1-1-2016

Gap and Tight Junction Stabilization in Cardiac Transplantation

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by

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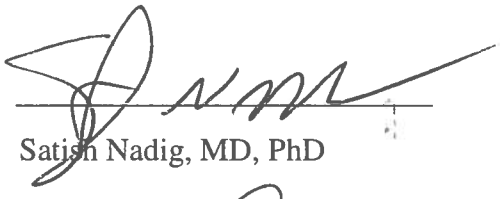
A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Master of Science in the College of Graduate Studies.

Department of Microbiology and Immunology

2016

Approved by:

Carl Atkinson, PhD, Chairperson



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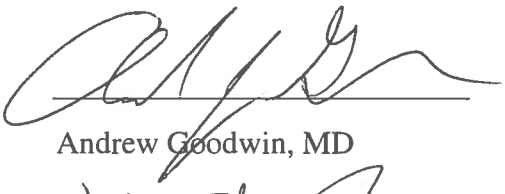

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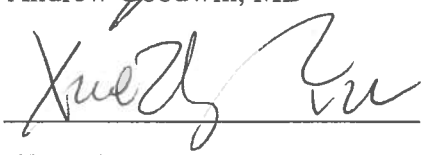

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Acknowledgements

I would like to express my deepest gratitude to my mentor Dr. Carl Atkinson for his continual support and guidance. I would also like to thank my committee members Drs. Satish Nadig, Laura Kasman, Xue-Zhong Yu, Andrew Goodwin for their feedback and helpful discussions. Additionally, I would like to thank my lab members for the occasional assistance.

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Abstract

Purpose: Organ procurement, cold storage and ischemia reperfusion injury (IRI) promote inflammation, which induces endothelial cell (EC) activation and dysfunction post transplantation. EC gap junctions (GJs) breakdown as a consequence of these injuries and play a key role in graft injury post transplantation. Here we explore the therapeutic potential of adding a novel gap junction (GJ) stabilizing peptide, ACT1, to UW preservation solution as a therapeutic agent to improve endothelial cell health. ACT1, is a small peptide Cx43 mimetic, which impairs the association of ZO-1 with Cx43 thus promoting GJ integrity.

Methods: Mouse cardiac ECs (MCECs) were exposed to 6 hrs of cold storage in UW or UW/ACT1 solution followed by reperfusion to mimic clinical cold storage and reperfusion. Efficacy was determined by trans-endothelial electrical resistance (TEER), a measure of GJ function, cell viability assays, and ELISAs for pro-inflammatory cytokines. In-vivo, utilizing cardiac and aortic allograft models, Balb/c donor hearts and aortas were stored in UW or UW/ACT1 for 6 hrs prior to transplantation into C57Bl/6 recipients. Grafts were harvested 48 hrs post-transplant, in the cardiac allograft model, to determine cardiac graft ischemia reperfusion as measured by serum cardiac troponin I and histological analyze. For aortic grafts, aortas were harvested at 28 days to determine the impact of graft pre-treatment on the development of chronic rejection. Efficacy was again determined by pathological indices. Grafts, hearts and aortas, were

assessed for innate inflammatory cells (neutrophils and macrophages) and T cell infiltration by immunohistochemistry at their specified harvest time points.

Results: In-vitro studies demonstrated that UW/ACT1 solution significantly reduced EC injury and inflammation, as measured by TEER, cell viability and ELISA. In-vivo studies showed that ACT1 pretreatment of the donor heart led to a reduction in IRI, as noted by reduced serum troponin and histological analysis. Furthermore, donor aortic graft pretreatment with ACT1 reduced the size and development of chronic rejection as shown by histological matrices. Subsequent analysis of neutrophil, macrophage, and T cell infiltration showed pretreatment significantly reduced graft infiltrates, acutely (48hrs) and chronically (28 days).

Conclusion: Taken together these novel findings propose a role for GJ in the pathogenesis of cold storage IRI, and further demonstrate that stabilization of GJ with a novel connexin 43 mimetic, ACT1, significantly inhibits post-transplant IRI, and the later development of chronic rejection.

1 Introduction

1.1 Overview

Organ transplantation is an accepted therapeutic modality in treating end-stage organ disease. According to the Global Observatory on Donation and Transplantation (GODT), there were approximately 106,879 solid organ transplants performed worldwide: 5582 heart, 3927 lung, 21,602 liver, 73,179 kidney, and 2,362 pancreas transplants reported in 2010 from an analysis of 95 countries representing 90% of the worldwide population [1]. Additionally, there were approximately 2,655 heart, 1,925 lung, 6,729 liver, 17,107 kidney, and 245 pancreas transplants occurring in the U.S. alone in 2014 (Based on OPTN data as of December 18, 2015) [2]. While there has been an increase in the number of transplant centers and people awaiting organ allografts, the number of available organs has not changed over the past 20 years [3]. Since the inception of transplantation as a bona fide field of practice, there has been an enormous amount of focus on improving the longevity of an allograft. Although many strides have been made in this arena over the last few decades the rate of chronic graft failure remains the same. The issues of organ availability and allograft survival are two of the most pressing issues that face transplantation today.

Chronic rejection, which develops over several months to years, can be categorized as either chronic parenchyma cell rejection, induced by immune mediated replacement fibrosis of the parenchyma in the graft, or chronic vascular rejection, induced by immune-mediated host response to the blood vessels of the graft, resulting in stenosis of these vessels [4]. Chronic graft failure is

multifactorial and, along with the obvious immunologic causes, graft loss is also related to alloimmune independent mechanisms as well as recipient related risk factors[5]. Robust investigations centered around the dampening of antigen presentation by professional antigen presenting cells (APC) to T and B lymphocytes have formed the basis of the current standard of care in immunosuppression. Although transplantation immunology has made many strides in blunting immune responses that are initiated by lymphocyte-APC interactions, it is becoming clear that early insults to the endothelium of graft are deleterious to the long-term outcome of transplanted organs.

Functions of the Endothelium

The endothelium is the first point of contact between the donor and recipient in transplantation and early insults in the transplantation process prime the endothelium for susceptibility to injury, and impact the entire allograft[6]. The endothelium is a multifunctional monolayer vascular lining that represents the initial barrier of recipient to graft communication. Endothelial cells (ECs) control the passage of various proteins, along with the passage of leukocytes into and out of the bloodstream and are capable of antigen presentation to the host. The barrier function of ECs is critical to natural immunity as well as graft tolerance and is accomplished, in part, through the coordinated opening and closing of intracellular cell to cell junctions[7-9]. ECs exhibit a tight regulation of the intracellular passage of molecules in order to maintain the integrity of the

endothelial lining and to protect from increased permeability leading to inflammation, fibrosis and thrombosis[10]. Endothelial cells have various functions such as programming the immune system, regulating blood pressure through vasoconstriction/vasodilation and act as antigen presenting cells. Strong regulation in membrane integrity is also essential in maintaining normal organ function and diminishing this barrier function may lead to organ dysfunction, resulting in serious pathological consequences such as neurodegenerative disorders, pulmonary edema, angioedema, ischemic stroke,[11] sepsis,[12] and cancer [8, 13, 14].

Immunology of the Endothelium

Many of these pathological conditions are induced by infiltrating leukocytes such as neutrophils, lymphocytes, monocytes and platelets into the permeable endothelium. Immune cells, such as neutrophils that interact with endothelial cells release mediators, such as cytokines, proteolytic enzymes, and reactive oxygen species (ROS), impairing endothelial barrier function. When exposed to ROS, the permeability of endothelial cells is greatly compromised, which has been linked with disruption of endothelial junctions, gap formation, actomyosin contraction, as well as an alteration of junctional adhesion molecule expression and phosphorylation [15-17]. The effects on the barrier function of ECs by ROS has also been linked to the bioavailability of nitric oxide (NO), an endothelium-derived relating factor (EDRF), which has been associated with both positive and

negative modulation of endothelial barrier function [18-20]. NO demonstrates a protective role by inhibiting the adhesion of leukocytes to endothelial cells and blocking NO synthase disrupts adherens junctions, increasing permeability of the endothelium [21, 22]. NO, endothelin, angiotensin II, and prostacyclin are the physiologic vasoregulators in the endothelium, allowing for the ability to detect of shear stress in the bloodstream [6, 23, 24]. Endothelial NO and endothelin have been shown to interact with inflammatory effectors[25, 26]. The vasodilatory effects of NO are mediated through multiple pathways such as cyclic guanosine monophosphate, inhibition of the endothelin, and redox-sensitive potassium channel interactions[6, 27]. These interactions with inflammatory effectors promote the migration of effector and regulatory T cells into the graft, essentially priming the recipient for rejection. Acetylcholine, a neurotransmitter associated with endothelium derived contracting factor (EDCF)-mediated responses, exhibits protective effects on cardiomyocytes from ischemia reperfusion injury (IRI) by inhibition of the formation of ROS, downregulation of inflammatory markers, as well as improving mitochondrial biogenesis [28-30]. While the anti-inflammatory effects of acetylcholine have not been extensively studied on endothelial cells, recent studies have demonstrated this neurotransmitter to be an effective modulator of mitochondrial and EC crosstalk as well as inducing endothelium-dependent, cyclooxygenase-dependent contractions in blood vessels [29-31]. Along with EDCFs, endothelium derived relaxing factors (EDRFs) are important for the endothelium to induce relaxations/dilations of the vascular smooth muscle for hyperpolarization.

Under normal conditions, the endothelium has the ability to inactivate the complement proteins. When the endothelium is damaged, activated complement proteins bind to receptors on the endothelium promoting inflammation, vasoregulation, and coagulation. Antithrombin III and thrombomodulin, are contributors to the anticoagulation properties of the endothelium and endothelial dysfunction has been shown to be associated with the downregulation of these proteins [32]. Thrombin, an essential enzymatic component in coagulation and blood clotting, has additional proinflammatory and mitogenic roles which are mediated through transmembrane signaling by utilizing protease-activated receptors expressed on the surface of endothelial cells [33, 34].

Acting as semi-professional antigen-presenting cells (APCs)

ECs interact with their environment by upregulating cell surface markers that interact with pathogens or leukocytes, resulting in the binding and migration of lymphocytes into the tissue through the vessel wall. Interferon-gamma (INFy) has been shown to induce the upregulation of major histocompatibility complex (MHC) class II molecules, resulting in the activation and proliferation of T-cells [35, 36]. Human endothelial cells express MHC class I and II molecules to allow for cross-presentation of exogenous antigens [36, 37]. Mouse cardiac endothelial cells (MCECs) only express MHC class II when stimulated by interferon. Once stimulated by ECs, a secondary signal is required by CD80 and CD86 to CD28, up regulated on naïve and activated T-cells, as well as CD152 expressed on

cytotoxic T cells. If there is no secondary signal received, the activated T-cell will undergo apoptosis or become anergic, functionally inactive [36]. CD152 preferentially binds to ligand B7-1, acting as an important negative regulator of activation whereas CD28 preferentially binds to B7-2 ligand, acting as a positive regulator for activation, leading to the upregulation of the IL-2 gene [36, 38, 39]. While direct contact between ECs and immune cells through MHC presentation occurs, another method of presentation through microparticles can occur, allowing for antigen presentation at longer distances [36, 40]. Molecular particles present antigen through surface antigens, such as phosphatidylserine and can activate CD4+ T cells in an allogeneic-dependent pathway [36, 40, 41].

Stages of transplantation: Cardiac Transplant Donors

Brain Dead Donors

The first major insult that impairs long term graft acceptance and function is donor brain death [42]. All donor hearts are procured from a brain dead donor, with a large majority of these hearts unusable due to brain death-induced organ damage. Among the damaged hearts that are used, the donor organ is immunologically primed for immune recognition by the recipient[43]. Brain death has been shown to be associated with hemodynamic variations, hypothermia, coagulopathies, pulmonary alterations, terminal hypertension, hormone depletion, electrolyte irregularities, as well as endothelial activation and end organ dysfunction[6, 44]. Additionally, brain death has been shown to up

regulate the expression of inflammatory mediators, adhesion molecules, cytokines and activate the complement system[43, 44]. Excessive or inapt activation of the complement system, through cleavage fragment mediated effector functions, initiates the pathogenesis of many inflammatory disease conditions by promoting cell lysis, migration of inflammatory cells, release of cytokines, and the modulation of adaptive immune cells [43, 45]. Proinflammatory cytokines interleukin 1 (IL-1), 6 (IL-6) and tumor necrosis factor-[alpha] expression in the donor heart have been linked to poor hemodynamics along with allograft dysfunction post-transplantation [46, 47].

Cold Storage and Warm Ischemia

Following this unavoidable damage, the donor heart is subjected to cold ischemia/storage (to facilitate organ transportation), which causes further organ damage, as well as creating metabolic instabilities that impair early graft function. Finally, upon implantation, the organ undergoes warm ischemia and reperfusion injury. The severity of these early injuries has been shown to prime the transplant heart for primary graft failure, acute and chronic rejection [43]. During cold storage, the cells are placed in hypoxia for up to six hours in preservation solution. In a time-dependent manner, hypoxic conditions alone are able to induce exocytosis of the Weibel-Palade bodies, expressing P-selectin and von Willebrand factor on the cell membrane [42]. Hypoxia significantly alters the

permeability of the endothelium, downregulates basal VCAM-1 and ICAM-1, which can be enhanced in combination with inflammatory stimuli [42, 48, 49].

During ischemia, endothelial cells switch to anaerobic metabolism, utilizing their energy stores for cell functions which are vital for cell survival [6]. Metabolic problems induced by ischemia are ATP depletion leading to the inhibition of ATP-dependent pumps controlling osmotic and ionic gradients in the membrane. Additionally, significant oxygen free radicals are formed by catabolism of high energy phosphate groups when followed by reperfusion[50], the generation of which are thought to cause immune cell infiltration, complement activation and direct cell damage.

Acute and Chronic Rejection

Acute rejection primarily consists of T lymphocyte mediated rejection and can appear as early as one week [51]. Upon transplantation, recipients receive lifelong immunosuppression to control acute allograft rejection. While these immunosuppressive strategies have been a successfully utilized therapy for many years, and have significantly improved short-term graft survival rates, these strategies have little impact on the development and severity of chronic allograft rejection [5].

