larger quantities of blood to pass (45). Disruption of arteriogenesis will prevent blood flow to damaged tissues, and consequently potentiating. Arteriogenesis not only requires the presence of VSMCs but relies upon the cellular attachment, cytoskeletal, and migration pathways to be unaffected. Currently, though elucidation of the molecular underpinnings potentiating these adverse vascular outcomes has not been investigated as it relates to arteriogenesis and vascular regeneration in CKD patients.

Attachment and migration are controlled by multiple different pathways that include focal adhesion attachment, and coordination of the cytoskeleton (12-15). Focal adhesions are complexes responsible for potentiating extracellular stimuli and convey that signal intracellularly. These complexes are dynamic and designed to respond to multiple stimuli. Responsiveness to these signals requires the coordination of canonical focal adhesion proteins including Focal Adhesion Kinase (FAK), Paxillin, Src, p130cas, actopaxillin, vinculin, talin, and many more. Alterations to these complexes would lead to ineffective response to stimulation of the focal adhesions and defective vascular repair (16-20). Elucidation of the role uremic toxin IS has on vascular regeneration and arteriogenesis, and the molecular interplay between the actin cytoskeleton, regulators of actin structure, and focal adhesions will provide vital insights into disease progression in CKD patients.

Buildup of uremic toxin IS resulted in alterations to the actin cytoskeleton and formation, focal adhesions, and motility that impairs vascular regeneration and arteriogenesis mechanisms.

3.3 Results

3.3.1 Adenine Diet Model of CKD

Firstly, it was necessary to examine the effect CKD has on vascular regeneration and arteriogenesis. We utilized a CKD mouse model fed 0.25% adenine diet for 1.5 weeks, and then induced a vascular injury through hindlimb femoral artery ligation (21). Laser doppler analysis of CKD mice exhibited impaired blood flow restoration (44.7% restoration) compared to controls (82.6% restoration) after 28 days of recovery in the ligated hindlimb (Figure 3A&B) (22). Defective vascular regeneration can lead to limb atrophy, increased chances of infection, and further complications ultimately resulting in mortality. Also, CKD mice presented with elevated levels of IS, Serum Nitrogen, and Creatine which demonstrate that kidney function is impaired (Figure 3C-E). Cardiovascular function was examined due to previously reported complications associated with CKD. Adenine diet induced CKD resulted in cardiovascular complications marked by increased pulse wave velocity (PWV), and decreased fraction shortening (FS) and ejection fraction (EF), but no change to strain (Figure 8A-D).

Finally, once it was determined that CKD impaired vascular regeneration the next step was to examine arteriogenesis of the damaged limb. Importantly, vasculature within tissues can be classified as capillaries, composed of endothelial cells, or complex vessels, composed of endothelial cells and VSMCs. Complex vessels, formed through the process of arteriogenesis, are necessary to carry higher volumes of blood compared to capillaries. Impaired arteriogenesis contributes to decreased recovery and blood flow capacity. Examination of damaged musculature in the ligated muscle occurred via immunofluorescent staining for vWF (endothelial cells) and α-SMA (VSMCs) (23). CKD mice muscles exhibited decreased arteriogenesis as demonstrated by lack of α-SMA in vessels while vWF was present (Figure 3F). Vessels lacking VSMCs were inadequate to compensate and repair the damage resulting from hindlimb ligation. Our data

demonstrated that arteriogenesis was impaired due to CKD progression and buildup of uremic toxins.

The role that tryptophan plays in the progression in CKD progression is well understood due to the role it plays in uremic toxin formation. Tryptophan deficient diet was utilized to understand the role in wound healing progression. It was determined that tryptophan deficient diet resulted in loss of weight and inability to recover from CKD induction (Data not shown).

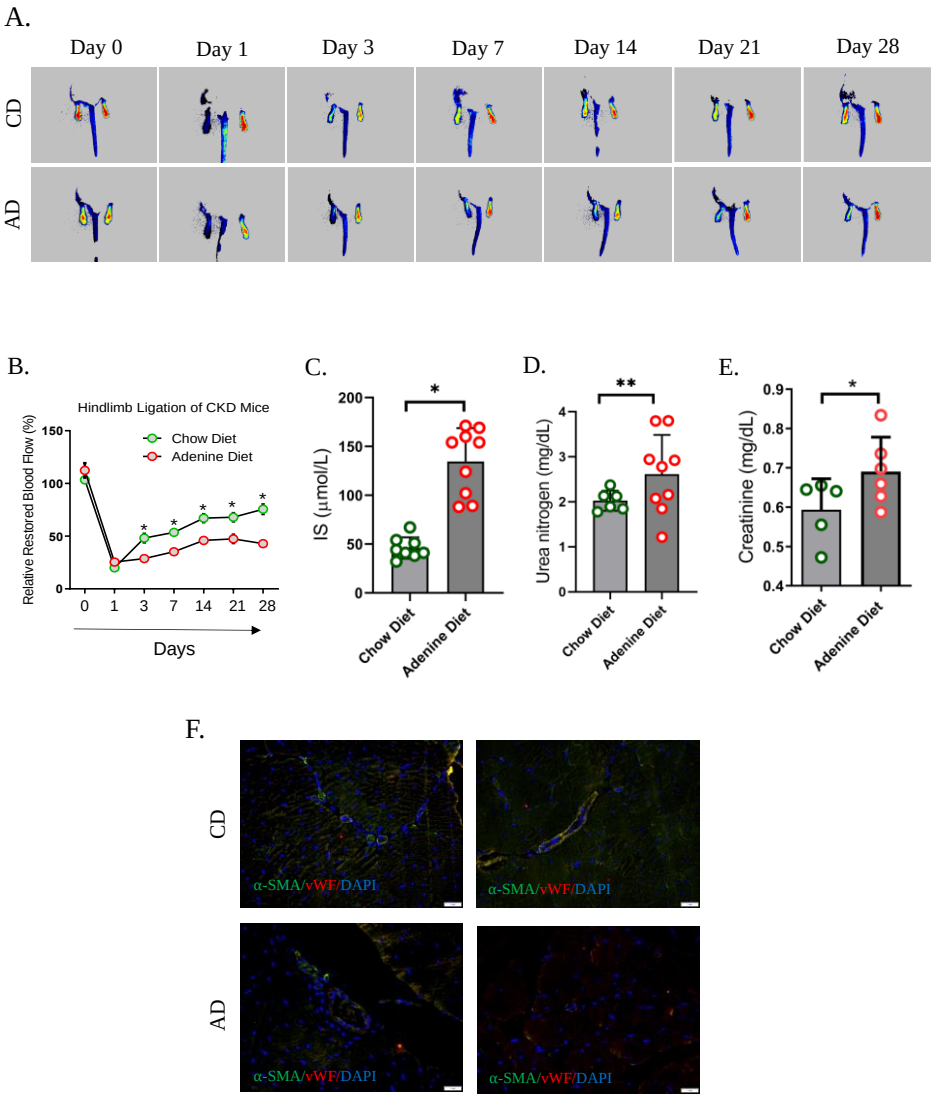

Figure 3: Hindlimb Ligation of CKD Mouse Model

A. Heat Map for blood flow restoration after 28 days post-surgery. **B.** Relative percentage of blood flow restoration after hindlimb ligation. Adenine diet (AD) mice exhibited approximately 44.7% blood flow restoration compared to Chow Diet (CD) Mice 82.6% restoration of blood flow (ND, n=9; AD, n=8). **C.** To be completed. **D.** serum Nitrogen levels are

significantly elevated in Adenine Diet mice. **E.** To be completed. **F.** Ligated muscle was stained for capillaries vWF (Red, endothelial cells), α-SMA (Green, VSMCs) *p<0.05, **p<0.005

3.3.2 Vascular Smooth Muscle Cell Motility

Uremic toxins, specifically IS, which accumulate during CKD progression, are known to has adverse effects on vascular cells that contribute to cardiovascular disease (44). Firstly, VSMCs underwent IS-exposure for 2 days and we utilized a wound healing assay to determine the effect on migratory ability. Examination the migratory efficiency of IS-exposed VSMCs demonstrated approximately 20% wound closure after 12hrs compared to approximately 35% wound closure in control samples (Figure 4A&B). Next, a transwell migration assay was employed further examine IS-exposed VSMC migratory ability. IS-exposed VSMCs were applied to the transwell and after 6hrs migration there was a marked reduction in migratory ability compared to the control. Migration activator PDGF-ββ was utilized to determine if the phenotype would be reversed upon stimulation (24). PDGF-ββ only partially rescued IS-exposed VSMC motility, lending evidence that IS-exposure (Figure 4C&D). It became necessary to examine whether other known activators could reverse the observed phenotype. Finally, we utilized migration activators FGF, VEGF, and ANGII were utilized and found to be incapable of reversing IS impaired motility (Figure 4E). This data demonstrated that IS impaired motility can be ameliorated but not completely reversed. To ensure that our observations are related to decreased migration and not cell death cell viability was examined. Cell viability decreases significantly until 4 days after exposure to IS but has minimal effect after 2 days of exposure (Figure 9A-D). IS-exposure does induce inflammation in VSMCs due to expression of IL-6 which is consistent with previous reports (25). Our data now lends compelling evidence that IS-exposure directly inhibits motility.

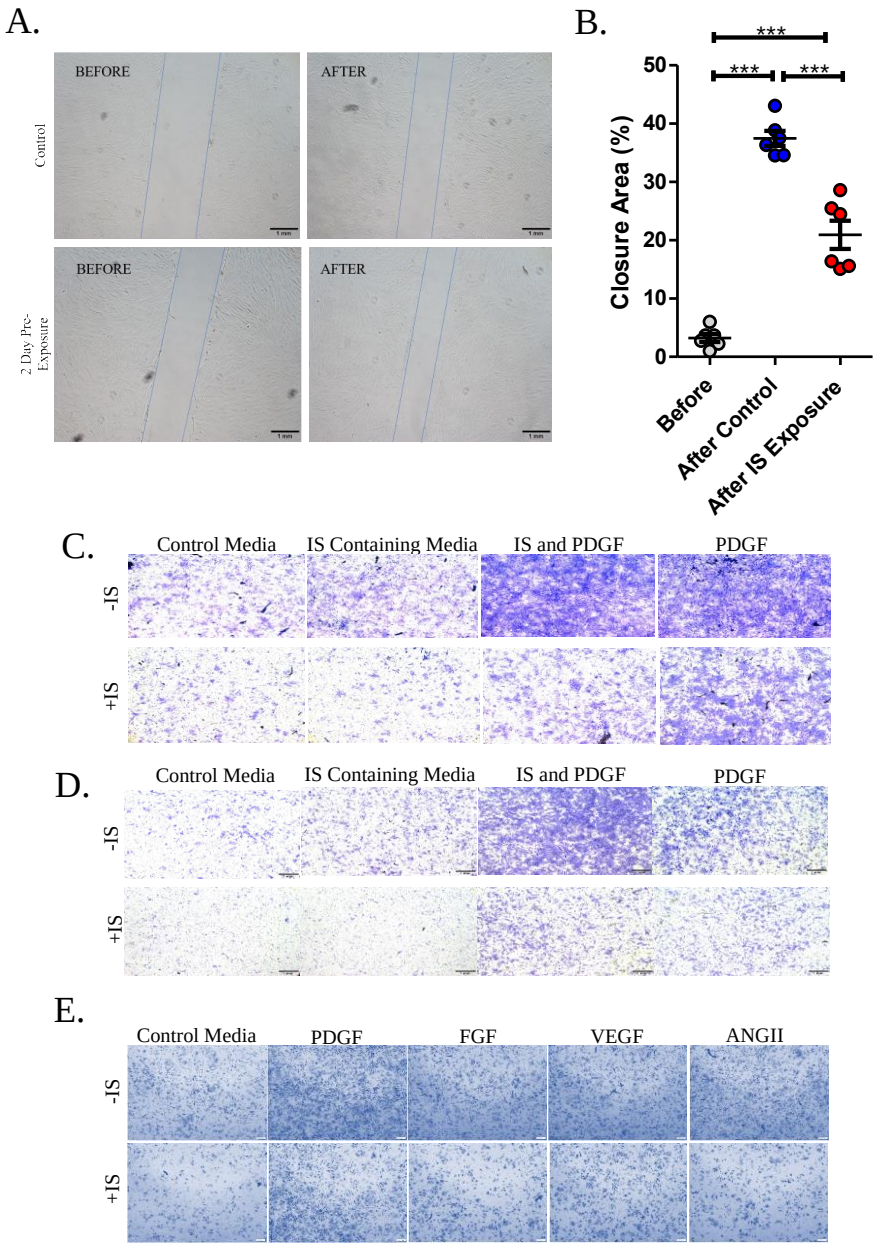

Figure 4: Indoxyl Sulfate Effect on VSMC Wound Healing and Migration

A&B. VSMCs exposed to IS had decreased wound healing capacity and results in wound closure of approximately 20% compared to approximately 40% closure after 12hrs. (1mm scale) **C.** Transwell migration assay was utilized for VSMCs exposed to IS for 24hrs. There was a

