EFFECT OF CYCLOSPORIN A ON THE TUMOR MICROENVIRONMENT

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A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2014

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DEDICATION

To my parents, Yu Yao and Suiping Zhou, who instilled in me a lifelong love of learning.

ACKNOWLEDGEMENTS

ABSTRACT

EFFECT OF CYCLOSPORIN A ON THE TUMOR MICROENVIRONMENT

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Tumor angiogenesis is a hallmark of cancer, and plays a critical role in tumor growth,

expansion, and metastasis. Both physiological and pathological angiogenesis is assumed

to be regulated by the balance between pro and anti-angiogenic factors. One of the best

characterized and most potent pro-angiogenic regulators is vascular endothelial growth

factor, or VEGF. Calcineurin signaling is an important mediator of VEGF signaling in

endothelial cells. Negative regulation of calcineurin by increased expression of its

endogenous inhibitor, Down Syndrome Candidate Region-1 (DSCR1), suppresses tumor

growth and angiogenesis. However, a potent pharmacological calcineurin inhibitor, the

commonly used immunosuppressant cyclosporin A (CsA), significantly increases the

incidence of cancer in organ transplant recipients. The mechanism by which CsA

promotes cancer in this patient population is not well understood and despite the

significance of calcineurin signaling in endothelial cells, the consequences of CsA on

tumor angiogenesis has not been investigated. Using an *in vivo* model of skin

carcinogenesis, we show that long-term CsA treatment promotes tumor growth and

angiogenesis. Further our data indicate that treatment of endothelial cells *in vitro* with

CsA increases proliferation and migration, in a calcineurin-independent manner. Our

studies reveal that CsA-induced endothelial cell activation was due to the interaction of

CsA with cyclophilin D located on the mitochondrial inner membrane. CsA treatment in
v

endothelial cells increased mitochondrial membrane potential and mitochondrial reactive oxygen species production, and was associated with sustained mitogen-activated protein kinase (MAPK) activity. Co-treatment with antioxidants significantly abrogated CsA-induced endothelial cell activation. Furthermore, mice treated with antioxidants were protected against CsA-mediated tumor progression. Taken together, these findings show that CsA functions independent of calcineurin to potentiate tumor growth by promoting tumor angiogenesis via mitochondrial reactive oxygen species production. This work identifies a previously undescribed mechanism underlying a significantly adverse off-target effect of CsA and suggests that co-treatment with antioxidants may inhibit the tumor promoting effects of CsA.

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CHAPTER 1: INTRODUCTION

Angiogenesis

Physiological angiogenesis

The circulatory system provides multi-cellular organisms with oxygen, nutrients, and signaling molecules necessary for growth and homeostasis. Vertebrate vasculature is defined as a closed system of elastic arteries that distribute blood to distant tissues, capillary beds where gas exchange occurs, a venous system that returns deoxygenated blood, and a muscular pump that moves the blood through at a rate adaptive to the metabolic demands of the organism. This mode of nutrient and oxygen delivery has been conserved with relatively few changes throughout evolution (Axnick & Lammert 2012).

Developmentally, the vertebrate vascular system appears early on in embryogenesis, starting in the primitive streak. Endothelial precursors develop into a primary plexus, dorsal aorta, and the cardinal vein, before joining together to form the preliminary circulatory system. The de novo formation of vessel from endothelial precursors early in development is termed vasculogenesis (Jain 2003). In addition to providing a rudimentary circulatory system for the developing embryo, vasculogenesis also plays a role in shaping organogenesis and has been shown to provide trophic signals for liver, pancreatic, and kidney development (Coultas et al. 2005).

Once a primitive vascular template has been established through vasculogenesis, the vascular plexus expands and grows into a mature circulatory system by means of

vascular sprouting and remodeling from existing vessels, a process known as angiogenesis (Jain 2003) which is responsible for the majority of vascular development. Angiogenesis is a tightly regulated process that continues throughout development, with increasing interactions and specificity towards the organ systems it supplies.

The angiogenic process can be roughly separated into four stages: initiation of sprouting, sprouting outgrowth, sprout fusion / lumen formation, and stabilization/ maturation of the vessel (Jain 2003; Carmeliet 2000) (Fig 1.1.). These stages are contiguous with one another and are tightly regulated by pro and anti-angiogenic regulators to ensure proper vessel growth. This process is most prominent in fetal development, but also occurs later in life during physiological and pathological angiogenesis in adults (Carmeliet 2005).

The first step of angiogenesis is the stimulation of angiogenic growth that is primarily mediated by the secreted vascular endothelial growth factor A (VEGFA), a critical pro-angiogenic molecule. VEGFA is part of a large family of angiogenic regulators that include VEGFB, VEGFC, VEGFD, and placental growth factor (PlGF) (Coultas et al. 2005) (Table 1.1). The predominant effect of VEGFA on nascent endothelial cells is to promote migration, proliferation, and differentiation. The effect of VEGFA on developing vessels is regulated by various splice variants and receptor signaling such as the anti-angiogenic β-isoform VEGFA splice variant and the decoy receptor VEGFR1, which sequesters VEGFA with minimal angiogenic signaling effects (Adams & Alitalo 2007) (Table 1.2). Chemotactic gradients, formed from pro- and

inhibitory signals, select individual endothelial cells to sprout from the parent vessel.

Sprouting endothelial cells actively degrade extracellular matrix in the migratory front,
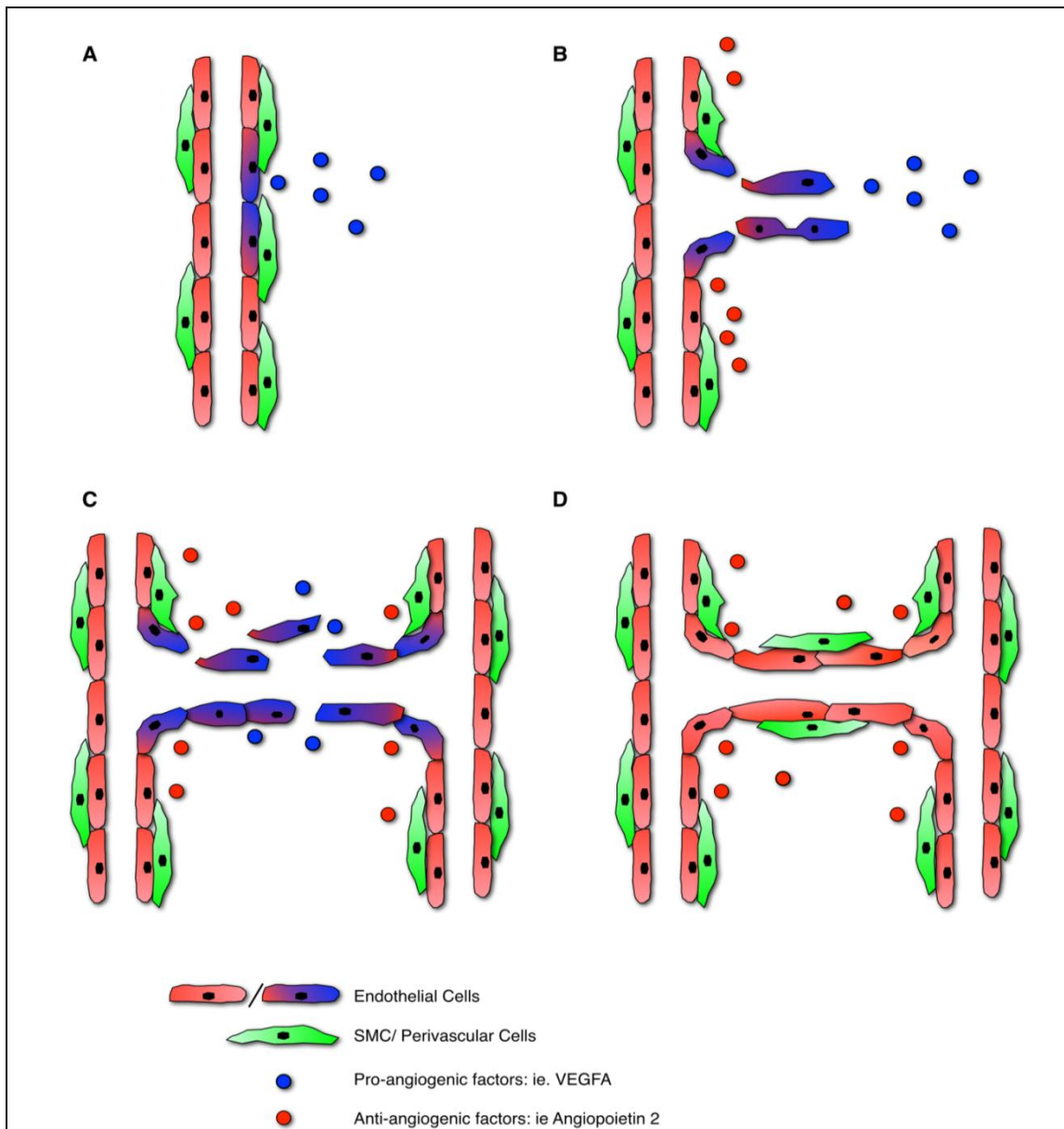
Fig. 1.1. Overview of physiological angiogenesis. (A) Activation of endothelial cells through chemotactic gradients, followed by (B) migration through the vascular smooth muscle cell barrier and proliferation, guided by pro- and anti-angiogenic signals. (C) The vessel continues to elongate and may fuse with another growing vessel before (D) excessive vessels are pruned, lumen formation finalizes, and vascular smooth muscle cells are recruited.

change their apical-basal cellular polarity, and laterally inhibit neighboring endothelial

Table 1.1 VEGF family members. Bolded terms indicate dominant form or effect

Ligand	Size – isoforms	Expression	Receptor	Effect on angiogenesis
VEGF-A	VEGFA$_{121}$ (soluble) **VEGFA$_{165}$ (intermediate solubility)** VEGFA$_{165b}$ (inhibitory) VEGFA$_{189}$ (heparin bound) VEGFA$_{206}$ (heparin bound)		VEGFR1 VEGFR2 NPR1	**Pro-angiogenesis** ↑ Permeability Bone turnover Monocyte chemokine HSC mobilization
VEGF-B	**VEGFB$_{167}$ (heparin bound)** VEGFB$_{186}$ (soluble)	Muscle Myocardium Brown fat	VEGFR1 NP1	Unclear Weakly mitogenic
VEGF-C	VEGFC $_{(29/31kDa – unprocessed)}$ VEGFC $_{(21kDa – processed)}$	Fetus: Lymph vessels	VEGFR2 VEGFR3	**Lymphangiogenesis**
VEGF-D		Fetus: lung, skin	VEGFR2 VEGFR3	Angiogenesis and lymphangiogenesis
PlGF	PlGF$_{131}$ PlGF$_{152}$ (heparin bound) PlGF$_{203}$ PlGF$_{224}$ (heparin bound)	Placenta Retina Heart Lung	VEGFR1 NP1	Placental, ovarian, and retinal angiogenesis

Table 1.2 VEGF receptors. Bolded terms indicate dominant effect.

Receptor	Alternative forms	Ligand	Expression	Pairing Partner	Signaling Outcome
VEGFR1 (Flt-1)	Soluble VEGFR1 (inhibitory)	VEGFA VEGFB PlGF	endothelium, osteoblasts, macrophages, renal cells, hematopoietic stem cells (HSC), placenta trophoblasts	VEGFR1 VEGFR2	+VEGFR2 = **weak angiogenesis** +VEGFR1 = inhibitory Soluble VEGFR1 = decoy receptor
VEGFR2 (KDR/Flk-1)		VEGFA VEGFC VEGFD	endothelium, neurons, osteoblasts, megakaryocytes and HSC	VEGFR1 VEGFR2 VEGFR3	VEGFR2 homodimerization major mediator of **angiogenesis**
VEGFR3 (Flt-4)		VEGFC VEGFD	Lymphatic endothelial cells	VEGFR2 VEGFR3	**Lymph-angiogenesis**
NP1	Soluble NP1 (inhibitory)	VEGFA$_{165}$ VEGFB and PlGF	arterial endothelium, lung, heart, liver, kidney, pancreas, bone marrow	VEGFR2	Enhances VEGFA-VEGFR2 signaling Soluble NP1=decoy receptor
NP2		VEGFA, VEGFC and PlGF	lymphatic, venous endothelium, lung, heart, liver, kidney, pancreas, bone marrow	VEGFR2	Unclear

cells from undergoing the same process. Interplay between the Notch-delta ligand like 4 (DLL4) and the VEGFA-VEGFR signaling plays a crucial part in this sprouting process (Adams & Alitalo 2007; Jain 2003). Tip cell selection is not completely understood, but is thought to be due to small differences in VEGFR2 expression or VEGFA in the local environment along with crosstalk with Notch signaling (Blanco & Gerhardt 2013).

The establishment of tip cells is closely associated with the conversion of cells immediately behind the tip cell into stalk cells, which follow the tip cells as the sprout extends outwards, and proliferate to lengthen the nascent vessel. Stalk cells will proliferate in response to guidance by the tip cell, which itself does not divide. Tip cell maintenance, like in sprout selection, is thought to be due to interplay between the Notch-DLL4 and VEGFA-VEGFR pathways, with increased expression of both in tip cells (Adams & Alitalo 2007).

When the budding vessel encounters existing capillaries or other growing endothelial sprouts, it establishes adhesive cellular junctions to fuse with the encountered vessel. Inappropriate junction formation is prevented by various adhesive and repulsive signals. Upon fusion with another vessel, the newly developed vessel then lumenizes to allow for eventual fluid flow (Adams & Alitalo 2007). This lumenization process is poorly understood, but recent evidence shows it occurs through 'cord hollowing' where a cylindrical column of cells drastically reshape around a hollow core to form a multicellular lumenized tube (Nelson & Beitel 2009).

Naked endothelial cells that constitute newly formed vessels are unstable and leaky and require stabilization through pericyte coverage. Pericytes and the closely

related vascular smooth muscle cells are contractile, mesenchymal cells that regulate vessel contraction or dilation, permeability, and immune cell infiltration. In the developing vessel, platelet derived growth factor (PDGF), secreted by endothelial cells, recruits and attracts pericytes. Subsequent pericyte coverage and stabilization of the vessel is, in part, regulated by the Angiopoietin-Tie2 system. Pericyte-derived soluble molecule angiopoietin 1 (Ang-1) promotes endothelial cell survival and mural cell attachment through binding to Tie-2 receptors on endothelial cells. Negative regulators such as angiopoietin 2 (Ang-2), which also binds Tie-2 receptors, act to antagonize vessel stability and work in conjunction with Ang-1 to prune and guide vessel formation and maturation (Jain 2003).

Angiogenesis is an active process that occurs largely during development. Once the vascular network have been formed and stabilized, it remains relatively static throughout life. Second only to cells in the nervous system, endothelial cells are one of the longest-lived cells in the body (Bergers & Benjamin 2003). Angiogenesis in adulthood is an infrequent event and occurs in response to a precipitating incident such as wound healing, endometrial growth during the menstrual cycle, or placental growth during pregnancy (Carmeliet 2005). When it does occur, physiological angiogenesis in adults is also tightly regulated and follows the same steps and pathways as in developmental angiogenesis.

Pathological angiogenesis

While physiological angiogenesis in adults is a rare event, many pathological conditions feature dysregulated vessel growth. In preeclampsia and ischemic heart disease, the angiogenic response is insufficient to meet metabolic needs, resulting in inadequate tissue perfusion. In the majority of disease states involving pathological angiogenesis the angiogenic switch is inappropriately activated, leading to aberrant vessel formation. While pathological angiogenesis occurs through a similar fashion as physiological angiogenesis, the tightly controlled factors and temporal steps that result in functional vessels are dysregulated, and vessel maturation in particular, is absent or abnormal in the disease state. The predominant pathological conditions that involve aberrant angiogenesis are either inflammatory or malignant.

Diabetic retinopathy

The retina has a highly regulated and organized vascular anatomy to balance perfusion with light detection. Areas of high photoreceptor density such as the fovea, have greatly decreased vascularization (Gariano & Gardner 2005). Pathological retinal angiogenesis that occurs in diabetes is the leading cause of irreversible vision impairment and blindness in adults. Diabetic retinopathy occurs in 20% of patients with diabetes, a systemic metabolic and inflammatory disease, and is characterized by microaneurysms and exudative changes in the retinal vessels, areas of ischemia, abnormal vessel growth, friable vessels, and hemorrhages (Gariano & Gardner 2005; Gardner 2012). This process is progressive, occurring over years, and is dependent on the overall metabolic health of

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the individual. Untreated, visual acuity will progressively decline and, in severe cases, marked by episodic events of hemorrhaging or retinal detachment (Gardner 2012). As the major inducer of angiogenesis, VEGFA plays a significant role in diabetic retinopathy. In combination with inflammatory cytokines and chemokines, accumulation of VEGFA in the vitreous humor results in increased vascular permeability of existing vessels and initiates the formation of new vessels (Gariano & Gardner 2005). However, pericyte coverage of the newly formed vessels is low, which in combination with pericyte loss from existing vessels, results in weak and unstable retinal vasculature that dilate and leak. This abnormal vessel growth paradoxically produces hypoxic conditions in the vitreous, which induces the hypoxia inducible factor (HIF) to further upregulate pro-angiogenic responses (Gariano & Gardner 2005). The most common treatment for diabetic retinopathy involves vessel ablation by laser treatment, which prevents hemorrhages and decreases exudate, but is a symptomatic treatment at best. Single target anti-angiogenic therapies with anti-VEGF treatments have shown only partial success, as diabetic retinopathy is complicated by the involvement of inflammatory molecules which also affects vessel function (Gardner 2012). Therefore, the development of anti-angiogenic treatments for diabetic retinopathy is still an area of active research.

Exudative age-related macular degeneration

The second most common cause of blindness in the elderly is age-related macular degeneration (AMD), an inflammatory disease confined to the retina. As the name implies, macular degeneration is the progressive and selective loss of vision in the macula,

or center of the visual field. This disease occurs in two types, dry/non-exudative AMD, characterized by plaque-like formations called drusden, and wet/exudative AMD, a more severe form characterized by neovascularization. As with diabetic retinopathy, the newly formed vessels associated with wet AMD is dysfunctional, leaky, and fragile, causing functional impairment of the retina and can lead to retinal detachment (Rattner & Nathans 2006). Previously, clinical management of wet AMD consists of symptom relief through laser ablation therapy. In recent years, treatment options have improved due to the use of anti-VEGF therapy to staunch the growth of new vessels (Dadgostar & Waheed 2008). Injections of anti-VEGF antibodies such as Bevacizumab and Ranibizumab directly into the retinal space have not only slowed the progression of disease but also significantly improved vision, and is now the gold standard treatment for wet AMD (CATT Research Group 2011; Dadgostar & Waheed 2008).

Tumor angiogenesis

Perhaps the most diverse and heterogeneous example of pathological angiogenesis is tumor angiogenesis. The vascular requirement of solid tumors was first recognized in the early 1970s by Judah Folkman. Given the rogue nature of tumor growth, perfusion of a solid malignant growth can be a rate-limiting step for its expansion. The importance of angiogenesis for malignant progression is reflected in its inclusion as one of the original six hallmarks of cancer (Hanahan & Weinberg 2000).