Chronic rejection, which develops over several months to years, can be categorized as either chronic parenchyma cell rejection, induced by immune

mediated replacement fibrosis of the parenchyma in the graft, or chronic vascular rejection, induced by immune-mediated host response to the blood vessels of the graft, resulting in stenosis of these vessels [4, 51]. Chronic graft failure is multifactorial and, along with the obvious immunologic causes, graft loss is also related to alloimmune independent mechanisms as well as recipient related risk factors [5]. ECs are central to transplant rejection and sit at the interface between preserving normal physiologic function and protection of this physiologic function can vastly impact the immunology of ECs.

Endothelial Gap and Tight Junctions

The endothelium plays a key role in the outcome of the transplanted organ. As outlined above the endothelial sits at the interface between the donor and the recipients immune system. While much attention has been paid to the immunological role of the endothelium, and how these functions contribute to the activation of the recipients immune response, little focus has been placed on modulating the endothelium early post transplantation. In addition to the many immunological functions the endothelial cells can promote, the endothelial layer is first and foremost a physical barrier, which by design is constructed to repel invaders, to provide protection to tissues contained in the sub-endothelial space. The barrier function of endothelial cells is in part modulated by the cell-cell connections that join EC-to-EC. Endothelial cells are anchored together by groups of intracellular junctional protein channels termed tight, gap and adherens

junctions. Tight junctions are located near the surface of the cell-cell junction and function in fusing adjacent endothelial cells tightly in order to limit the transportation of fluid through the intracellular space[52]. Having a water-tight connection between adjacent endothelial cells is crucial for proper endothelial function while forcing molecules and liquid to pass through the cell, rather than through the intercellular space. Tight junctions are comprised of approximately 40 proteins which can be categorized into three groups of transmembrane proteins claudins, occludens, and adhesion proteins. The claudin family of proteins, which form a charge selective opening, allow entry to only small uncharged molecules and ions [52]. Zonula Occludens (ZO) proteins are capable of modulating cell progression thorough the cell cycle by associating with regulatory molecules such as adapter proteins, signaling molecules, and growth factors [53]. Gap junctions are a group of intercellular channels between two adjacent endothelial cells, essentially connecting the cytoplasm of each cell. Relatively large (~20 A), they allow the passage of organic and inorganic ions into each cell, allowing for intercellular communication and electrical coupling of cardiomyocytes [54, 55]. An individual gap junction is comprised of two connexons (hemichannels), each originating from its own respective cell. Connexins are a family of proteins which form the individual hemichannels of gap junctions and contain four transmembrane spanning motifs on the cytoplasmic side of the plasma membrane, composed of two amino-(NT) termini and two carboxyl-(CT) termini [55]. An individual hemichannel consists of six connexins. These proteins have immunological involvement in intercellular

spread of injury signal, immune cell infiltration through vessel walls (barrier function), inflammation and re-endothelialization. The coordinated closure of these gap junctions are essential for proper endothelial function and can be influenced by high concentrations of calcium ions, low pH, phosphorylation induced by special hormones, and change in voltage difference.

Adherens junctions are adhesive complexes that comprised primarily of cadherins (VE-cadherin) and catenins which are attached to actin filaments in the cytoplasm [10]. Adherens junctions function similarly to tight junctions whereas adhesion between cells is facilitated by specific transmembrane proteins. Catenins are the primary scaffolding proteins and function in connecting these junctions to the cytoskeleton of the cell.

Tight and Gap junction interactions

The interaction between gap and tight junction proteins is crucial for their functions. Zona occludens (ZO) are important scaffolding proteins that interact with F-actin and other intracellular components which effect cytoskeleton mobility, as well as involvement in intracellular signaling and gene expression [53, 56]. ZO-1 is comprised three PDZ domains an SH3, GUK, and a proline-rich domain at the C-terminus, which function in binding to junctional transmembrane proteins, such as connexins on gap junctions, as well as the other cell junction proteins, and anchoring them to the cytoplasmic actin. ZO-1 mediates the size and stability of gap junction aggregates by binding Cx43, the most abundant

connexin protein on the cardiac endothelium, at the C-terminus to the PDZ2 domain of ZO-1[53, 57, 58].

Endothelial Barrier function and Leukocyte Infiltration

Endothelial cells express integrins on their surface which can interact with some inflammatory cells allowing for transendothelial migration into tissues. Neutrophils, lymphocytes and monocytes are a few examples of important inflammatory cells involved with endothelial barrier function. Neutrophils are fast acting leukocytes and one of the first to arrive at any site of infection, including diseased and injured tissue. These granulocytes have been associated with many pathological conditions that lead to increased endothelial permeability, such as ischemia reperfusion [59]. Once activated, neutrophils release a large amount of various cytokines, enzymes and reactive oxygen species that interfere with the cell-cell junctions and alter the expression and phosphorylation of the junctional adhesion molecules[17].

While brain death and ischemia/reperfusion injury lead to primary graft dysfunction and chronic rejection, many treatments have shown to abolish IRI through microvascular protection. Statins, cholesterol lowering drugs used to prevent cardiovascular diseases, have been shown to reduce myocardial IRI in several studies [60-62]. The effectiveness of statins has been observed even when added after reperfusion, although these protective effects are more related to their lipid lower effects than directly on the amelioration of IRI [62]. This not

withstanding, studies have shown that simvastatin treatment of rat cardiac allograft donors 2 hours before allograft procurement provides vasculoprotective effects on IRI as well as subsequent tissue remodeling [63]. The authors demonstrated that donor simvastatin treatment, in combination with post transplant recipient simvastatin therapy, protected against both renal and cardiac microvascular dysfunction and IRI. The authors determined that the simvastatins therapeutic benefits were modulated through a preservation of the microvascular barrier function, which resulted in reduced adaptive immune and early fibroproliferative responses [64]. Simvastatin through inhibition of Rho GTPases regulated cytoskeleton and intracellular pathways, independent of its cholesterol lowering properties, modulated gap junction functions, and thereby improved gap junction stability, and reduced subsequent endothelial cell activation.

It is important to note, that simvastatin therapy was only effective when applied to both donor and recipient. While recipient simvastatin therapy is easy, and clinically utilized, systemic therapy of the donor with a therapeutic intervention is not without challenges. Most donor organs are procured from brain dead donors. These donors give rise to multiple solid organs, such as heart, lung, liver and kidney, and while simvastatin donor therapy has been shown to provide protection to kidney and heart grafts, these beneficial effects may not necessarily translate to liver, small intestine and lung for instance. Therefore, while donor therapy is feasible, its application clinically is fraught with difficulties. While the approaches used in these studies is not necessarily clinically relevant they do highlight the important role of gap junction stability in graft outcomes, and

therefore, the identification of novel therapies that will more specifically and effectively modify gap junctions may yield promise for clinical translation.

A number of small molecule and antibody therapies have been shown to specifically interact with connexins (Cx) that compose the main structures of gap, tight and hemichannels [10, 65, 66]. One such small molecule is ACT1, or α-connexin carboxyl-terminal 1. ACT1 is a novel gap junction modifying peptide designed to mimic the C-terminus of Cx43 and can outcompete ZO-1 for binding to Cx43, in doing so reduces the natural turnover of gap junctions providing protection from injury. The ability of ACT1 to stabilize gap junctions has been demonstrated in a number of in-vitro, rodent, and large animal models [55, 57, 67-69]. ACT1 was first characterized in models of acute and chronic wound healing, and is now in clinical trials for the treatment of diabetic wound healing.

ACT1 therapy has been shown to improve cell-cell communication, dampen inflammatory responses, and reduce fibroblast proliferation [68-70]. More detailed analysis of ACT1 mechanism of action showed that ACT1 inhibited ZO-1/Cx43 interactions which leads to an increase in the size of gap junctions, formation of gap junctional plaques by promoting the recruitment of hemichannels. The net result is improved cell-cell communications, which facilitate the spreading of injury signals across larger group of cells, thus dissipating injury a single cell receives [58]. Therefore given ACT1 overall

mechanism of action, the role of gap junctions in post transplant endothelial injury, and that stabilization of gap junctions can improve post transplant outcomes we hypothesized that the application of ACT1 to donor organs pre-transplantation would improve post transplant outcomes.

2. Materials and Methods

2.1 Materials

Therapeutic peptides

α–connexin carboxyl-terminal (ACT1) peptide and its reverse sequence peptide (R-pep) were synthesized by the American Peptide Company (Sunnyvale, CA). The ACT1 peptide corresponds to a short sequence at the Cx43 C-terminus linked to an antennapedia internalization sequence (RQPKIWFPNRRKPWKKRPRPDDLEI). Antennapedia internalization peptide sequence is RQPKIWFPNRRKPWKK. R-pep sequence consists of the reverse sequence of ACT1 attached to an anntennapedia internalization sequence.

Cell Culture

Mouse Cardiac Endothelial Cells (MCEC) (CELLutions Biosystems Inc, Canada) were used in all experimental procedures. Cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, CA) supplemented with 10% fetal bovine serum (FBS) in a 37°C incubator with humidified room air and 5% CO2.

Novel in-vitro organ preservation model

A novel simulated cold ischemia warm reperfusion model of organ preservation was used to mimic the oxidative stress and subsequent injury that occurs during organ storage and post heart transplantation[71, 72]. Mouse Cardiac Endothelial cells (MCEC) were cultured to confluence and stored in a hypoxic chamber in University of Wisconsin (UW) or UW/1ACT1 solution at varying concentrations of ACT1 at 4 °C for 6 hours and then reperfused with Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, CA, USA) with 10% fetal bovine serum (FBS). This model was utilized to model the clinical scenario of organ preservation and provide clinical relevance in our in-vitro experiments.

Heterotopic heart transplantation model of acute rejection

C57BL/6 mouse hearts were transplanted into the abdomens of C57BL/6 recipients by anastomosis of the donor and recipient aortas and the donor pulmonary artery with recipient inferior vena cava. All animals were 8-10 weeks old and between 20-30 grams, and purchased from the Jackson laboratory. After ligating superior vena cava and supra-hepatic vena cava, stainless steel micro serrefines (Fine Science Tools, CA, USA) were used to clamp thoracic aorta, following a small incision on the pulmonary artery, they were perfused with 0.5 mL ice cold UW or UW/ACT1 solution through the proximal of thoracic aorta after performing a small cut on pulmonary artery. Harvested donor hearts were stored in UW or UW/ACT1 solutions at 4°C for 6h

prior to implantation. Anastomosis time was standardized to 30 min. After the operation, 1 ml of sterile saline was administered to the recipient animal by subcutaneous injection. Transplanted hearts were explanted 48 h post-implantation and processed for further evaluation.

In-vivo aortic chronic rejection transplant model

Infrarenal abdominal aortic interposition transplantation were performed between Balb/c to C57Bl/6 allograft recipients by a surgeon in our lab as previously described [73, 74]. Specifically, the donor operation commenced once mice (Balb/c) are lethally anesthetized. A midline incision was made and extended into a cruciate abdominal incision for improved access. The bowels were reflected to the side and 1ml of heparinized saline (diluted 1:50 heparin to saline) was infused into the inferior vena cava. Using the same syringe, 1ml of blood was removed and discarded from the mouse. The ribs were then incised laterally and the entire chest shield including sternum was be excised providing full exposure to the thoracic cavity. The heart and left lung were reflected into the right chest and the thoracic aorta was identified and dissected out from the aortic root to diaphragm. The thoracic aorta was then flushed with saline and heparinized saline (1:50 dilution) and stored on ice in heparinized saline until grafting. Once ready to implant, recipient mice (C57Bl/6) were anesthetized and a midline abdominal incision was made. The skin and peritoneum was held open using body wall retractors for increased accessibility and visualization. The large and small bowels were then reflected cephalad, and the infrarenal IVC and aorta identified. The aorta was then carefully dissected off of the IVC and

all branches were ligated using 10-0 nylon monofilament suture (Sharpoint, Reading, PA) or thermally cauterized. Once the aorta was isolated, aneurysm clips were then used to obtain proximal and distal hemostatic control. After the clips were applied, the aorta was transected midway between the clips using microscissors. The proximal and distal aortic ends as well as the peritoneal cavity were flushed with heparinized saline. The aortic graft was then cut to size and trimmed of fat and excess tissue. Additionally, all side branches of the graft itself were cauterized. Using an end-to end anastomosis, 10-0 nylon monofilament interrupted sutures were placed circumferentially between the graft and the native aorta. The clips were then removed and hemostasis/reperfusion was achieved. After hemostasis was confirmed, the abdominal cavity was then flushed with warm saline and the peritoneum and skin were closed with 4-0 vicryl suture. The anticipated morbidity and mortality rates for aortic transplantation is 10% with a 90% success rate.

Methods

2.2 In-Vitro Trans-endothelial electrical resistance (TEER)

Trans-endothelial electrical resistance was used to directly measure the endothelial and gap junction integrity in-vitro, allowing us to see if ACT1 pretreatment maintains endothelial integrity by stabilizing GJ and TJ. Mouse cardiac endothelial cells were seeded ($1x10^5$ cells per well). Once confluent, MCECs were stored in a hypoxic chamber in UW or UW/100uM ACT1 solution at 4 °C for 6 hours and then reperfused into normal media as previously described. Gap junction communication was inhibited by adding 18-β-glycyrrhetinic acid while hemichannel activity was blocked by the addition of apyrase to the endothelial cells [75, 76]. TEER measurements were taken prior to initial treatment along with 1, 3, 12 and 24 hours post-reperfusion using a Millicell ERS-2 Epithelial Volt-Ohm Meter (EMD Millipore, MA, USA). The TEER outcome was analyzed to determine the impact of ACT1 on gap junction and hemichannel function.