signification decrease in VSMC migration. PDGF (10ng/mL) was utilized as a positive control. PDGF stimulation did stimulate migration however IS-treated cells had less migration than untreated cells. (1mm scale) **D.** 48hrs of IS exposure was utilized. Transwell migration yielded similar results to the 24hrs exposure. Migration was inhibited. (1mm scale) **E.** Various other migration stimulators were utilized PDGF, FGF (1ng/mL), VEGF (10ng/mL), and Angiotensis II (ANGII, 5μM), ***$p<0.0005$, ****$p<0.0001$

3.3.3 Indoxyl Sulfate Alters Actin Cytoskeleton and Attachment

Once it was understood that IS-exposure impairs VSMC motility the next question that needed to be asked was how the actin cytoskeleton is affected. The actin cytoskeleton is directly involved in motility and due to the impaired motility, it this logically appears to be the next step of examination (12&13). First, we utilized IS-exposed VSMCS to examine effects on actin structure and arrangement. IS-exposure induced actin cytoskeletal disorganization as evidenced through actin staining (Figure 5A). Alteration to the actin cytoskeleton would interfere with normal cellular functions. Elucidation of the disruptions to the actin cytoskeletons necessitated examination of actin polymerization in IS-exposure conditions. Within all cells actin exists in two states either Filamentous-actin (F-actin) or Globular-actin (G-actin). Isolation and examination of actin states demonstrated IS inhibited polymerization which will decrease the number of actin fibers (Figure 5B). Further confirmation was sought through IF staining of F- and G-actin. Actin staining further reinforced the observation that IS-exposure inhibited actin polymerization and decreased levels of F-actin (Figure 5C). Lending further evidence that IS-exposure alters the VSMC actin cytoskeleton Inhibition of actin polymerization will result in alterations to morphology and directly drive the loss of motility previously observed.

Understanding that IS-induces alterations to the actin cytoskeleton it was necessary to examine the effect this had on VSMC morphology. Extracellular matrix (ECM) components are utilized by all cell types to attach and interact with the cellular environment. VSMCs when healthy are known to prefer binding to Laminin however, in disease conditions ECM affinity has been shown to be altered. To this end we employed standard ECM or Laminin coated plates to examine cell attachment, size, and substrate affinity. IS-exposure significantly reduced cell size by as much as 50% on laminin or standard ECM plates (Figure 5D&E) (26). Alterations to the actin cytoskeleton led to smaller cells due to lack of structural support to maintain larger cell sizes. Further analysis of VSMC ECM affinity was undertaken utilizing collagen and fibronectin coated slides. We found that VSMCs have an increased affinity for collagen and cell size (Supplementary Figure 3A-C). VSMCs exposed to IS also experienced a change the integrin profile that affected attachment and response to the extracellular environment (Figure 10D&E). Finally, to examine focal adhesions, which are attachment points for actin fibers, we utilized IF staining for FAK and Paxillin (Figure 5F) (27). Focal adhesions are decreased after IS exposure leading to decreased actin attachment, and size. Taken together this data demonstrated that IS-exposure directly interferes in the actin cytoskeleton structure, directly leading to alterations of cell size and attachment ability.

Further analysis demonstrated that VSMCs plated on Fibronectin and Gelatin exhibited altered cell size (Figure 10A&B). These alterations resulted changed interaction with the extracellular environment as evidenced by the alteration in focal adhesions numbers (Figure 10C). Examination of integrins necessary for attachment to the extracellular matrix was undertaken. It is demonstrated that there decreases in multiple alpha and beta integrins due to IS-exposure (Figure 10D&E). Finally, we examined the activity of MMPs necessary for ECM degradation was

undertaken. IS-exposure results in decreased activation of MMP2 as IS levels increase (Figure 11A). It was also demonstrated that ECM component collagen experienced an increased expression as well. These data lend further evidence that IS-exposure alters VSMC ECM interaction.

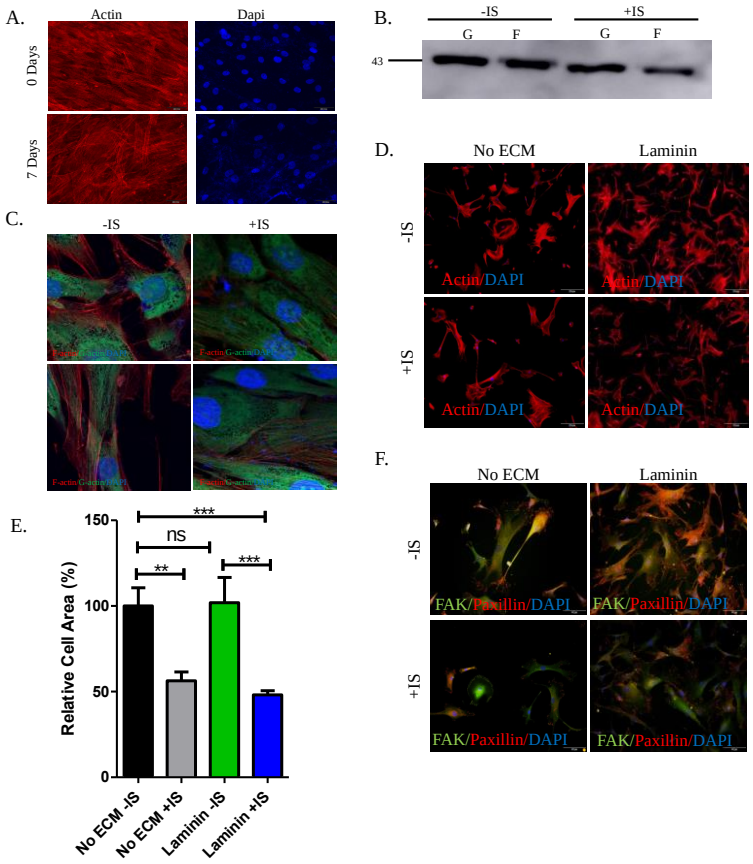

Figure 5: Indoxyl Sulfate Effect on Actin Cytoskeleton, Cell Size, and Attachment

A. Prolonged exposure to IS results in disorganization of the VSMC actin cytoskeleton. **B.** IS also results in decrease actin polymerization thus inhibiting actin fiber formation. **C.** IF staining of VSMCs for F- (Red) and G-Actin (Green). IS exposed cells have decreased F-Actin

and increased G-actin pool. **D.** VSMCs were plated on Standard Coating or Laminin (20µg/mL) coated plates. **E.** Percentage of cell area with or without IS exposure. **F.** Focal adhesion in response to IS exposure. Focal adhesions decrease on Standard Coating, and Laminin coated plates. **p<0.005, ***p<0.0005

3.3.4 Focal Adhesion Loss Due to Indoxyl Sulfate

Understanding how IS-exposure affected cellular structure and attachment it was necessary to examine vital attachment known as focal adhesions. Focal adhesions are necessary for attachment and signal transduction from the extracellular environment. Firstly, we examined how IS affect the expression of canonical focal adhesion proteins Focal Adhesion Kinase (FAK) and Paxillin. Dose dependent IS-exposure resulted in significant decrease in the expression of FAK and Paxillin, and the activation of FAK at Tyr397 (Figure 6A-C) (28). Microscopy demonstrated that FAK and Paxillin were depleted at the leading edge of VSMCs and throughout the cell (Figure 6D&E). Without these points at the leading edge there will be reduced actin polymerization and impaired forward motility. FAK and Paxillin are vital components of focal adhesions however they are not the only proteins present thus leading to question if IS-induced loss if confined to these proteins. Another two canonical focal adhesion proteins Src and p130cas were examined for activation and expression after IS-exposure. Analysis determined there was no alteration to activation or expression of these proteins due to IS-exposure (Figure 6F). *In vivo* analysis of the effect CKD has on FAK and Paxillin was necessary. Aortas were isolated from CKD mice, perivascular adipose tissue and endothelial cells were removed, and were examined via western blot. Analysis of aortic samples recapitulated the *in vitro* samples demonstrating a significant loss of FAK and Paxillin in CKD conditions (Figure 6G). Ligated muscle was examined, and it was found that vessels lacked FAK expression after CKD induction (Figure 6H)

Following observations that IS-exposed VSMCs exhibit decreased motility and loss of focal adhesion proteins it was necessary to elucidate if the loss of focal adhesions directly contributed to lost motility. FAK or Paxillin were silenced in VSMCs, and the effect on migration was examined via transwell migration assay. Silencing of FAK resulted in significantly decreased VSMC motility however, loss of Paxillin had no effect on VSMC motility (Figure 6I). Taken together these data demonstrate loss of focal adhesion proteins specifically FAK impair vascular motility and play a role in alterations to the actin cytoskeleton.

We must also consider the microenvironment that is produced during vascular damage and that is hypoxia. To determine if the microenvironment caused by vascular damage would alter the mechanism being proposed we utilized hypoxic conditions. Analysis of VSMCs demonstrated that hypoxia did not alter the expression profile of FAK and Paxillin with or without IS exposure (Figure 12A). Finally, we also examined the mechanism by which IS induced the loss of FAK and Paxillin. Utilizing inhibitors for proteasome, early- and late-stage lysosome degradation. We found that IS induced FAK and Paxillin degradation through the proteasome degradation system (Figure 12B).