Neoplastic angiogenesis is varied and heterogeneous depending on tumor type, tumor stage, and other microenvironment cues. It is, however, almost always

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disorganized, tortuous, and inefficient. Tumor vasculature does not follow hierarchical

divisions that mark normal vasculature such as arteries, capillary beds, and veins, with

hemodynamic considerations. Instead, neoplastic vessels are irregularly shaped and

dilated, with little or no distinction between arterioles, venules, or capillaries. Vessel

anastomosis may be abnormal or absent, explaining the presence of dead-end vessels.

Due to the chaotic nature of tumor vessels, perfusion is often poor with areas of

hemostasis or even reversal of flow. In addition to abnormal structure, the vessel itself is

often vastly haphazard and irregular with variable cellular organization and improper cell

junctions. These vessels display patchy and irregular mural cell coverage which, when

present, is also abnormal and dysfunctional. In combination with irregular hemodynamics,

these erratic vessels with poor vessel structure are often leaky, exudative, and

paradoxically under-perfused (Bergers & Benjamin 2003). Due to the dysfunctional

nature of neoplastic vessels and continuous remodeling that occurs in tumor vessels,

tumor perfusion can range from very hypoxic to very oxygenated, and can vary spatially

and temporally (Bergers & Benjamin 2003). The tumor microenvironment is a harsh and

variable environment; therefore, cancer cells that survive in this environment often have

decreased metabolic and oxygen demands, enabling them to survive within the inefficient

vascular network.

As a tissue expands, it outgrows its oxygen and nutrient supply and becomes

hypoxic, at which point hypoxia-responsive genes, regulated by HIF, are upregulated.

Growing tumor tissue, under the duress of hypoxia, secretes pro-angiogenic factors, the

most notable being VEGFA, but also can include fibroblast growth factor (FGF), placenta

induced growth factor (PIGF), and transforming growth factor-β (TGF-β). VEGFA alone is able to initiate the angiogenic response, promoting sprouting from nearby vessels. Since tumor growth is not organized or patterned, its promotion of angiogenesis is similarly disorganized due to a misbalance between pro and anti-angiogenic factors (Hickey & Simon 2006; Bergers & Benjamin 2003). While pericyte coverage in tumor vasculature is found to be variable depending on the tumor subtype and the study methods, the general consensus is that they are abnormal and dysfunctional (Raza et al. 2010). Tumor associated pericyte-like cells have weak endothelial cell contacts, and have been shown to secrete VEGF, further aggravating vessel growth and leakiness (Raza et al. 2010; Bergers & Benjamin 2003).

The angiogenic process is a tight balance of pro and anti-angiogenic regulators. In physiological angiogenesis, endogenous anti-angiogenic proteins help guide and structure new vessel growth, as well as maintain vascular quiescence and prevent excessive growth. Endogenous anti-angiogenic proteins originate from multiple sources; many are derived from extracellular matrix proteins, others are inflammatory molecules, and some are soluble decoy receptors (Folkman 2004). The extracellular matrix and stroma surrounding existing vessels exert multiple layers of control and regulation in vessel growth. As angiogenesis is a rare event in adult vasculature, the stromal environment strongly inhibit angiogenesis, and cleaved fragments of many extracellular matrix proteins, such as endostatin from collagen XVIII and tumstatin from collagen IV (O'Reilly et al. 1997; Maeshima et al. 2000), act to inhibit new vessel growth. Inflammatory molecules such as IFN-α, IL-1, IL-4, and IL-8 have also been shown to

have anti-angiogenic properties (Ribatti 2009). Endostatin, angiostatin, and thrombospondin are the best characterized endogenous anti-angiogenic factors. Loss of these factors, as in thrombospondin-1 null mice, results in accelerated tumor growth with increased angiogenesis (Good et al. 1990). Therefore, endogenous anti-angiogenic factors act as a natural defense system against pathological angiogenesis. Several drugs exploiting these endogenous anti-angiogenic factors have shown promise in clinical trials. For example, endostatin, in combination with chemotherapy, showed efficacy in several clinical trials (Rong et al. 2012). The use of these drugs, however, is complicated by the high dose needed, short half-life, repeated administration, and potential toxicities. While they have potential for treating solid cancers, more pharmacological and clinical studies are needed.

Anti-angiogenic therapy

The neovascular requirement of solid tumors opens a promising field for the development of new anti-cancer drugs that target angiogenesis. A common misunderstanding of anti-angiogenic therapy is that it serves only to cause vessel regression, starving the tumor of oxygen and preventing tumor progression. While this does occur, anti-angiogenic therapy has also been shown to cause vessel normalization (Carmeliet & Jain 2011). Tumor vessels are inefficient in delivering oxygen and nutrients, and in the setting of treatment, chemotherapeutic drugs. Therefore, in combination with chemotherapeutic treatment, improving tumor vessel function actually results in better drug delivery to the intended tissue (Carmeliet & Jain 2011). Therapies that target tumor

vessels are focused on vessel maturation and stabilization. VEGF withdrawal occurs in

the maturation stages of physiological angiogenesis and contributes to endothelial cell

quiescence. In line with this, multiple anti-angiogenic therapies were put into clinical trial

with the intent of vessel normalization, better drug delivery, and ultimately tumor

regression. In targeting the VEGF pathway, several anti-angiogenic drugs have been

developed: bevacizumab is a ligand-trapping monoclonal antibody, sorafenib and

sunitinib are kinase inhibitors that inhibit VEGFR2 (Bergers & Hanahan 2008).

Given the complexity of angiogenesis, its dysregulation in tumor angiogenesis,

and the mutational propensity of cancer cells, it is not surprising that anti-angiogenic

therapy has been clinically disappointing. While clinical trials utilizing anti-angiogenic

therapies initially showed promise with tumoristatic activity and rare tumoricidal activity,

most, if not all anti-angiogenic therapies eventually fail with tumor progression and

growth (Bergers & Hanahan 2008; Weis & Cheresh 2011). This has been attributed to a

number of resistance mechanisms. In response to anti-angiogenic treatment, tumors often

develop evasive resistance to the anti-VEGF agent used, switching from reliance on the

VEGF pathway to alternative pro-angiogenic signals, such as FGF, PDGF, or

angiopoietins. Secondly, hypoxia induced by the anti-angiogenic agents not only

upregulates pro-angiogenic signals, but also induces the recruitment of various bone

marrow derived cells such as vascular progenitors and vascular modulatory cells, which

can independently contribute to the tumor vasculature. Thirdly, anti-angiogenic therapy

in the absence of other chemotherapeutic agents will promote vessel normalization by

increasing pericyte coverage. This increases oxygen and nutrient delivery to the tumor

despite lowering microvessel density. Lastly, tumors by their nature are highly adaptive. With the loss of de novo vessel growth, cancers cells that are more invasive have a survival advantage, resulting in increased local invasion and distant metastasis. For example, in response to regression of the anti-VEGF-sensitive tumor vessels, gliomal cells have been shown to co-opt normal vessels that are insensitive to anti-VEGF therapy, and track along those conduits to invade locally (Bergers & Hanahan 2008). The failure of the initial trials using anti-angiogenic therapy highlights the complexity of tumor angiogenesis, and targeting it successfully requires further understanding of the interplay between tumor cells and their microenvironment.

Calcineurin and Angiogenesis

Calcineurin biology

Calcineurin is a calcium regulated ser/thr phosphatase conserved from yeast to humans (Krinks 1979). It is a ubiquitous signaling pathway downstream of many extracellular signals with tissue-specific functions (Rusnak & Mertz 2000). Discovered as a binding partner to the immunosuppressant cyclosporin A (CsA), the calcineurin pathway (Liu et al. 1991) (Fig. 1.2.) was first delineated in T cells (Ho et al. 1996; Clipstone & Crabtree 1992), and was later found to have important roles in neuronal survival, myocardiocyte response to stress, vascular patterning, and angiogenesis (Crabtree & Olson 2002). In response to an activating signal, such as T cell receptor

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(TCR) activation, there is an increase in intracellular calcium that binds to the calcium

modulated protein, calmodulin, which in turn activates the calcineurin complex.

Fig. 1.2. Overview of calcineurin signaling in endothelial cells.
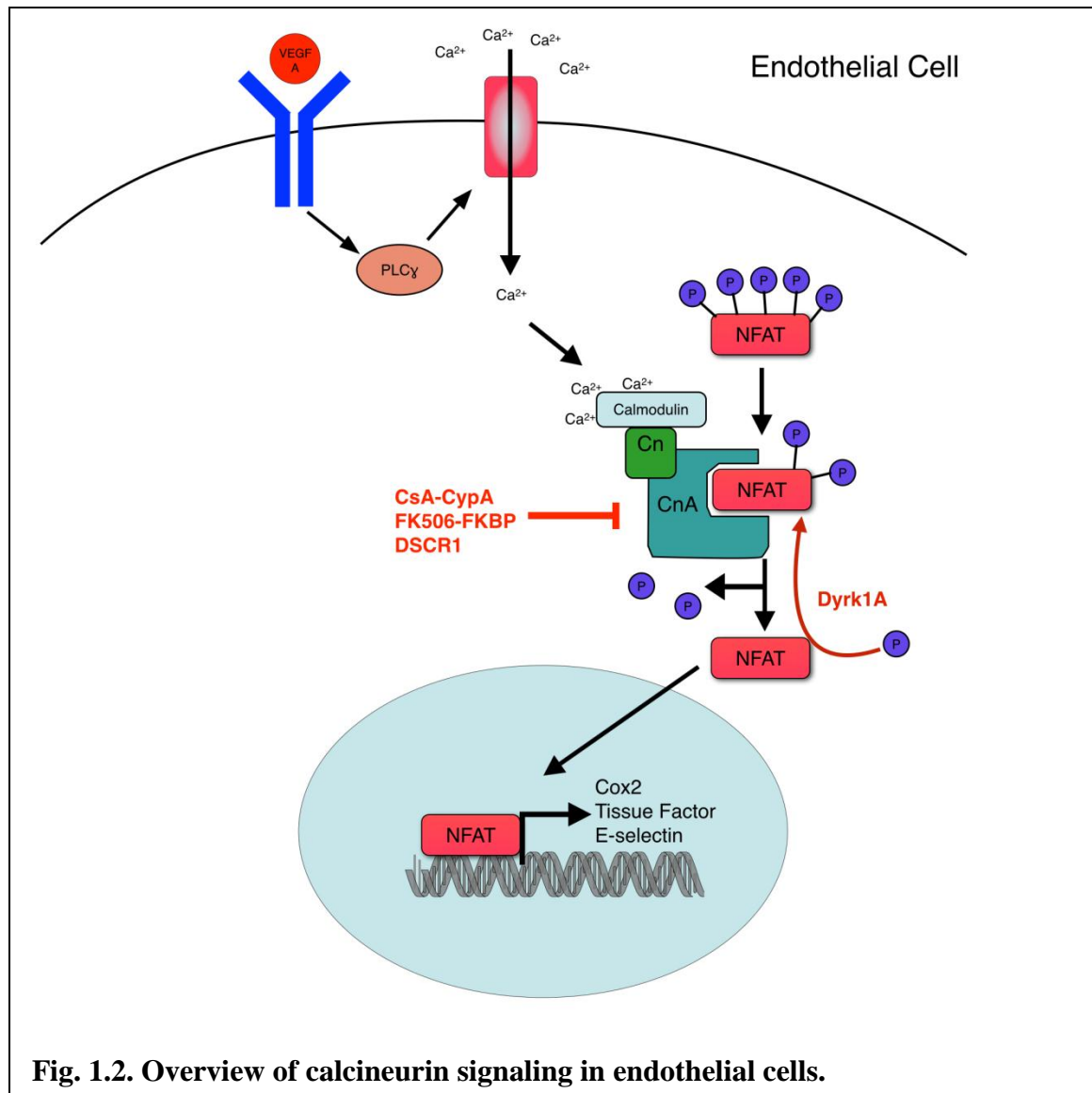
Calcineurin is composed of the catalytic subunit calcineurin A and the regulatory

subunit B. In absence of calcium and calmodulin binding, calcineurin A is kept inactive

with an auto-inhibitory loop. When calmodulin is bound to calcium, it binds to

calcineurin B, which moves the auto-inhibitory loop out of position, allowing calcineurin

to be enzymatically active. When active, calcineurin dephosphorylates its downstream

substrates, the most notable being the nuclear factor of activated T-cells (NFAT) family

of transcription factors (NFAT1-5) (Table 1.3), although a number of other calcineurin

substrates have also been identified. After dephosphorylation by calcineurin, NFAT

shuttles from the cytosol into the nucleus, where it acts as a transcription factor. (Rusnak

& Mertz 2000; Jain et al. 1993)

Table 1.3 NFAT family members.

Name	Alternate names	Expression Pattern	Knockout phenotype
NFAT1	NFATc2 NFATp	Immune cells, olfactory cells, endothelial cells, skeletal muscle, chondrocytes, adipocytes	Immune hyperactivation and allergic responses Suppression of chondrogenesis (NFAT1+2 double knockout = failure of T cell activation and immune response)
NFAT2	NFATc1 NFATc	Immune cells, endothelial cells, skeletal muscle	Lethal failure of cardiac morphogenesis Defects in thymic development and T cell activation in RAG1-/- chimeras
NFAT3	NFATc4	Embryonic: vascular precursor Endothelial cells, adipocytes	No apparent defects (NFAT3+4 double knockout = lethal vascular patterning defects)
NFAT4	NFATc3 NFATx	Embryonic: vascular precursor Immune cells, endothelial cells, skeletal muscle, keratinocytes	Defects in thymic development and hyperproliferation of lymphocytes
NFAT5	TonEBP OREBP	Immune and tumor cells	Unknown

In T-cells, nuclear NFAT transactivates genes that are required for T-cell activity,

such as IL-2 and the IL-2 receptor (Hogan et al. 2003). In other cell types, NFAT is

responsible for cell-specific gene transactivation or repression. For example, calcineurin

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is highly expressed in neuronal tissue and plays a role in neuronal plasticity and memory consolidation (Mansuy 2003). The role of calcineurin in the vasculature is an active and important area of research (Nilsson et al. 2008). Calcium signaling is highly regulated in the myocardium. In embryonic cardiovascular development, calcineurin signaling is involved in the migration of the myocardial precursors and the formation of the heart valves (Chang et al. 2004). Post-natally, the calcineurin-NFAT pathway appears to dichotomously regulate heart size and hypertrophic responses as well as exert a protective effect on the heart (Molkentin 2004). Specific loss of calcineurin in the heart results in mice with smaller hearts at baseline that are also refractory to hypertrophic growth (Frey & Olson 2003). This and other studies indicate the potential for targeting calcineurin signaling for developing therapies for hypertrophic cardiomyopathy (Frey & Olson 2003).

Calcineurin signaling in tumorigenesis

As the calcineurin-NFAT pathway regulates a variety of physiological tissue-specific cellular effects, it similarly has been found to be dysregulated in cancers with a multitude of effects. There are five NFAT family members with varying degrees of redundancy and specificity in their downstream effects (Table 1.3). NFAT1 and 2 have similar DNA binding domains and are mostly functionally redundant. NFAT3 and 4 also share similar roles (Crabtree & Olson 2002). Unlike other NFAT proteins, NFAT 5, is not regulated by calcineurin (Mancini & Toker 2009). Dysregulation of NFAT expression or signaling has been found in various cancers and reflects its tissue-specific functions. Consistent with its role in the immune system, activated nuclear NFAT2 has been found

in cases of hematological malignancies such as Burkitts lymphoma, diffuse large cell B

cell lymphoma, and T cell lymphomas, and responds to calcineurin inhibition (Mancini &

Toker 2009). Additionally, NFAT2 has been found to be overexpressed in pancreatic

cancers. Expression of NFAT1 in breast cancer promotes invasion and migration through

increased transactivation of the direct NFAT target, COX-2. Furthermore, the

calcineurin-independent NFAT5 has also been shown to be an effector in integrin

mediated migration but not invasion in breast cancer, demonstrating multiple and

complex roles of NFAT family members in tumorigenesis (Jauliac et al. 2002).

Calcineurin signaling in tumor angiogenesis

The calcineurin pathway in the tumor microenvironment plays a critical role in

tumor angiogenesis. Calcineurin is highly expressed in endothelial cells and is

downstream of VEGF signaling. Activation of VEGFR2 by its ligands increases

intracellular calcium, which activates calcineurin and subsequently causes NFAT nuclear

localization. In endothelial cells, NFAT promotes the transcription of angiogenesis

responsive genes, such as cyclooxygenase-2 (COX-2), E-selection, and tissue factor

(Hesser et al. 2004). The physiological relevance of the calcineurin-NFAT pathway in

tumor angiogenesis is illustrated in the Down Syndrome (trisomy 21) population.

Individuals with Down Syndrome have an extra copy of chromosome 21 and are

protected from up to 90% of solid tumors when compared to age-matched controls (Yang

et al. 2002), suggesting the increased expression of tumor suppressor genes. Alternatively,

as solid tumors require angiogenesis for progression, overexpression of anti-angiogenic

regulators can tip the angiogenic balance against the growth of tumor vessel, halting solid

tumor growth. Indeed, chromosome 21 encodes DSCR-1, dual specificity tyrosine-

phosphorylation-regulated kinase 1A (DYRK-1A), and PCP-4, which are three negative

regulators of calcineurin. A mouse model of Down Syndrome also demonstrated

decreased tumor growth in a transplantable tumor model (Baek et al. 2009). Modest

overexpression of just one gene that negatively regulate calcineurin, DSCR-1, from three

alleles as opposed to two, was sufficient to inhibit calcineurin activity in endothelial cells,

resulting in decreased tumor growth and tumor angiogenesis. In light of this work, it is

clear the calcineurin pathway is a critical determinant in tumor angiogenesis and

inhibition by the endogenous protein DSCR1 can be potentially exploited to develop new

anti-angiogenic therapies for solid cancers. Furthermore, studies of trisomy 21 highlight

additional molecules such as DYRK-1A and PCP-4, also involved in the negative

regulation of calcineurin signaling, that may yield potential anti-angiogenic therapies.

Cyclosporin A and Tumorigenesis

      Pharmacological calcineurin inhibitors have been used clinically for decades as

immunosuppressive drugs. Cyclosporin A (CsA), a cyclic fungal peptide, was first

identified in 1970s as a potent T cell inhibitor, and subsequently the entire calcineurin-

NFAT pathway was elucidated based on its interactions (Rusnak & Mertz 2000;

Clipstone & Crabtree 1992; Jain et al. 1993). As a potent immunosuppressant, CsA is

used primarily in organ transplant patients to prevent graft rejection, but is also used for

dermatological, ocular, and various autoimmune conditions. Within a cell, CsA

complexes with intracellular cyclophilins, a family of peptidy-propyl isomerases (PPI). When CsA is bound to the cyclophilin family member cyclophilin A, it forms a complex that sits at the substrate docking site of calcineurin, acting as a competitive inhibitor (Rusnak & Mertz 2000; Huai et al. 2002). Binding of CsA to other cyclophilins does not affect calcineurin activity, but is responsible for calcineurin-independent effects of CsA.