2.3 Cx43 and ZO-1 Expression Post-Reperfusion

Mouse Cardiac Endothelial cells (MCEC) were cultured on glass bottom petri dishes at 100,000 cells per well. Once confluence, MCEC were stored in a hypoxic chamber in UW or UW/100uM ACT1 solution at 4 °C for 6 hours, to model organ cold storage and reperfusion, then reperfused with Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, CA, USA) with 10%

fetal bovine serum (FBS). After 1-hour post-reperfusion, MCEC were fixed overnight at 4 °C with 4% Paraformaldehyde in PBS (Alfa Aesar, MA, USA). Dishes were washed with phosphate-buffered solution (PBS) three times for 5 minutes followed by the addition of Image-iT FX signal enhancer (Life Technologies, OR, USA). Primary antibodies, connexin43 (1:200; Sigma Aldrich), and ZO-1 (1:200; Invitrogen), occludin (1:200; Invitrogen), rabbit polyclonal were added for 1 hour at 4 °C. Dishes were then washed with PBS three times for 5 minutes. Secondary antibody Alexa Fluor 488 goat-anti-rabbit (1:500; Invitrogen) were then added overnight and analyzed with confocal microscopy. Quantitative analysis of membrane associated fluorescence was measured via the cell profiler software (cellprofiler.com).

2.4 Fluorescein Diacetate- Propidium Iodide Staining

MCECs were treated under hypoxic conditions in UW or UW/100uM ACT1 solution at 4 °C for 6 hours, to model organ cold storage and reperfusion. MCECs were stained with stock solutions of 5mg/mL FDA (live staining) and 2mg/mL of propidium iodide (dead staining) at 1, 6, 12, and 24 hours' post-reperfusion. Once stained, these cells were observed with a confocal microscope with 520 and 590 nm filters [72]. Quantitatification of membrane associated fluorescence was measured via the cell profiler software (cellprofiler.com).

2.5 Co-culture and Co-stimulatory Molecule Expression Assay

Balb/c allogeneic mice were innoculated with MCEC i.p. as previously reported [77]. Three weeks later the spleens were harvested and T cells were sorted by CD3 bead isolation using a Millipore Guava EasyCyte 8HT flow cytometer. MCECs were cultured and exposed in our in-vitro model to UW or UW+ACT1 and upon reperfusion were exposed to 1 x 10^5 allogeneic T cells. Supernatants were collected at 72 hrs and assessed for the presence of IFN gamma and KC. ECs were collected, quantified, washed with FACS buffer. 100 µl of cells, 1 x 10^5 cells were transferred to each well of a 96 well, round bottom plate (Corning, Inc, USA). Cells were incubated in FACS blocked for 5 minutes on ice and antibodies for PD-L2, CD80, CD86, PD-L1 and corresponding isotype control antibodies FITC, PE, APC, and PE-Cy7 were added to the appropriate wells and incubated for 20 minutes in the dark on ice. Cells were washed twice with 200 µl of FACS Buffer and cell-death marker 7AAD was added 10 minutes prior to running the flow cytometer. Flow cytometry was utilized to look at PD-L1, PD-L2, CD40, CD80, and CD86 expression.

2.6 ELISA measurement of proinflammatory cytokines with modulation of inflammation post reperfusion

Using the culture model outlined above, an ELISA for KC, and IFN gamma were used to measure cytokine release to assess the impact of GJ and TJ, and endothelial health on pro-inflammatory cytokine release.

2.7 Evans Blue staining following heterotopic transplant model

Utilizing our heterotopic heart allograft model, Balb/c donor hearts were stored in UW preservation solution and UW solution augmented with ACT1 and transplanted into a C57Bl/6 recipient. This model allowed us to observe the impact of IRI and act1 on the onset of acute rejection. To assess the impact of ACT1 augmentation of cold storage preservation solution on heart vascular permeability, C57Bl/6 recipients were injected with Evan's Blue Dye immediately post-reperfusion and harvested 30 minutes later to analyze uptake.

2.8 Histopathology and ELISA measurement of Acute rejection in heterotopic transplant model

Utilizing our heterotopic heart allograft model of chronic rejection, Balb/c donor hearts were stored in UW preservation solution and UW solution supplemented with ACT1 and transplanted into a C57Bl/6 recipient. 48 hours' post-transplant, grafts were harvested and cardiac graft injury was measured with an ELISA for cardiac troponin I. Graft damage was further scored by a pathologist in our lab using a semi quantitative scoring system. Heart sections were scored 0-3 in four groups for epicardial, myocardial, endocardial damage, and inflammatory cell accumulation, where 0 represents no damage and 3 represents severe damage. These results were combined as a cumulative score from 0 to 12. Inflammatory cell infiltration was analyzed by immunohistochemistry for GR1 and MAC-3 positive cells.

2.9 Histopathology for Chronic Rejection

Aortic grafts from mice were harvested at three and twenty-eight days postoperatively as previously described and snap frozen in liquid nitrogen until further analysis. Aortic samples were sectioned transversely using a cryostat at a thickness of 6μm. Each section was stained with Elastin van Gieson (EvG) stain. Evaluation of neointimal hyperplasia was performed on aortic sections after visualization imaging using light microscopy. Photoshop (version 6.0, Adobe, San Jose, CA) software was then used to morphometrically analyze each EVG- stained image for luminal occlusion. The luminal area and area within the internal elastic lamina was both circumscribed and measured for pixel counts. The thickness of the intima and percentage intimal expansion was then calculated using absolute pixel counts with the following formula[73, 74, 78, 79].

$\%\text{Intimal Expansion} = ((A_{lamina}-A_{intima})/A_{lamina}) \times 100$; A=area.

2.10 Inflammatory infiltrate

Infiltrating inflammatory cells were immunostained using an antibody to CD3 positive T cells and cell numbers quantified[43, 80, 81] at days seven and twenty-eight post transplantation.

Pretreatment of endothelial cells with ACT1 promotes endothelial stabilization of gap and tight junctions, and protection from cold storage and reperfusion injury induced damage.

Transplantation is a widely accepted and highly successful therapy for end-stage diseases. While success rates and survival have risen steadily since its inception some 50 years ago, due largely to improved immunosuppression regimes, there is a growing appreciate that factors associated with organ donation, procurement and storage, prior to implantation greatly effect outcomes post transplantation. The donor organ is exposed to a series of injurious events prior to and during the transplant operative period, such as brain death, cold storage, cold and warm ischemia reperfusion. These events cause damage and immunologically prime the donor organ for allo-immune recognition. The removal, storage, and transplantation of a solid organ from a donor profoundly alter the homeostasis of the interior milieu of the organ. These effects impact how long the donor organ is delayed in returning to normal function after the transplantation is completed. The injury an organ sustains during recovery, preservation, and transplantation occurs primarily as a result of ischemia and hypothermia. Techniques for organ preservation serve to minimize this damage to promote optimal graft survival and function. Damage to donor organs prior to transplantation occurs in 2 main phases. The first (cold ischemic) phase, occurs when the organ is flushed *in situ*, then procured and preserved in a static or pulsatile hypothermic state prior to transplantation into the recipient. The second (warm ischemic) phase includes

the time from organ removal from the preservation solution to the time it is sewn into the organ recipient.

Cold preservation has greatly facilitated the use of cadaveric kidneys for transplantation but, clearly, damage occurs during both the preservation episode (Taverna, 2006; Henke, 1995; Sammut, 2000) and the reperfusion phase (following transplantation). While metabolic additives have been incorporated into preservation solutions to prolong storage times and improve organ viability no additives have been incorporated into the preservation solutions to minimize injury associated with cold storage or reperfusion injury. One advantage of cold preservation is the opportunity to add agents to the preservation solution that might offer protection to the organ during cold storage and prior to reperfusion following transplantation. Here we interested in determining the impact of cold preservation and warm reperfusion on gap junctions and further to determine the feasibility of limiting endothelial cell damage with ACT1 therapy. Hypothermic heart storage before transplantation adds to ischemic tissue damage with the final stage of ischemic injury occurring during reperfusion after transplantation[82-84]. Ischemia-reperfusion injury (IRI) may be ameliorated by stabilization of junctional complexes during hypothermic storage. Disruption of the apical cytoskeleton by ATP depletion results in loss of junctions. Reduced expression, redistribution, and abnormal aggregation of a number of key proteins that constitute junctions have been documented after ischemic injury in cell culture, animal models, and human studies [6, 43, 62, 63, 85]. The consequent loss of tight junction barrier function can potentially magnify the thrombotic complications seen post-transplant and reduce EC barrier functions, which can promote recipient immune cell infiltration into the donor organ leading to injury. Stabilization of epithelial cell polarity using polyclonal

antibodies in kidney transplantation has been reported to improve delayed graft function [86]. Similarly, in the heart, stabilization of the microvascular gap junctions using simvastatin led to reduced IRI and chronic rejection [87], although this therapy was only effective when given to the donor hours prior to harvest of the donor organ [87]. These studies demonstrate a clear role for GJ and TJ in early graft injury, pre and post transplantation and support our hypothesis that injuries to the donor organ prior to transplantation, as a consequence of cold preservation, induces cell junctional damage, which leads to endothelial activation, inflammation and heart damage upon reperfusion. An approved therapeutic to improve the quality of donor heart, doesn't currently exist, and therefore these studies herein will explore the therapeutic potential of a novel therapeutic compound, ACT1, to prevent/reduce cold storage induced EC injury, and thereby improve donor heart quality prior to transplantation. Here we explore these questions using a novel in-vitro model of cold static hypoxic storage, and warm reperfusion injury to model the in-vivo clinical scenario.

3.1 Results

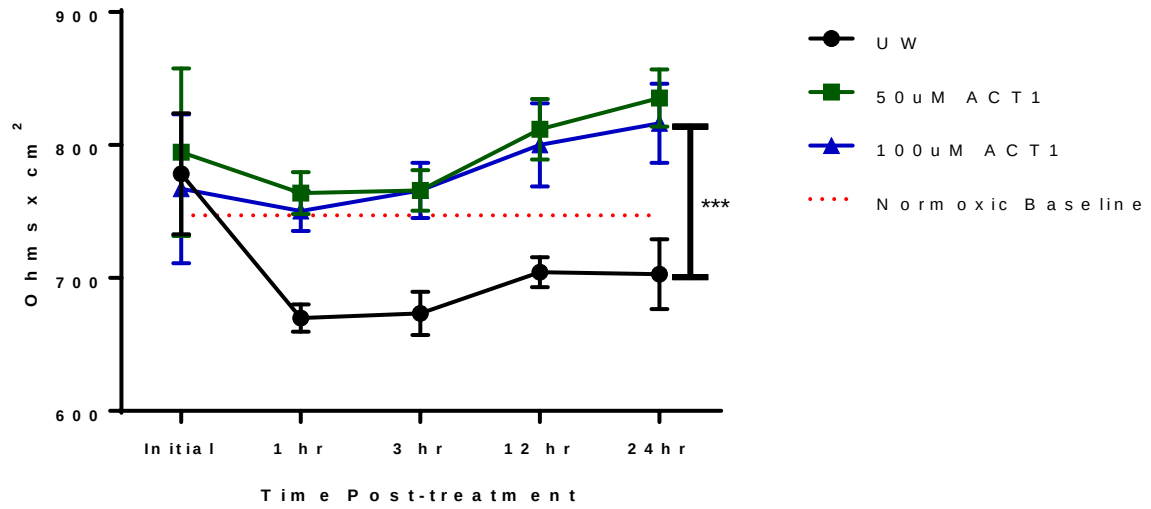
In-Vitro Trans-endothelial electrical resistance (TEER) to determine barrier function

To determine the impact of ACT1 on barrier function, trans-endothelial electrical resistance was used to directly measure the EC-EC junctional integrity using our novel in-vitro model of hypoxic cold storage and warm reperfusions, which is designed to mimic in-vivo organ storage. Endothelial resistance is a measure of cellular gap and tight junction integrity, and therefore a confluent monolayer of endothelial cells is associated with a high electrical resistance. Cold storage in UW solution allow followed by warm reperfusion with standard culture media results in a rapid drop in electrical conductivity, which over the 24 hr observation period never fully recovers (Fig. 1). Pre-treatment of MCECs with UW/ACT1 in this clinically relevant model significantly blunts the initial loss of electrical conductivity as compared to UW allow, and over the 24 hrs quickly returns to normal levels (Fig. 1).

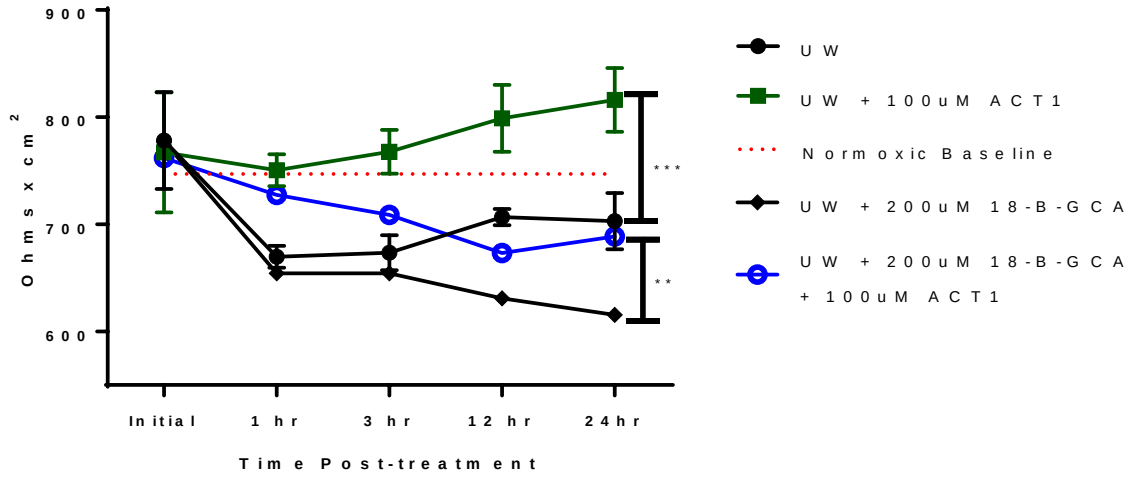
Previous studies have determined that ACT1 mechanism of action is through stabilization of gap junction proteins [55, 67, 69]. To determine whether gap junctions play a role in this loss of electric resistance post reperfusion we used two inhibitors that block gap junctions, 18- β-GCA, and hemichannel inhibitor, apyrase. In these experiments MCECs were cold stored in UW solution containing 18- β-GCA or apyrase. When 18- β-GCA was added to the UW solution, we observed a significantly increased drop in electrical

resistance, as compared to UW alone, suggested that augmented gap junction loss was associated with worsening injury. We next explored whether augmentation of ACT1 to 18- β-GCA/UW at the time of cold storage could competitively rescue these cells. Addition of ACT1 significantly improved TEER, although only to levels seen in the UW alone groups (Fig. 1B). We observed a similar decrease in electrical resistance when apyrase was added to the UW solution, suggesting that loss of hemichannel functions play a role in the loss of EC communication post cold storage and warm reperfusion. Interestingly, augmentation of ACT1 in these experimental conditions lead to a complete resolution of TEER to levels not significantly different from control samples ACT1 (Fig. 1C).