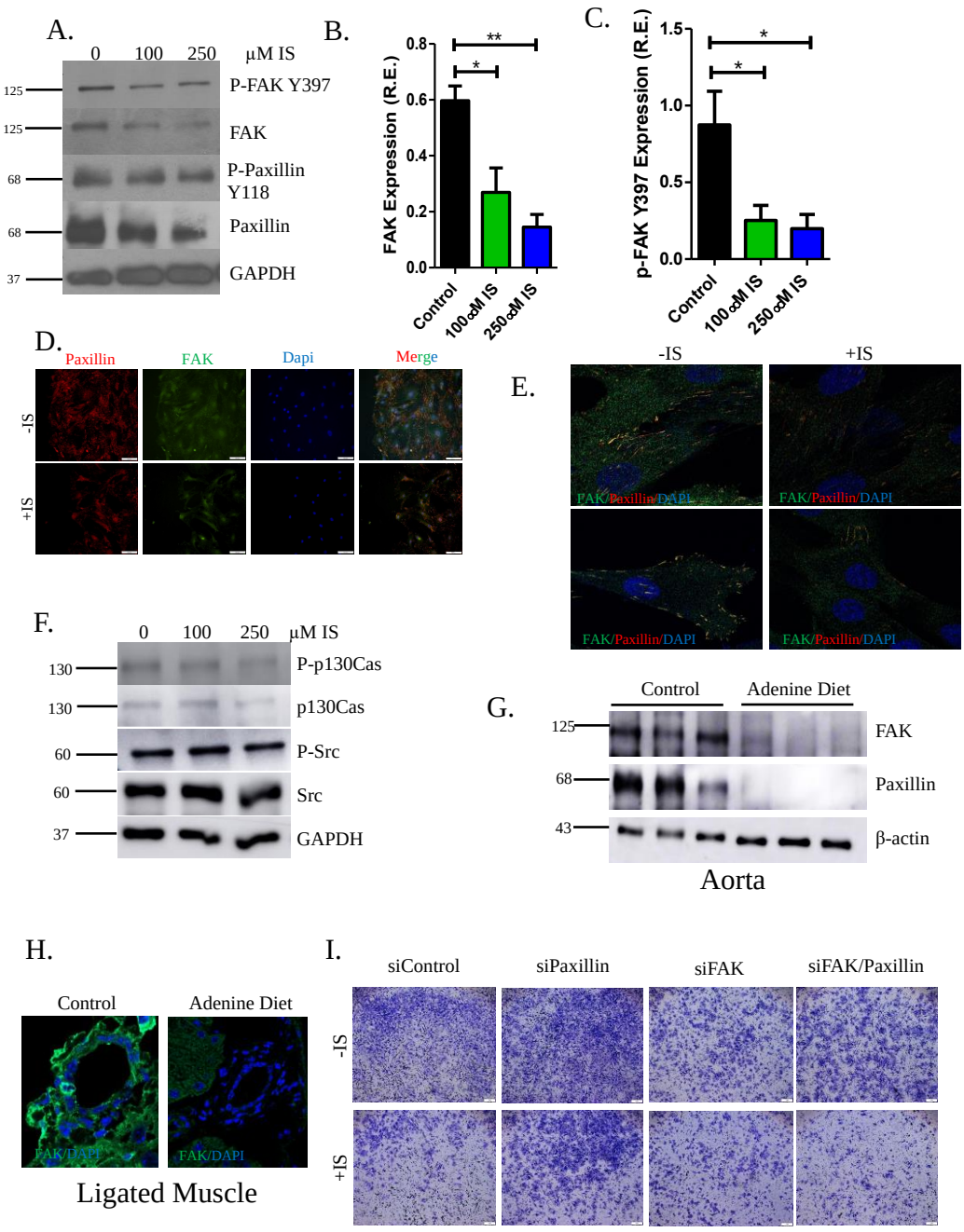

Figure 6: Indoxyl Sulfate Effect on Focal Adhesions

A. FAK and Paxillin decrease as IS concentration increases. Also results in loss of phosphorylation. **B.** FAK relative expression after IS-exposure. C. Phosphorylation of FAK at Y397 was decreased after IS-exposure **D.** Focal Adhesion made of FAK and Paxillin are

decreased after IS-exposure at the leading edge. **E.** Confocal microscopy further demonstrates the loss of focal adhesions. **F.** Related focal adhesion protein p130Cas and Src are not affected by IS exposure. **G.** Mice aortas were examined after Adenine diet. FAK and Paxillin is decreased mimicking *in vitro* VSMC profile (n=3). **H.** Ligated muscle from adenine diet fed mice demonstrated lower expression of FAK in vessels **I.** Silencing of either FAK, Paxillin, or both was examined via transwell migration. Silencing FAK had a marked decrease in migration however, this was not the case with Paxillin silencing. (R.E.- Relative Expression) *$p<0.05$, **$p<0.005$

After understanding that Focal adhesions were involved in the loss of cell motility after IS-exposure we needed to understand how loss of FAK and alterations to the cytoskeleton are propagated. We examined what could be a downstream effector for FAK, Neuronal Wiskott-Aldrich Syndrome Protein (N-WASP) which has been implicated in actin polymerization (28&30). Examination of N-WASP phosphorylation (S484/485) and total expression after IS-exposure. *In vitro* and *in vivo* samples we determined that IS exposure decreased N-WASP expression as well as cause the loss of the phosphorylation state (Figure 7A&C). We also utilized staining for FAK and N-WASP to demonstrate the result of IS exposure. VSMCs exhibited reduced expression of N-WASP as well as alterations to cellular localization (Figure 7B). Further demonstrating that IS-exposure has a direct effect on the ability of VSMCs to construct and utilize the actin cytoskeleton. To ensure that loss of N-WASP was involved in decreased motility a transwell assay was again utilized. Silencing of N-WASP severely inhibited migration in the same fashion as loss of FAK (Figure 7D). Demonstrating that the FAK-N-WASP pathway is directly involved in VSMC

migration. Now that we understand that IS affects a particular actin polymerization pathway the next step is to examine the Arp2/3 complex responsible for actin polymerization.

Expression of Arp2 and Arp3 was directly downregulated after IS exposure in both *in vitro* and *in vivo* models. Once it was determined that another link in the chain involved in actin nucleation was disrupted, we finally examined the Arp2/3 complex that has been directly tied to actin nucleation in cells (31&32). It was observed after IS exposure there was a decrease in the expression of Arp 2 and 3 *in vitro* and *in vivo* (Figure 7C, E&F; Figure 13A). This ties directly into the previous observations of decreased polymerization after IS exposure. Loss of these vital components of the Arp2/3 complex results in decreased actin nucleation. Taken together it has been demonstrated that aberrations to actin polymerization are responsible for lost motility.

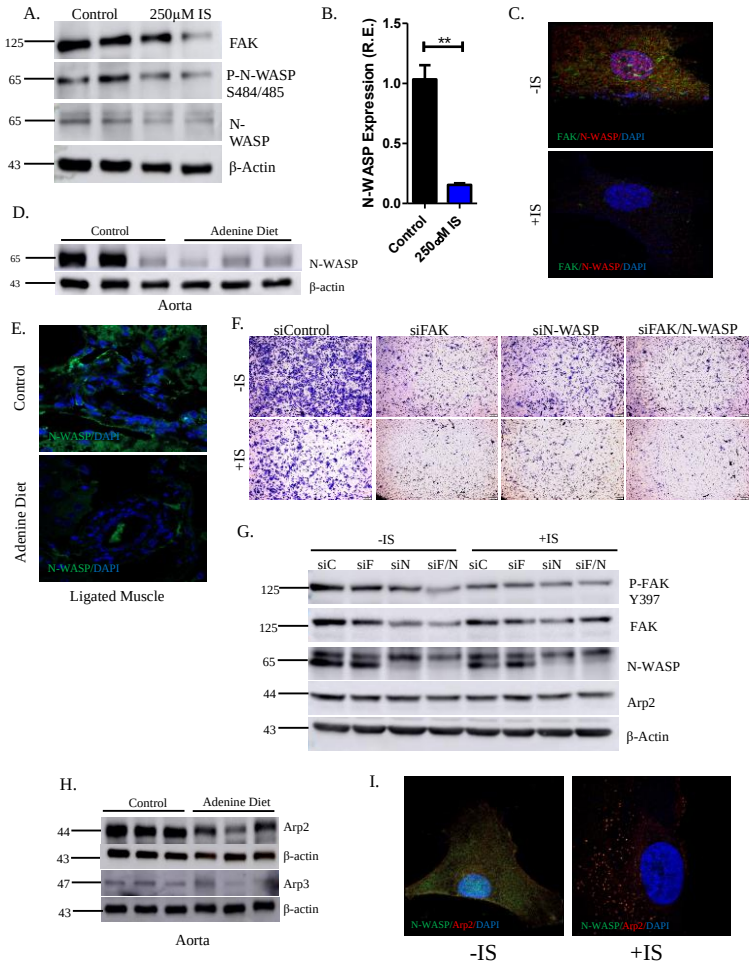

Figure 7: Downstream Effect of Indoxyl Sulfate on FAK Pathway

A. N-WASP, a downstream effector FAK, has decreased expression and activation after IS-exposure. **B.** N-WASP statistically decreased expression. (n=3) **C.** FAK and N-WASP colocalization is decreased due to IS exposure. **D.** Examination of N-WASP expression in aortic tissue from adenine diet treated mice. **E.** N-WASP expression was decreased in vessels of ligated muscle due to Adenine diet induced CKD. **F.** FAK and N-WASP loss directly contribute to loss of VSMC migration after IS exposure. **G.** Silencing of FAK, N-WASP, or both were utilized to examine the effect on Arp2. Arp2 decreased with N-WASP silencing which results in decreased

actin polymerization. **H.** Arp2 and Arp3 decreased expression in adenine diet mice. (n=3) **I.** N-WASP and Arp2 colocalization also decreases after IS exposure. **p<0.005

3.4 Supplementary Figures

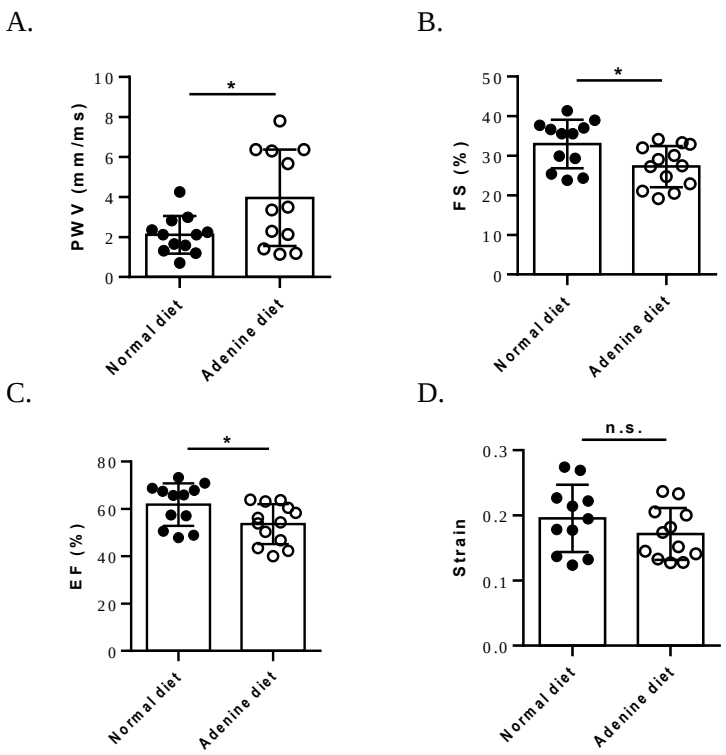

Figure 8: Cardiovascular Complications

A. Pulse Wave Velocity (PWV) was increased due to CKD. B. Fraction Shortening (FS) was elevated in CKD conditions. C. Ejection Fraction (EF) was decreased in CKD conditions. D. Vascular strain was not significantly affected by CKD conditions.