Based on the positive role of calcineurin signaling in tumor angiogenesis, it would be reasonable to assume that use of CsA, a calcineurin inhibitor, would decrease solid tumor incidence. In contrast, there is a significant increase in the incidence of solid tumors in the organ transplant population who are on long-term CsA therapy when compared to aged matched controls. This increase in cancer risk is selective, with an increase in virally associated lymphomas and leukemia, and more prominently, skin cancers. Depending on the study, CsA therapy increases the risk of developing squamous cell carcinomas by 65-250 times compared to the general population, followed by basal cell carcinomas and melanomas (Kauffman et al. 2006). While non-transplant associated squamous cell carcinomas and basal cell carcinomas can be fatal if left untreated, they generally present as a slow growing tumors that are amenable to surgical excision and have a good prognosis. CsA-associated skin cancers, however, are more locally invasive, with much greater metastatic potential and a poorer prognosis (Dantal et al. 1998; Dantal & Soulillou 2005).

This increase in cancer incidence after long-term CsA therapy is at odds with its role as a calcineurin inhibitor. Since CsA is a potent immunosuppressant, it was assumed that the increased cancer risk with CsA treatment was due to loss of immunosurveillance.

However, loss of immunosurveillance is not sufficient to account for the significant increase in tumorigenesis. Rapamycin, also an effective immunosuppressant used for organ transplants, is not associated with increased cancer risk, and actually has been shown to decrease tumor growth in some models (Guba et al. 2002). Additionally, CsA treatment has been shown to promote tumorigenesis in an immune-independent manner. In athymic nude mice and SCID mice, which lack functional B and T cells, CsA treatment still promotes the growth of subcutaneous xenograft tumors (Hojo et al. 1999). Several studies have investigated the mechanisms of CsA-associated tumorigenesis and are discussed in the following sections.

CsA treatment and immunosuppression

Cancers are often referred to as "wounds that never heal," and the inflammatory contribution of immune cells to tumorigenesis is appreciated as a major regulator of tumor progression (Dunn et al. 2002), both in promoting and inhibiting cancer progression. The theory of immunosurveillance, first elaborated by Burnet and Thomas in 1957 (Burnet 1957; Burnet 1970), depicts the immune system as anti-carcinogenic, recognizing cancer cells as "non-self" to be destroyed. Cancer cells, by their very nature, have escaped the normal constraints of their role in a multi-cellular organism, and are genetically and phenotypically different from normal cells. These differences allow for immunological recognition, which preludes their destruction by the adaptive immune system (Dunn et al. 2004). Additionally, infections by specific viruses such as human papilloma viruses, hepatitis B and C viruses, and herpes viruses, can elevate the risk of

associated cancer, especially if the infection is chronic and unresolved (Moore & Chang 2010). Loss of immunosurveillance, as in the context of acquired immunodeficiency syndrome (AIDS) and various congenital defects, also elevates the risk of cancers (Gatti & Good 1971; Simard et al. 2011). Since calcineurin is important for T-cell activation, systemic CsA therapy results in profound immunosuppression. While CsA treatment in immunodeficient mice has been shown to promote tumorigenesis (Hojo et al. 1999), it does not absolutely exclude the contribution of CsA-induced immunosuppression to tumorigenesis. The importance of immunosuppression in CsA and cancer risk is highlighted by the elevated risk of virally-associated lymphoproliferative disorders (Dantal & Soulillou 2005) in transplant patients. Furthermore, viral load of human papilloma virus (HPV), which is associated with skin cancer risk, has been found to be elevated in individuals on CsA therapy (Harwood et al. 2000). The causative contribution of HPV to CsA-associated skin cancers, as it stands, is currently only correlative. The high association of HPV presence with CsA-associated cancers may be a direct cause-and-effect, or two separate and unrelated manifestations of CsA treatment. More studies are required to elucidate the relationship between HPV infection, immunosuppression, and CsA-associated cancers.

CsA treatment and TGF-β

One of the hallmarks of CsA-associated cancers is the markedly increased invasiveness and high metastatic potential of these normally indolent tumor subtypes. The first study investigating the tumorigenic effects of CsA explored its role as a

metastatic promoter. CsA treatment in a transformed tumorigenic cell line induced an invasive phenotype in vitro and increased metastasis *in vivo*. This was associated with increased TGF-β production from tumor cells. TGF-β has a double-edged effect on tumorigenesis; it suppresses cell proliferation and promotes differentiation, potentially halting tumor initiation, but it also promotes epithelial-to-mesenchymal transdifferentiation (EMT), which increases tumor invasion and cancer spread. Therefore, TGF-β acts as a tumor suppressor early on in tumor progression and a tumor promoter at later stages. In transformed malignant cells, CsA-induced TGF-β signals in a paracrine or autocrine manner to increase migration and invasion. CsA-induced TGF-β was also responsible for increased tumor metastasis *in vivo*, which was reduced to baseline with TGF-β neutralizing antibodies (Hojo et al. 1999). In a xenograft squamous cell carcinoma model, CsA-induced TGF-β caused EMT in tumor cells, marked by decreased E-cadherin and increased N-cadherin, vimentin, snail, twist, fibronectin, and α-SMA (Walsh et al. 2011). In addition to promoting metastasis in mouse models of cancer, CsA-induced TGF-β contributes to nephrotoxicity and gingival hyperplasia, also side effects of CsA therapy (Naesens et al. 2009; Chung & Fu 2013). While TGF-β signaling can regulate NFAT activity, it is not entirely clear whether CsA induces TGF-β through calcineurin-NFAT inhibition or by a calcineurin-independent mechanism.

CsA treatment and cell proliferation

In addition to promoting metastasis, CsA has also been shown to increase cell proliferation through Ras activation in renal carcinoma cells in vitro. By inhibiting

carabin, a negative regulator of Ras, CsA treatment results in greater Ras activity, offering an explanation for the rapid tumor growth seen in CsA-associated tumors (Datta et al. 2009). CsA has also been shown to increase cell proliferation in non-transformed cells. Gingival hyperplasia resulting from CsA treatment is thought to be due to a combination of TGF-β production and fibroblast proliferation (Chung & Fu 2013). Additionally, CsA-induced nephrotoxicity is at least partially attributed to glomerular cell proliferation (O'Connell et al. 2012). There is little consensus in the literature on the mechanism by which CsA promotes cellular proliferation. Several studies in non-transformed cells have demonstrated CsA can affect cell proliferation through a reactive oxygen species (ROS) mediated hepatocyte growth factor pathway (O'Connell et al. 2012) or through AKT activation (Han et al. 2010). It is therefore most appropriate to conclude that CsA's effect on cell cycle is complex and likely affecting multiple pathways in a cell and context dependent manner.

DNA damage

UV rays are electromagnetic radiation with wavelength between 100-400nm and is invisible to the naked eye but is emitted from many sources, with solar UV representing the bulk of exposure to humans. UVA is a longer wavelength UV radiation that can penetrate deep into the skin, but does not cause severe cellular damage, although prolonged exposure results in photoaging. UVB, on the other hand, is a shorter wavelength UV that can penetrate into the upper dermal layers, and has sufficient energy to cause cellular damage, resulting in sunburns (Novarina et al. 2011). DNA will absorb

UVB energy, which causes adjacent thymidine bases to bond together into pyrimidine dimers. Left unrepaired, pyrimidine dimers will block transcription and replication, stalling RNA and DNA polymerases, and can lead to double-stranded DNA breaks. Pyrimidine dimers are repaired by the nucleotide excision repair (NER) pathway, a conserved DNA repair pathway which specifically addresses UV-induced DNA damage. Defects in the NER pathway lead to several diseases that predispose individuals to cancer and early aging (Novarina et al. 2011).

Because CsA-associated tumors are predominately skin cancers, it had been suggested that CsA may also negatively affect the NER pathway. The first support for this hypothesis was seen in lymphocytes, where CsA treatment allowed UV irradiated cells to re-enter the cell cycle without removal of the DNA damage product (Kuschal et al. 2009; Ahlers et al. 1999). Further work in fibroblasts demonstrated that CsA downregulates two of the proteins involved in NER, XP-A and XP-G, and this effect was phenocopied by calcineurin knockdown (Kuschal et al. 2011). Lastly, CsA treatment potentiated the carcinogenic effect of UV as shown by an increased incidence of skin tumors in UV-irradiated mice on CsA therapy (Han et al. 2012). Taken together, these studies convincingly show that CsA is involved in skin tumor initiation in the setting of UV irradiation by amplifying the mutational potential of UV-induced DNA damage.

Inhibition of apoptosis by CsA

The inhibitory effect of CsA on calcineurin is mediated through its interaction with cyclophilin A, but CsA can also bind to other cyclophilin family members, resulting

in calcineurin-independent effects. The cellular functions of most cyclophilin proteins are poorly characterized, making it difficult to determine the calcineurin-independent outcomes of cyclophilin-cyclosporin interactions. The function of one cyclophilin protein, cyclophilin D, however, is very well characterized, as is its binding with CsA. This particular cyclophilin family member is localized to the mitochondria inner membrane and is a regulatory component of the mitochondrial permeability transition pore (MPTP) (Fig. 1.3.).

Fig 1.3. Current model of the mitochondrial permeability transition pore (MPTP). Adapted from (Halestrap & Richardson 2014). The definitive structural identity of the MPTP is under investigation, but the latest proposed model consists of an outer pore formed by the voltage-dependent anion channel (VDAC), various members of the BCL-2 family, and hexokinase (HK). The inner pore consists of adenine nucleotide transporter (ANT), phosphate carrier (PiC), the c-subunit of the FoF1-ATP synthase, and cyclophilin D (CypD). Creatine kinase (CK) bridge the outer and inner pore. Other molecules such as translocator protein and protein kinase C may also be associated (not shown).

The MPTP is a calcium and ROS responsive nonselective channel that, when open, releases mitochondrial contents into the cytosol. Opening of the MPTP propagates

the apoptotic cascade and also aids the removal of aged mitochondria. Cyclophilin D regulates the stimulus threshold required for MPTP opening (Halestrap 2009).

Binding of CsA to cyclophilin D raises the stimulus threshold required for pore opening, thereby decreasing pore opening. During conditions that trigger cell death pathways, CsA decreases apoptosis by preventing mitochondrial content efflux. This calcineurin-independent effect of CsA on the mitochondria has been confirmed through studies with the non-immunosuppressive cyclosporin analog, NIM811, which preferentially binds to cyclophilin D (Waldmeier et al. 2002). The anti-apoptotic effect of CsA is consistent across cell types, and it is currently being investigated as a potential therapeutic agent to attenuate ischemia-reperfusion injury in cardiac tissue (Piot et al. 2008). Considering evasion of apoptosis is a significant factor in tumorigenesis, the ability of CsA to raise the apoptotic threshold allows for precancerous cells that would otherwise be eliminated through cell death to survive.

This carcinogenic effect of CsA has been demonstrated in keratinocytes, the cells of origin for epidermal squamous cell carcinomas, where CsA treatment decreased UV-induced apoptosis in vitro. This was found to occur in a calcineurin-independent manner as only CsA and NIM811, but not FK506, an unrelated calcineurin inhibitor, decreased UV-induced apoptosis (Norman et al. 2010). CsA-mediated evasion of apoptosis after UV irradiation may potentiate skin cancer initiation, as CsA-associated cancers generally occur in sun exposed areas and in individuals with low skin pigmentation (Moosa & Gralla 2005).

ATF3

Oncogenic Ras mutations, specifically H-ras, are found in 10-20% of skin cancers (Boukamp 2005). Additionally, hyperactive Ras (beyond physiological levels), through DNA damage response secondary to hyperproliferation, can induce irreversible cell cycle arrest and senescence (Di Micco et al. 2006). Defects in oncogene-induced senescence allows for propagation of cancer cells into an expanding tumor. Using H-ras transformed human keratinocytes xenografted into immunocompromised mice; a recent paper showed calcineurin expression is required for oncogene-induced senescence. Calcineurin knockdown by siRNA or inhibition by CsA or FK506 bypassed oncogene-induced senescence and resulted in large and poorly differentiated tumors. This previously undescribed calcineurin-mediated oncogene-induced senescence was found to occur through NFAT transrepression of activating transcription factor 3 (ATF3). ATF3 is a stress-induced transcription factor found to both positively and negatively regulate the cell cycle (Boyd & Yan 2006; Thompson et al. 2010), and in this study ATF3 was found to function as a negative regulator of p53. Loss of calcineurin, or suppression by CsA, removed this inhibition on ATF3 transcription, whose expression decreased p53 levels bypassing Ras-induced p53-mediated senescence (Wu et al. 2010). The significance of oncogene-induced senescence in cutaneous squamous cell carcinoma, however, is controversial as it has only been described *in vitro*, but not in *in vivo* murine models (Tarutani et al. 2003; Dajee et al. 2002). Therefore, while CsA and calcineurin are shown to regulate ATF3 and senescence in mice, future studies are required to determine its contribution to CsA-associated cancers in humans.

CHAPTER 2: CYCLOSPORIN A PROMOTES TUMOR ANGIOGENESIS AND ENDOTHELIAL CELL ACTIVATION IN A CALCINEURIN INDEPENDENT MANNER

Introduction

Cyclosporin A (CsA) has been commonly prescribed for post-organ transplant immunosuppression since 1983. A well-documented adverse event for patients on CsA containing immunosuppressive regimens is a vastly increased risk of malignancy (Dantal & Soulillou 2005; Euvrard et al. 2003). With improvements in the organ-transplant field over the last few decades, allograft recipients are living longer, and CsA-associated tumorigenesis is an important cause of morbidity and mortality in that population. Newer generation immunosuppressants such as rapamycin and FK506 are replacing CsA as transplant immunosuppressants due to decreased side effects such as renal toxicity and malignancy risk. Many transplant recipients, however, have had past exposure to CsA prior to medication adjustment or are still on CsA therapies due to prescriber preference. Delineating the mechanisms by which CsA promotes tumorigenesis is critical for management and prevention of this adverse drug effect and may provide a greater insight on the role of the calcineurin pathway in cancer.

It has long been assumed that CsA increases cancer risk through its immunosuppressive effects, leading to the loss of immunosurveillance, and escape of transformed tumor cells from immunoediting (Dunn et al. 2002). This hypothesis is supported by the fact that virally induced leukemias and lymphomas are found among CsA-associated malignancies (Durnian et al. 2007). However, the use of other equally

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effective immunosuppressants for organ transplant patients, such as FK506 or rapamycin, is not associated with a similar cancer risk (Kauffman et al. 2006). Additionally, CsA treatment of immunocompromised mice promotes tumor growth and progression (Hojo et al. 1999), suggesting that at least one mechanism of CsA-induced tumorigenesis is independent of immunosuppression.

There are several known pathways through which CsA can promote tumorigenesis discussed in detail in Chapter 1 and summarized in Figure 2.1. With the exception of increasing the apoptotic threshold, the effects of CsA on tumorigenesis have been shown or assumed to occur through calcineurin inhibition. Calcineurin dysregulation has been found in cancer cells as well as in the tumor microenvironment. The role for the calcineurin pathway in mediating tumor angiogenesis has been well characterized (Baek et al. 2009; Yao & Duh 2004; Nilsson et al. 2008). The calcineurin-NFAT pathway is downstream of VEGF receptor activation in endothelial cells and transactivates angiogenesis-responsive genes. Suppression of calcineurin activity by its endogenous inhibitor Down syndrome candidate region 1 (DSCR1) results in a significant decrease in tumor growth due to inhibition of tumor angiogenesis (Baek et al. 2009; Hesser et al. 2004; Chan et al. 2005; Yao & Duh 2004; Iizuka et al. 2004), suggesting a potential role for calcineurin inhibitors as anti-angiogenic therapy. As a pharmacological calcineurin inhibitor, the pro-tumorigenic effect of CsA is inconsistent with the established role for calcineurin in tumorigenesis. Studies examining the ability of CsA to negatively regulate tumor angiogenesis in a manner similar to DSCR1 are

limited. We therefore sought to examine the potential effects of CsA on tumor

vasculature.

Figure 2.1. Overview of the known mechanisms of cyclosporin A on tumorigenesis. The pathway or factors directly affected by CsA are highlighted in yellow. Abbreviations: MPTP = mitochondrial permeability transition pore, NER = nucleotide excision repair, ATF3 = activating transcription factor 3, TGF-β =transformation growth factor β
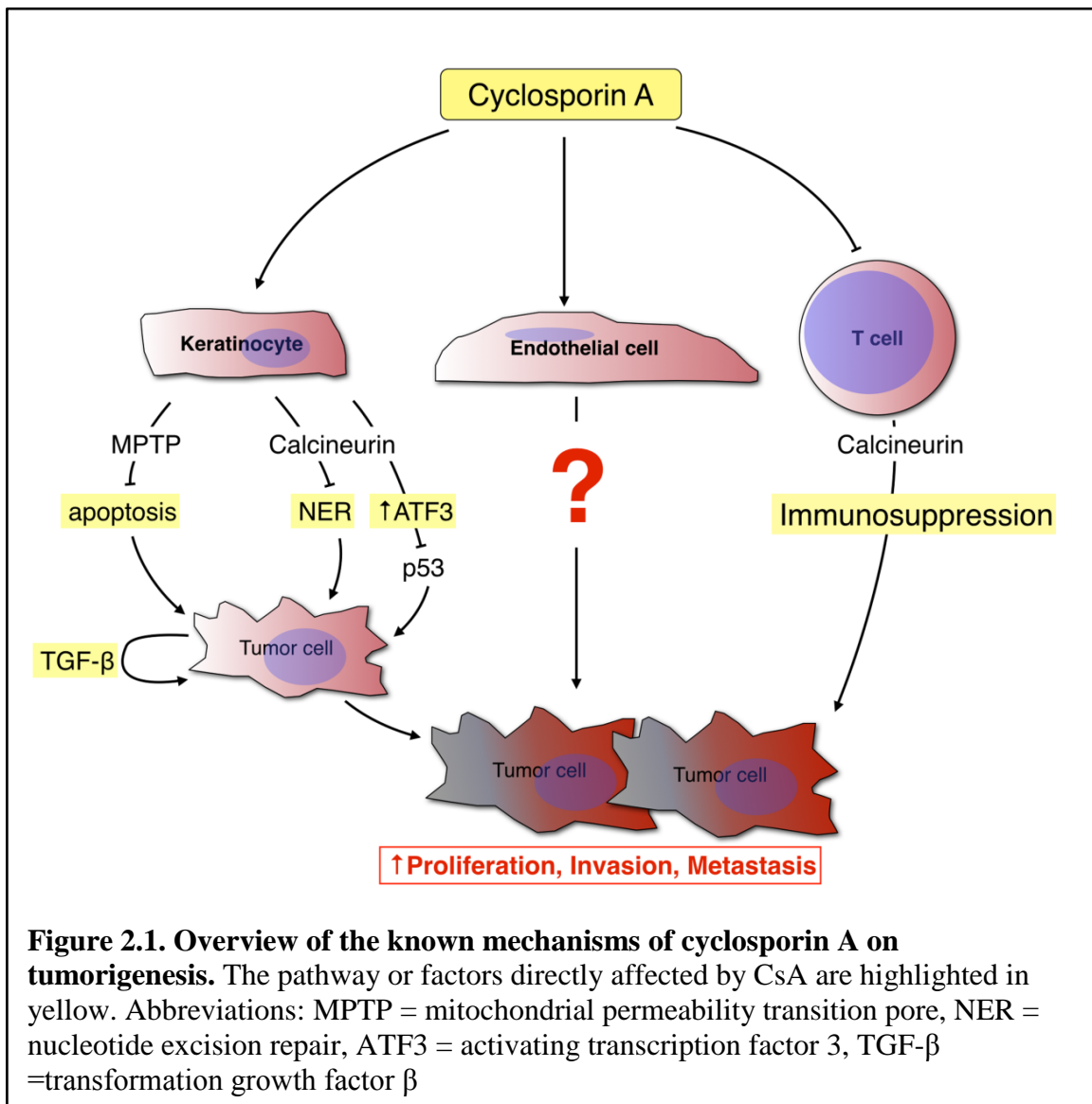
FK506, or tacrolimus, is another calcineurin inhibitor also used in post-organ

transplant immunosuppression. Patients on FK506-containing immunosuppressive

regimens experience significantly decreased rates of malignancy compared to patients on

CsA (Kauffman et al. 2006), suggesting CsA's ability to promote tumorigenesis, at least in part, is calcineurin-independent.