A


900
800
700
600
Ohms x cm2
Initial
1 hr
3 hr
12 hr
24hr
Time Post-treatment
***
UW
50uM ACT1
100uM ACT1
Normoxic Baseline

B


900
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Ohms x cm2
Initial
1 hr
3 hr
12 hr
24hr
Time Post-treatment
***
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UW
UW + 100uM ACT1
Normoxic Baseline
UW + 200uM 18-B-GCA
UW + 200uM 18-B-GCA + 100uM ACT1

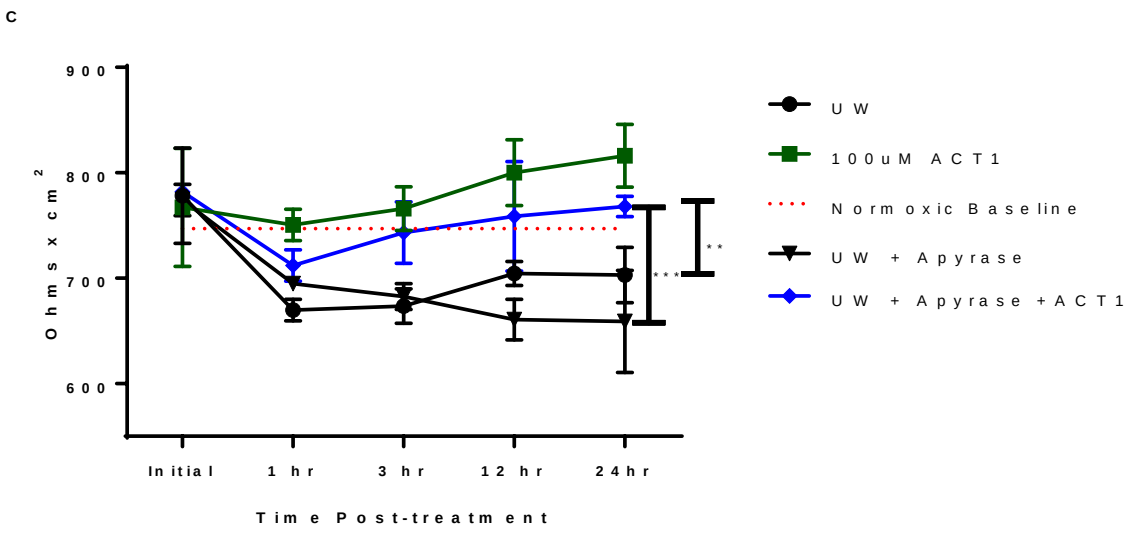


Figure 1: *Augmentation of University of Wisconsin (UW) solution with ACT1 provides protection from hypoxic cold storage ischemia reperfusion injury induced endothelial cell damage.* **A.** Trans-endothelial electrical resistance (TEER) measurements demonstrate that cold hypoxic storage in UW solution, and reperfusion, leads to a rapid decline in electrical resistance, an indicator of loss of gap junction (GJ) functions. Pretreatment with ACT1 appears to stabilize GJ functions and provides protection from injury in a dose dependent manner. **B.** Pre-treatment of ECs with the GJ inhibitor 18- β-GCA exacerbates cold hypoxic storage and reperfusion injury as compared to UW alone. Co-incubation of ACT1 with 18- β-GCA partially rescues injury, which supports ACT1 proposed mechanism of action is through stabilization of GJ. **C.** Pre-treatment of ECs with the hemichannel inhibitor 18- apyrase aggravates cold hypoxic storage and reperfusion injury as compared to UW alone. Co-incubation of ACT1 with apyrase partially rescues injury, which reveals that modulation of hemichannels

function is important in the mechanism of action of ACT1. N=3 in all experiments ***$p<0.001$, **$p<0.01$.

Given the stabilization effect observed in TEER, we sought to determine whether ACT1 therapy lead to a reduction in EC cell death. We used fluorescein diacetate and propidium iodide staining to determine cell viability of MCECs post-reperfusion. In this assay healthy, viable cells fluoresced green with the vital dye FDA, while dying, nonviable cells fluoresced red. FDA-PI viability staining is a reliable way to determine the damage induced on these cells by ischemia reperfusion injury. Non-fluorescent fluorescein diacetate is converted by living cells into fluorescein, a green fluorescent compound, whereas propidium iodide will enter the nucleus of membrane-compromised cells and fluoresce red [88].As previously described, MCECs were treated under hypoxic conditions in UW or UW/100uM ACT1 solution at 4 °C for 6 hours, to model organ cold storage and reperfusion. MCECs were stained with stock solutions of 5mg/mL FDA (live staining) and 2mg/mL of propidium iodide (dead staining) at 1, 6, 12 and 24 hours post-reperfusion (Fig. 2A) to correlate with TEER data and fixed in paraformaldehyde at 4° overnight. There was a notable time-associated decrease in adherent cells post ischemia reperfusion with UW preservation alone, while this effect was not observed with UW preservation augmented with ACT1. Furthermore, there is a significant decrease in red, dead cells, more visible at earlier timepoints post reperfusion in UW preserved cells compared to the cells preserved in ACT1 augmented UW preservation (Fig. 2a). To confirm our visual observations we performed image analysis to quantify the number of adherent living and floating dead cells (Fig. 2b).

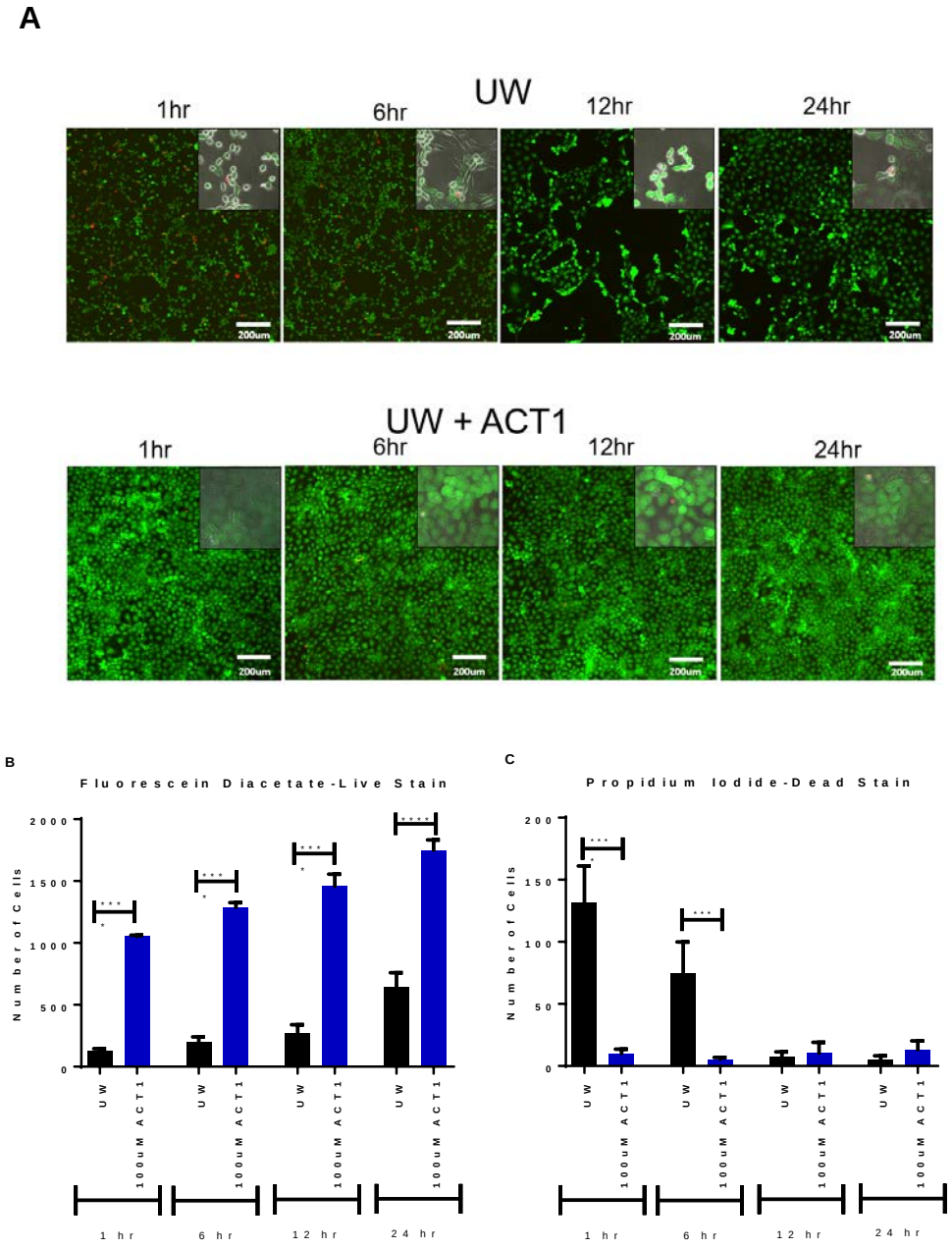


Figure 2: *Cell viability as determined by fluorescein diacetate-propidium iodide (FDA-PI) staining.* **A.** MCECs were dual-stained in vitro with FDA-PI and imaged with an Olympus Fv10i confocal microscope. Living cells stained

green, and dead cells stained red. Imaged representative of n=3 per timepoint. Note the loss of cells with time post ischemia reperfusion with UW preservation alone as compared to UW+ACT1. Further note the significant increase in red cells (dead cells) seen early post reperfusion in UW preserved cells, compared to UW+ACT1 stored cells. **B.** Quantification of attached living cells with FDA **(B)** and detached dead cells, PI **(C).** FDA-PI stained cells demonstrate a significant reduction in cell death following ACT1 pre-treatment and increased total live cells. Images and quantification of live/dead analysis representative of n=3 experiments, n=5 images per time point/group. ****$p<0.0001$, ***$p<0.001$.

We hypothesize that ACT1 stabilizes EC junctions through modulation of ZO-1 binding to Cx43. To determine the impact of cold storage on junctional stability we performed fluorescent staining to analysis the expression pattern of Cx43 at different time points post reperfusion Cx43 is a key gap junctional protein that is vital in cell-cell communication, the integrity of which is important in modulating the spread of cell communications and injury across EC monolayer. Cold hypoxic storage and warm reperfusion lead to an increase in Cx43 expression at 1 hour post reperfusion, but expression significantly decreased in UW stored MCECs, as compared to controls at 24 hours, post reperfusion. ACT1 augmentation of UW preservation solution promoted an increase in Cx43 presence as well as an enlargement and accumulation of Cx43 at GJ and membrane surface at 1 hour post reperfusion, with the effect still noted at 24 hours post reperfusion (Fig. 3A). We again quantified our immunostaining with an automated cell profiler image analysis process, which confirmed that cold storage and reperfusion injury leads to a time dependent loss of membrane associated Cx43 staining, and thus EC junctions. ACT1 therapy led to an increase in Cx43 expression as compared to UW and controls.

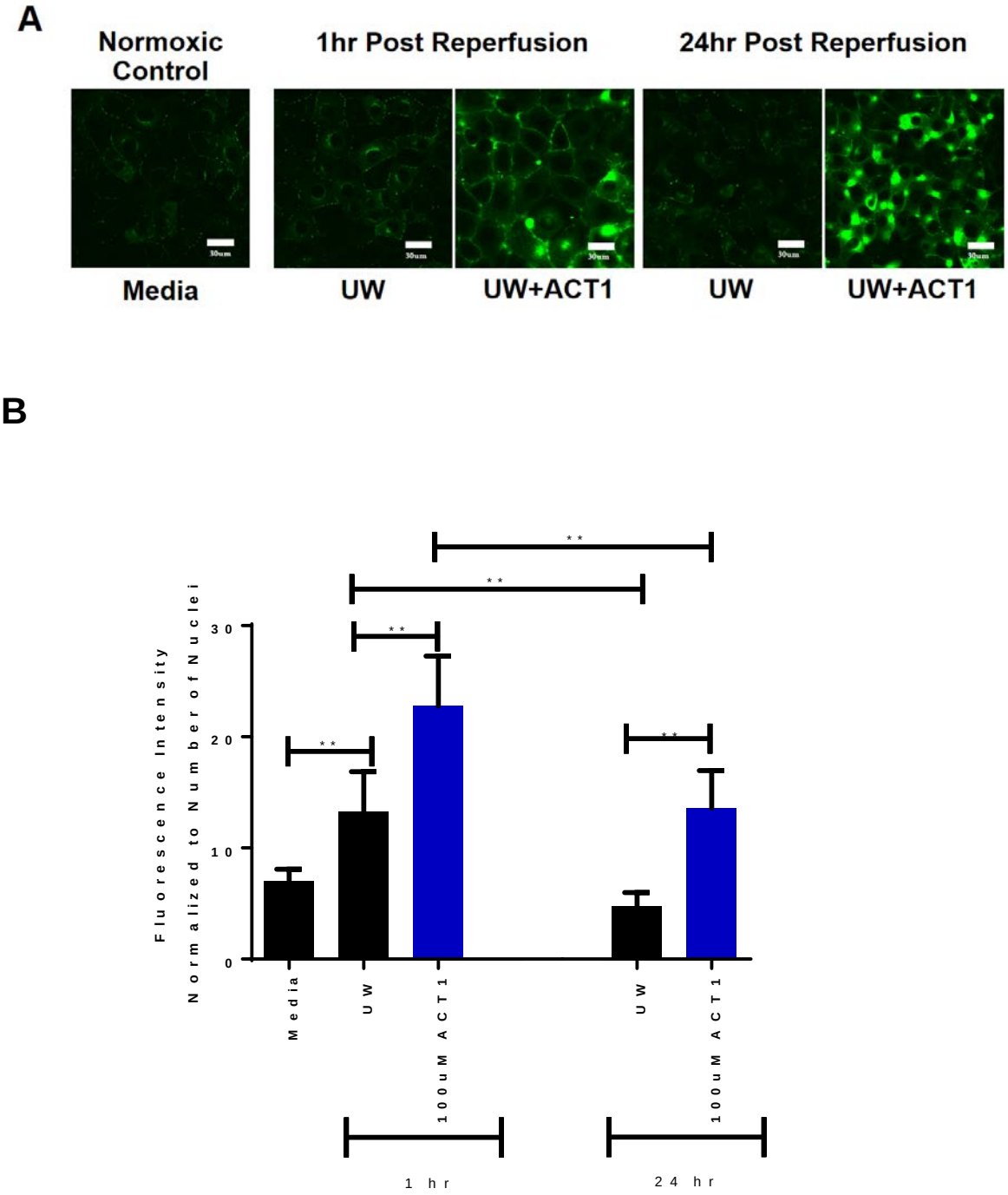
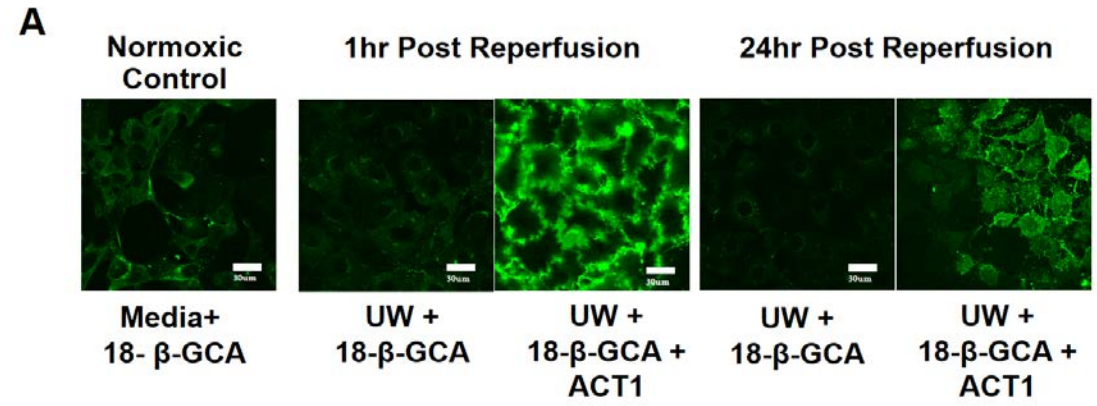