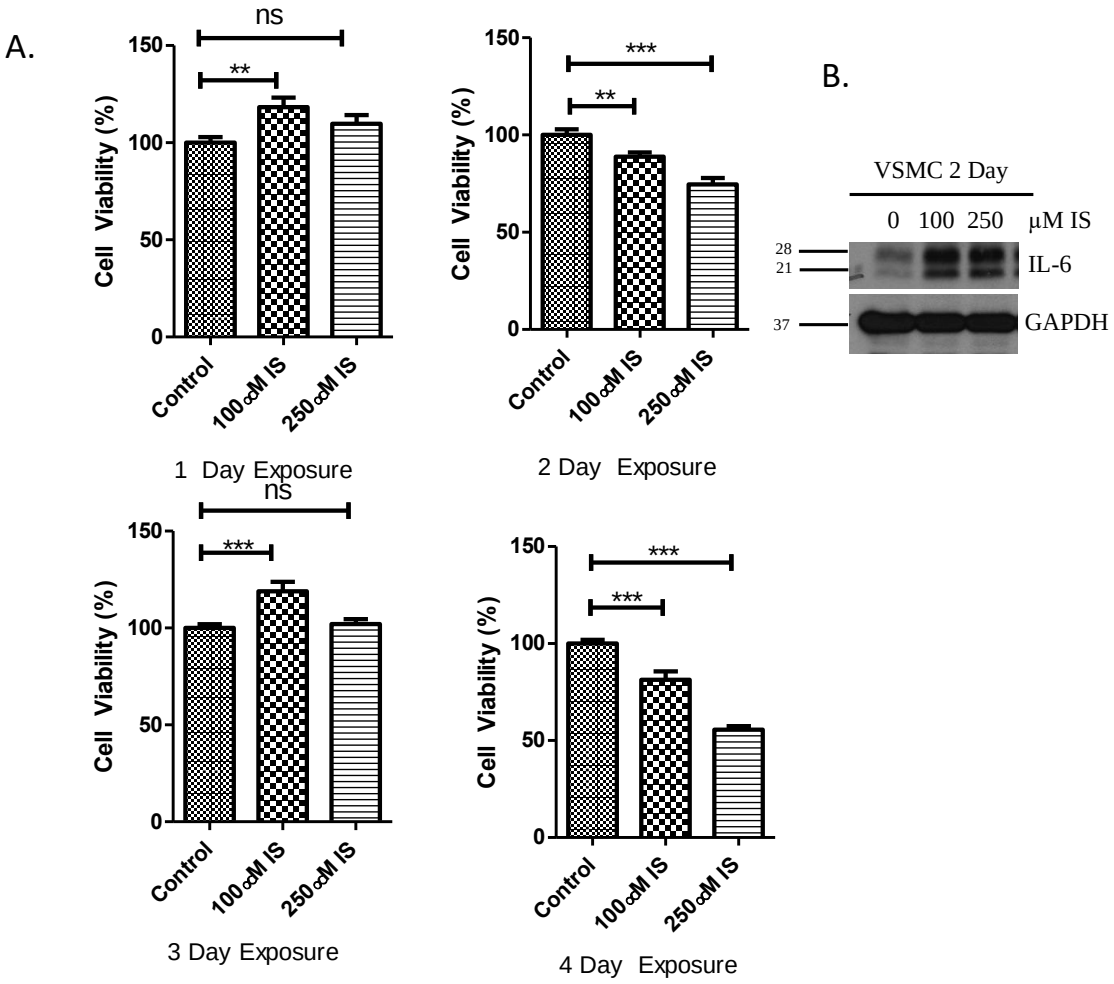

Figure 9: VSMC Cell Viability

A. VSMC viability rate is ok until 4 days of exposure and then it decreases. B. VSMCs experience inflammation due to IS but not cell death.

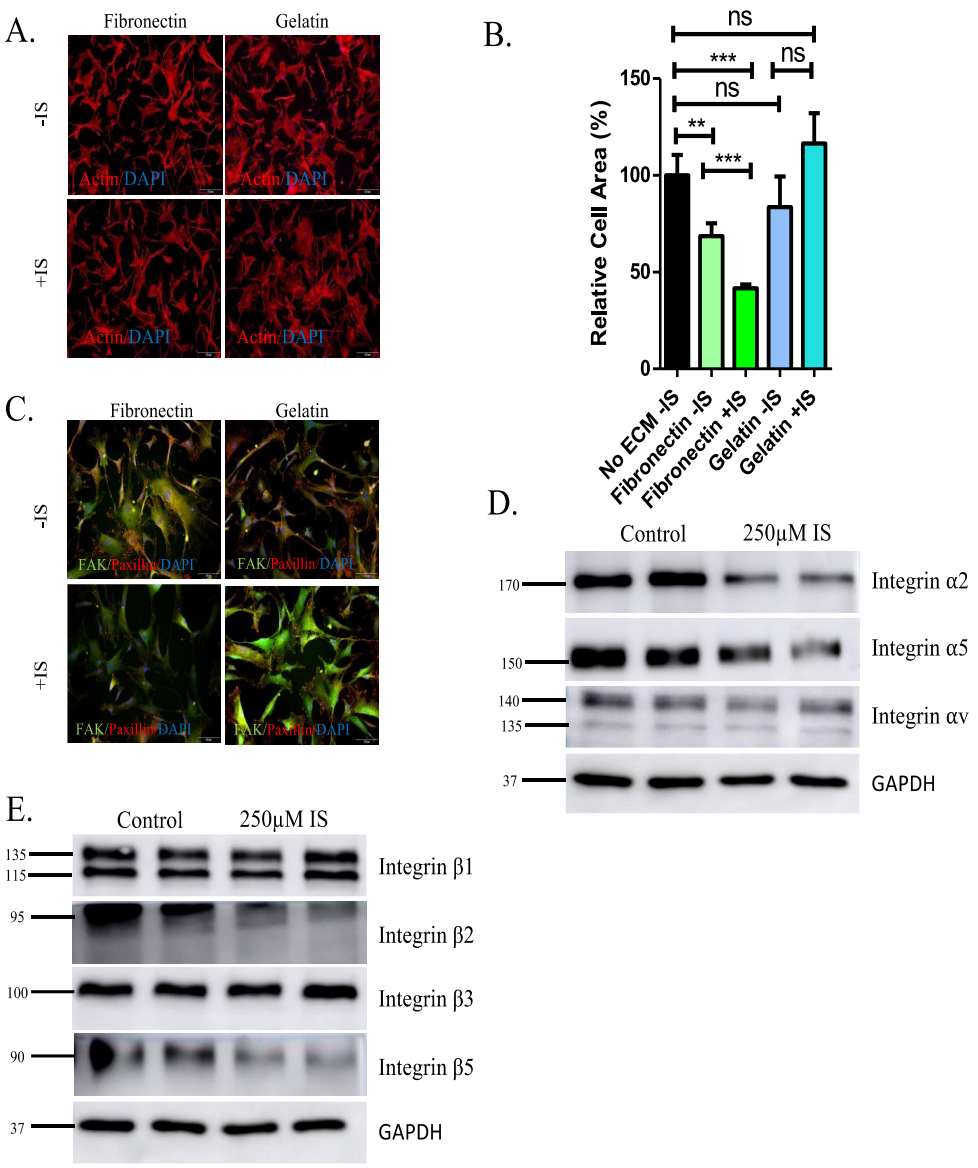

Figure 10: Further Analysis of VSMC Attachment

A. Fibronectin (10ng/mL) and Gelatin (Cellbiologic). Cell size decreased on Fibronectin after IS exposure and increased on Gelatin. B. Graph of cell size for fibronectin and gelatin. C. Focal adhesions change on Fibronectin and Gelatin. D&E. Integrins profile for both alpha and beta subunitss shifted in VSMCs after IS exposure. **p<0.01, ***p<0.005

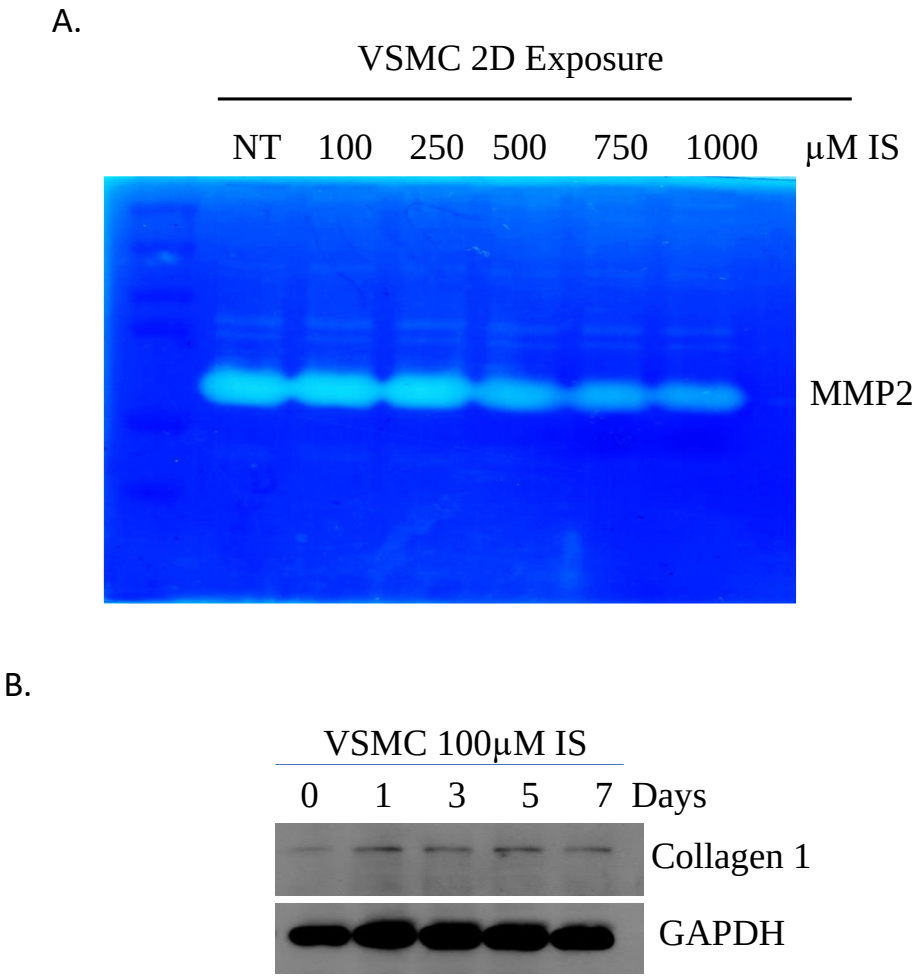

Figure 11: Examination of ECM Degradation

A. Zymography of media from IS exposed VSMCs. Illustrating a decrease in MMP2 activity with increased IS exposure B. Analysis of Collagen I expression with IS exposure. Collagen expression increases with IS exposure

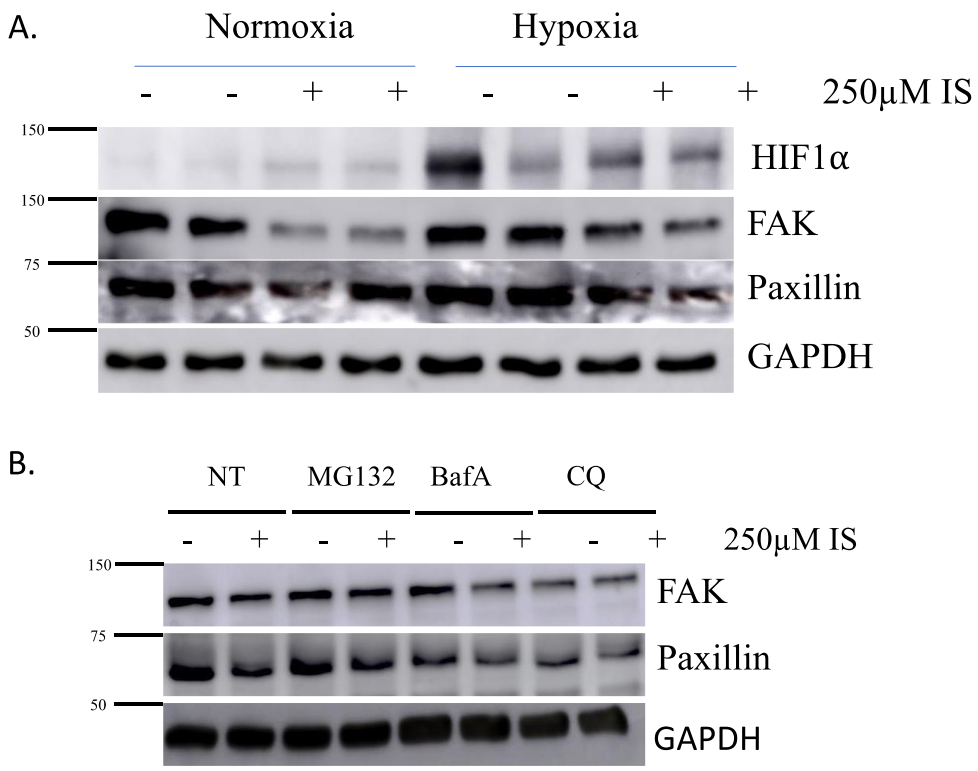

Figure 12: Hypoxic Effect on VSMC FAK

A. Examination of the effect of 12hrs Hypoxia on VSMCs exposed to IS. B. Inhibition of protein degradation after IS exposure.