While CsA forms a calcineurin-inhibitory complex with the cyclophilin family member cyclophilin A (CypA), it may also bind to other intracellular cyclophilins and mediate calcineurin-independent effects. For example, when bound to cyclophilin D, CsA increases the apoptotic threshold (Halestrap 2009). Therefore, we wanted to investigate the role of CsA in the tumor microenvironment and determine whether these effects depend on calcineurin.

Based on previous reports showing that CsA treatment promotes tumor angiogenesis in a colon cancer model (Guba et al. 2002), we expect that chronic CsA treatment will also increase tumor angiogenesis in a skin cancer model. In order to evaluate the effect of CsA on angiogenesis, we characterized the effect of CsA on solid tumor growth and angiogenesis *in vivo* and on endothelial cell proliferation and migration *in vitro*. To assess for the calcineurin-dependence of these effects of CsA on angiogenesis, we utilized endothelial cells with a genetic deletion of calcineurin as well as cyclosporin analogs and other small molecule calcineurin inhibitors.

We show that chronic CsA treatment increases tumor growth in two separate mouse models of skin cancer with increased tumor microvessel density. Treatment of primary mouse endothelial cells in vitro with CsA results in increased proliferation and migration. CsA-induced endothelial cell activation is calcineurin-independent, as it still occurs following genetic deletion of calcineurin and is not seen following treatment with other calcineurin inhibitors.

Results

Cyclosporin A treatment increases skin tumorigenesis in vivo

Previous studies investigating the *in vivo* effect of CsA on tumor growth were conducted in diverse tumor types including renal and lung carcinoma models. However, patients on long-term CsA treatment predominantly have an elevated risk of skin cancers. Therefore, to study the effect of CsA on tumor angiogenesis in a cancer type most relevant to the patient population on CsA therapy, we examined these effects in a chemically induced mouse model of skin cancer and in mice allografted with murine melanoma cells.

To model squamous cell carcinoma, we used a well-characterized two-step chemical carcinogenesis model (Abel et al. 2011; Verma & Boutwell 1980) that results in de novo skin cancers. This model involves the one time topical application of the tumor initiator 7,12-dimethylbenz[α]anthracene (DMBA), which causes DNA damage and the emergence of H-ras mutant keratinocytes in the epidermis, followed by twice weekly application of the tumor promoter 12-*O*-Tetradecanoylphorbol-13-acetate (TPA). With continual carcinogen treatments, oncogenic mutations accumulate in keratinocytes, resulting in premalignant papilloma growth (Fig. 2.2.A). These papillomas are benign, but have pathological features similar to squamous cell carcinoma, such as the expansion of the basal cell layer, nests of tumor cells, and keratin pearls. Over time, they may progress into invasive carcinomas, although this is dependent on the genetic background of the mouse (Kemp et al. 1993). In the mouse strain used here (C57/BL6), 40 weeks of

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TPA treatment did not result in carcinoma conversion. However, chronic CsA treatment

of these mice at the clinical dose of 10mg/kg five days a week resulted in a significant

increase in the number of dermal papillomas (Fig 2.2.B). The number of large papillomas

was also greater, although not significantly so, in CsA treated mice (Fig 2.2.C).

Because CsA also increases the incidence and progression of melanomas in organ
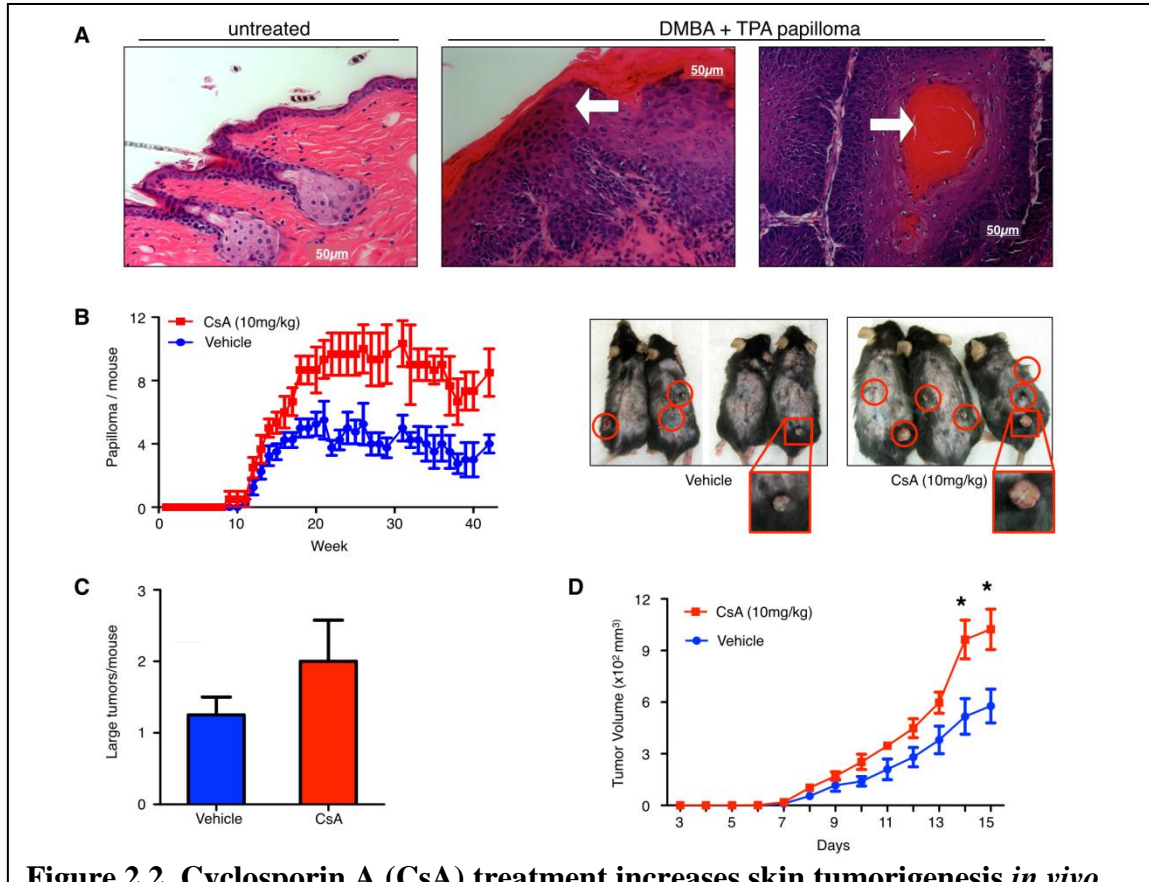
Figure 2.2. Cyclosporin A (CsA) treatment increases skin tumorigenesis *in vivo*.
(A) Hematoxylin and eosin staining of WT C57/BL6 mouse skin. The left image is of
normal skin. The middle and right images are of DMBA-TPA induced skin
papillomas 40 weeks after treatment initiation with arrows pointing to expansion of
the basal cell layer and keratin pearls, respectively. Quantification of (B) total
papilloma per mouse or (C) only large papillomas in mice treated daily with peanut
oil (vehicle) or 10mg/kg CsA. Representative photos are shown on the right (B) with
papilloma highlighted in red. N=5 per group. (D) Tumor growth over time of
subcutaneous xenograft tumors in mice treated with vehicle or CsA. WT mice were
pre-treated with CsA or vehicle for 14 days prior to tumor inoculation and maintained
on drug for duration of study. Results are representative of three experiments, with
n=3-5 per group. Error bars indicate SEM, and * P <0.05 for all experiments.

transplant patients (Euvrard et al. 2003), we investigated the tumorigenic effects of CsA on melanoma in the B16-F10 transplantable melanoma mouse model. Chronic CsA treatment in wild type mice with allografted B16-F10 melanoma cells increased tumor growth compared to untreated mice (Fig 2.2.D). These data show that CsA treatment in mice potentiates skin tumorigenicity in at least two *in vivo* models of skin cancer.

Cyclosporin A increases tumor angiogenesis

Expansion of solid tumors is in part limited by the growth of new blood vessels in the tumor. Since calcineurin inhibition by DSCR1 suppresses neoplastic angiogenesis, we wanted to study the effect of CsA in the tumor microenvironment. Its function as a calcineurin inhibitor should block VEGF signaling in endothelial cells, decreasing tumor angiogenesis. The increase in tumor growth seen with CsA treatment, however, is inconsistent with that hypothesis.

Chemically induced dermal papillomas from mice treated with CsA or vehicle control were harvested and sectioned for histopathological analysis. Vessel-like structures, characterized by a conspicuous lumen lined with end-to-end elongated cells that feature flat thin nuclei, are easily distinguishable (Fig 2.3.A), and their distribution in the tumor is heterogeneous. Analysis based on the number of vessels per tumor shows a trend towards more total vessels per mouse (Fig. 2.3.B), but no difference in vessel number per tumor if tumor size is taken into consideration (Fig 2.3.C & D). In our hands, immunofluorescence of tumor sections with anti-CD31 and anti-VE-Cadherin antibodies,

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specific markers of endothelial cells was unsuccessful due to a high degree of autofluorescence in the skin tissue.

A major limitation of the DMBA-TPA carcinogenesis model is that while it accurately portrays skin tumor progression, the tumors generated have heterogeneous vessel distribution that is difficult to quantify. Differences between treatment groups may be obscured by the varied structure of this particular tumor tissue. Additionally, papillomas on the C57/BL6 genetic background, which is relatively resistant to carcinoma conversion, do not grow very large and may not provide sufficient hypoxic stimulus for angiogenesis.

Figure 2.3. CsA treatment on tumor angiogenesis in the DMBA-TPA induced skin cancer model. (A) Representative H&E staining of DMBA-TPA induced skin papilloma. Black triangles indicate putative endothelial cells lining tumor vessels, and scale bars indicate 50µm. (B) Quantification of total vessels counted per mouse in each cohort, each dot represents a mouse. (C) Quantification of total vessels counted per tumor in each cohort, each dot represents a tumor. (D) Quantification of vessels stratified by tumor size in each cohort.

B16-F10 allograft melanoma tumors also demonstrated increased tumor growth when treated with CsA, so we also assessed the role of CsA in tumor angiogenesis in this model. Melanoma allograft tumors were harvested from mice maintained on CsA or vehicle therapy and frozen sections were immunostained with anti-CD31 antibody and Hoechst. Random low powered images of the tumors were taken (Fig. 2.4.A) and CD31 positive units, vessel number, lumenized vessels, and large vessels (≥100µm) were counted and analyzed. We found an increase in CD31 positive units and total vessel number in tumors from mice that were treated with CsA (Fig. 2.4.B). There was no

Figure 2.4. CsA treatment increase tumor angiogenesis in the B16-F10 allograft tumor model. (A) Representative immunofluorescence images of B16-F10 tumor sections stained with anti-CD31 (red) and Hoechst (blue), scale bars indicate 100µm. (B) Quantification of microvessel density, number and size. Microvessel density is quantified by the total number of CD31$^+$ units in a high powered field (hpf), lumenized CD31$^+$ structures and CD31$^+$ structures measuring 100µm or longer are also counted. (C) Quantification of Evans blue dye in tumor tissue normalized to tumor weight. B16-F10 tumor-bearing mice were treated with either vehicle or CsA. Error bars indicate SEM, and * P <0.05 for all experiments.

significant change in the number of lumenized vessels, and only a small increase in the

number of large vessels ($\geq 100\mu m$) in tumors from mice treated with CsA compared to

vehicle (Fig. 2.4.B). The majority of the increased microvascular density found in CsA-

treated tumors consisted of small, short CD31$^+$ structures. These findings where CsA

treatment leads to an increase in small short vessels without affecting larger or lumenized

vessels suggests that CsA may promote the early stages of tumor angiogenesis, which is

marked by cell proliferation and sprout formation.

It remains undetermined whether CsA has an effect on vessel maturation and

leakiness. We sought to test the effect of CsA on tumor vessel permeability by injecting

Evans blue dye into the systemic circulation, then quantifying its tissue distribution.

There were no differences in tumor vessel permeability between groups (Fig. 2.4.C).

However, there was high variation in each group, potentially obscuring subtle differences.

Cyclosporin A treatment promotes endothelial cell activation in vitro

To isolate the specific effects of CsA on the process of angiogenesis, we treated

primary endothelial cells isolated from the lungs of adult mice with CsA. These

microvascular endothelial cells are isolated using anti-CD31-conjugated magnetic beads

and generally are 90-95% CD31$^+$. CsA treatment of primary endothelial cells led to

increased cell number over time (Fig. 2.5.A) with increased 5-bromo-2'-deoxyuridine

(BrdU) incorporation (Fig. 2.5.B), a synthetic nucleotide whose incorporation into a cell

indicates active DNA replication. We also assessed for apoptosis using terminal

deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), an apoptotic marker
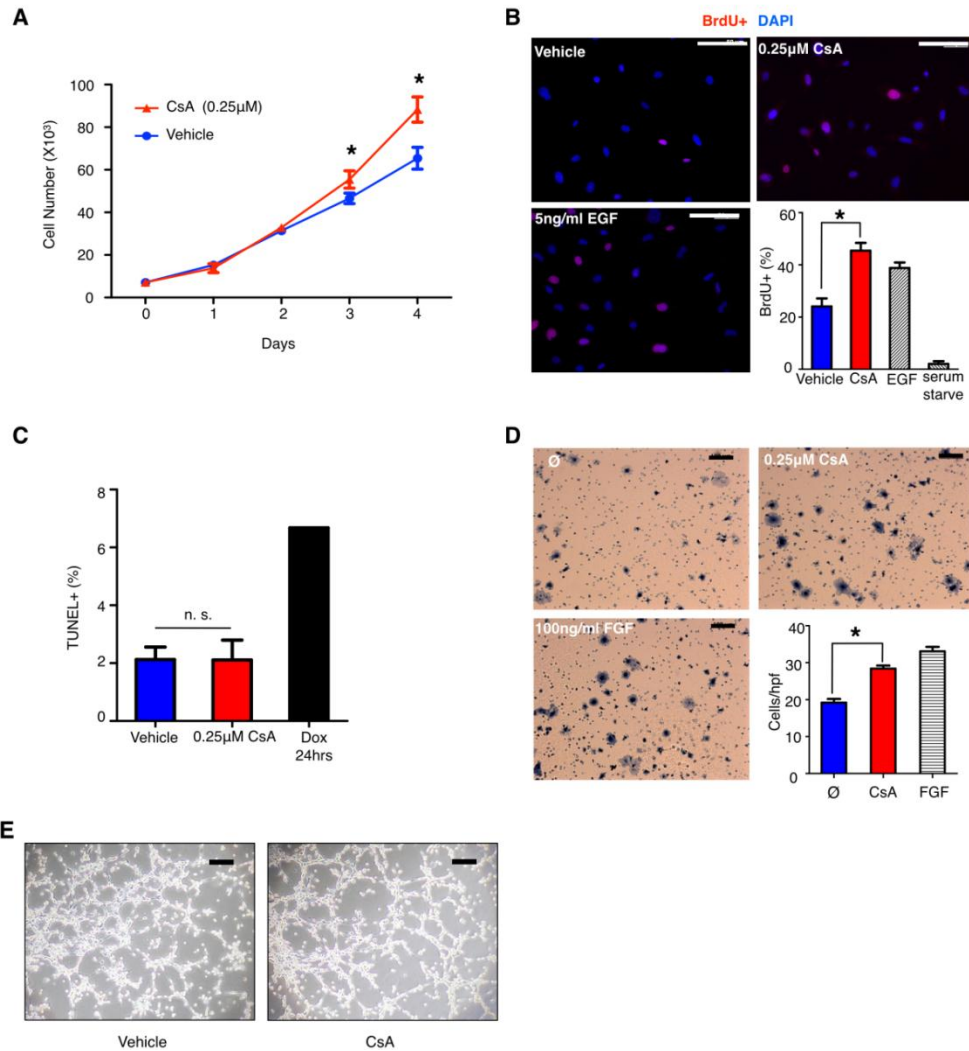
Figure 2.5. Cyclosporin A potentiates endothelial cell proliferation and migration *in vitro*. (A) Cell numbers of endothelial cells with 0.25µM CsA or vehicle treatment. Results are representative from three experiments, with n=3 per group. (B) Representative images of endothelial cells treated with 0.25µM CsA, 5ng/mL EGF or vehicle for 48 hours, pulsed with BrdU (red), and stained with Hoechst (blue). Quantification of BrdU$^+$ cells is shown in the graph. (C) Quantification of TUNEL$^+$ endothelial cells after treatment with vehicle or CsA for 72 hours or doxorubicin (Dox) for 24 hours, n=5 per group. (D) Representative images of endothelial cells migrating through a transwell filter with vehicle or 0.25µM CsA treatment. Quantification of cells counted per hpf is shown on the bottom. FGF (100ng/ml) is used as a positive control. Results are representative of three experiments. (E) WT endothelial cells were treated with vehicle or CsA and then seeded onto Matrigel and images were taken 5.5 hours later. Error bars indicate SEM, and * P <0.05 for all experiments. n.s. = not significant. Scale bars in (B), (D), and (E) indicate 100µm.

which labels fragmented DNA, and found no difference in TUNEL staining between vehicle and CsA treated cells (Fig. 2.5.C). These data indicate that CsA increases endothelial cell proliferation.

During the early stages of angiogenesis, endothelial cells migrate towards chemotactic gradients to form the nascent vessel before coalescing together to form a tube (Carmeliet 2000). To test the effect of CsA on endothelial cell migration, we pre-treated endothelial cells with CsA for two hours and created a CsA gradient in the lower well of the Boyden chamber. We found more cells migrated through the transwell filter with CsA treatment (Fig. 2.5.D) after 4 hours. Lastly, we tested the effect of CsA on endothelial cell tube formation, an *in vitro* measurement of endothelial cell adhesion, migration, protease activity, and tubule formation. Endothelial cells were plated onto a Matrigel matrix, and over the next 4-12 hours they invade and coalesce together to form tube-like structures. CsA treatment had no effect on endothelial cell tube formation compared to control treated cells (Fig. 2.5.E). These data suggest that CsA promotes angiogenesis through proliferation and migration with little effect on adhesion and tube formation.

Calcineurin inhibitors (CNI) have differential effects on endothelial cell activation

The calcineurin pathway is found downstream of VEGFR2 signaling in endothelial cells, and its activity in these cells results in transactivation of pro-angiogenic genes like E-selectin, COX-2, and tissue factor (Armesilla et al. 1999; Hernández et al. 2001). The endogenous calcineurin inhibitor DSCR-1 has been shown to inhibit

endothelial cell proliferation (Baek et al. 2009; Hesser et al. 2004; Chan et al. 2005; Yao & Duh 2004; Iizuka et al. 2004). It is therefore inconsistent that CsA, a potent calcineurin inhibitor, increases endothelial cell proliferation and migration. As a small molecule inhibitor, CsA may have additional effects independent of calcineurin inhibition.