Figure 3. *ACT1 pretreatment increases connexin 43 expression post cold hypoxic storage and reperfusion injury.* **A.** Connexin 43 (Cx43) is a key gap

junctional protein to improve cell-cell communication and promote injury spread across monolayers. Under normoxic conditions Cx43 localizes to discrete GJ at the cell surface (green). Hypoxia increases Cx43 expression at 1hr, but rapidly reduces by 24 hrs, in UW stored groups. ACT1 augmentation of UW promotes increased Cx43 presence, and enlargement of Cx43 GJ at the membrane surface at both 1 and 24 hrs post reperfusion. Images representative of n=5 at each time point. **B.** Quantification of Cx43 fluorescence intensity demonstrates that ACT1 augmentation of UW solution promotes increased Cx43 expression. N=5 at each timepoint/group. ***$p<0.001$, **$p<0.01$.

To determine whether the increase in Cx43 at the membrane surface is associated with ACT1 stabilization and/or increased Cx43 hemichannels to the membrane surface we treated cells with the gap junction inhibitor, 18-β-glycyrrhetinic acid, and performed Cx43 immunostaining. Somewhat surprisingly 18- β-GCA therapy had no significant impact on Cx43 as compared to UW alone, although it was reduced as compared to normal cells. Co-treatment with ACT1 not only increased immunreactivity, but significantly altered the immunostaining pattern at both 1 and 24 hrs post reperfusion to levels above that seen in UW, and ACT1 alone samples.



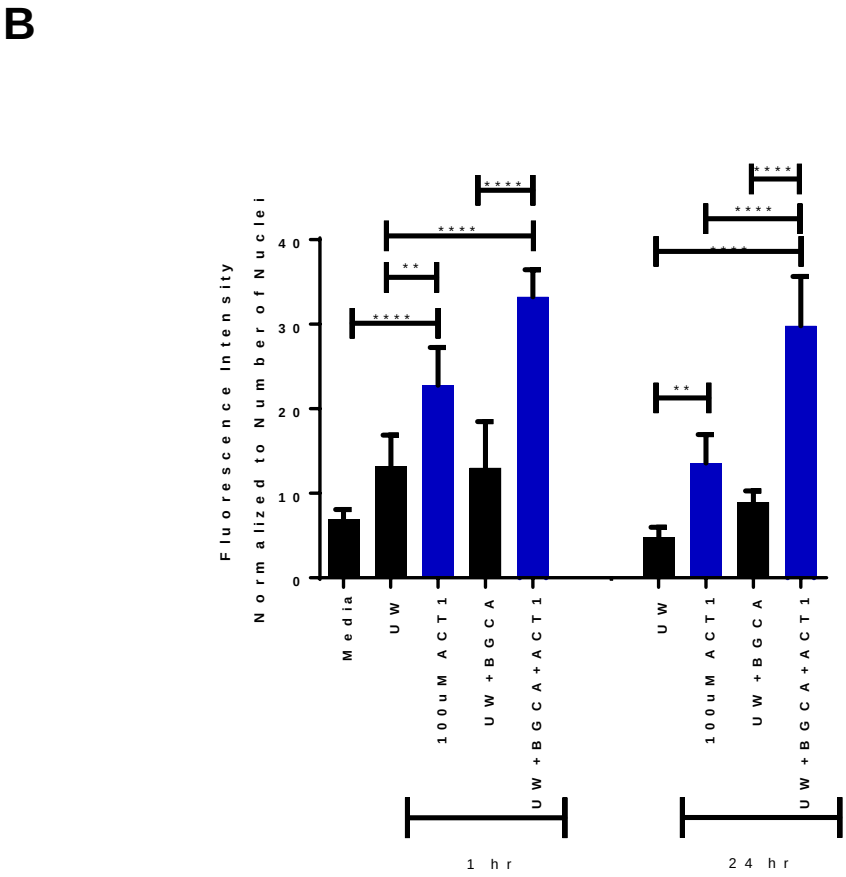
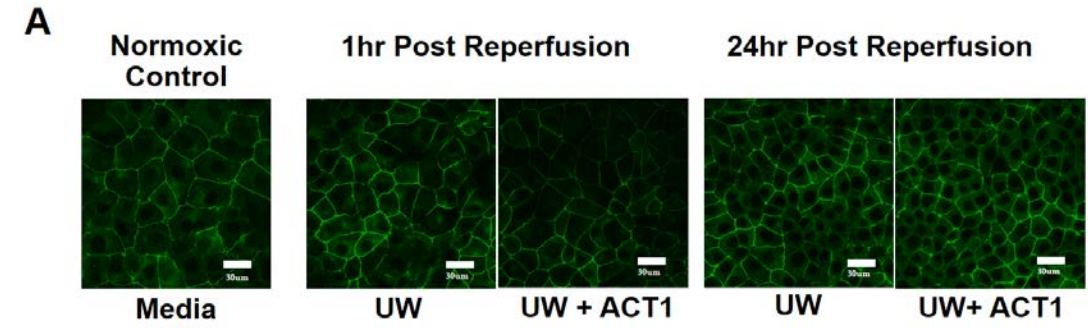


Figure 4: *ACT1 pretreatment increases connexin 43 expression post cold hypoxic storage and reperfusion injury with gap junction inhibition.* **A.** Pre-

treatment of ECs with the GJ inhibitor 18- β-GCA alters the stability and expression of gap junctional protein, Cx43. In UW solution alone, there is no difference in membrane associated Cx43 expression. Co-incubation of ACT1 with 18- β-GCA increases presence and enlargement of Cx43 GJ at the membrane surface at both 1 and 24 hrs post reperfusion. Images representative of n=3 at each time point. **B.** Quantification of Cx43 fluorescence intensity demonstrates that ACT1 augmentation of UW solution promotes increased Cx43 expression, further increasing when inhibitor is added. N=5 at each timepoint/group. ***$p<0.001$, **$p<0.01$.

We have thus shown that cold storage and warm reperfusion injury leads to EC damage, loss EC communication, EC cell death and a time dependent loss of Cx43. We propose that ACT1 stabilizes these injuries by altering promoting cell survival and improved cell-cell interactions. ACT1 functions by inhibiting ZO-1, a key tight junction protein, interaction with the N-Terminus of Cx43, thus inhibiting normal physiological gap junction turnover and injury induced gap junction turnover. Given this proposed mechanism of action we explored the expression of ZO-1 post reperfusion using immunofluorescent staining. Under normoxic conditions, ZO-1 formed a relatively continuous membranous staining pattern, seen adjoining cell membranes (Fig. 5A). When stored in UW solution, ZO-1 staining demonstrates a slight decrease at 1 hr post-reperfusion in UW, with staining significantly reduced in UW/ACT1 groups as compared to control and UW samples. Expression levels were comparable in all groups at 24 hrs post-reperfusion. Treatment with 18- β-GCA, gap junction inhibitor, reduced the expression of ZO-1 at 1 hour, and unlike UW samples expression was still suppressed at 24 hours post reperfusion. Augmentation with ACT1 rescued the phenotype slightly, but levels were still below that of normal cells.



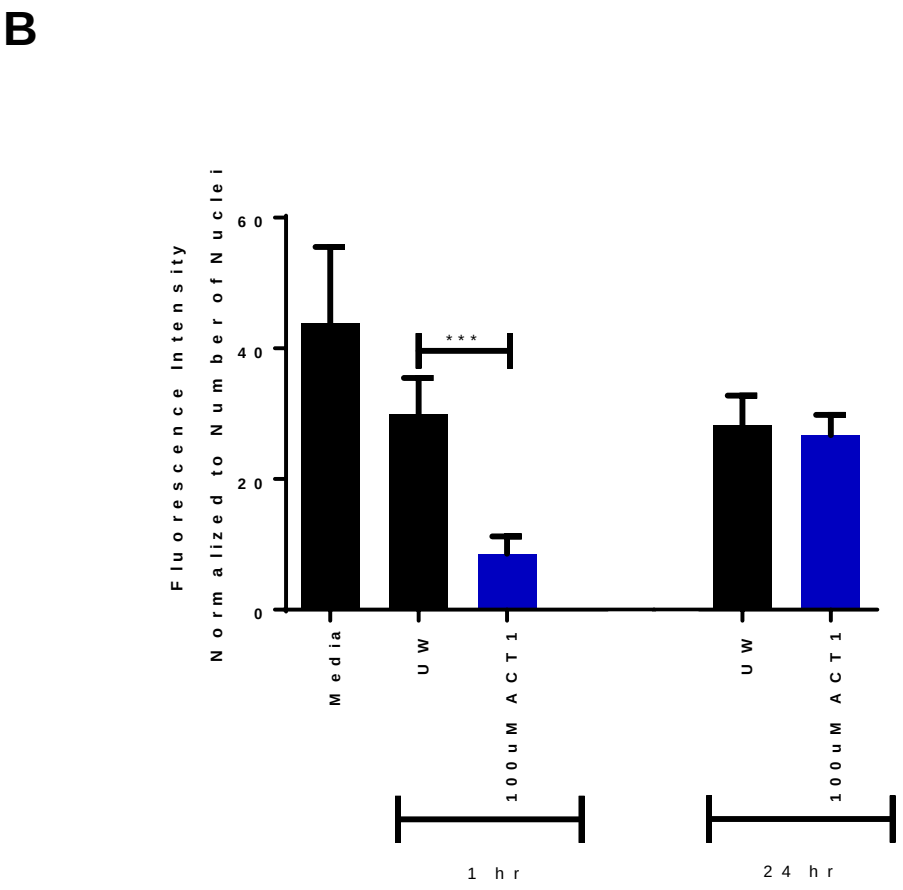
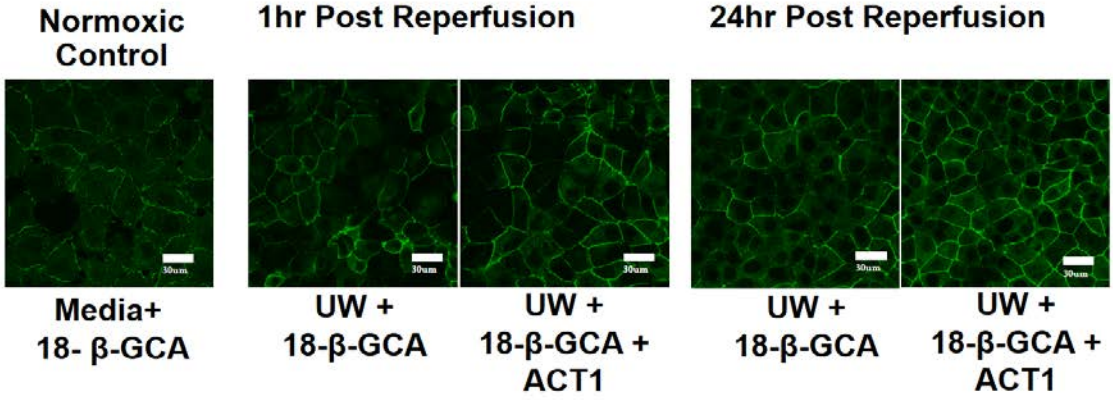


Figure 5: *Cold Storage, ischemia, and reperfusion injury transiently reduce ZO-1 expression.* **A.** Representative immunofluorescent ZO-1 stained cells (Green) demonstrating contiguous cell membrane broader pattern for ZO-1 in normoxic control cells. Images representative of n=3 for all groups. **B.**

Quantification of ZO-1 staining demonstrates a marked decrease at 1 hr in UW and UW/ACT1 groups, which is restored by 24 hrs post-reperfusion. ACT1 augmentation further abolishes ZO-1 staining, supporting ACT1 mechanism of action, which is proposed to competitively inhibit ZO-1's association with Cx43, thus stabilizing GJs. N=3 in all groups, ***$p<0.001$, **$p<0.01$, *$p<0.01$.