A.

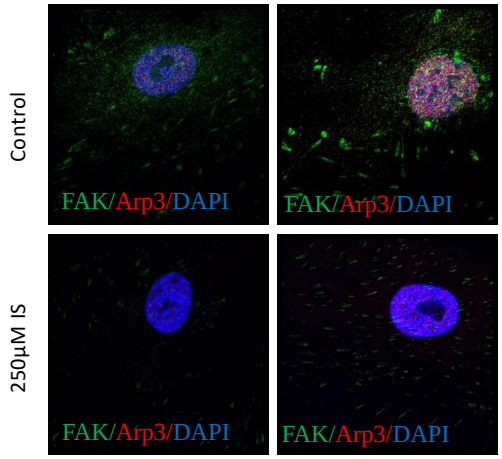

Figure 13: Arp3 Expression

A. FAK and Arp3 were stained in VSMCs after IS-exposure.

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4 NEUROPILIN-1 (NRP1) PROTEIN IS A MODULATOR OF INSULIN SIGNALING FUNCTIONS

4.1 Abstract

Transmembrane neuropilin-1 (Nrp1) receptor is a single-pass glycoprotein with well-established roles in vascular events, including tumor angiogenesis, hypoxia, and growth factor-mediated signal transduction. Here we propose a previously unidentified role of the Nrp1 protein on insulin signaling functions. Our data showed that in obese conditions, high fat diet or insulin stimulation promoted the expression of Nrp1 in adipose and liver tissues, as well as vascular and cancer cells. Consequently, abundant Nrp1 availability resulted in increased co-localization with insulin substrates, thereby modulating the insulin signaling pathway. Interestingly, improved vascular cell functions were observed under Nrp1-depleted and insulin-challenged conditions. Nrp1-depletion results in stronger and faster activation of the insulin signaling cascade. For the first time, these observations uncover novel association and subsequent modulation of insulin signaling substrates by Nrp1. Potential implications of these findings with respect to cancer risk and links to metabolic perturbations, such as obesity and insulin resistance, are hereby reported.

4.2 Introduction

Type II Diabetes (T2D) a metabolic condition that is characterized by high blood glucose levels, insulin insensitivity, and/or decreased insulin levels. Risk factors for T2D can include family genetic history, age, and/or obesity or a combination of multiple factors. Obesity results in adipose tissue inflammation and a concomitant increase in serum insulin levels and insulin insensitivity. Recent evidence has demonstrated that T2D patients are at increased risk

of cancer development. As insulin insensitivity progresses and insulin signaling is altered cancer progression is exacerbated leading to adverse outcomes. By examining the underlying mechanisms that exacerbate T2D more effective treatments can be devised.

Cellular effects of insulin are mediated through binding with its canonical receptors, namely inulin receptor (IR) and insulin growth factor-1 receptor (IGF-1R) (15). Specifically, insulin binds to the insulin receptor's alpha subunit, which activates tyrosine kinase residues within the beta subunit (16). Insulin receptor substrates (IRS) mediate intracellular signaling in response to upstream activation of insulin receptors (17-19). Subsequent downstream events are mediated by recruiting several adaptor molecules with unique motifs that guide specificity and cellular outcomes (15-20). For example, cytoplasmic IRS-1 proteins contain multi 'docking' sites with Src-homology-2 (SH2) and SH3 domains that mediate signal transduction (16). Activation of phosphatidylinositol 3-kinase (PI3K)/Akt, ERK1/2, mTOR, and AMPK have been associated with IRS-1 (17; 21; 22). Furthermore, IRS-1 has been implicated in several pathologies, including type 2 diabetes and cancer (22).

Neuropilin 1 (Nrp1) was initially a coreceptor for class 3 semaphorins (Sema3), and growth factors including vascular endothelial growth factor, transforming growth factor-β, hepatocyte growth factor, and platelet-derived growth factor (8). Nrp1 is highly expressed in vascular and myeloid cells (9; 10). This report proposes novel metabolic Neuropilin-1 (Nrp1) functions as a major hub of well-coordinated and adaptive mechanism that supports tumor growth and progression. Transmembrane Nrp1 receptor demonstrates pleiotropic cellular functions, including independent and co-receptor interactions with endocrine growth factors, such as VEGF, EGF, HGF, and Sema3 (11-14). Expectedly, Nrp1 functions may be responsive to cellular microenvironments and the availability of secreted growth factors.

With the ever-increasing prevalence of T2D and the associated metabolic perturbations will heighten cancer incidence. Strong epidemiologic evidence also links T2D with increased incidence of several types of cancer (1). Most importantly, aggressive growth and aberrant cellular properties associated with malignant tumors benefit from metabolic and vascular alterations. Insulin signaling proteins, mainly insulin receptor-A (INSRA) and Akt, often demonstrate upregulated expression, as well as increased activation in tumor cells **(2), (3)**. Interestingly, abundant expression and phosphorylation of INSR have been observed in mammary tumors from diabetic mice **(4)**. While various animal and human studies and epidemiological data recognize the association between obesity and cancer risk, molecular mechanisms linking obesity, metabolic syndromes, and cancer remain incomplete.

In this study, we investigated potential links between insulin-related substrates and Nrp1 receptor protein in response to metabolic perturbations associated with obesity. Here, we report novel metabolic roles of Nrp1 receptor with direct relevance to insulin signaling.

4.3 Results

4.3.1 Insulin Instigates Enhanced Nrp1 Expression and Vascular Function.

Vascular Nrp1 expression and functions have been well described in cancer biology (7; 12). To test the response of vascular Nrp1 to circulating insulin, HUVECs and VSMCs were exposed to insulin. Increased Nrp1 expression was evident upon insulin stimulation, with stronger levels observed in endothelial (HUVEC) relative to smooth muscle cells (VSMC) (Fig. 14*A*-*C*). Akt, a critical downstream substrate of the insulin signaling pathway, was activated in response to insulin stimulation in HUVECs as well as VSMCs (Fig. 14*A*). Furthermore, Nrp1

mRNA expression was elevated by insulin in a time and dose dependent manner in HUVEC (Fig. 14*D* and *E*). HFD induced T2D results in increased Nrp1 expression in the endothelial and smooth muscle layers (Fig. 14*F*). To test whether Nrp1 upregulation was specific to insulin, HUVECs were exposed to different growth factors, including IGF-1, EGF, FGF2, HGF, VEGF, and TGFβ1. Results indicated that Nrp1 expression was enhanced further by the presence of several growth factors (Fig. 14*G*) and is consistent with earlier described pleiotropic Nrp1 co-receptor functions (24). However, downstream activation of Akt in response to growth factors was negligible, except for HGF and insulin-treated cells (Fig. 14*G*). Insulin stimulation can cause the activation of ENOS leading to vascular dilation however, Nrp1 silencing inhibitions ENOS which can lead to decreased vasodilation.

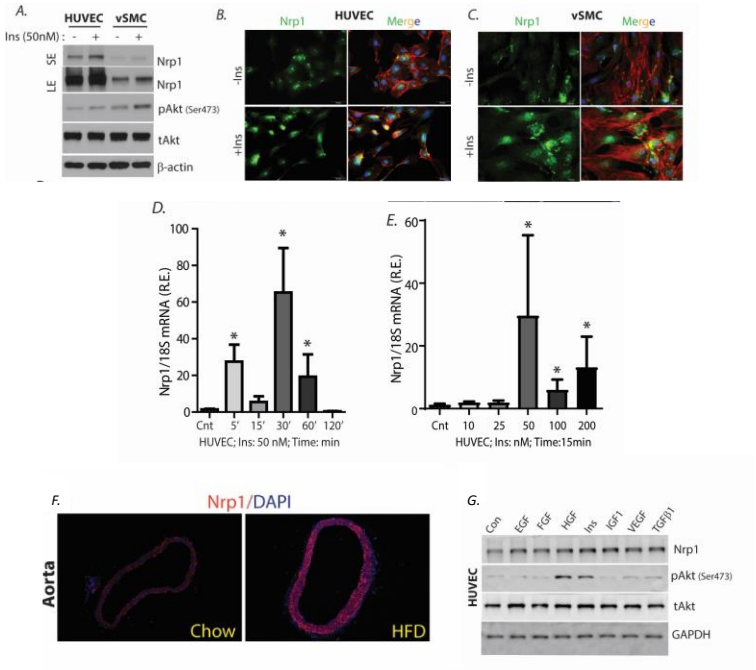

Figure 14: Insulin enhances Nrp1 expression and vascular function

(A) HUVEC and HVSMCs were challenged with insulin (50 nM). Nrp1 and Akt protein levels were assessed by Western blot. (B/C) Nrp1 staining (green) of HUVEC and SMC after

challenging with insulin. Actin filament (red). (D/E) HUVECs were challenged with insulin at different time and doses and Nrp1 mRNA expression was assessed. (F) IF staining aorta of Chow and HFD fed mice for Nrp1 Expression. (G) Immunoblot analysis of HUVECs challenged with different growth factors.

4.3.2 In vivo Deletion of Nrp1 on Obesity-Induced Type II Diabetes

To understand the role that Nrp1 plays in the pathology of T2D *in vivo,* age-matched mice littermates were placed on either a high-fat diet (HFD; ~60kCal) or a normal chow diet (NCD) for 6 months. Body weights were routinely recorded, and blood glucose (GTT) levels were measured following overnight fasting. HFD resulted in a marked increase in body weight and blood glucose levels relative to chow fed mice (Fig. 15*A*-*D*) in both male and female mice. The liver samples of high fat diet fed mice showed strongly upregulated Nrp1 expression (Fig. 2*F*). To understand the effect of Nrp1 deficiency and glucose tolerance *in vivo* $Nrp1^{endo-/-}$ mice were utilized. These mice and their littermates were fed with chow or HFD for ~6months and glucose and insulin tolerance tests (GTT and ITT) were carried out. The results showed that endothelial specific Nrp1 deficiency improved the glucose tolerance (Fig. 15*A*-*D*). IHC staining of Nrp1 in these mice demonstrate an increase in expression after HFD in WT mice compared to $Nrp1^{endo-/-}$ mice. Together, these data suggest that vascular Nrp1 expression and functions are modulated by insulin. Once we determined that Nrp1 deletion could provide a protective effect on T2D mice needed to examine the how Nrp1 modulates insulin pathway activation. Nrp1 was silenced in HUVECs and exposed to insulin a time and dose dependent manner. Nrp1 silencing resulted in elevated Akt phosphorylation (Ser473) compared to control cells. It can be observed that Akt phosphorylation exhibited prolonged activation compared to the sinusoidal

phosphorylation in the control. Thus, demonstrating that Nrp1 can inhibit insulin pathway activation. Finally, we examined the effect of Nrp1 expression in white adipose tissue (WAT), a highly metabolic tissue. T2D resulted in accumulation of Nrp1 in the adipose tissue. As shown via WB analysis of WAT (Fig 19*A&B*).