To test whether the endothelial cell phenotype induced by CsA is due to calcineurin inhibition, we compared the effects of CsA with those of two other calcineurin inhibitors. First, we made use of transgenic *Dscr1* mice which express three alleles of the endogenous calcineurin inhibitor *Dscr1*, resulting in increased protein levels (Baek et al. 2009). There were fewer DMBA-TPA induced papillomas in transgenic *Dscr1* mice compared to wild-type mice treated with CsA (Fig. 2.6.A). Additionally, CsA treatment of DSCR1 overexpressing endothelial cells increased cell proliferation (Fig. 2.6.B) whereas DSCR1 overexpression by itself decreased cell numbers compared to wild type cells (Baek et al. 2009), suggesting separate and distinct underlying mechanisms by which these two calcineurin inhibitors act in endothelial cells. We also assessed the effects of the structurally dissimilar but functionally comparable small molecule inhibitor FK506, which is also used clinically as an immunosuppressant. Whereas CsA binds to cyclophilin A to inhibit calcineurin, FK506 instead binds to FK binding proteins and forms an independent but convergent calcineurin inhibitory complex. FK506 treatment of B16-F10 allograft tumor-bearing mice resulted in smaller tumors compared to CsA-treated B16-F10 allograft tumor-bearing mice (Fig. 2.6.C), consistent with the hypothesis that CsA promotes tumorigenesis in a calcineurin-independent manner. FK506 treatment decreased endothelial cell numbers and inhibited VEGF-

induced migration *in vitro* when compared to CsA (Fig. 2.6.D&E) The differential effects

seen with CsA and FK506 suggests these two molecules, despite both being calcineurin

Fig. 2.6. Calcineurin inhibitors have opposing effects on tumorigenesis and endothelial cell activity. (A) Quantification of DMBA-TPA induced skin papilloma measured over time in *Dscr1* transgenic (Tg) or WT mice treated with vehicle or CsA. (B) Proliferation of WT and *Dscr1* Tg primary endothelial cells after treatment with vehicle or 0.25μM CsA. Cell numbers are counted every other day. (C) Quantification of B16-F10 melanoma tumor growth in WT mice in either control group, treated with 10mg/kg CsA or 0.15mg/kg FK506, tumor size is shown on the right. (D) Proliferation of WT primary endothelial cells after treatment with vehicle, 0.1μM FK506, or 0.25μM CsA. (E) Quantification of endothelial cells migrating through a transwell filter in response to vehicle, 100ng/mL VEGF, and 0.1μM FK506 or 0.25μM CsA. Error bars indicate SEM, and * $P < 0.05$ for all experiments.

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inhibitors, have separate effects. We expected that FK506, as a calcineurin inhibitor, would decrease endothelial cell numbers but instead found it had no effect on cell numbers when compared to the vehicle treated group. This result suggests a calcineurin-independent action of FK506 on FK binding proteins, which are highly expressed in endothelial cells (Higgins et al. 2003) with poorly delineated functions, but its inhibition by FK506 may independently affect angiogenesis. FK506 is known to be a more potent calcineurin inhibitor than CsA (Woo & Propper 1990), and the concentration of FK506 used *in vitro* was adjusted to account for this discrepancy. A dose-curve, however, was not performed and may further explain the differences. Taken together, these data are highly suggestive of a pro-angiogenic effect of CsA that occurs independent of calcineurin signaling.

Cyclosporin A treatment activates endothelial cells in a calcineurin-independent manner.

To investigate the effects of CsA treatment in absence of calcineurin, a transgenic mouse strain with a conditional calcineurin deletion was utilized. This transgenic mouse

Figure 2.7. Adenovirus Cre infection in *Calcineurin B^{f/f}* (*CnB^{f/f}*) endothelial cells (EC) results in the loss of calcineurin A protein. Western blot showing calcineurin A protein in endothelial cells without Adeno-Cre infection, or with Adeno-Cre infection at the indicated infection ratio and passage (P) number. Endothelial cells were infected at P1 and *Calcineurin B* deletion was assessed by presence of calcineurin A protein at P2 and P6.

has loxP sites flanking the *calcineurin B* (*CnB*) gene, which is excised upon Cre recombinase administration. Without CnB, the catalytic calcineurin A subunit degrades. Primary lung endothelial cells were isolated from $CnB^{flox/flox}$ mice and treated *in vitro* with adenovirus carrying Cre recombinase (Adeno-Cre). Upon infection with Adeno-Cre, calcineurin A protein is lost from endothelial cells within 1-2 passages and remains absent for up to four additional passages in vitro (Fig. 2.7.).

In agreement with our earlier pharmacologic data, CsA treatment in calcineurin-null endothelial cells increases cell numbers, comparable to its effect in wild type endothelial cells (Fig. 2.8.A&B). Calcineurin deletion in endothelial cells results in a near complete loss of migration through a transwell filter (Fig. 2.8.C&D). These data demonstrate that calcineurin is not required for the pro-proliferative phenotype induced by CsA in endothelial cells.

Fig. 2.8. Calcineurin is not required for cyclosporin A-induced endothelial cell proliferation and migration (A, B) Proliferation of uninfected (A) or adenovirus-Cre infected (B) *Calcineurin B*$^{flox/flox}$ *(CnB*$^{f/f}$*)* primary murine endothelial cells in the presence of 0.25μM CsA or vehicle alone. Cell numbers were counted every other day. (C, D) Representative images of uninfected (C) or adenovirus-Cre infected (D) *CnB*$^{f/f}$ primary murine endothelial cells migrating through a transwell filter in response to 0.25μM CsA or vehicle alone. FGF is used as a positive control. Quantification of cells per high-powered field (hpf) is shown on the bottom. Scale bars indicate 100μm. Results are representative of two experiments, with n=3 per group. Error bars indicate SEM, and * P <0.05 for all experiments.

Discussion

Our data show that chronic CsA treatment *in vivo* promotes skin tumorigenesis in two separate murine skin cancer models. This is accompanied by increased tumor angiogenesis with amplification in the number of short small vessels and minimal change in the number of large lumenized vessels. In vitro, CsA increases the proliferation and migration of primary endothelial cells. This occurs in a calcineurin-independent manner, as calcineurin-null endothelial cells responds to CsA in a manner similar to wild-type endothelial cells, and these effects were not seen with other calcineurin inhibitors.

Considering the cancer profile associated with CsA therapy in transplant populations, it would be pertinent to use a skin cancer model to study this phenomenon in mice. Since CsA-associated cancers skew significantly towards squamous cell carcinomas (SCC), *in vivo* mouse models of SCC would be ideal for studying CsA's effect on tumorigenesis. There are several *in vivo* SCC progression mouse models; the chemical carcinogenesis (DMBA-TPA) model described earlier in this chapter and UV irradiation of hairless/shaved mice (Gruijl & Forbes 1995). Additionally, genetic manipulation of p53 and Ras in the dermis, in combination with chemical carcinogens, has also been shown to produce SCC (Caulin et al. 2007; Zhang et al. 2005). Lastly, SCC xenografts, comprised of human SCC cell lines or H-ras overexpressing human keratinocyte grafts (Wu et al. 2010) have also been used to study skin cancer progression.

Given the multi-stage nature of the DMBA-TPA skin carcinogenesis model and its similarity to human disease (Abel et al. 2011; Verma & Boutwell 1980), it was a

reasonable model to study the effects of CsA on cancer. CsA treatment in C57/Bl6 mice subjected to multiple rounds of topical carcinogens had more papilloma growth than vehicle treated mice. However, the genetic background of the mouse has a significant impact on cancer progression. In the C57/Bl6 mouse strain in our studies, these papillomas never converted to carcinomas nor grew very large, making it difficult to assess the angiogenic requirements of the tumors. It is also unclear whether the increase in papilloma number found in CsA-treated mice is due to increased angiogenesis or if it is a direct effect of CsA on keratinocytes, as CsA has also been shown to suppress cell death and DNA damage repair in DNA-damaged keratinocytes (Yarosh et al. 2005; Norman et al. 2010; Han et al. 2012). Although the cytochrome P450 isozyme responsible for converting DMBA from a prodrug to its more carcinogenic form DMBA-DE is distinct and separate from the P450 isozymes which metabolize CsA (Gao et al. 2005; Staatz et al. 2010), we cannot rule out a drug interaction between DMBA and CsA through the induction of P450 enzymes. It is also interesting to note that in our experiments, the number of papillomas per mouse increased steadily over time until week 20, then remained relatively stable for the duration of the experiment (20 additional weeks) despite continued TPA administration, suggesting a limitation in papilloma numbers imposed by the initial number of DMBA transformed cells. Moreover, the tumor structure of the papillomas was heterogeneous, and randomly taken images showed high variation in vessel-like structures, making vessel density highly variable and quantification of angiogenesis difficult.

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Subcutaneous xenograft injections of the human squamous cell carcinoma cell line SCC-13 (Okano et al. 2000) resulted in high variability in tumor size within each group (data not shown), potentially obscuring any treatment effects. Due to these limitations, we were not able to assess the effect of CsA on angiogenesis in this *in vivo* model of SCC.

While transplant patients on CsA therapy have an increased risk of squamous cell carcinoma, they are also at elevated risk for basal cell carcinoma and melanoma. The B16-F10 allograft mouse model of melanoma is a well-characterized model for studying melanoma, angiogenesis, and metastasis (Ryeom et al. 2008; Nicolson et al. 1978). Therefore, we utilized the transplantable B16-F10 melanoma cells in an allograft tumor model to determine the effect of CsA on tumor angiogenesis. CsA treatment increased tumor angiogenesis as well as tumor size, which was consistent with our hypothesis. However, there are limitations to this model. The subcutaneous allograft injection of already transformed B16-F10 tumor cells into the mice allows for studying tumorigenesis only in the late progression stage, as tumor initiation and early progression is bypassed. Additionally, while B16-F10 allograft tumor model have been used by other groups to study metastasis, in our hands, this metastasis model showed high variability from experiment to experiment and could not be used to assess the effects of CsA on metastasis.

To best understand whether CsA has a pro or anti-angiogenic effect on tumorigenesis, we used the clinically relevant dose of 10mg/kg CsA for our *in vivo* experiments, a comparable dose used for transplant patients. We did not, however, assess

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the serum levels of CsA in the mice to compare to human populations. When used at this dose, CsA therapy consistently increased the number and size of solid tumor formation (Yajima et al. 2008; Guba et al. 2002) as well as tumor angiogenesis (Guba et al. 2002). In the B16-F10 allograft melanoma mouse model, CsA treatment had a specific effect on the quantity and phenotypic appearance of tumor vessels.

While neoplastic vessels are generally considered to be inferior conduits for nutrient and oxygen delivery compared to normal vessels, they are still able to meet some of the metabolic demands of the tumors. Although tumor vasculature varies depending on the tumor type, the microvessel density in most solid tumors has been shown to be correlated with greater tumor growth, decreased patient survival, and poorer prognosis (Hlatky et al. 2002).

In the B16-F10 allograft melanoma model, the tumor vasculature consists of a mixture of immature and mature vessels, including lumenized larger vessels. CsA treatment increased the overall microvessel density of the tumors, which would raise the metabolic limit on the tumor cells imposed by vessel density. Additionally, CsA treatment resulted in a striking increase in small and short vessels, with little or modest change in the number of lumenized vessels or larger vessels. In a hypoxic tumor, increases in vessel density, even small and immature vessels, still aid in nutrient delivery.

Due to technical limitations, we were unable to determine the effects of these additional small vessels on tumor and tissue perfusion. While we have shown by staining that there are increased vessel structures within the tumors, *in vivo* labeling of tumors with FITC-lectin or FITC-dextrin would be helpful to determine *in vivo* functional quality

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of these vessels. Alternatively, Doppler ultrasound could be used to assess fluid/blood flow within the tumor, and tumor pO_2 could be measured with an Eppendorf fine needle probe (Menon et al. 2003). While the time frame of our tumor model did not allow for metastasis, immature small vessels often show a paucity of pericytes and leakier cell to cell junctions compared to mature vessels and could promote increased tumor cell escape into the periphery. Future studies using metastatic cancer models could determine whether the aberrant tumor vessels induced by CsA treatment contribute to the metastatic potential of solid tumors.

Because CsA treatment likely affects multiple signaling pathways in endothelial cells, we conducted our experiments using a low dose of CsA that fell within the pro-proliferative range in order to best dissect the pro-angiogenic effects of CsA. Using this dose, we show that CsA-treated endothelial cells have greater proliferation and migration in vitro compared to vehicle treated cells, but no difference in tube formation in Matrigel. The tube formation assay is an *in vitro* model for the later stages of angiogenesis, such as adhesion, matrix invasion, and capillary tube formation. However, due to the short time frame and reduced growth factor conditions, endothelial cell tube formation does not accurately reflect changes in proliferation. These data suggest that CsA specifically affects the early stages of angiogenesis, especially endothelial cell proliferation and migration. Future characterization of protease production and activation, as well as cell surface receptors (adhesion molecules, VEGFR, Tie receptors) in endothelial cells would be helpful to further elucidate the specific angiogenic pathways affected by CsA.

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Systemic CsA treatment results in drug accumulation in multiple tissues and cell types in addition to tumor and endothelial cells. Pericytes or vascular smooth muscle cells are important vascular regulators and are often abnormal in neoplastic vessels. While the effect of CsA on tumor-associated vascular smooth muscle cells is not known, previous studies have demonstrated that CsA can affect proliferation, adhesion, and cellular orientation of vascular smooth muscle cells (Garvey et al. 2010). Our study demonstrates a direct effect of CsA on endothelial cells *in vitro* but does not rule out the potential involvement of other cell types such as vascular smooth muscle cells. Staining of tumors from CsA-treated mice for vascular smooth muscle markers in combination with permeability studies and *in vitro* characterization of vascular smooth muscle cells after CsA treatment in co-culture experiments with endothelial cells or tumor cells may help clarify the contribution of vascular smooth muscle cells to the CsA-associated angiogenesis.

While we are studying the effect of CsA in the context of tumor angiogenesis, it has been investigated by other groups in nonmalignant pathological angiogenic settings, although the results are varied and appear to be dose-dependent. Generally, high doses (>5μM) of CsA *in vitro* has been found to be anti-angiogenic. One study found that CsA, in combination with the antifungal drug itraconazole, is selectively toxic to endothelial cells (Nacev & Liu 2011). The same group found that CsA was toxic to human umbilical vein endothelial cells in a calcineurin-independent manner, presumably through binding to cyclophilins (Nacev et al. 2011). In a mouse model of neovascular (wet) macular degeneration, both CsA and a non-immunosuppressive analog *N*-MeVal-4-CsA

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decreased choroidal neovascularization (Nacev et al. 2011). Low doses (<0.5-1μM) of CsA, on the other hand, have been shown to exert a cytoprotective effect on endothelial cells *in vitro* by increasing VEGFR2 levels (Alvarez-Arroyo 2002). If CsA has dose-dependent cellular effects on endothelial cells, there must be a combination of independent pathways affected, one of which is the inhibition of the angiogenic calcineurin-NFAT axis. Therefore, in order to produce the pro-angiogenic effects observed, CsA must evoke an alternative, activating pathway in endothelial cells.

Most studies involving CsA assume that the effects seen occur in a calcineurin-dependent fashion. In our experiments, the pro-proliferative effects induced by CsA in endothelial cells are at odds with the known effects of calcineurin inhibition in this cell type. Therefore, we hypothesized that these events occur in a calcineurin-independent manner. In agreement with this, other CNIs have dissimilar effects on tumorigenesis and endothelial cell activity when compared to CsA. It is important to note, however, that calcineurin inhibition by DSCR1 and FK506 in *in vivo* murine tumor models is not limited to endothelial cells but extends to other cell types. Therefore, we also assessed the effect of different CNIs on endothelial cells *in vitro*. Additionally, while we used a lower concentration of FK506 for *in vitro* studies to partially reflect its greater potency (Kino et al. 1987), ideally a dose curve would be more informative.

We found that calcineurin loss in endothelial cells had no effect on the ability of CsA to increase proliferation; therefore, we concluded that CsA promotes pro-angiogenic behavior in endothelial cells in a calcineurin-independent manner. However, this does not necessarily mean that calcineurin inhibition by CsA does not have a significant effect in

endothelial cells, as it still occurs regardless of off-target effects and likely explains the toxicity seen at high doses.

In summary, our data show that CsA increases tumor growth and angiogenesis *in vivo*, and it promotes endothelial cell proliferation and migration *in vitro* in a calcineurin independent manner. In chapter 3, we will explore potential calcineurin-independent pathways stimulated by CsA treatment that may be responsible for the pro-proliferative and migratory effects seen in endothelial cells.

CHAPTER 3: CYCLOSPORIN A INCREASES ENDOTHELIAL CELL ACTIVITY THROUGH MITOCHONDRIAL REACTIVE OXYGEN SPECIES

Introduction

Our studies indicate that cyclosporin A (CsA) treatment increases tumor growth and angiogenesis in a mouse model of skin cancer. CsA treatment of endothelial cells *in vitro* increases proliferation and migration. These CsA-induced endothelial cell effects occurred in a calcineurin-independent manner, as CsA still affects calcineurin-null cells in a similar manner. Furthermore, treatment with other calcineurin inhibitors does not recapitulate this phenotype. Therefore, we investigated the underlying mechanism of CsA-induced endothelial cell activation in the absence of calcineurin.

CsA is a fungal cyclic decapeptide with a high affinity for a family of intracellular poly-propyl-isomerases (PPI) called cyclophilins (Hemenway & Heitman 1999). This family of sixteen proteins catalyzes the *cis* to *trans* isomerization reaction in folding proteins. They act mostly as chaperone proteins and are implicated in both physiological and pathological processes (Wang & Heitman 2005). CsA binds to the cyclophilin family member cyclophilin A and forms a calcineurin-inhibitory complex, which occupies the substrate docking site and competitively displaces calcineurin substrates such as NFAT from docking and being dephosphorylated. The selectivity of this CsA-cyclophilin A complex for calcineurin inhibition is purely by structural chance, as neither CsA or cyclophilin A alone affects calcineurin activity (Hemenway & Heitman 1999). The

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cellular roles of the other cyclophilins are not well defined, and the calcineurin-independent effects of CsA binding to these cyclophilin proteins are not well known.

The binding of CsA to cyclophilin D, a mitochondrially targeted PPI family member, has been well characterized (Friberg et al. 1998). Cyclophilin D is a part of the mitochondrial permeability transition pore (MPTP), a large calcium-responsive complex spanning the mitochondrial inner and outer membrane.

The structural components of the MPTP is a subject of debate and the latest proposed model is illustrated in Figure 1.3; the voltage-dependent anion channel (VDAC) has been proposed as the outer membrane subunit and either the adenosine nucleotide translocase (ANT) or the mitochondrial phosphate carrier (PiC) have been proposed as the inner mitochondrial membrane subunit (Halestrap 2009). Hexokinase and translocator protein (TSPO, also known as peripheral benzodiazepine receptor) are also thought to be part of the MPTP (Brenner & Moulin 2012). Genetic deletions of these various components, however, does not result in mitochondrial permeability phenotypes, (Brenner & Moulin 2012), illustrating the controversy of the MPTP identity. Most recently, the c-subunit ring of the ATP synthase is highlighted as the putative inner mitochondrial membrane channel portion of the MPTP (Alavian et al. 2014). The structural identity of the MPTP remains an active area of research, and only cyclophilin D is agreed upon as an obligatory pore component.