A


Normoxic Control
1hr Post Reperfusion
24hr Post Reperfusion
Media+ 18- β-GCA
UW + 18-β-GCA
UW + 18-β-GCA + ACT1
UW + 18-β-GCA
UW + 18-β-GCA + ACT1

B

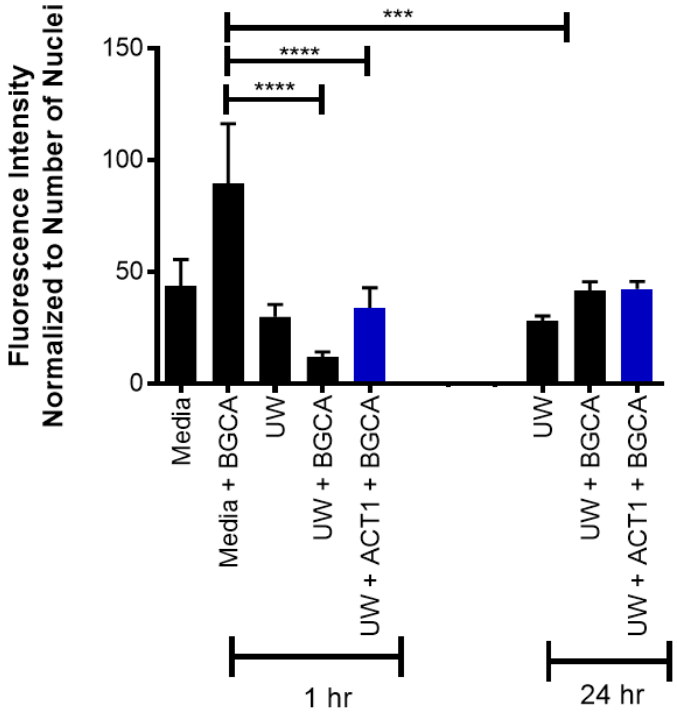

Fluorescence Intensity Normalized to Number of Nuclei
150
100
50
0
***
****
****
Media
Media + BGCA
UW
UW + BGCA
UW + ACT1 + BGCA
UW
UW + BGCA
UW + ACT1 + BGCA
1 hr
24 hr

Figure 6. *Cold Storage, ischemia, and reperfusion injury transiently reduce ZO-1 expression.* **A.** Representative immunofluorescent ZO-1 stained cells (Green) demonstrating contiguous cell membrane broader pattern for ZO-1 in normoxic control cells. Images representative of n=3 for all groups. **B.** Quantification of ZO-1 staining demonstrates a marked decrease at 1 hr in UW and UW/ACT1 groups, which is restored by 24 hrs post-reperfusion. ACT1 augmentation further abolishes ZO-1 staining, supporting ACT1 mechanism of action, which is proposed to competitively inhibit ZO-1's association with Cx43, thus stabilizing GJs. N=3 in all groups, ***$p<0.001$, **$p<0.01$, *$p<0.01$. Pre-treatment of ECs with the GJ inhibitor 18- β-GCA alters the stability and expression of gap junctional protein, Cx43. In UW solution alone, there is no difference in membrane associated Cx43 expression. Co-incubation of ACT1 with 18- β-GCA increases presence and enlargement of Cx43 GJ at the membrane surface at both 1 and 24 hrs post reperfusion. Images representative of n=3 at each time point.

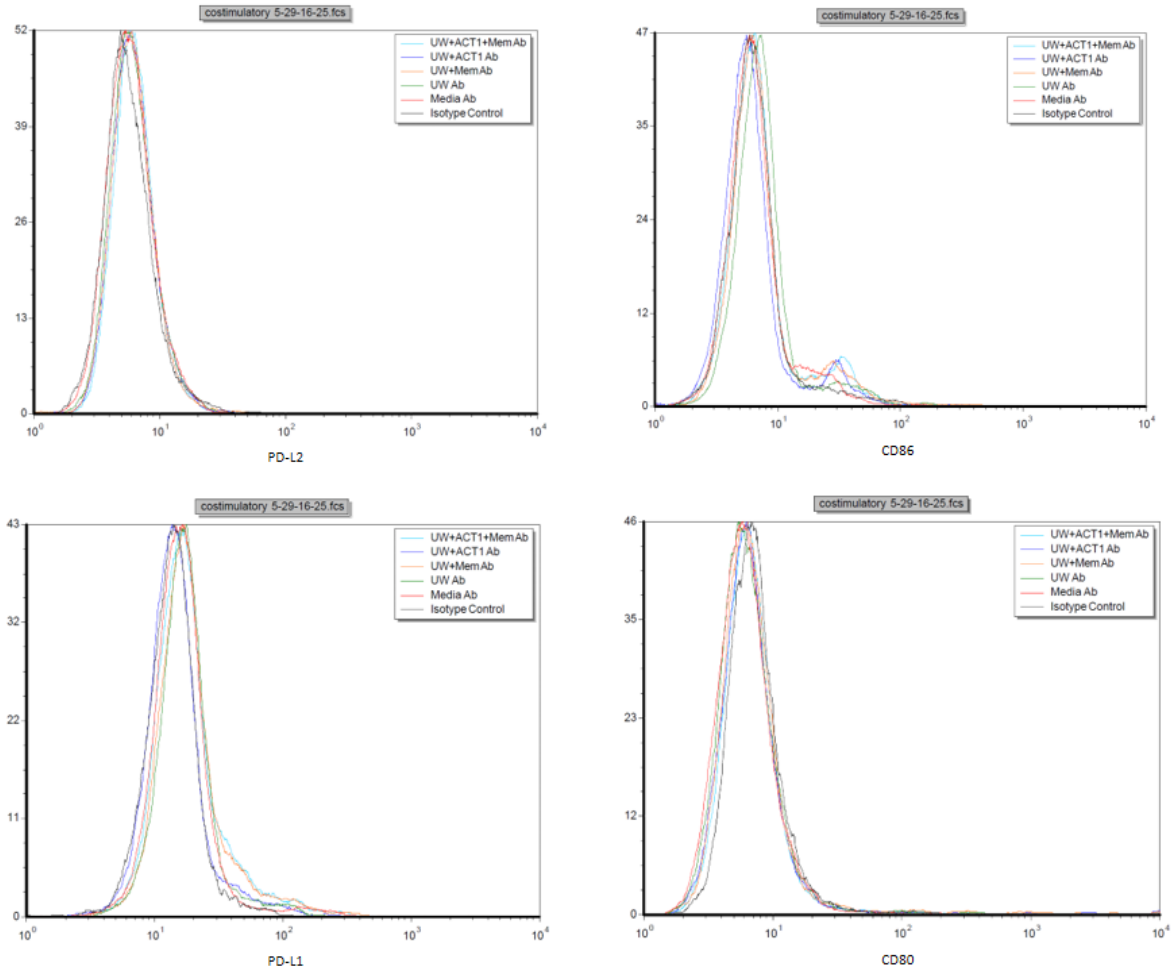
Impact of Hypoxia Reoxygenation and ACT1 on Co-stimulatory molecule expression

The first point of contact of the transplanted organ is the endothelium. We have shown that ACT1 can positively impact barrier functions by improve cell-cell communication through gap junction stabilization. In addition to barrier functions ECs play a pivotal role in immune functions post transplantation. Following reperfusion the EC becomes activated by injury, ROS, and cytokines all of which primes the EC to promote immune cell activation or infiltration. To determine any potential immunological impact of junctional protection with ACT1 we determined the impact of ACT1 pre-treatment on T cell priming and EC co-stimulatory molecule expression. ECs act as antigen presenting cells post transplantation and are thought to play a pivotal role in recipient memory T cell activation. Memory T-cells, cells that have an alloreactivity to the graft, interact with the endothelium and proliferate, generating anti-graft T-cells which injury the graft, and are unresponsive to standard immunosuppressive regimes.

ECs express a series of co-stimulatory molecules, such as, CD80/CD86 which present to CD8 T cells as part of the signal 2.. ECs also express inhibitory molecules/signals, such as PD-L1 and PD-L2, which can promote the induction of T regulatory cell phenotypes. Given that the expression of many of these molecules are thought to be modulated by EC activation and injury we hypothesized that ACT1 EC protective effects may reduce pro- T effector co-stimulatory molecules and promote pro- T regulatory cell co-stimulatory molecule expression, such as PD-L1. We therefore performed co-culture studies

with our in-vitro model of cold storage and reperfusion injury. However, in these experiments we introduced T cells from mice that had been pre-sensitized with MCECs to develop alloreactive T cells and analyzed T cell proliferation by measuring IFN gamma release and co-stimulatory molecule expression by performing EC flow cytometry. In these the studies we could demonstrate no increase in expression of any of the analyzed co-stimulatory molecules in any of the groups at any time point. ACT1 had no impact on expression positive or negative (Fig. 7A and B).

A


costimulatory 5-29-16-25.fcs
UW+ACT1+Mem Ab
UW+ACT1 Ab
UW+Mem Ab
UW Ab
Media Ab
Isotype Control
PD-L2
CD86
PD-L1
CD80

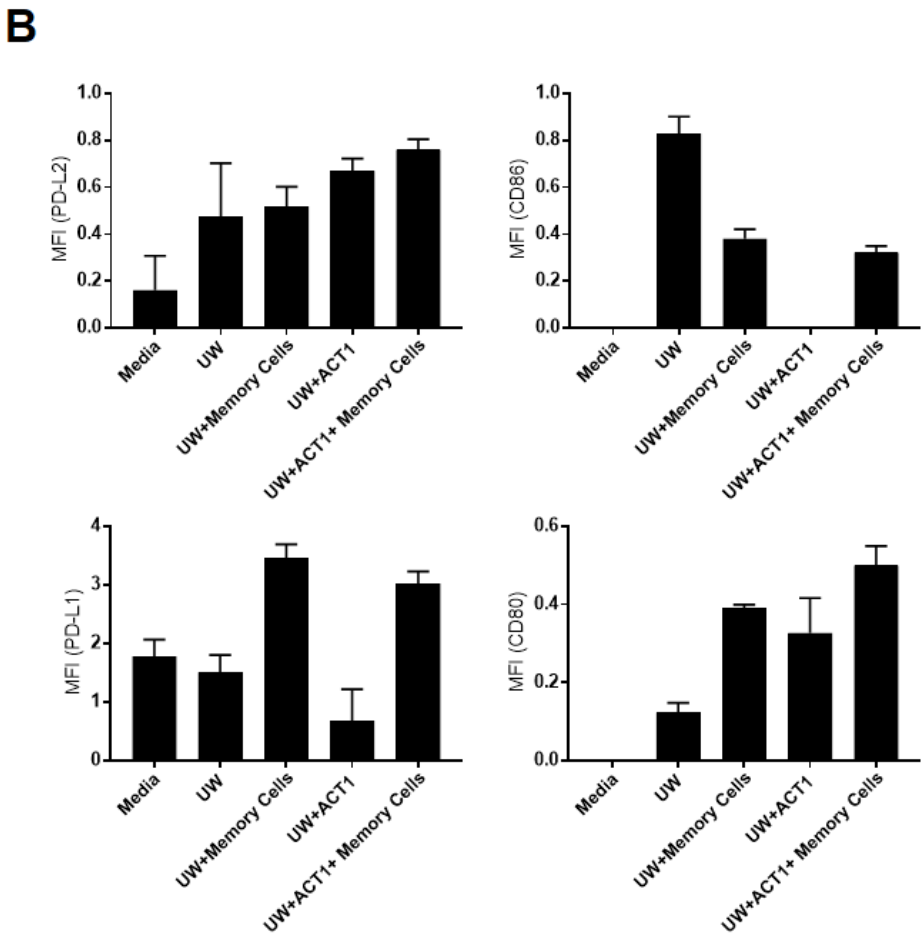


Figure 7. *Hypoxia reoxygenation and ACT1 has little impact on co-stimulatory molecule expression.* **A.** Flow cytometry analysis of PD-L2, PD-L1, CD80, and CD86 expression on MCECs following a 72 hr co-culture with FACs purified CD4+ T cells. **B.** Median fluorescence intensity (MFI) values were quantified to analyze expression of these co-stimulatory molecules. Graphs representative of n=3 for each group.

ELISA measurement of proinflammatory cytokines with modulation of inflammation post reperfusion

Co-culture supernatants were collected at 72 hrs and assessed for the presence of KC and IFN gamma. Cold storage and reperfusion have shown to injure ECs, resulting in immunological priming and co-stimulation of memory T cells. We expected that ACT1 treatment of endothelial cells would reduce co-stimulation leading to reduced KC and IFN gamma levels and previous experiments have demonstrated that 72 hrs is the optimum time point for analysis. ELISA analysis showed co-culture and hypoxic cold-storage to have no significant impact on interferon gamma and KC expression.

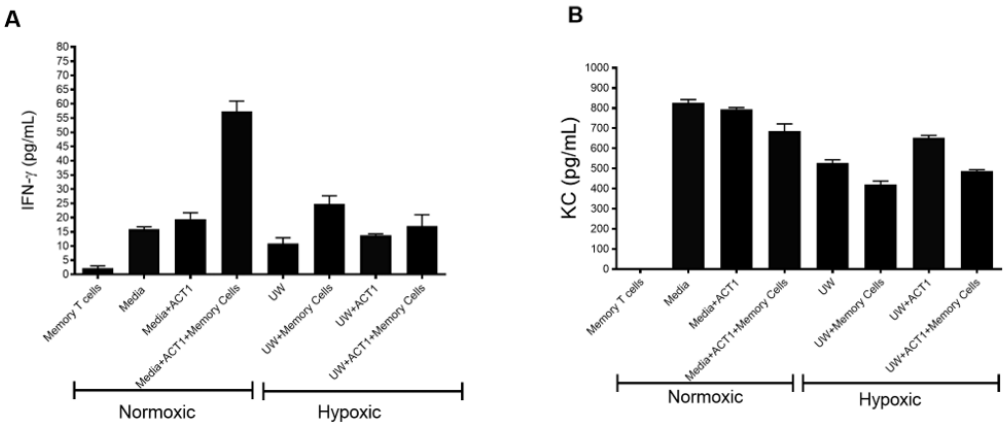


Figure 8. ELISA Measurement of proinflammatory cytokines with modulation of inflammation post reperfusion yields no significant impact on interferon gamma expression (Fig. 8a) and KC expression (Fig. 8b). Graphs representative of n=3 for each group.