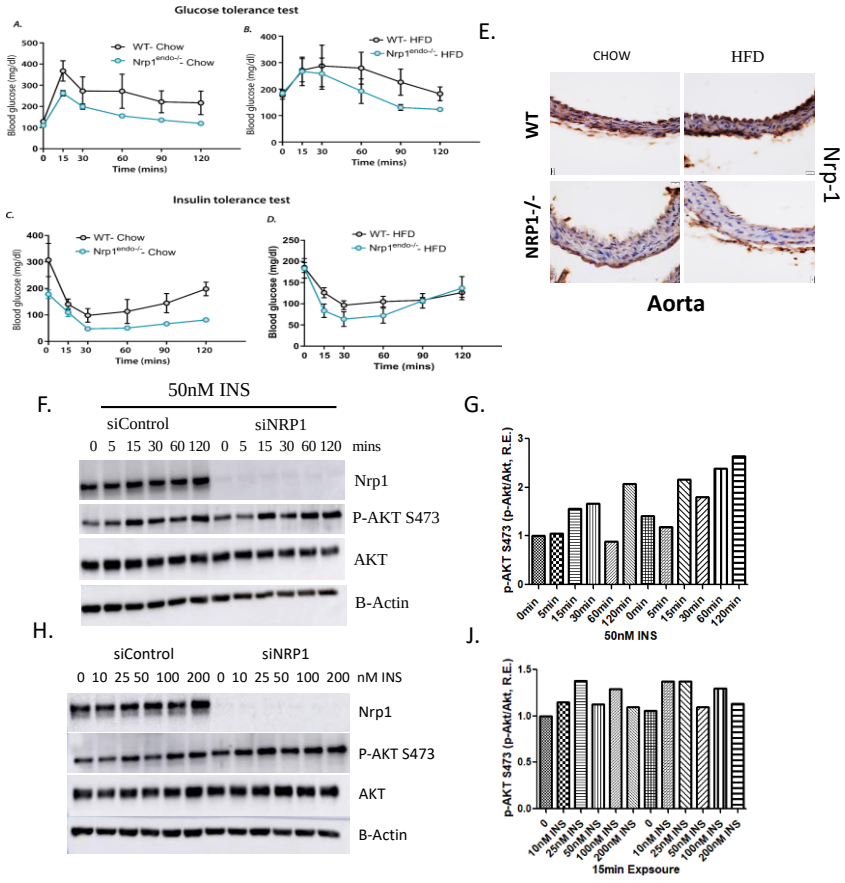

Figure 15: In vivo, Analysis of Nrp1 Expression in Type II Diabetic Conditions

(A-D) Glucose tolerance test for Chow (CD) and high fat diet (HFD) for WT and Nrp1$^{endo-/-}$. (E) IHC of Nrp1 in aortas from WT and Nrp1$^{endo-/-}$ demonstrating Nrp1 is elevated in HFD. (F) Immunoblot for Nrp1, p-AKT S473, AKT, and β-actin for insulin time dependent exposure. (G) Relative p-AKT S473 levels for time dependent exposure. (H) Immunoblot for

Nrp1, p-AKT S473, AKT, and β-actin for insulin dose dependent exposure. (I) Relative p-AKT S473 levels for dose dependent exposure.

4.3.3 Nrp1 Colocalization and Effect on Insulin Pathway Activation in Endothelial Cells

Potential interaction of Nrp1 with insulin-related substrates was explored beyond the upregulated expression of Nrp1 in response to insulin. First, co-localization of Nrp1 with insulin receptor β (IRβ), insulin-like growth factor 1b receptor (IGF1Rβ), and IRS-1 was analyzed in HUVEC cells. Relative to non-stimulated cells, Nrp1 upregulation and enhanced co-localization with IRβ was evident upon insulin stimulation (Fig. 16*A*). A similar trend was confirmed between Nrp1 and IRS-1, and Nrp1 and IGF1Rβ (Fig. 16*B-C*). These data indicate increased interaction between Nrp1 and insulin-related targets in response to insulin stimulation.

To further unravel the complex signaling interplay mediated by the availability of several upstream insulin receptors, we focused on immediate IRS-1 downstream substrate of the insulin receptors, given its well-established roles in cancer biology (22). Once we identified the colocalization of Nrp1 and IRS1 the next step was to determine if there was direct interaction. Also, insulin stimulation results in increased IRS1 phosphorylation at Ser612 recapitulating previously reported data. Pulldown of IRS1 was utilized to determine if there was a direct interaction with Nrp1. In HUVECs, the interaction was confirmed following the pull-down of IRS-1 under basal conditions and immunoblotting for Nrp1 (Fig. 16*D*). Thus, lending evidence that Nrp1 has direct effects on the insulin signaling pathway. Further analysis

of this interaction is necessary to understand the function. Given the abundance of Nrp1 expression in H1792 cancer cells, a similar pull-down of IRS-1 protein was performed in the presence or absence of insulin stimulation. Immunoblot of Nrp1 again demonstrated close interaction with IRS-1 in insulin-treated or untreated cells (Fig. 20*A*). However, reverse immunoprecipitation of Nrp1 showed decreased interaction with IRS-1 within insulin-treated cells (Fig. 20*B*). The data suggest decreased availability of IRS-1 protein following stimulation with insulin. IRS-1 possess multiple phosphorylation sites that mediate the complex regulation of specific downstream targets (28). In vascular cells, the IRS-1/PI3K/Akt pathway has been linked with endothelial function *via* insulin-mediated synthesis of eNOS (28). It was determined that Nrp1 alters activation of eNOS in a time and dose dependent manner (Fig. 20*C&D*). Specifically, phosphorylation of IRS-1 at serine-612 has been implicated with dissociation of p85 subunit of PI3K, resulting in further inhibition of signaling (28).

When it comes to insulin signaling, we need to first examine the effect loss of Nrp1 has on activation of IRβ. Nrp1-silencing results in alterations activation of IRβ at activation sites Y11146, and Y1150/1151 in a time dependent manner. Y1150/1151 phosphorylation was prolonged after Nrp1 loss, and Y1146 activation was blunted (Fig. 16*E*). Nrp1 appears to alter the phosphorylation and activity of IRβ. Dose dependent insulin stimulation also alters the phosphorylation states of IRβ after Nrp1 loss (Fig. 16*F*). IRβ phosphorylation directly activates downstream receptors that propagate insulin signals such as IRS1. We, therefore, further analyzed phospho-IRS1 (Ser612 & Ser636/639) in Nrp1-expressing or deficient HUVECs following time-dependent insulin stimulation. As shown above of Nrp1, insulin stimulation enhanced further the expression of Nrp1 but attenuated phosphorylation of IRS-1 (Fig. 15*E* and Fig. 16*E*). In Nrp1-depleted cells, phospho-IRS-1 was decreased but demonstrated stronger

activation of Akt in response to insulin (Fig. 14*F&H*). Finally, dose-dependent insulin stimulation elicited a similar enhanced IRS-1 and Akt activation pattern in Nrp1-deficient cells relative to Nrp1-expressing cells (Figure 14*H* & 16*F*). Upregulated Nrp1 expression in response to dose-dependent insulin stimulation correlated with diminished phospho- IRS-1 and elevated Akt (Fig. 16*F*). These data suggest that Nrp1 attenuates the canonical insulin signaling pathway and uncovers novel connections between Nrp1 and insulin signaling.

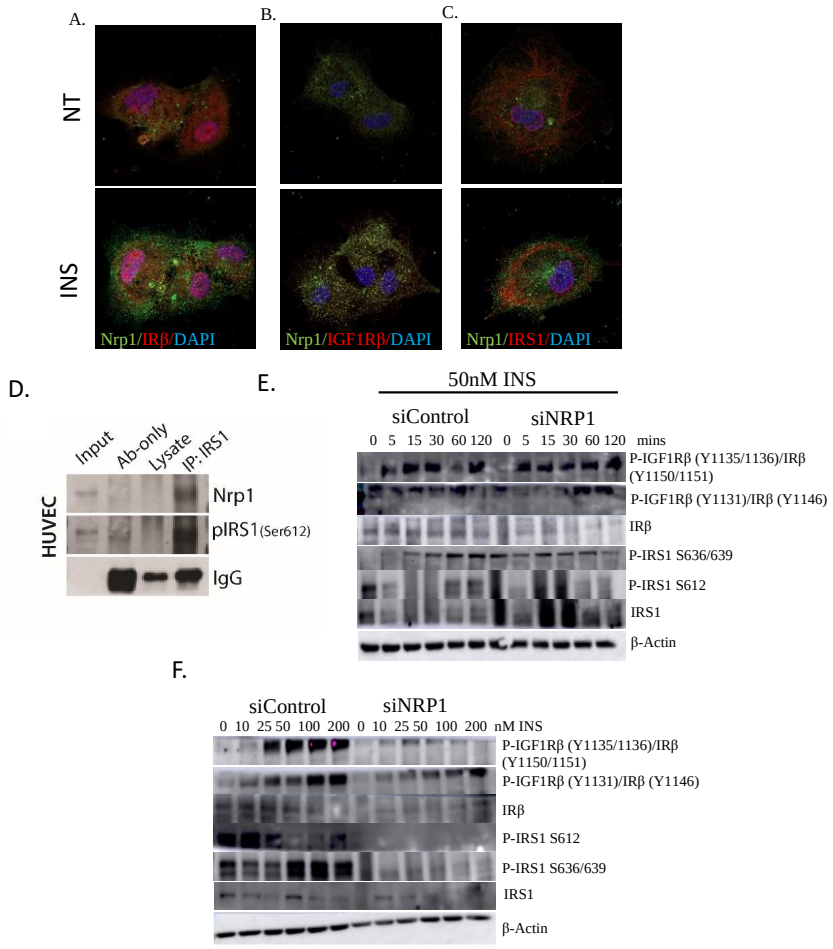

Figure 16: Nrp1 modulates insulin signaling pathway

(A) IF costaining of HUVECs for Nrp1 (Green) and IRβ (red) (B) IF costaining of HUVECs for Nrp1 (Green) and IGF1Rβ (red). (C) IF costaining of HUVECs for Nrp1 (Green) and IRS1 (red). (D) At basal condition the HUVEC cells homogenates were used to pull down the pIRS1 (Ser612) and Nrp1 binding was demonstrated. (E) Examination of insulin signaling pathway perturbations in a time dependent manner. (F) Examination of insulin signaling pathway perturbations in a dose dependent manner

4.3.4 Nrp1 Attenuates the Availability and Distribution of Intracellular Insulin.

To gain further insights into novel associations between Nrp1 and insulin family members, FITC-labelled insulin was employed either alone or following 15 minutes pre-treatment of serum-starved HUVEC cells with unlabeled insulin. As expected, non-treated and unlabeled cells displayed negligible signal detection by flow cytometry (Fig. 17*A&B*). However, FITC-insulin treatments displayed attenuated signal detection in Nrp1-expressing HUVECs relative to Nrp1-deficient cells (Fig. 17*A&B*). Conversely, when cells were pretreated with unlabeled insulin before FITC-insulin stimulation, signal detection in the presence or absence of Nrp1 was attenuated (Fig. 17*A&B*). The data suggest that pretreatment with unlabeled insulin promotes internalization of cell surface insulin receptors, resulting in depleted insulin receptor and availability for binding upon subsequent stimulation with FITC-labeled insulin. Subsequent gain-of-function experiments employed transfection of Nrp1-expressing plasmid in H1299 cells. Interestingly, a similar trend of attenuated FITC-insulin signals was detected in Nrp1-expressing cells relative to empty-vector controls (Fig. 16*C*&D). Immunofluorescence microscopy further revealed a distinct perinuclear distribution of FITC-insulin in Nrp1-deficient cells; however, the distribution pattern was disrupted by the presence

of Nrp1 expression (Fig. 16*E*). Taken together, dispersed insulin distribution and attenuated availability in the presence of Nrp1 support the modulation of insulin signaling by Nrp1.