Despite the ambiguity of MPTP structural identity, its function is well known. The MPTP is triggered to open by mitochondrial matrix calcium and its sensitivity to calcium-induced opening can be affected by multiple factors. Stressors, such as apoptotic

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stimulus, low ATP (Crompton 1999), UV irradiation (Norman et al. 2010), high pH, and high mitochondrial reactive oxygen species (ROS) increases the sensitivity of the MPTP to calcium-induced opening. Opening of the MPTP leads to equilibration of mitochondrial contents such as ROS, calcium, and other small molecules (<1.5kDa) across the mitochondrial membranes. This subsequently results in mitochondrial osmotic imbalance and swelling (Halestrap 2009).

The MPTP is believed have multiple physiological functions. As mitochondria age, mutations accumulate in the mitochondrial DNA, which encodes components of the electron-transport chain. Over time, the mitochondrial bioenergetic circuit becomes progressively uncoupled, leading to elevated ROS production, ion imbalances, and low ATP:ADP ratio. These signals can promote MPTP opening, which then causes destruction and removal of the unhealthy mitochondria (Wallace 2005). This process selectively removes old or unhealthy mitochondria from a cell. If occurring on a more widespread, cell-wide scale, it can also contribute to cell death. In those incidences, Bax/Bad megachannels can form on the swollen mitochondria, leading to cytochrome c release as part of the apoptotic cascade.

Cyclophilin D plays a central role in regulating MPTP opening. Loss of cyclophilin D raises the stimulus threshold required to open the pore. CsA treatment phencopies cyclophilin D deletion and also results in decreased pore opening in response to stimulus. It's not entirely clear how CsA binding to cyclophilin D affects its regulation of the MPTP, but it may occur through inhibition of the catalytic PPI activity of cyclophilin D (Halestrap & Davidson 1990), which has been shown to be important in

MPTP opening (Machida et al. 2006). CsA treatment consistently increases the apoptotic threshold of cells, and this off-target effect of CsA has been exploited experimentally to investigate mitochondrial function and apoptosis. Since mitochondrial content efflux is a major component of ischemia-perfusion injury, inhibition of the MPTP by CsA in cardiomyocytes decreases the severity of myocardial damage and is currently being investigated as potential therapy for myocardial infarction (Piot et al. 2008).

Though CsA treatment decreases MPTP opening, the downstream effects on mitochondrial biology and specifically ROS levels appear to be context-dependent. Maintaining pore closure in the presence of an apoptotic stimulus impedes the release of mitochondrial ROS. For example, CsA treatment decreases the efflux of mitochondrial ROS in keratinocytes in response to UV irradiation (Norman et al. 2010). CsA treatment in non-apoptotic conditions, however, leads to increased cellular ROS levels (O'Connell et al. 2012; Longoni et al. 2001; Krauskopf et al. 2005).

The mitochondria is a major producer of endogenous ROS as it is a byproduct of the electron transport chain (Adam-Vizi & Chinopoulos 2006). Endogenous ROS can also be generated through specific enzymatic processes such as the NAD(P)H oxidase, found in phagocytotic cells, endothelial cells, and smooth muscle cells (Touyz & Briones 2011).

In the body, ROS participates in the inflammatory response to neutralize pathogens. They are also involved in the pathogenesis of many conditions such as diabetes, atherosclerosis, and cancer (Bartosz 2009). ROS and other free radicals were initially shown to be damaging to cellular components due to their unstable atomic

structure, which causes protein and lipid oxidation and DNA damage. More recently, however, ROS have also been shown to act as a signaling molecule at low levels (Bartosz 2009) and can affect a number of cellular processes, including mitogenic pathways (Finkel 2011). In the circulatory system, ROS and the closely related molecules reactive nitrogen species (RNS) are potent modulators of vascular tone (Yung et al. 2006; Maulik & Das 2002). Therefore, endothelial cells are very sensitive to changes in ROS and RNS. These compounds also affect endothelial cell junctions, which in turn alter vascular permeability (Monaghan-Benson & Burridge 2009). Furthermore, ROS signaling has also been shown to affect other processes such as angiogenesis by promoting endothelial cell migration and proliferation (Maulik & Das 2002; Ushio-Fukai 2006).

We have shown that CsA treatment of endothelial cells promotes proliferation and migration in a calcineurin-independent manner. CsA is known to induce a strong calcineurin-independent effect by binding to the mitochondrially located cyclophilin D. Mitochondrial contents such as reactive oxygen species can trigger a variety of signaling pathways in the vasculature, including pro-angiogenic effects. Therefore, we hypothesize that CsA exerts a pro-proliferative and migratory effect in endothelial cells by stimulating the production of mitochondrial ROS.

Here we show that treatment of endothelial cells with the non-immunosuppressive cyclosporin analog NIM811 phenocopies the effects of CsA. Furthermore, CsA treatment increases mitochondrial ROS levels and is associated with an increase in mitochondrial membrane potential. Antioxidant co-treatment with CsA abrogates the pro-proliferative

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and migratory effects induced by CsA *in vitro* and decreases CsA-induced tumor growth

in vivo.

Results

Cyclosporin A increases endothelial cell proliferation and migration via its interaction with cyclophilins

We showed previously that CsA is able to promote proliferation and migration of endothelial cells in the absence of calcineurin. Since CsA is not known to have cyclophilin-independent effects, we speculated that this calcineurin-independent effect of CsA is derived from its binding with other cyclophilin proteins. To test this hypothesis, we utilized NIM811, a non-immunosuppressive cyclosporin analog that is unable to bind calcineurin, but has similar binding profiles to cyclophilin proteins as CsA. In a manner similar to CsA, NIM811 interacts with the well-characterized cyclophilin D and it is often used as a research tool to study cyclophilin D and MPTP biology.

Using WT endothelial cells, we compared the effect of NIM811 to CsA on endothelial proliferation. Dose-response proliferation curves for both drugs were bell-shaped (Fig. 3.1A) after six days of treatment; low concentrations of CsA or NIM811 (10nM – 1µM) increased endothelial cell numbers whereas higher concentrations ($\geq$1µM) decreased cell numbers. Treatment with low concentration of either NIM811 or CsA increased endothelial cell growth over time compared to vehicle treated controls (Fig. 3.1B). Furthermore, both CsA and NIM811 increased endothelial cell migration through a transwell filter (Fig. 3.1C). The comparable growth and migratory phenotypes following CsA and NIM811 treatment suggest that both molecules affect a common pathway.

Fig. 3.1. CsA and NIM811 promote endothelial cell proliferation and migration *in vitro*. (A) Proliferation of primary murine endothelial cells in response to increasing concentrations of CsA or NIM811. Cell numbers were counted after 6 days of treatment, n=3. (B) Proliferation of endothelial cells treated with 0.25µM CsA, 0.1µM NIM811, or vehicle. Cell numbers were counted every other day. * P <0.05 (between vehicle and CsA, and between vehicle and NIM811). Results are representative of two experiments, n=3 per group. (C) Representative images of endothelial cells migrating through a transwell filter in response to 0.25µM CsA, 0.1µM NIM811 or vehicle alone. Quantification of cell numbers per high-powered field (hpf) is shown below. Results are pooled from two experiments. Error bars indicate SEM, and * P <0.05 for all experiments. Scale bars indicate 100µm.

Cyclosporin A treatment increases mitochondrial ROS

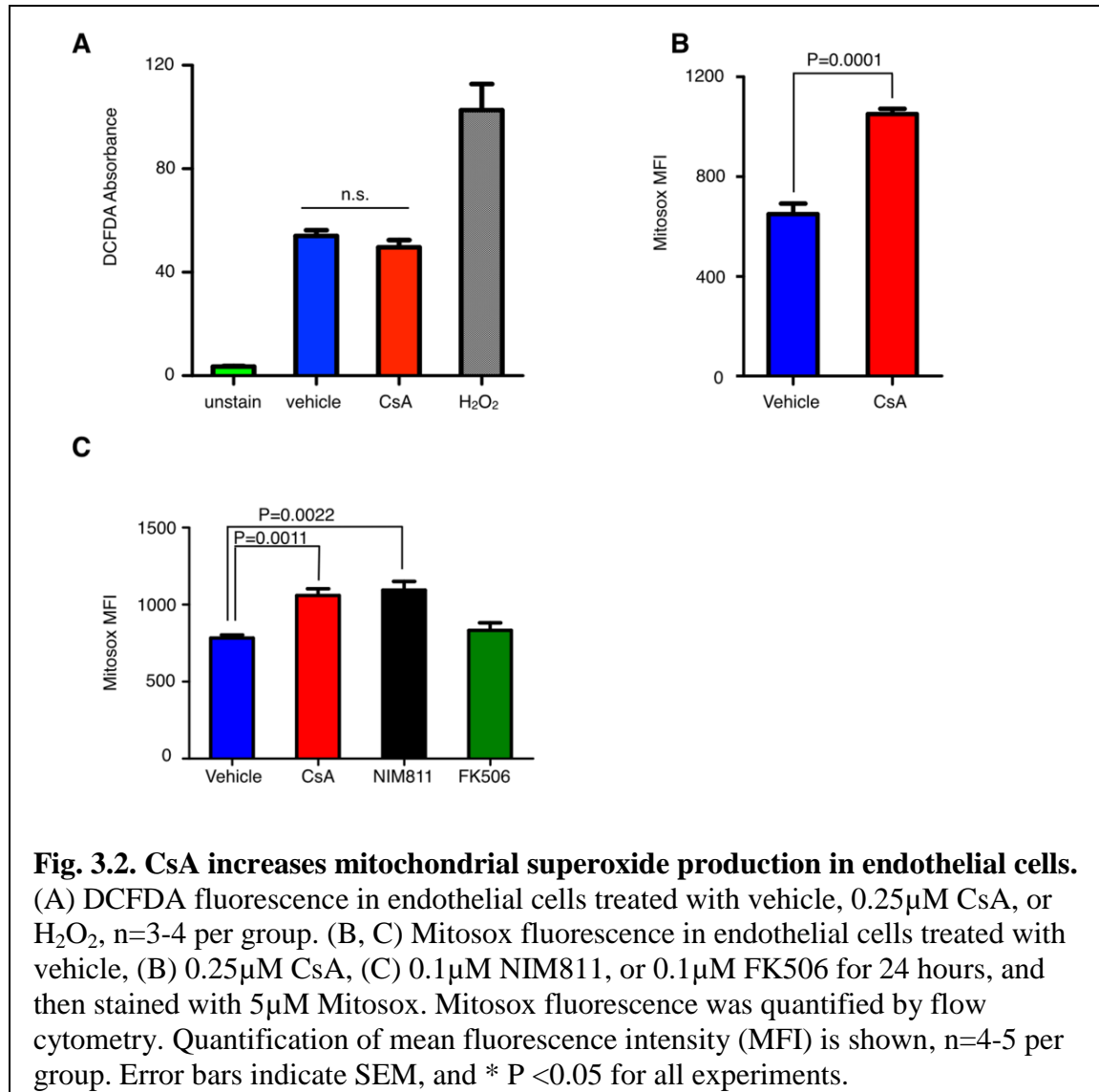Mitochondrial-derived ROS have been demonstrated to affect a number of cellular processes including cell growth (Hamanaka & Chandel 2010) and adhesion (Zhang & Gutterman 2007). We hypothesize that CsA treatment leads to an increase in mitochondrial ROS, leading to the observed endothelial cell changes. The exact effects of CsA on ROS are still unclear and may reflect differential MPTP activity in apoptotic vs.

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non-apoptotic contexts. Therefore, we sought to determine the effects of CsA on mitochondrial ROS in our experimental model.

Cellular ROS was measured using the commonly used ROS indicator DCFDA, a broad nonspecific indicator of multiple reactive oxygen species. We found endothelial cells treated with CsA showed no difference in DCFDA staining when compared to vehicle treated cells (Fig. 3.2A).

While we expected to see an increase in cytosolic ROS following CsA treatment, we hypothesized that the levels of ROS induced by the concentration of CsA we used was low and non-damaging. Routinely used to measure the oxidative burst found in neutrophils and macrophages, DCFDA may not have the sensitivity to detect the low level of ROS generated by CsA in our system (Gomes et al. 2005). Additionally, the use of DCFDA for the detection of overall oxidative stress has been undermined by its inability to detect specific ROS (Myhre et al. 2003), such as H_2O_2 (in low peroxidase contexts). To investigate the effect of CsA specifically on mitochondrial ROS, we stained live cells with Mitosox, a mitochondrial targeted superoxide detector that fluoresces upon superoxide binding and is a sensitive and specific indicator of mitochondrial superoxide levels (Robinson et al. 2006), which can converted to the membrane permeant and angiogenic H_2O_2 (Urao et al. 2013; Boveris & Cadenas 2000). When compared to vehicle-treated cells, endothelial cells treated with CsA showed increased Mitosox staining as detected by flow cytometry (Fig. 3.2B). Furthermore, endothelial cells treated with NIM811 but not FK506 showed increased Mitosox staining when compared to

vehicle-treated controls (Fig. 3.2C). These results are consistent with our hypothesis that

CsA increases mitochondrial ROS in a calcineurin-independent manner.

Fig. 3.2. CsA increases mitochondrial superoxide production in endothelial cells.
(A) DCFDA fluorescence in endothelial cells treated with vehicle, 0.25μM CsA, or
H_2O_2, n=3-4 per group. (B, C) Mitosox fluorescence in endothelial cells treated with
vehicle, (B) 0.25μM CsA, (C) 0.1μM NIM811, or 0.1μM FK506 for 24 hours, and
then stained with 5μM Mitosox. Mitosox fluorescence was quantified by flow
cytometry. Quantification of mean fluorescence intensity (MFI) is shown, n=4-5 per
group. Error bars indicate SEM, and * P <0.05 for all experiments.

Cyclosporin A treatment increases mitochondrial membrane potential

While we and other groups have demonstrated that CsA increases ROS, the

mechanism by which this occurs is not completely understood. It is known that CsA

decreases MPTP opening in response to apoptotic stimuli, reducing the efflux of

mitochondrial contents, including ROS. It is less clear how CsA increase ROS in the absence of these stimuli.

The MPTP is a nonselective calcium and ROS-responsive channel bridging the mitochondrial inner and outer membrane and is well known for its role in propagating cell death (Halestrap 2009). This opening is irreversible and spreads to neighboring MPTP complexes, causing depolarization waves across the mitochondrial membrane and collapsing the mitochondrial membrane potential. This irreversible MPTP activity is well-characterized in pathological settings, such as ischemia-reperfusion injury in cardiomyocytes and hypoglycemia-triggered neuronal cell death (Crompton 1999). More recently, the MPTP also has been shown to function under physiological conditions. In this state, the MPTP undergoes individual reversible openings that recover quickly without membrane depolarization or mitochondrial swelling (Halestrap 2009; Rasola et al. 2010; Wang et al. 2012; Wang et al. 2008). This mode, termed "physiological flickering" or "superoxide flash," is thought to have signaling or metabolic functions (Rasola et al. 2010). One consequence of a superoxide flash is a brief dip in mitochondrial membrane potential, followed by recovery upon MPTP closure (Wang et al. 2012; Kowaltowski et al. 2000). Therefore, physiological flickering of the MPTP may act as a potential release-valve for regulating the mitochondrial membrane potential.

The mitochondrial membrane potential is closely linked to mitochondrial metabolism, and studies in isolated mitochondria have shown a direct correlation between the mitochondrial membrane potential and mitochondrial ROS production (Korshunov et al. 1997). Thus, we hypothesize that during non-apoptotic conditions, CsA increases ROS

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by acting on the MPTP to decrease physiological flickering, resulting in increased

mitochondrial membrane potential, leading to increased mitochondrial ROS production.

To test this hypothesis, we utilized tetramethylrhodamine ethyl ester (TMRE), a

cationic fluorescent dye that is readily sequestered by active mitochondria. Due to its

cationic nature, the binding of TMRE to the mitochondria is membrane potential-

dependent (Wang et al. 2008). Endothelial cells treated with either CsA or NIM811 for

48 hours showed substantially increased TMRE staining compared to vehicle treatment

(Fig 3.3.). These data are consistent with the hypothesis that CsA increases mitochondrial

ROS by increasing mitochondrial membrane potential.
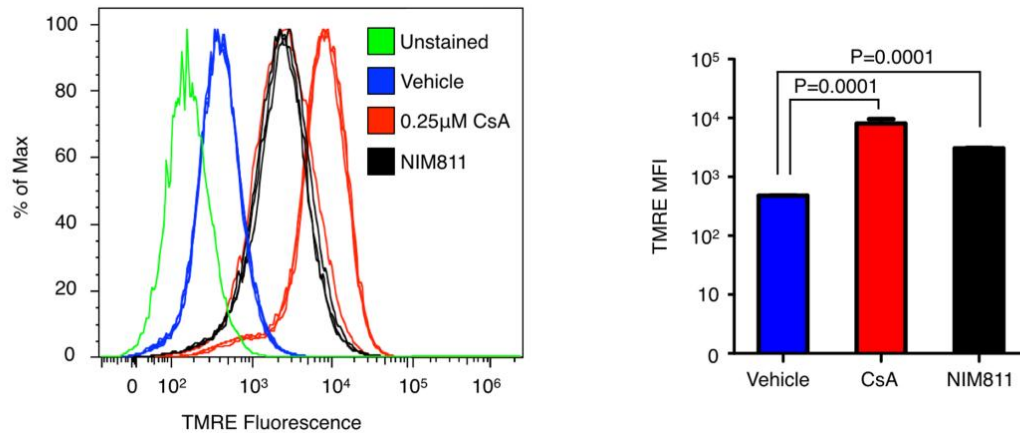
Fig. 3.3. CsA treatment increases mitochondrial membrane potential in endothelial cells. TMRE fluorescence in endothelial cells treated with vehicle, 0.25µM CsA, 0.1µM NIM811 for 24 hours, and then stained with 100nM TMRE to detect mitochondrial membrane potential. TMRE fluorescence is detected by flow cytometry, quantification of mean fluorescence intensity is shown on the right with n=3-5 per group. Error bars indicate SEM, and * $P < 0.05$.

Antioxidant treatment abolishes the pro-angiogenic effect of cyclosporin A on endothelial cells **in vitro.**

While our data show that CsA increases mitochondrial ROS in endothelial cells, it is unclear whether the elevated ROS is responsible for the pro-angiogenic effects observed. ROS is known to be involved in angiogenesis as VEGFR activation result in NAPDH oxidase stimulation, which increases intracellular levels of the mitogenic H_2O_2, leading to endothelial cell proliferation and migration (Ushio-Fukai 2006).