3.2 Discussion

Cold storage and ischemia reperfusion injury cause a distinct loss of barrier function in MCECs [8, 43]. One of the most important properties of ECs is that they act as a barrier, controlling the passage of various proteins in the bloodstream as well as into the sub-endothelial space. This barrier function is accomplished through the coordinated opening and closing of intracellular cell-cell junctions. Degradation of this function could potentially lead to an increase in coagulation, injury signals and immune cell infiltration into the graft. Our

objective was to investigate the protective effects of gap and tight junction stabilization in the form of an additive to cold storage solutions in solid organ transplantation. We investigated the protective effects of ACT1 on endothelial gap and tight junctions by measuring the permeability of the membrane post-cold storage and reperfusion.

Our first conclusion from the in-vitro components is that pretreatment of UW preservation solution with ACT1 protects cells from hypoxic cold storage and ischemia reperfusion injury induced damage, resulting in improved barrier function and increased cell viability. Trans-endothelial electrical resistance allowed us to measure the endothelial and gap junction integrity. We show that pre-treatment of MCECs with ACT1 in this clinically relevant model greatly improves and protects endothelial gap and tight junction integrity. To further our understand the mechanism of action of ACT1 on endothelial cells, we used gap junction and hemichannel inhibitors individually and in combination with UW and UW/ACT1 in order to analyze the communication and activity in MCECs. Studies on regenerative wound healing and heart injury showed that the organization of gap junctions and associated electrical properties can be improved by modulation of connexin43 at the site of injury [67]. This study allowed us to determine whether ACT1 has a direct impact on gap, tight junctions or hemichannels. Gap junction communication was inhibited by 18-β-glycyrrhetinic acid (18- β-GCA). We demonstrated that gap junctions play a key role in post injury in maintaining electrical conductivity, and thus junction properties. 18-β- glycyrrhetinic acid, a gap junction inhibitor, increased the

injury seen post reperfusion injury. Co-incubation with ACT1 in part rescued the response, suggesting that ACT1 functions are mediated through its gap junction properties. In these experiments we co-administered ACT1 and 18-β-glycyrrhetinic acid at the same time. An alternative strategy could be to have added ACT1 at a timepoint post reperfusion. While this approach would have provided some additional mechanistic data the approach would not not be clinically translatable and therefore we opted for the single time point approach. Interestingly, apyrase administration also decreased TEER as compared to control, and while not significantly reduced TEER below that seen in the UW alone group. Cx43 Hemichannels are crucial for mediating intercellular communication by the release of small extracellular messenger molecules, such as ATP [75, 76],[85]. Apyrase blocks hemichannel activity by functioning as a catalyst for the hydrolysis of ATP, increasing the rate of hydrolysis of ATP results in blocking its ability to bind to Cx43-P2 receptors when released by hemichannels. Our findings with apyrase support a role for hemichannels in ischemic injury. Cold storage and hypoxia significantly impact on the energy metabolism of ECs and therefore a further loss of ATP may induce damage and injury. In addition to inhibiting ZO1-Cx43 interactions it has been proposed that ACT1 can induce phosphorylation of Cx43 at serine 328. This phosphorylation is thought to lead to a recruitment of hemichannels to the gap junction complex leading to the formation of a peri-nexus, a structure that is essentially an enlarged gap junction which is thought to more efficiently spread injury signals protecting against cell injury and activation. ACT1 co-incubation with apyrase

rescued EC from injury to a level that was comparable to that of ACT1 alone. Taken together these data might support a role of hemichannels in cold storage induced injury and further, the need for the formation of a perinexus for injury to be inhibited by ACT1.

We further demonstrated that ACT1 pretreatment increased Cx43 expression post cold hypoxic storage and reperfusion injury, while temporarily reducing ZO-1 expression. These data should however been interpreted with caution. The polyclonal antibody used to detect Cx43 will also detect ACT1 and therefore the increased signal could be a consequence of localization of the peptide giving the appearance of increased Cx43 gap junctions. We had hope to resolve this potential with western blot analysis, however, antibodies for western blot similarly lack the ability to discriminate between peptide and Cx43. A potential solution would be to perform FRET studies, however, we feel these studies fall beyond the scope of the current study.

In conclusion augmentation of UW solution with ACT1 improves EC barrier function and reduces EC cell injury. Augmentation has no impact on EC immunological phenotype, nor does it reduce pro-inflammatory cytokine expression.

4. Therapeutic impact of ACT1 therapy on post-transplant outcome for clinical relevance

We have established that EC pretreatment with ACT1 significantly reduces EC injury and strengthens the barrier function in-vitro. To build upon these observations here we extrapolated our in-vitro findings into two rodent models of cardiac transplantation. Our overarching hypothesis is that pretreatment of donor organs ex-vivo during the cold static hypoxic preservation period will reduce post-transplant ischemia reperfusion injury and the later development of chronic rejection. Additionally, we hypothesize that pre-treatment will enable longer organ storage times, which may enable expansion of the donor pool given that a number of organs are lost due to the need for extended transportation times to suitable recipients, which often time renders the organs unusable. Here we explore these questions in two animal models of allogeneic transplantation. The heterotopic cardiac allograft model of ischemia reperfusion injury and the aortic interposition allograft chronic rejection model.

4.1 Results

Evans Blue Permeability assay

Given in-vitro data that cold storage and ischemia reperfusion injury lead to loss of junction function and endothelial cell permeability, we performed an Evans blue permeability assay to assess whether the endothelium was permeable post-

transplant by looking at microvascular leakage into the heart. We utilized the heterotopic heart allograft model. Balb/c donor hearts were harvested and flushed with UW or UW preservation solution augmented with ACT1 followed by 6 hours of static storage in the same solutions. Hearts were then again flushed with fresh UW solution before being transplanted into C57Bl/6 recipients. Immediately post reperfusion recipient C57Bl/6 mice were injected with Evan's Blue Dye and allografts and native hearts harvested 30 minutes later to analyze uptake. This model allowed us to observe the impact ACT1 static storage on endothelial injury and permeability. Figure 9 shows that in UW alone, there was a large amount of dye leakage visible in the heart (Fig. 9B). When hearts were static stored in UW solution augmented with ACT1, there is a significant reduction in dye uptake (Fig. 9A and C), and that the dye that did incorporate was not significantly different from that seen in the native heart (Fig.9A). These data suggest that ACT1 promotes gap and tight junction stability and subsequently improve endothelial cell integrity in-vivo.

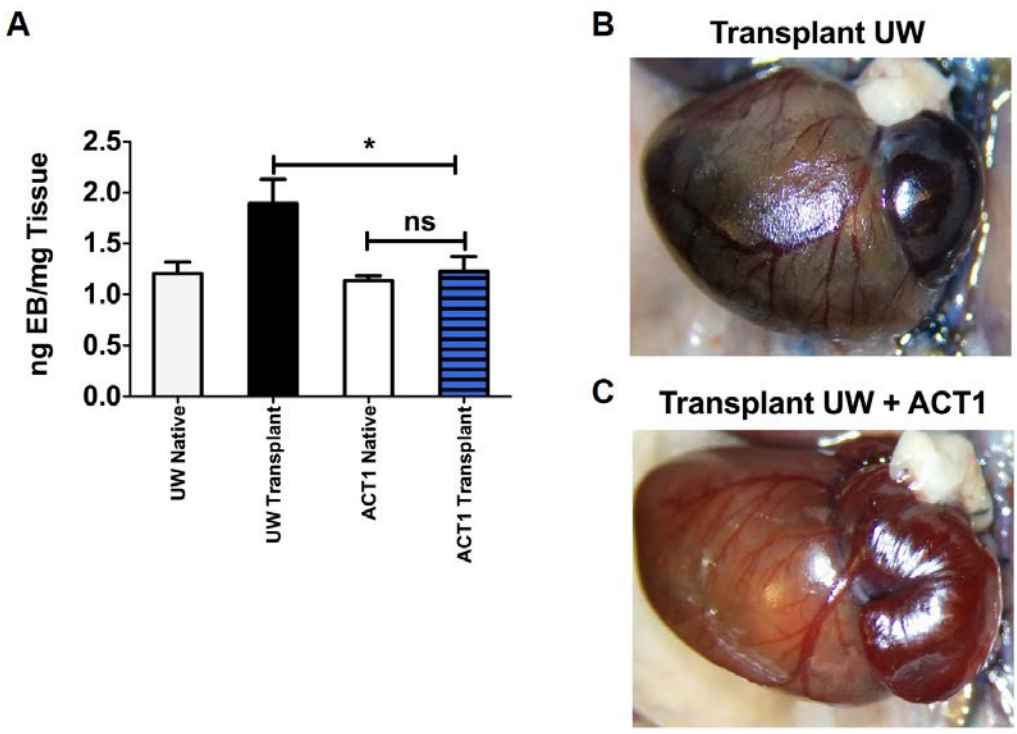


Figure 9*. Donor heart ACT1 preservation solution treatment prevents microvascular leakage following heterotopic heart transplantation.* **A.** Measurement of extravasted Evan's blue dye spectrophotometry 30 minutes post cardiac allograft reperfusion demonstrates a significant reduction in dye uptake in ACT1 pretreated hearts with levels of dye not significantly different from that seen in the native animal heart. **B.** No treatment UW alone, and **C.** UW+ACT1 treatment.

Immune cell infiltration in Acute Heterotopic Heart Transplantation Model

EC activation, injury and permeability all aid the recruitment of inflammatory cells into the allograft which contribute to graft injury and failure. Given our data showing that pre-treatment inhibits microvascular leakage, a marker of endothelial barrier function we next analyzed the impact of pretreatment on ischemia reperfusion injury. We performed allogeneic transplants as described above and harvested heterotopic heart transplants at 48 hrs post transplantation and analyzed for histological evidence of injury, serum cardiac troponin I levels, and the infiltration of inflammatory cells. Cold storage in UW solution augmented with ACT1 significantly reduced cardiac histological evidence of injury (Fig. 10A). Standard of care stored hearts (hearts stored in UW solution) showed evidence of myocardial and endocardial myocyte injury with frequent signs of endothelial activation, such as endothelial swelling. These features were markedly reduced in ACT1 pretreated hearts. To confirm these subjective histological findings we performed serum analysis to measure cardiac troponin I levels, a marker of cardiac myocyte injury (Fig. 10B). In keeping with our histological findings serum cardiac troponin I levels were elevated in UW stored hearts as compared to ACT1 stored donor organs. Finally, we analyzed the presence of graft infiltrating neutrophils (Fig. 10C) and macrophages (Fig.10D). Using immunohistochemistry for cell type specific cell markers we quantified the number of immune cells per/unit area of tissues and demonstrated that both neutrophil and macrophages numbers were significantly elevated in UW versus ACT1 stored hearts.

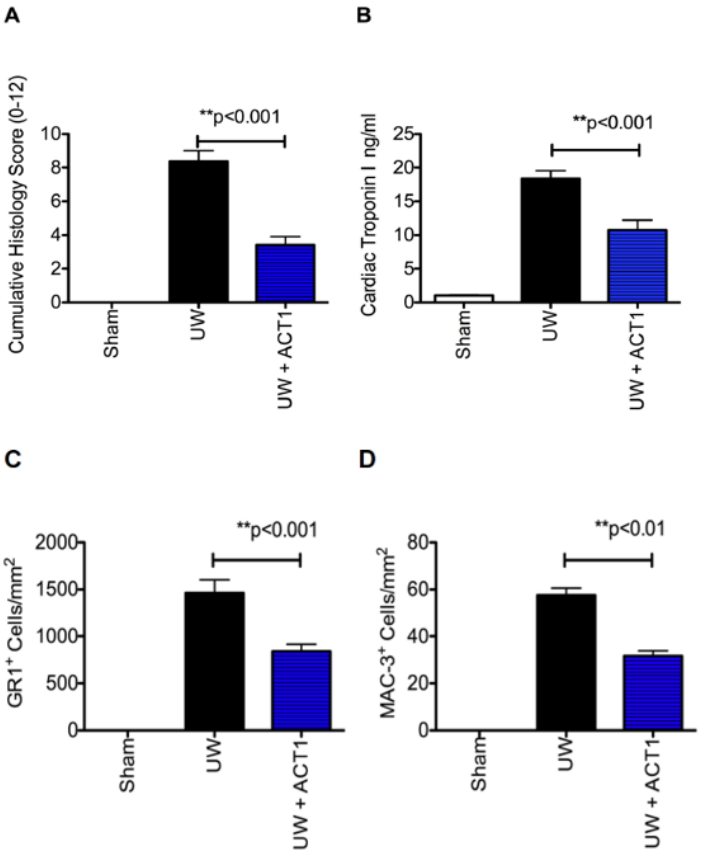


Figure 10. *Assessment of cardiac injury in recipients of donor hearts pretreatment with ACT1*. **A**. Histological quantification of cardiac injury in grafts harvested 48 hours after transplantation, quantified by use of a 0 to 12 cumulative injury score. Pairwise comparisons between UW and UW+ACT1 (#P<0.001). **B**. Serum cardiac troponin I levels in recipient mice 48 hours after transplantation. Pairwise comparisons between UW and UW+ACT1 (#P<0.001). Results are representative of n=6–8. **C** and **D**. Immunohistochemistry localization and quantification of neutrophils (Gr-1) and

macrophages (MAC-3) in cardiac allografts 48 hrs post transplantation. Immunopositive cells were quantified by computerized image analysis and cell counts expressed as cells per mm2. Results expressed as mean ± SE (n=6-8) #p<0.001 and p<0.01, UW vs UW + ACT1.