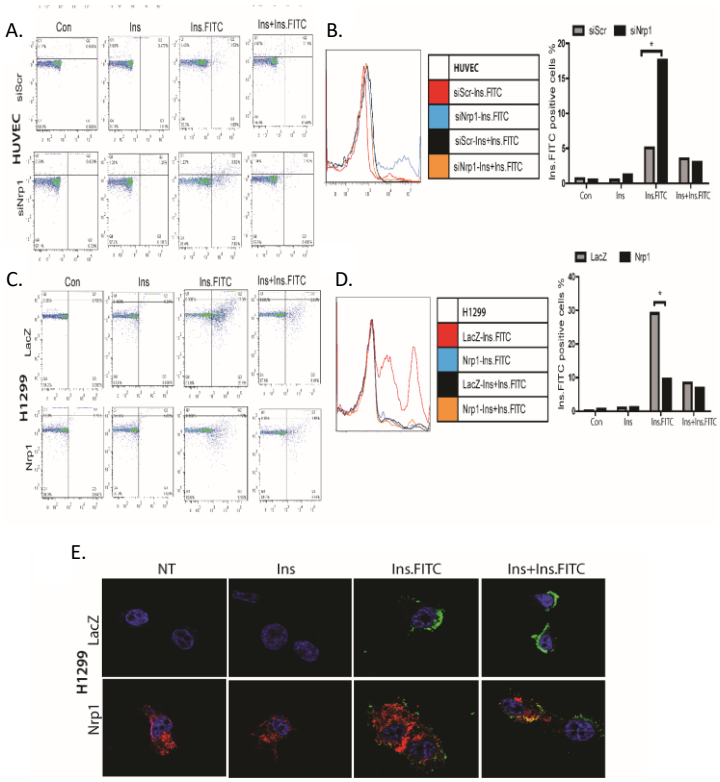

Figure 17: Nrp1 attenuates availability and distribution of intracellular insulin

(A,B). FACS analysis and measurement of insulin internalization after siRNA mediated depletion of Nrp1 in HUVEC cells. (C,D) FACS analysis and measurement of insulin internalization after Nrp1 overexpression in Nrp1-deficient H1299 cells. (E) H1299 cells were transfected with Nrp1 overexpressing plasmid and stimulated with insulin. FITC-conjugated insulin was further used to observe the localization of Nrp1. Nucleus (DAPI, blue), Insulin-FITC (green) and Nrp1 (red).

4.3.5 Insulin Promotes Nrp1 Upregulation in Cancer Cells

Based on established links between Nrp1, cancer, and vascular tissue (25-27), we analyzed the response of Nrp1 to insulin stimulation across multiple cancer cells. First, heightened Nrp1 protein expression was confirmed in H1792 lung cancer cells relative to metabolically relevant adipose cells (Fig. 18*A*). Next, we validated basal Nrp1 expression in multiple cancer cell lines and MRC9 cells (non-cancer lung cells). With exception of H1299 cells, varying Nrp1 protein levels were detected across several other cell lines including MRC9, A549, H1792 and H1650 (Fig. 18*B*). Subsequently, transient Nrp1 deletion was performed in HepG2, A549, H1650, and H1972 cells and exposed to insulin (Fig. 18*C&D*). The results consistently demonstrated insulin-specific upregulation of Nrp1 expression (Fig. 18*C*&*D*). Surprisingly, Akt activation in response to insulin was blunted in Nrp1-expressing HepG2 cells, suggesting that Nrp1 may interfere with insulin-mediated signaling (Fig. 18*C*). Gain-of-function analysis involved exogenous Nrp1 expression in H1299-deficient cells; however, Nrp1 levels were comparable in the presence or absence of insulin (Fig. 18*D*). At the mRNA level, rapid Nrp1 upregulation was evident by insulin stimulation in HepG2, H1792, H1650 and A549 cells (Fig. 18*E*&*F*). Conversely, negligible Nrp1 mRNA expression was observed following insulin stimulation of LacZ-transfected H1299 cells but peaked at 60 minutes upon exogenous Nrp1 transfection (Fig. 15*H*). Together, these data strongly suggest modulation of Nrp1 expression by insulin.

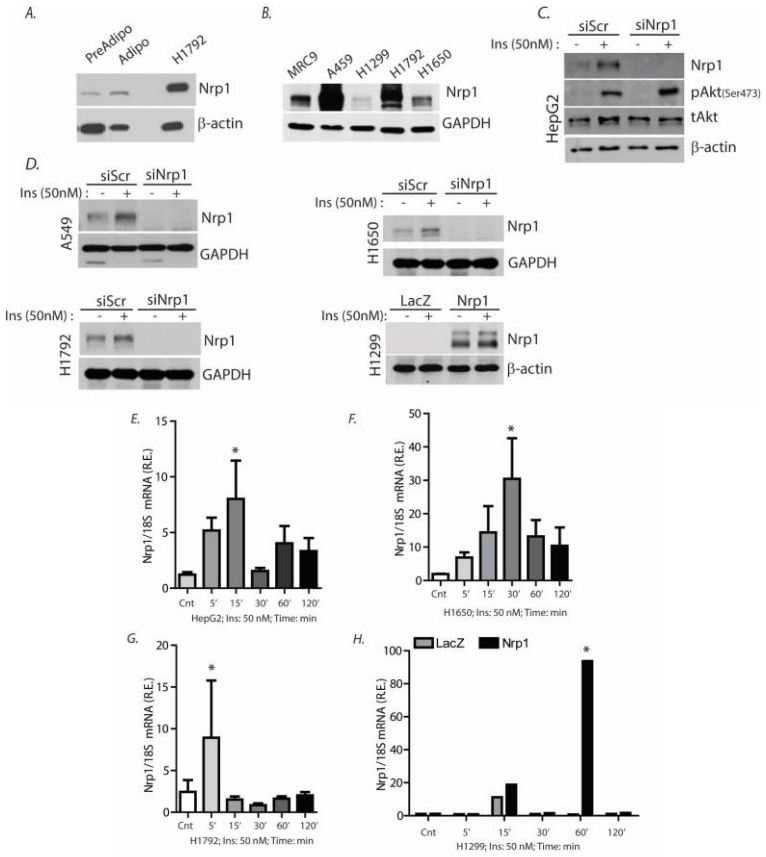

Figure 18: Insulin promotes Nrp1 upregulation in multiple cells

(A) Immunoblot analysis of Nrp1 expression in pre and post adipocytes, with control H1792 lung cancer cells. (B) Immunoblot analysis of Nrp1 expression across multiple cancer cell lines (A459, H1299, H1792 and H1650). H1299 is Nrp1-deficient, and MRC9 is non-cancer lung cell line. (C/D) HepG2, A549, H1650 and H1792 cells were transfected with siNrp1 to silence the Nrp1 expression and challenged with insulin. Nrp1 and GAPDH expressions were assessed by Western blot. Exogenous Nrp1 expression was undertaken in H1299-deficient cells. (E-G) Nrp1 mRNA in response to insulin stimulation across multiple cancer cell lines. (H) Insulin stimulation following exogenous Nrp1 expression in H1299-deficient cells.

4.4 Supplementary Figures

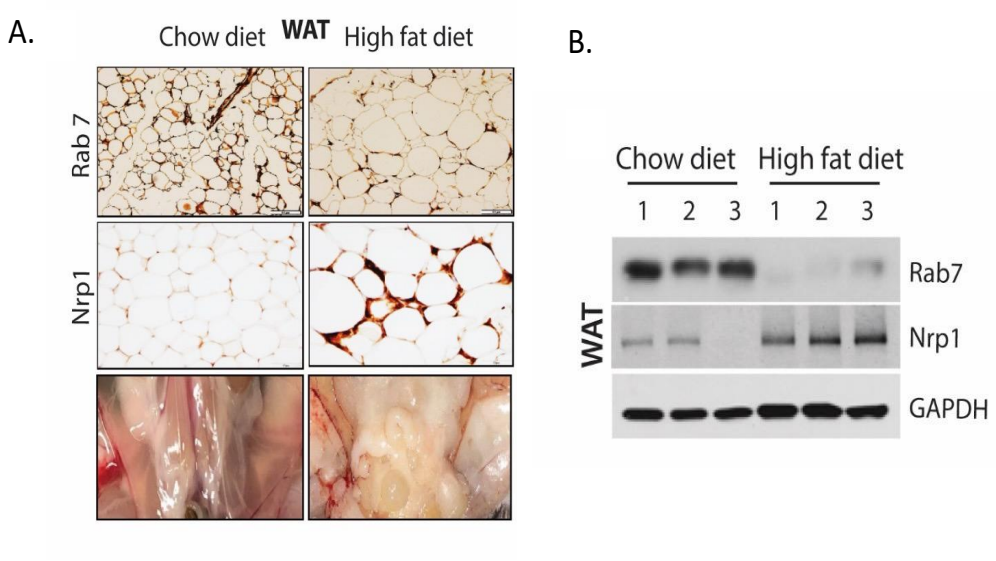

Figure 19: High Fat Diet Effect on Nrp1 Expression

(A&B) High Fate Diet results in accumulation of Nrp1 in WAT.

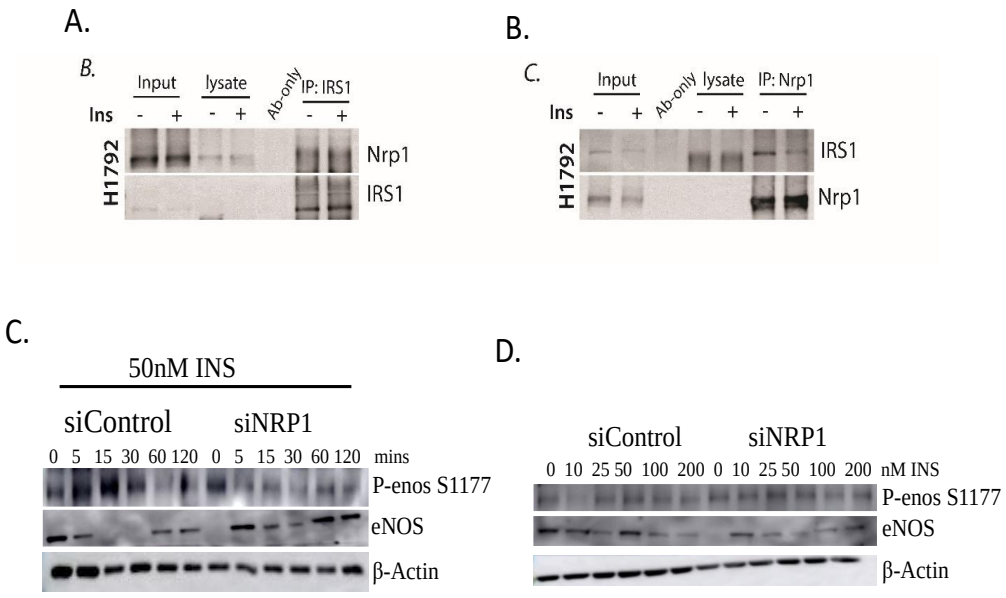

Figure 20: Nrp1 Interaction with IRS1

(A) H1792 cells challenged with insulin (50nM; 15min) and IRS1 was pulled down and Nrp1 binding was confirmed **(B)** H1792 cells challenged with insulin (50nM; 15min) and reverse pull down was done for Nrp1 and IRS1 binding was confirmed. **(C**) Time dependent analysis of p-ENOS S1177. **(D)** Dose dependent analysis of p-ENOS S1177 due to Nrp1 silencing.

4.5 References

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5 DISCUSSION AND CONCLUSION

5.1 Indoxyl Sulfate Effect on Vascular Repair

Vascular complications are a known hallmark of CKD progression (1). This study demonstrated that uremic toxin IS directly inhibited vascular regeneration and arteriogenesis through alterations to the actin cytoskeleton, disruptions to actin polymerization, loss of focal adhesions, and cellular attachment. IS-exposure drastically altered the expression of canonical focal adhesion proteins FAK, and Paxillin in both *in vitro* and *in vivo* models. These major components of focal adhesions are necessary for proper formation and structure. Loss of focal adhesions, due to uremic toxin accumulation, will interfere in response to extracellular stimuli necessary for vascular regeneration. Impaired vascular regeneration due to the loss focal adhesions would account for data from CKD mice which exhibited defective blood flow restoration. Understanding how focal adhesion are involved in vascular regeneration during CKD progression is necessary.