We hypothesize the CsA-induced ROS are responsible for the proliferative and migratory effect observed in CsA-treated endothelial cells. To test this hypothesis, we utilized both cellular and mitochondrial antioxidants to quench ROS. Co-treatment of endothelial cells on CsA therapy with the cellular antioxidant N-acetyl-cysteine (NAC) abolishes CsA-induced proliferation *in vitro* (Fig. 3.4.A). NAC treatment alone had no effect on either wild type or *Dscr1* transgenic endothelial cells, which overexpress the endogenous calcineurin inhibitor DSCR1. Co-treatment with the mitochondrially permeant superoxide dismutase mimetic and peroxynitrate scavenger manganese(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) also attenuates CsA-induced proliferation (Fig.3.4.B). Treatment with both NAC and MnTBAP decreased CsA-induced migration (Fig.3.4.C & D). Mitosox staining confirms that MnTBAP co-treatment with CsA reduces mitochondrial generated ROS (Fig.3.4.E). These results are consistent with the hypothesis that CsA promotes angiogenesis through a ROS-dependent mechanism.
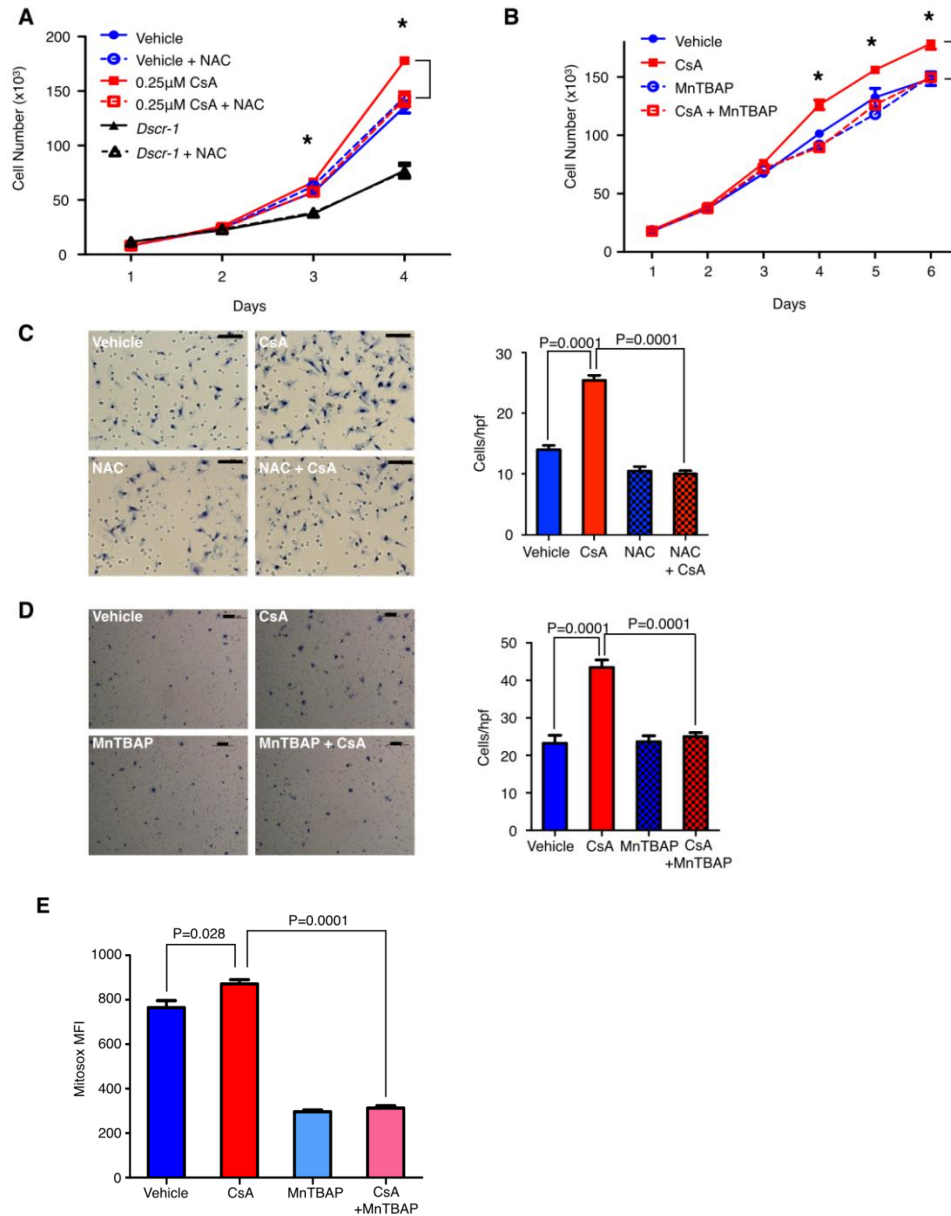
Fig. 3.4. Antioxidant treatment abolishes cyclosporin A-induced endothelial cell activation, A-D. (A, B) Proliferation of primary endothelial cells after treatment with 0.25μM CsA plus (A) 5mM NAC, or (B) 10μM MnTBAP. Cell numbers were counted daily. * P <0.05 (between CsA and CsA + antioxidant groups). Results are representative of two experiments, with n=3 per group. (C, D) Representative images of endothelial cells migrating through a transwell filter in response to vehicle, 0.25μM CsA, (C) 5mM NAC, or (D) 10μM MnTBAP. Black scale bars indicate 100μm. Quantification of cell numbers per high-powered field (hpf) is shown below. Results are pooled from two experiments. (E) Quantification of Mitosox fluorescence in endothelial cells treated with vehicle or 0.25μM CsA for 24 hours with 10μM MnTBAP co-treatment.

Antioxidant treatment in melanoma tumor models decreases cyclosporin A-induced

tumorigenesis

Our experiments show that CsA treatment leads to increased endothelial cell

proliferation and migration, which can be abolished with antioxidants. These results raise

the possibility of *in vivo* therapy with antioxidants to treat CsA-associated cancers. NAC

is an FDA approved drug commonly used as a mucolytic agent or to treat acetaminophen

toxicity. In the body, NAC is converted to a precursor of the cellular antioxidant

glutathione; external administration of NAC augments and boosts cellular antioxidant

capabilities (Samuni et al. 2013). Clinically, NAC is well tolerated in single, intermittent,

(Atkuri et al. 2007) or chronic use (Grandjean et al. 2000).

We sought to determine whether antioxidant therapy has an effect on CsA-
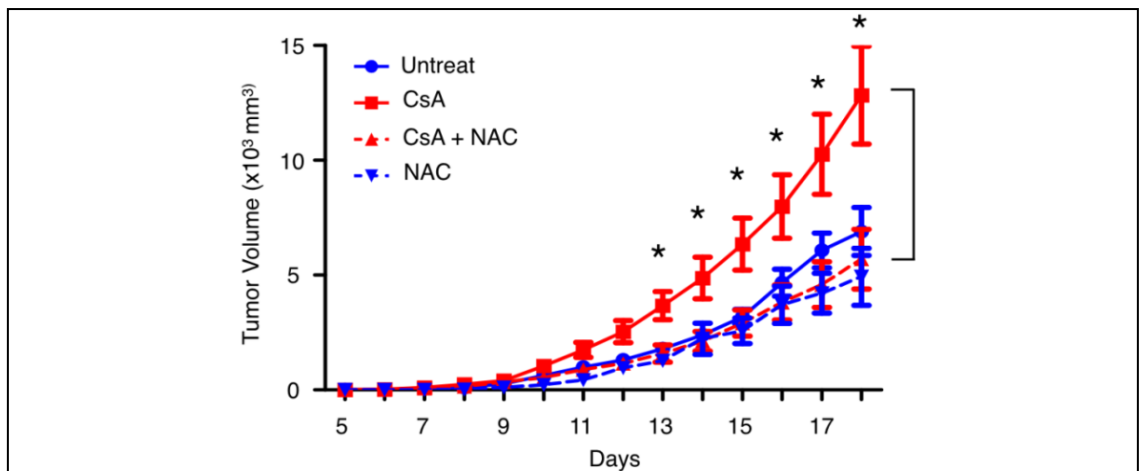
Fig. 3.5. Antioxidant treatment abolishes cyclosporin A-induced tumorigenesis *in vivo*. Tumor growth over time in mice treated with or without CsA and with or without 40mM NAC. WT C57/BL6 mice were supplied with NAC for 14 days, and then treated daily with 10mg/kg CsA for 14 days before inoculating subcutaneously with B16-F10 melanoma cells. Animals were maintained on CsA and or NAC treatment for the duration of the experiment. Tumor volume was measured on the indicated days. * P <0.05 (between CsA and CsA + NAC groups). Representative experiment shown from two experiments, n=5-9. Error bars indicate SEM, and * P <0.05 for all experiments.

associated tumorigenesis *in vivo* using the B16-F10 allograft melanoma model in WT

mice. Oral NAC was delivered to mice in the drinking water at 40mM, a routinely used

investigational dose (Aoi et al. 2014). NAC co-treatment in tumor-bearing mice on CsA

therapy decreased tumor growth compared to CsA alone (Fig.3.5.). Tumor growth in

mice on NAC and CsA co-treatment were indistinguishable from untreated mice. NAC

treatment by itself also did not affect tumor growth. These data demonstrate the

significance of CsA-induced ROS in an *in vivo* tumor model and the therapeutic potential

for prophylactic prevention of CsA-induced tumorigenesis by administering antioxidants.

Cyclosporin A increases ERK signaling in a ROS-dependent manner

In endothelial cells, ROS can differentially affect numerous pathways depending

on the particular ROS agent, its concentration and location (Maulik & Das 2002). Given

the integration of ROS signaling in many essential endothelial cell processes, it is unclear

which pathways CsA affects to promote ROS-dependent pro-angiogenic effects.

To determine which pro-proliferative pathway CsA-induced ROS affects in

primary endothelial cells, we assessed for activation in the mitogenic AKT and MAPK

pathways and found that CsA treatment increased ERK but not AKT phosphorylation

(Fig.3.6.A). Pre-treatment of endothelial cells with NAC prior to CsA treatment

abolished CsA-induced ERK phosphorylation (Fig. 3.6.B), indicating this is a ROS-

dependent process. These data suggest that CsA-induced ROS promotes MAPK activity.

ROS can change the oxidative-reductive state in a cell, affecting the enzymatic

activity of proteins such as phosphatases, including those involved in mitogenic pathways

(Forman et al. 2004; Thannickal & Fanburg 2000). Low levels of ROS have been shown

to deactivate phosphatases that negatively regulate mitogenic intermediaries, resulting in

prolonged proliferative signaling (Meng et al. 2002). Consistent with this notion, we

found that CsA treatment in endothelial cells prolonged ERK activation over time
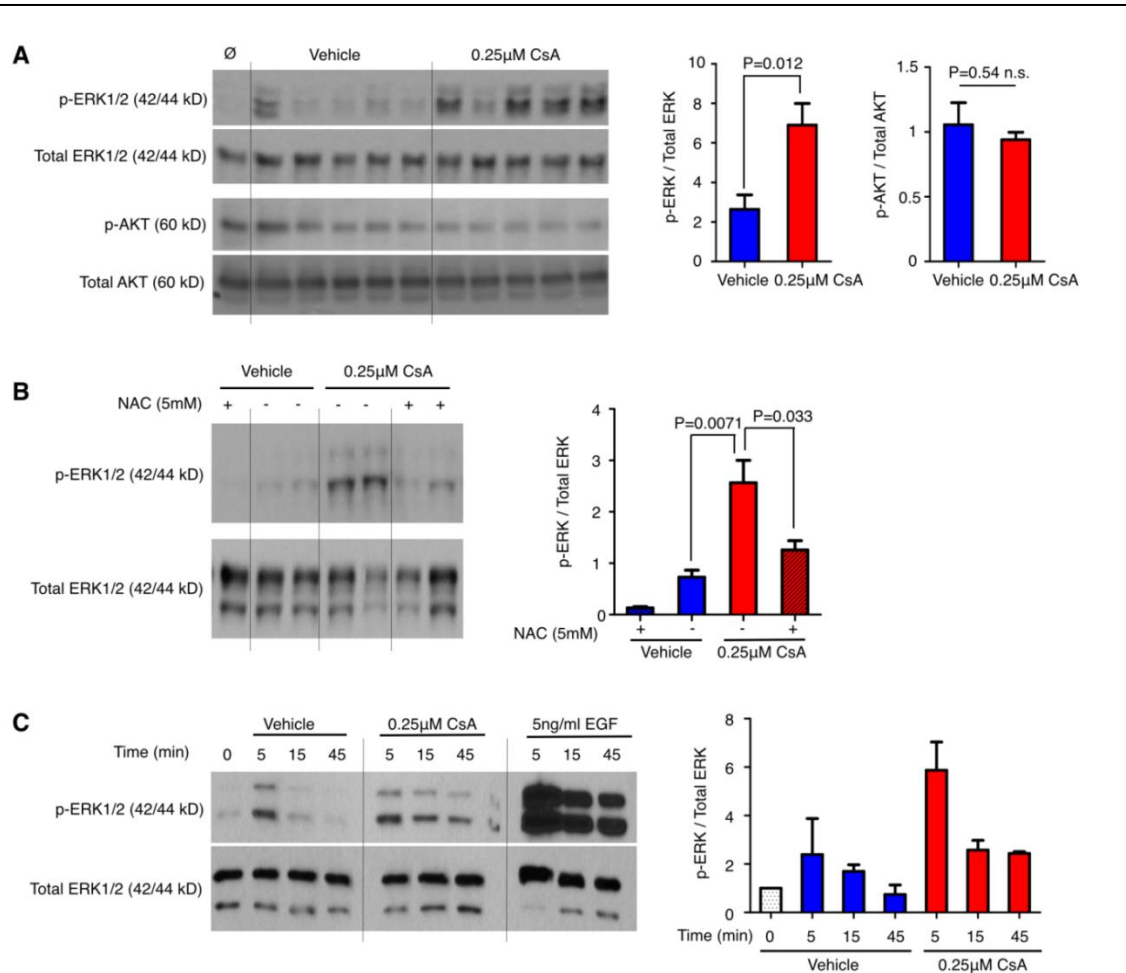
Fig. 3.6. Cyclosporin A-induced reactive oxygen species upregulates MAPK signaling. (A) Western blot of phospho and total ERK and AKT protein in endothelial cells after treatment with 0.25µM CsA or vehicle for 5min. (B, C) ERK phosphorylation in endothelial cells was examined by Western blot after treatment with (B) 5mM NAC for 30 min followed by 0.25µM CsA or vehicle for 5min, or (C) 0.25µM CsA or vehicle for the indicated time. Phosphoproteins were normalized to their respective total protein levels with relative expression levels on the right for all experiments. (A-C) each lane is an individual experiment. Results are pooled from 4-5 experiments. Error bars indicate SEM, and * P >0.05 for all experiments.

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compared to vehicle treatment (Fig.3.6.C). Treatment with the supra-physiological

5ng/mL EGF (physiological range: 10^{-12}g/mL (Joh et al. 1986)) was used as a positive

control to demonstrate both ERK activation as well as its decline over time.

Discussion

In Chapter 2, we showed that CsA promotes endothelial cell proliferation and migration in a calcineurin-independent manner. Here we argue that CsA achieves these effects through elevated mitochondrial ROS. In these experiments, we demonstrate that CsA and its non-immunosuppressive analog, NIM811, both act on the mitochondria to increase mitochondrial ROS and promote endothelial cell proliferation and migration. Neutralization of elevated ROS by either cellular or mitochondrial targeted antioxidants abrogates CsA-induced endothelial cell proliferation and migration. Prophylactic antioxidant treatment in tumor-bearing mice on CsA regimens significantly reduces the increased tumorigenesis seen with CsA treatment. *In vitro* studies in endothelial cells showed that CsA promotes ERK phosphorylation in a ROS dependent manner.

A number of calcineurin-independent effects have been ascribed to CsA. CsA has been shown to increase p38 and JNK signaling in T cells (Matsuda et al. 2000) and inhibit mitochondrial calcium uptake in HeLa cells (Montero et al. 2004), effects not shared by other calcineurin inhibitors, either pharmacological or endogenous. CsA has previously been shown to bind and inhibit P-glycoprotein (Morjani & Madoulet 2010). Brain and testis endothelial cells express high levels of P-glycoprotein in order to maintain the blood-brain or blood-testis barrier. Tumor cells can also express P-glycoprotein as a means of drug resistance (Morjani & Madoulet 2010), however, this does not always extend to tumor endothelial cells (Tóth et al. 1996). Moreover, our experiments utilized lung endothelial cells, which do not express P-glycoprotein

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(Demeule et al. 2001). Therefore, we do not expect the inhibition of P-glycoprotein by CsA to be a confounding factor in our results.

While we show that antioxidant treatment of tumor-bearing mice *in vivo* decreases CsA-induced tumor growth, it is still unclear whether this is due to a decrease in tumor angiogenesis. This ambiguity could be addressed by staining for endothelial cell markers such as CD31 to reveal whether antioxidant treatment has any effect on CsA-induced tumor angiogenesis.

Oral ingestion of both CsA and NAC results in elevated drug levels in both tumor cells as well as the tumor microenvironment, all of which may also affect tumor angiogenesis. We have shown *in vitro* that CsA elicits pro-angiogenic responses directly in endothelial cells, but have not excluded the possibility that it may also act in a tumor cell-autonomous manner *in vivo* to induce angiogenesis as tumor-derived ROS can also promote neoplastic angiogenesis (Xia et al. 2007). Likewise, systemic administration of NAC could be affecting both the tumor tissue and also the tumor microenvironment. Since we postulate that CsA promotes ROS in a cyclophilin D-dependent manner in endothelial cells, the deletion of cyclophilin D in the host tissue should abrogate the effects of both CsA and antioxidant treatment on tumorigenesis. Utilizing a whole body cyclophilin D knockout transgenic mouse model would address whether CsA-induced effects are tumor cell autonomous or microenvironment based. Similarly, transgenic mice with conditional deletion of cyclophilin D specifically in endothelial cells could be used to determine whether CsA-induced tumorigenesis occurs only through its activity in endothelial cells.

CsA has not been described to have any cyclophilin-independent effects. Scant functional characterization of some cyclophilin proteins has been performed, but their specific functions are mostly unknown. Correspondingly, the effect of CsA binding to these proteins is unknown. An exception is cyclophilin D, where both its function and interaction to CsA has been thoroughly studied with biological relevance in multiple disease models (Giorgio et al. 2010; Elrod & Molkentin 2013). Since the effect of the CsA-cyclophilin D interaction on mitochondrial biology is well known, cyclophilin D is an attractive candidate as a calcineurin-independent mechanism responsible for the pro-angiogenic effect of CsA in endothelial cells. However, NIM811 and CsA still bind to other intracellular cyclophilins. Therefore, the possibility of other CsA binding partners contributing to this effect cannot be ruled out and will be discussed in further detail in chapter 4.

While we suspect that CsA acts through a ROS-dependent mitochondrial mechanism to increase endothelial cell proliferation and migration, we have not completely ruled out the contribution of ROS-independent mitochondrial effects. We did establish that CsA has no effect on cell death in the absence of apoptotic stimulus, as it known that CsA decreases apoptosis under cell death conditions (Norman et al. 2010). The effects of CsA-cyclophilin D interaction on other mitochondrial functions were not addressed in this study and future studies assessing mitochondrial metabolism, calcium signaling, fission/fusion, and mitophagy would be helpful in tackling this concern.