Elastin van Gieson (EvG) staining to evaluate Neointimal hyperplasia in Aortas

Early insults to the graft have been shown to lead to microvascular injury and dysfunction, which have been argued contribute to the exacerbation of chronic rejection [64, 89]. Given our data that ACT1 protects against ischemia reperfusion injury in our heart transplant model, and that IRI predisposes the graft to chronic rejection, we hypothesized that ACT1 augmentation would reduce the severity of chronic rejection seen in an aortic allograft model of chronic rejection. To further analyze the potential clinically translatability of these studies we further explored the efficacy of ACT1 augmentation of UW solution by storing aortas for prolonged times, 18 and 24 hours, in addition to the standard 6 hour storage time. Grafts were harvested at 7 or 28 hours in the 6 hour storage group to assess chronic rejection and early pathological changes in the standard of care storage time. Grafts static stored for 18 and 24 hrs were harvested at 28 days only. Grafts harvested at 7 days post transplantation had very little evidence of chronic rejection which is seen by expansion of the intima. Small intimal growth were noted in the UW alone group. Intimal expansion was not obviously evident in the ACT1/UW group at 7 days,

although no significant difference overall could be demonstrated (Fig. 11A and C). Pre-treatment with ACT1 for 6 and 24 hours significantly reduced the intimal hyperplasia as compared to control UW, or UW scrambled ACT1 peptide controls (Fig. 11B and C). Eighteen hour stored samples were not significantly different. However, for some reason this group did not develop vasculopathy to the degree that was expected given the storage time and previously published observations.

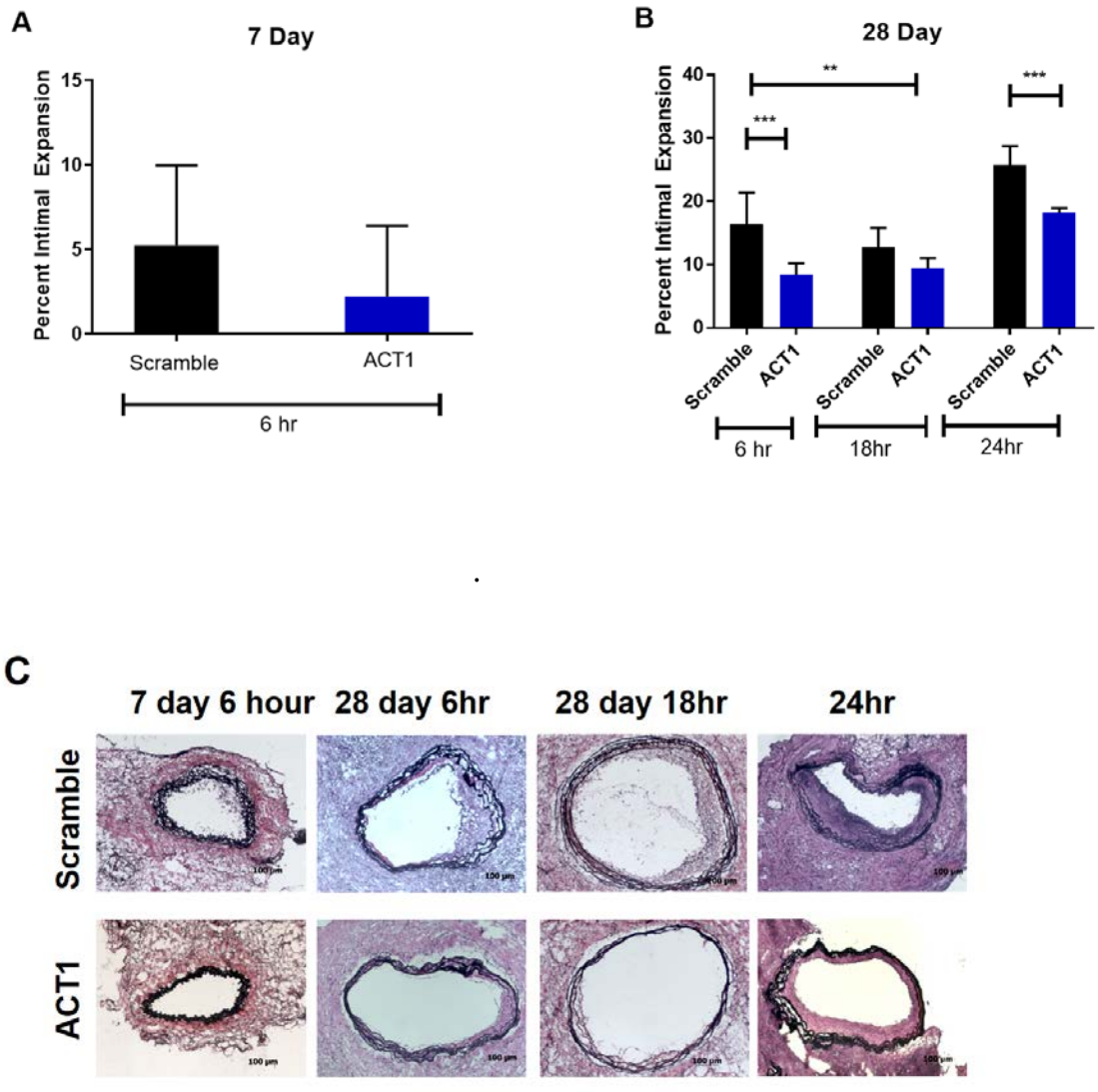


Figure 11: Donor aorta ACT1 preservation solution treatment ameliorates neointimal hyperplasia following heterotopic heart transplantation. **A.** Measurement of aortic intimal proliferation 7 days' post cardiac allograft reperfusion demonstrates a reduction in accumulated cells in the intima with ACT1 pretreated aortas compared with UW treatment alone. **B.** Percent intimal expansion is significantly reduced at 28 days and 6 hrs post-transplantation

including those stored for 24 hours. **C.** Scale as indicated, representative of n=3-6.

Immunohistochemistry to evaluate inflammatory immune cell infiltration

Previous in-vivo and in-vitro studies have shown that brain death, cold storage, and ischemia reperfusion injury activates the endothelium and prime the endothelial cell to facilitate immune cell diapedesis and T cell priming through the expression of co-stimulatory and adhesion markers on the cells surface. To look at immune cell migration into the aorta, immunohistochemistry for CD3 was utilized on aortas harvested at 7 and 28 days' post transplantation. The number of T cells present in the intima was significantly reduced by ACT1 pretreatment.

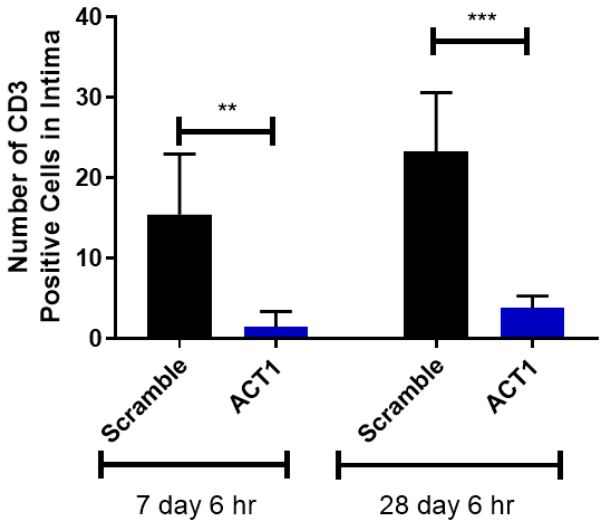


Figure 12. Augmentation of University of Wisconsin Solution with ACT1 reduces innate immune cell allograft infiltration. Immunohistochemistry quantification of CD3 positive T cells in aorta allografts 7 and 28 days post transplantation. Immunopositive cells were quantified by cell counts in the intima of the graft. Results expressed as mean ± SE (n=6-8) #$p<0.001$ and $p<0.01$, UW + Scramble vs UW + ACT1. Representative of n=3-6 per group.

4.2 Discussion

Endothelial cells have a powerful impact on the immune system and its involvement in transplantation. Due to this impact, it is important to understand how these endothelial gap and tight junctions function in graft injury and endothelial interaction with the immune system post-transplantation. Brain death, cold storage and ischemia reperfusion injury (IRI) have a direct impact on endothelial cells, priming the transplanted heart for immunological attack, primary graft failure, acute and chronic rejection [6, 43, 63]. While immunosuppressive treatments reduce acute rejection, these pharmacotherapeutics have little impact on the incidence and severity of chronic rejection. ACT1 is a peptide-based therapeutic that modulates the activity and signaling pathways mediated by the gap junction transmembrane protein, Cx43. Here we analyzed the effects of ACT1 in vivo as a therapeutic to stabilize gap and tight junctions in organs prior to transplantation during static cold preservation in order to reduce organ inflammation, immune cell diapedesis, and ischemic injury to the graft upon transplantation.

As our previous in-vitro data suggested, cold storage and ischemia reperfusion injury lead to endothelial cell permeability and cell death. To test this in vivo, an Evans blue permeability assay was used to see whether the endothelium was permeable post-transplant by looking at microvascular leakage into the heart. ACT1 therapy significantly reduces Evan's Blue extravasation into the transplanted heart as compared to controls, suggesting that ACT1 promotes GJ and TJ stability, and improved endothelial cell integrity in-vivo. Improving the

integrity and barrier function reduces peri-vascular odema which is associated with serum factor induced injury. Factors such as complement components can become activated in the sub-endothelial space leading to immune cell infiltration and activation of smooth muscle and endothelial cells, setting of a cascade of events that ultimately injuries the graft. Thus reduced intra-vascular odema likely contributes to reduced injury. Furthermore, our in-vitro and in-vivo studies show that reduced gap junction injury is associated with decreased EC activation and cell infiltration. Augmentation of UW solution with ACT1 lead to a significant reduction in cardiac graft injury, and further a reduction in immune cell infiltration. Neutrophils are fast acting leukocytes and one of the first to arrive at any site of infection, including diseased and injured tissue. These granulocytes have been associated with many pathological conditions that lead to increased endothelial permeability, such as ischemia reperfusion [59]. Once activated, neutrophils release a large amount of various cytokines, enzymes and reactive oxygen species that interfere with the cell-cell junctions and alter the expression and phosphorylation of the junctional adhesion molecules[17]. Therefore pre-transplant protection of gap junctions likely provides protection from neutrophil induced injury by reducing neutrophil chemotaxis and infiltration.

Given that we had shown a decrease in ischemia reperfusion in-vitro and in-vivo in the cardiac allograft model of ischemia reperfusion injury, and the proposed association with IRI and chronic rejection, we explored the impact of

ACT1 mediated IRI inhibition on the development of chronic rejection. There are a number of rodent models of chronic rejection, all with their associated caveats [4, 50, 63, 90]. Here we utilized the well described aortic model of chronic rejection. This model demonstrates the many features of chronic rejection, such as intimal proliferation, T cell infiltration, and medial injury. While a minor mismatch chronic rejection cardiac transplantation model exists, the vasculopathy they develop, develops in the absence of acute rejection, and the lesions bare little resemblance to the disease seen in aortic model and the human scenario. The literature shows that chronic rejection takes the form of progressive narrowing of the graft arterial lumen, allograft vasculopathy, leading to graft ischemia, replacement fibrosis of the parenchyma of the graft, ultimately resulting in late graft failure [6, 90]. This proliferation of cells in the intima resulting in thinning of the lumen of the vessel, categorized as chronic rejection. Aortic chronic rejection model confirms the features of chronic rejection, characterized by this progressive neointimal thickening and associated luminal occlusion as well as the infiltration of various cell types.

Wang and associates have demonstrated that IRI alone, is sufficient in inducing and accelerating cardiac allograft vasculopathy in their murine model of transplantation. We demonstrate convincingly that ACT1 pretreatment blunts the vasculopathy seen in 6 and 24 hr cold static stored aortic allografts as compared to controls. The data with 18 hrs is less convincing and difficult to interpret. We had expected that 18hrs would follow a similar trajectory of increased vasculopathy associated with increased storage. However, this did not

manifest. It is possible that the n number was insufficient to achieve significant results as only 3 animals were performed in each group, as such these experiments are being repeated to determine the validity of our findings. Nevertheless, the results are clearer with 6, which is the standard of care, and 24hrs that ACT1 not only reduces chronic rejection, but further allows for prolonged storage of organs prior to transplantation.

Cold storage and ischemia reperfusion injury cause a distinct loss of barrier function in MCECs [8, 43]. This could potentially lead to an increase in coagulation, injury signals and immune cell infiltration into the graft. ACT1 augmentation reduced T cell allograft infiltration. Storage in UW solution supplemented with ACT1 significantly reduced T cell infiltration into the graft. These data support our hypothesis that pre-treatment of donor organs with ACT1, a gap and tight junction-stabilizing agent will protect against the pro-inflammatory environment by maintaining endothelial integrity, resulting in improved post-transplant outcomes.

5. Summary and Conclusions

ACT1 is a novel gap and tight junction modifying peptide that interacts with the transmembrane gap junction protein, Cx43, and modulates the activity and signaling of endothelial junctions. We utilized ACT1 as a therapeutic to stabilize gap and tight junctions in organs prior to transplantation during static cold preservation to improve donor organ quality by reducing heart organ injury, inhibiting/reducing endothelial dysfunction caused by cold storage, and reducing organ inflammation, immune cell diapedesis, and ischemic injury to the graft upon transplantation. While many therapies have been applied to preservation solutions, most of these therapies have been used to augment metabolic functions of cells and none of these therapies have been specifically used to improve cell-cell communication as a strategy to reduce donor organ injury during storage and transportation. The mechanism of action of ACT1 is centered around modulating the interactions between the C-terminus of gap junction protein Cx43 and tight junction protein ZO-1, impacting the cellular distribution of Cx43 and altering gap junction and hemichannel communication. This peptide has allowed us to prevent UW cold storage induced endothelial injury, reduce endothelial pro-inflammatory cytokine release, endothelial permeability post transplantation, heart graft injury post transplantation, post transplantation inflammation, with the net effect to reduce the severity of chronic rejection. These results have significance, as current immunosuppressive adequately control acute rejection but do little to impact

chronic rejection. It is well established that the severity of ischemia reperfusion injury has been shown to correlate with early, and more severe chronic rejection. The studies presented here clearly demonstrate that protection of EC junctions can reduce ischemia reperfusion injury whilst further reducing chronic rejection. Given these novel findings we are actively pursuing the application of ACT1 as a therapeutic contained in the preservation solution in large animal models of transplantation.

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