Vascular regeneration and arteriogenesis are necessary processes responsible for blood vessel expansion and repair of damaged vessels. Vascular damage would lead to multiple complications including but not limited to ischemia, necrosis, amputations, infections, and ultimately patient mortality (2). Blood vessel expansion is achieved through the recruitment of VSMCs, which provide structural support. Without this support vessels would experience blood leakage into tissues and decreased extremity flow. Recovery of CKD patients would experience increased recovery time or in extreme cases infection, amputation, and death (2-3). Examination of the role CKD and the resultant accumulation of uremic toxins has on vascular regeneration is necessary

Particular attention was paid to FAK which plays roles in cytoskeleton formation, activation, cellular motility, and potentiation of extracellular signals. IS-induced loss of FAK directly contributed to lost motility in VSMCs. VSMCs that are defective in migratory ability would significantly contribute to impaired vascular regeneration and arteriogenesis. Thus, our data lent compelling evidence that CKD-induced loss of FAK has a direct effect over vascular regeneration. Understanding that loss of FAK plays a significant role in impaired motility the next step was to examine downstream effectors interact with actin. Examination demonstrated that N-WASP was significantly decreased by CKD progression and IS-expsoure. Wiskott-Aldrich Syndrome (WAS) a known genetic condition that is characterized defective actin fiber formation. N-WASP is a member of the (WAS) family of proteins and deficiency can lead to similar abrogation's of the actin cytoskeleton. However, abrogation of the actin cytoskeleton does not rest with N-WASP alone. N-WASP activates the Arp2/3 complex, a well described actin regulation complex. Alterations to Arp2/3 activation or expression as, demonstrated in our analysis, would directly inhibit actin fiber formation (4-5). Knowing, that IS-exposure and CKD progression negative impact activation of actin polymerization, our observations of inhibited actin polymerization and altered morphology are understandable. Without input from control pathways actin would be unable to respond to impaired stimulation and will exacerbate vascular complications. Taken together, our data demonstrated that CKD progression has a negative impact on focal adhesion formation all the way cytoskeletal control. If these pathways are inhibited, then VSMCs would be able to participate in vascular regeneration and arteriogenesis necessary for blood flow restoration and prevention of ischemic tissue injury.

Demonstration of the adverse effects CKD progression and IS accumulation has on vascular regeneration provides insights into previous clinical reports of vascular damage. CKD

patients who were afflicted by multiple vascular complications that can be related to impaired vascular regeneration. Firstly, CKD patients presenting with AAAs, and have undergone surgical repair, are increased mortality risk due to repair failure. Secondly, vascular canula for hemodialysis of CKD patients are vulnerable to vessel leakage (6). Finally, CKD/diabetic patients who undergo extremity amputation decreased would healing and elevated rates of mortality (7). Our data could provide insights into the molecular basis for these phenomena, from the loss of FAK to impaired actin polymerization, and provide a window into possible mitigation strategies or treatments.

By demonstrating that uremic toxins directly impact vascular health and repair capacity it becomes necessary to postulate future mitigation strategies. We in this study detail an outcome that has been observed partially in clinical settings but has not been examined on a mechanistic, or phenotypic level. Renal specialists have noticed numerous complications that arise from vascular injuries but have not completely confirmed a link between CKD and defective repair mechanisms. By understanding how this process occurs due to IS accumulation we can then better direct treatments and possibly decrease the mortality rate in CKD patients further. These can be achieved through employing new dialysis methods to remove uremic toxins or direct inhibition of IS production by the liver by targeting sulfotransferase Sult1A1 (8-10). Inhibition of this enzyme will decrease the levels of circulation IS and mitigate the damage that could occur. By understanding the role IS plays in vascular injury it is hoped that this data will open new therapeutic avenues to treat or in certain cases prevent the injury all together. Along with inhibiting the formation of IS by sulfotransferases it is possible to prevent precursor formation via tryptophan restricted diet. However, tryptophan depletion as a treatment model is controversial. Data has demonstrated both beneficial and adverse complications that arise from dietary restriction

(11-12). Tryptophan as an essential amino acid is necessary for the productions of multiple biologically relevant metabolites. Insufficient level of tryptophan lead to alterations in metabolite production and can have adverse effects on mental health and sleep. Understanding how to regulate the role and function of tryptophan and its metabolites is essential for effective treatment. More work is necessary to elucidate the roles each metabolite has in CKD pathology.

When examining arteriogenesis for this study there were a few limitations for this study. Currently there are short coming in fully examining arteriogenesis due to the simple reason no sufficient model has been found to allow direct examination of the process. *In vitro* models are insufficient in replicating arteriole formation due to lacking a viable matrix to induce formation. Arteriole formation is a complex process that involves first capillary formation through single layer endothelial cells followed by expansion support from VSMCs. However, there are many questions that remaining concerning the exact formation and environmental components necessary to achieve vessel expansion and blow flow capacity. We are also limited in understanding CKD mitigation strategies including, hemodialysis and oral sorbents to remove uremic toxins from the intestinal tract, will influence observations in this study.

In summary, our study has provided compelling evidence that uremic toxins, *in vitro* and *in vivo,* have deleterious effects on vascular regeneration and arteriogenesis. By elucidating the molecular mechanisms altered by IS-exposure, our study provides novel insights into CKD progression and opening new avenues for treatment strategies.

5.2 Nrp1 Effect on Vascular Endothelial cell Response to Insulin Signaling

In this study, we demonstrate that obesity induced T2D, or elevated insulin secretion promotes the expression of oncogene Nrp1 in vascular, adipose, and liver tissues, as well as

vascular and cancer cells. The resulting increase of Nrp1 availability promotes further interaction with insulin receptors and substrates, thereby modulating insulin signaling functions in cancer and metabolically active tissues. As observed in our findings, Nrp1 responds to insulin exposure in vascular and epithelial cancer cells. Therefore, increased Nrp1 expression under obese conditions can alter the activity and responsiveness of insulin receptors. In a process reminiscent to a negative feedback mechanism, Nrp1 acts as a roughe receptor protein that interferes with insulin substrates, culminating in the modulation of insulin signaling functions. This sequence of events supports metabolic dysregulation, such as insulin resistance and oncogenic signaling and proliferation of cancer cells. Collectively, our data for the first time uncover novel Nrp1 functions at the nexus of obesity, insulin resistance and cancer metabolism with profound implications for diagnostics and therapeutics.

Across multiple cell types, including vascular (HUVEC and VSMC) and cancer cells, insulin stimulation resulted in rapid upregulation of Nrp1 at the mRNA and protein levels. Co-localization of Nrp1 with insulin substrates, namely IGF1Rβ, IRβ and IRS-1 enhanced aggressive Nrp1 colocalization. Novel links between Nrp1 and insulin family members are highly significant, directly relevant to several pathological conditions. Further analysis of insulin receptors demonstrates that Nrp1 has a direct effect on activation and propagation of signals. As alterations to the insulin signaling occur due to Nrp1 loss this can open new avenues for understanding its role in T2D. Recent global events have demonstrated the importance of understanding the broad functions that Nrp1 is involved. Nrp1 acting as a coreceptor to ACE2 for Sars-CoV-2 infectivity there is new urgency to delineate the metabolic implications these interactions are involved in. Based on our findings it is necessary to examine the effects that newly discovered Nrp1 pharmacological inhibitors that have been utilized to impeded Sars-

CoV-2 infections. If these inhibitors can ameliorate the symptoms of T2D then we will have opened new avenues to treat this ever-increasing disease condition.

The high fat diet fed, endothelial specific Nrp1 deficient mice showed improved glucose tolerance. This finding significantly impacts current knowledge relevant to disease etiology, diagnosis, and treatments of metabolic disorders associated with T2D. For example, vascular and metastatic alterations that promote tumor angiogenesis depend on the utilization of cellular nutrients that support the rapid development and spread of vessel networks (13-14). Beyond maintenance of glucose and lipid homeostasis, insulin promotes vasodilatation via PI3K/Akt mediated activation of endothelial nitric oxide (NO) synthase (eNOS) (15). Loss of Nrp1 decrease activation of eNOS within endothelial cells. If this blockage occurs, due to the loss of Nrp1, vasodilation will not occur preventing nutrient flow to cancer cells, or augment tumor microenvironments and metastasis. Independently, Nrp1 and insulin substrates have been implicated in multiple cellular pathways, including mTOR, which plays established roles in autophagy and cancer (16-17). As Nrp1 is an oncogene and obesity/diabetes are known to have elevated risks of cancers, it is plausible that insulin (growth factors) induces Nrp1 expression resulting in insulin resistance and elevated risk of cancers.

For the first time, these findings implicate Nrp1 as a novel insulin receptor and expand further the repertoire of growth factors that interact with Nrp1 to mediate cellular events. However, additional studies are required to fully understand confounding observations. Wilson *et al*, reported that Nrp1 expression in adipose tissue macrophages (ATMs) protect against obesity and metabolic syndromes (18) and is consistent with our study in myeloid-specific Nrp1 deficient mice (19). Secreted factors influenced by obesity include circulating free fatty acids (20). However, cellular responses by ATMs may be context dependent. Given the lethality of

global Nrp1 deletion, tissue-specific Nrp1 ablation in macrophages, endothelial and adipose cells (*in vivo* and *in vitro*) will be necessary to decipher cell-specific Nrp1 functions completely.

Furthermore, specific Nrp1 protein domain(s) critical for interaction with insulin substrates, as well as modulation of insulin signaling requires elucidation. Potential codomain cooperativity between SH3-PDZ domains of Nrp1 and effector binding sites of insulin-related substrates, such as IRS-1, will be investigated. To this end, we have generated Nrp1 constructs that lack cytoplasmic and/or extracellular domains to clarify specific regions involved with insulin substrates. Additionally, the contributions of various phosphatases that inhibit aberrant insulin phosphorylation remain unknown (21). Findings from this study raise thought-provoking questions related to the expression and function of Nrp1 in obese or insulin-resistant cancer patients. As growth factors such as HGF and insulin can activate PI-3 kinase and PI3 kinase is known to promote cancers, increased Nrp1 expression in insulin/HGF-stimulated cells might be PI-3 kinase-dependent. Thus, clarifying the contribution of PI-3 kinase in growth factors-induced Nrp1 is essential.

Elucidation of the role Nrp1 plays in insulin signaling will open new avenues of treatment. Recent developments in Nrp1 inhibitors could provide effective treatments to improve T2D progression.

5.3 CONCLUSION

Prevalence of chronic kidney disease has been steadily increasing over the last couple of decades to where it affects approximately 1 in 7 Americans. Examination and understanding of underlying conditions and subsequent complications will provide valuable knowledge during treatment. Firstly, we demonstrate that accumulation of uremic toxin IS results in defective vascular repair potential. Disruption of focal adhesion related proteins, namely FAK, leads to

decreased actin polymerization and decreased wound repair. Patients have been observed exhibiting adverse outcomes after various vascular injuries from lower extremity amputations to AAA repairs. Understanding the deleterious effects of uremic toxin accumulation on CKD patients allows for targets treatment strategies. Along with examining the effects of CKD it is important to examine comorbidities, including Type II Diabetes, associated with disease progression.

Type II Diabetes a condition known to be exacerbate CKD progression was examined. T2D prevalence has exploded over the last couple of decades. We demonstrate that as T2D progresses there is a significant increase in oncogenic protein Nrp1. Increased expression of Nrp1 leads to alterations in the insulin signaling pathway and exacerbation of T2D. However, we demonstrated that loss of Nrp1 could help protect against T2D and lessen the symptoms. Recently, there have been multiple Nrp1 inhibitors developed to help combat Sars-CoV-2 infections. Examination of the effectiveness of these inhibitors could open new strategies to treat T2D and help prevent CKD progression.

5.4 References

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