Our data shows elevated Mitosox but not DCFDA staining in endothelial cells following CsA treatment. We hypothesize that CsA leads to an increase in the production

of mitochondrial ROS, which could enter the cytosol in the membrane-permeant form H_2O_2 to affect cellular redox signaling. Therefore, we expected to see elevated ROS levels in both compartments. As DCFDA staining shows no change with CsA treatment, it suggests cellular ROS levels are unchanged. However, the DCFDA marker may not be a representative readout of cytosolic ROS. Upon entering a cell, cleavage of DCFDA by cellular esterases converts it into a non-fluorescent ROS indicator, which becomes fluorescent when oxidized. This intracellular ROS probe is sensitive to oxidation by peroxyl, alkoyl, NO_2, carbonate, and $OH^.$ radicals, but not to O_2^- or H_2O_2 (in the absence of peroxidases) (Eruslanov & Kusmartsev 2010). H_2O_2 is a relatively stable ROS family member which can diffuse freely across membranes such as the mitochondrial membrane and has been implicated in physiological processes such as proliferation and growth arrest (Giorgio et al. 2007). The unreliability of the DCFDA probe to detect H_2O_2 causes it to be a less relevant indicator of the specific reactive oxygen species that may be responsible for the proliferative changes studied here. The addition of horseradish peroxidase or myeloperoxidase, which oxidizes DCFDA in the presence of H_2O_2, would allow us to determine the total cytosolic ROS in a cell. Alternatively, other hydrogen peroxide indicators such as Amplex Red (in combination with horseradish peroxidase) or Peroxy Green 1 and Peroxy Crimson 1 (Miller et al. 2007) could also be used.

The mitochondrial membrane potential, which contributes to the proton-motive force to drive the regeneration of ATP from ADP, is generated and maintained by the oxidative-phosphorylative chain. Changes in the mitochondrial membrane potential can significantly affect mitochondrial bioenergetics, including the production of reactive

oxygen species from the ox-phos chain. Increasing the mitochondrial membrane potential through chemical means increases the production of hydrogen peroxide from the mitochondria (Korshunov et al. 1997). Our studies indicate that this is a mechanism by which CsA could potentially stimulate mitochondrial ROS production. This concept, as well as the background concerning relevant mitochondrial bioenergetics, are complex and will be discussed in depth in the summary chapter.

While our data is consistent with the hypothesis that the increased mitochondrial potential is the cause of CsA-induced ROS, but we have not ruled out other potential means responsible for elevated ROS. A previous study in endothelial cells found that CsA treatment perpetuated ROS damage by inactivating the mitochondrial antioxidant manganese superoxide dismutase through an endothelial cell nitric oxide synthetase-dependent mechanism (Redondo-Horcajo et al. 2010). Additionally, CsA has been shown to upregulate the expression of TXNIP, an inhibitor of the ROS scavenger thioredoxin, in renal mesangial cells (O'Connell et al. 2012). It is possible that all these sources could potentially contribute to CsA-associated ROS elevation, and future studies examining the effect CsA on each pathway in a single system would help evaluate the contribution of each of these ROS-producing pathways to CsA-associated ROS elevation.

The major physiological source of ROS in endothelial cells is from the NADPH oxidase, where it is produced in response to pro-angiogenic stimulus (Maulik & Das 2002). ROS serves as a signaling molecule which can induce VEGF receptor autophosphorylation, c-Src activation, and VE-cadherin phosphorylation (Ushio-Fukai & Nakamura 2008). Additionally, ROS by itself can induce VEGF expression (Xia et al.

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2007). The studies presented here did not assess whether CsA-induced ROS has any effect on endogenous ROS-activated endothelial cell processes. Characterization of VEGF expression levels and downstream signaling using NIM811 (to bypass the effect of CsA on the VEGF-calcineurin-NFAT axis) and antioxidants would address this point.

Our data showed that CsA treatment leads to prolonged activation of the MAPK effector ERK1/2 *in vitro*, which is highly suggestive that CsA may act to reduce its negative regulation, but does not show a causal relationship. Probing the activity of the mitogen-activated protein phosphatases (MKP1-10) with CsA and antioxidant treatment would help fill in the gap in knowledge. Furthermore, future experiments examining and manipulating the downstream ERK1/2 effectors are needed to show a direct effect of CsA-induced ERK activation and CsA-induced proliferative changes.

CHAPTER 4: SUMMARY AND DISCUSSION

Summary of findings: overview

In this thesis, we have identified a novel effect of the commonly used immunosuppressant cyclosporin A (CsA) in tumor angiogenesis. CsA is a potent inhibitor of the calcineurin pathway, which is involved in immune defense, memory consolidation, cardiovascular biology, and angiogenesis (Rusnak & Mertz 2000; Liu et al. 1991; Graef et al. 2001; Molkentin et al. 1998; Miyakawa et al. 2003). Our lab previously demonstrated that suppression of calcineurin in endothelial cells by its endogenous inhibitor Down Syndrome Candidate Region 1 (DSCR1) results in suppression of tumor angiogenesis and tumor growth (Baek et al. 2009). Paradoxically, chronic therapy with the calcineurin inhibitor CsA for transplant patients is associated with increased tumor incidence and progression. Here, we demonstrate for the first time that CsA treatment promotes tumor angiogenesis *in vivo* as well as endothelial cell proliferation and migration *in vitro*. We found that the pro-proliferative and migratory effects of CsA in endothelial cells occur in a calcineurin-independent manner, and is associated with increased mitochondrial reactive oxygen species (ROS). Additionally, treatment with antioxidants decreased CsA-induced proliferation and migration *in vitro* and CsA-induced tumorigenesis *in vivo*. Our model is summarized in figure 4.1.

Our studies have uncovered a previously unknown and targetable pathway through which CsA promotes tumorigenesis. However, our results raise many new questions, which will be discussed in the following pages along with future directions.

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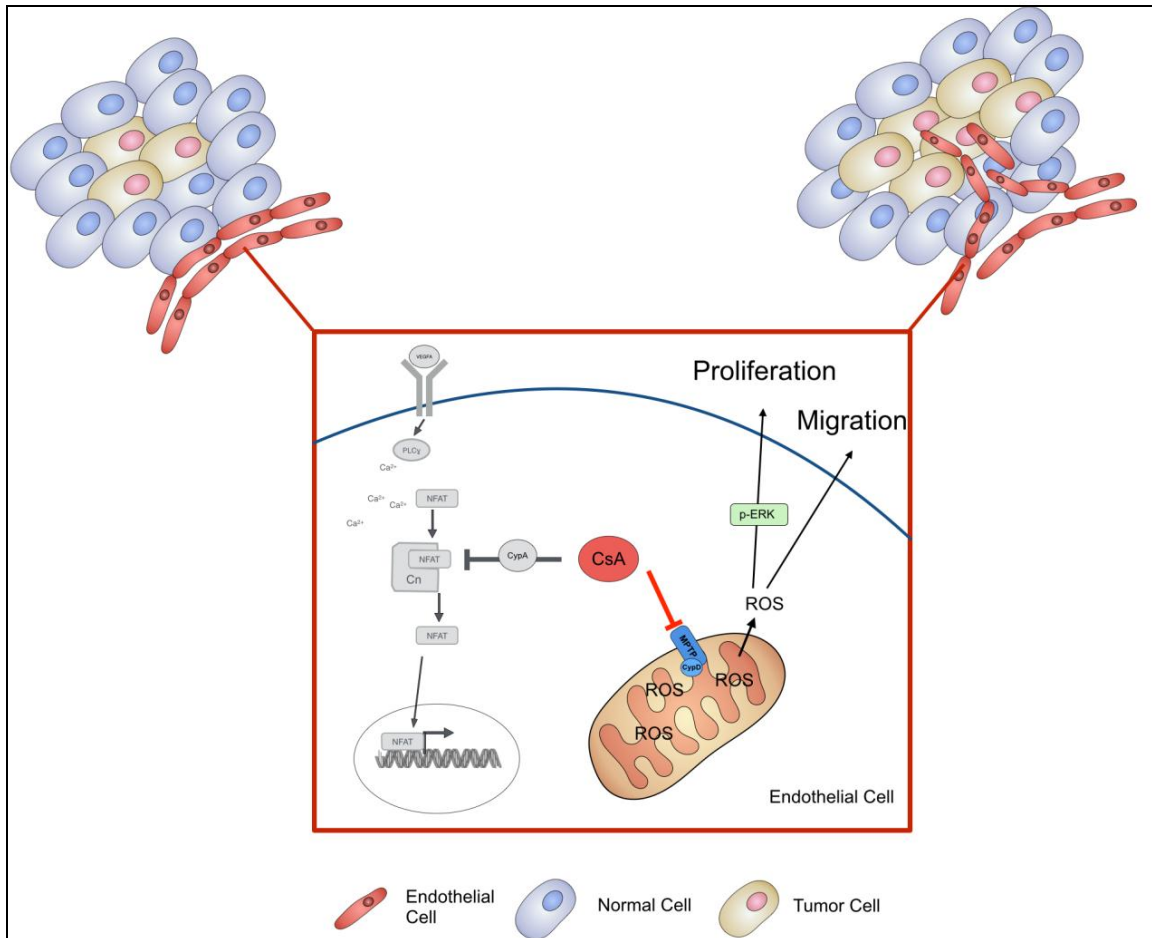
Fig. 4.1. Proposed model of CsA-induced tumor angiogenesis. CsA treatment in endothelial cells induces mitochondrial ROS in a calcineurin-independent manner, which promotes cell migration and proliferation. Increased endothelial cell proliferation and migration due to CsA treatment leads to increased tumor angiogenesis. Calcineurin (grey) inhibition still occurs, but the pro-proliferative effects of CsA is due to mitochondrial ROS.

CsA, cancer, and angiogenesis

Transplant-associated cancers: beyond skin cancers

To accurately reflect on the cancer profile of patients on CsA therapy, we utilized skin cancer models to study the effects of CsA on tumorigenesis. Most studies of CsA-

associated cancers also examine CsA in the context of skin tumorigenesis. There are a scattering of studies, however, that investigates the effects of CsA primarily on renal carcinoma or lymphocytes (Datta et al. 2009; Hojo et al. 1999; Mazière et al. 2005). A study by Guba et al in 2007 examined the risk of malignancy after kidney transplant in 2419 transplant patients and found an elevated risk of skin cancers, but also a 17.6-fold increase in the relative risk of developing kidney cancer (Wimmer et al. 2007). This was similarly mirrored by another study in 35,000 kidney transplant patients, who had a 15-fold increase in relative risk of kidney cancer (Kasiske et al. 2004). This elevation in kidney cancers in kidney transplant patients may be due to the kidney as the transplanted organ, as there is an organ-specific cancer risk arising from the transplanted organ (Engels et al. 2011).

Non-hogkins lymphoma in transplant patients is termed posttransplant lymphoproliferative disorder (PTLD) (Engels et al. 2011), and is associated with reactivation of latent Epstein-Barr virus and T cell dysfunction, presumably due to immunosuppression. PTLD often resolves upon discontinuation or reduction of immunosuppressive therapy, but can be also treated with radiation or chemotherapy (Tsai et al. 2001; Gottschalk et al. 2005).

Since kidney cancers are associated with an organ-specific etiology, and PTLD is strongly tied to immunosuppression, skin cancers appear to best represent a direct oncogenic effect of CsA treatment. Therefore, we conducted our study on CsA specifically in the context of skin cancers. Nonetheless, one should not marginalize the morbidity and mortality of non-skin solid tumors in organ transplanted patients.

Thorough investigation of the pathogenesis behind those tumors is important for decreasing the incidence and for the development of therapies.

Cancer cell autonomous effects of CsA on tumorigenesis: CsA and ROS on tumor cells.

The direct effect of CsA on tumor cells have been examined by other groups and described earlier in Chapter 1 and summarized in Figure 2.0. CsA is known to promote TGF-β production and Ras signaling in transformed tumor cells, induce ATF3 expression, and suppress DNA repair proteins and UV induced apoptosis in keratinocytes (the tumor cells of origin).

Our studies show CsA can act on the tumor microenvironment, specifically endothelial cells, through a ROS-dependent manner to increase cell proliferation and migration. Reactive oxygen species are often elevated in tumors and can affect a number of tumorigenic pathways in a cell-autonomous manner.

ROS molecules are highly reactive can interact with DNA, leading to DNA damage. If left unrepaired or mis-repaired, this DNA damage results in mutations, paving the way for oncogenic transformation.

ROS can also regulate various mitogenic signaling pathways through redox signaling. The MAPK pathway, which was demonstrated to be activated by CsA in endothelial cells, is overactive in many cancers. Elevated ROS in cancer cells has been shown to augment MAPK signaling, promoting tumor cell proliferation in a cell autonomous manner (Weinberg et al. 2010; Pelicano et al. 2004). ROS can also affect redox-sensitive transcription factors, for example, NF-κB and HIF-1α, which are

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important in tumorigenesis (Bonello et al. 2007). Additionally, p53, a critical tumor suppressor, forms disulfide bonds when oxidized that interfere with its ability to bind to p53 response elements, negatively affecting its transcriptional activities (Sun et al. 2003).

It is important to note that the relationship between ROS and tumorigenesis is complex, as it has also been found to negatively affect tumorigenesis. Radiation therapy exerts its tumoricidal effect by generating high levels of ROS. Elevated ROS has also been shown to lead to p38-dependent apoptosis in the setting of oncogene activation (Dolado et al. 2007). Therefore, while CsA-induced ROS in cancer cell could potentially lead to increased tumor growth, its actual contribution to tumorigenesis needs to be evaluated.

Non-cancer cell autonomous effects of CsA on tumorigenesis: immune cells and fibroblasts

We show that CsA promotes tumorigenesis through its effect on endothelial cells. The tumor microenvironment is a diverse milieu with multiple cell types which can affect tumorigenesis. Therefore, it is important to consider that CsA may also affect these other cell types to indirectly influence tumorigenesis.

Immune cells infiltrate tumors and secrete a number of inflammatory regulators. As CsA inhibits calcineurin-dependent T cell activation, it potently suppresses the adaptive immune response. It has been postulated that loss of immunosurveillance due to CsA treatment is a means by which transformed tumor cells can escape suppression by the immune system. Additionally, immunosuppression may also allow the unchecked

activity of oncogenic viruses. This is supported by greater HPV presence, which can cause skin cancers, in CsA-associated squamous cell carcinomas (Queille et al. 2007). However, no studies have shown a direct causal link between HPV and CsA-associated cancers, or whether they are two independent consequences of CsA therapy.

Cancer immunity is a complicated and multifactorial interaction between immune cells, tumor cells, stromal and endothelial cells. Immune cells can both antagonize and promote tumor growth. Generally, prolonged presence of activated innate immune cells such as neutrophils and macrophages is seen as pro-cancerous. However, innate immune cells are also under regulation by the adaptive immune system, which has paradoxical effects on tumorigenesis that can vary depending on cancer type, cancer progression, cell type, and experimental conditions (de Visser et al. 2006). So while suppression of adaptive immune cells in the tumor microenvironment by CsA is likely to affect tumorigenesis, it is unknown what the effect is. Comparing the effects of CsA with other immunosuppressants such as rapamycin or corticosteroids on tumor growth in immunocompetent and immunodeficient mouse models would be helpful in delineating the immunosuppressive contribution of CsA to tumorigenesis.

A large component of the tumor microenvironment is fibroblasts, often referred to as carcinoma-associated fibroblasts (CAF). These CAFs are poorly understood, including their cells of origin, but are believed to promote oncogenic signals to tumor cells. The effects of CsA on CAFs are not known. CsA has been shown to promote TGF-β production in tumor cells, which may activate fibroblasts to a CAF-like phenotype. However, unpublished data in our lab shows calcineurin activity may be important in

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TGF-β stimulated fibroblast activation, and inhibition of calcineurin by CsA prevents fibroblast activation. It would be interesting to determine the cumulative effect of CsA on the stromal fibroblasts and the fibroblast mediated contribution of CsA to tumor growth.

Functional effect of CsA on tumor angiogenesis

Given the disorganized nature of tumor vasculature, the pattern of CD31 positive structures in CsA treated tumors is by no means reflective of more functional vessels, but suggests an increase in nutrient or oxygen delivery that could provide a benefit to solid tumors (Bergers & Benjamin 2003). Future experiments involving the injection of FITC-lectin or FITC-dextrin, fluorescent markers of vessel permeability, into the circulation of tumor-bearing mice treated with CsA prior to harvesting would provide information on the fluid flow and permeability of these tumor vessels. Furthermore, *in vivo* assessment of tumor perfusion and oxygenation can be achieved through Doppler ultrasound or electrodes (Eppendorf electrodes or Recessed Microelectrodes) (Menon et al. 2003)

Our studies show CsA treatment *in vivo* results in a higher number of short CD31 positive structures in xenograft tumors, and *in vitro* CsA promotes endothelial cell proliferation and migration with no effect on matrigel tube formation. The data suggests that CsA promotes the earlier stages of angiogenesis, specifically proliferation and migration. Unlike physiological angiogenesis, the process of tumor angiogenesis places a greater emphasis on endothelial cell proliferation and vessel elongation compared to vessel remodeling and maturation (Nishida et al. 2006). Importantly, CsA treatment, through the upregulation of ROS, may further aggravate the imbalance of pro- to anti-

angiogenic pathways, amplifying the dysfunctional but aggressive nature of tumor angiogenesis. Future studies providing more detailed characterization of CsA-associated vessels, such as the expression of angiogenic regulators VEGF, TSP-1, or activation of Notch, would contribute more fully to our understanding of the role of calcineurin in tumor angiogenesis.

Effect of CsA on angiogenesis: contribution of tumor cells

Tumor cells are able to exert changes in its microenvironment through either secreted factors or cell-to-cell contact. The major secreted promoter of angiogenesis is VEGF (Carmeliet 2005). CsA treatment in renal carcinoma cells has been shown to directly promote the transcription and expression of VEGF (Basu et al. 2009). The significance of VEGF in CsA-associated skin carcinoma *in vivo* would be an interesting area to explore with the anti-VEGF antibody bevacizumab. . It is not clear whether CsA-induced VEGF production is due to calcineurin inhibition. Loss of calcineurin activity, through genetic deletion or pharmacological inhibition, in tumor cells could be used to evaluate the calcineurin-dependence of CsA-induced VEGF.

CsA also induces the production of TGF-β in tumor cells (Hojo et al. 1999). While a potent enhancer of cancer metastasis, TGF-β also has been described to have both pro- and anti-angiogenic effects. High levels of TGF-β is known to inhibit angiogenesis but low levels of TGF-β signaling can promote endothelial cell proliferation and enhance VEGF induced angiogenesis (Walshe 2010). Additionally, in nutrient deprived contexts, TGF-β promotes endothelial cell survival (Lu 2008), which can

provide an advantage in the harsh tumor microenvironment. Treating conditioned media from tumor cells with neutralizing TGF-β antibodies prior to application to endothelial cells *in vitro* would be helpful in determining the effects of tumor derived-TGF-β on endothelial cells.

Although it is known that CsA treatment induces TGF-β production, it is unclear whether it occurs through calcineurin-NFAT inhibition or through calcineurin-independent cyclophilin binding. There are NFAT and AP-1 binding sites present in the TGF-β promoter (Nakano et al. 2007). Additionally, calcineurin-NFAT signaling has also been shown to regulate a switch in the response to TGF-β, from cell cycle inhibitory to proliferative (Singh et al. 2010). It is possible that CsA not only promotes TGF-β production in tumor cells, but also regulates the response to TGF-β signaling.

While we found CsA increases ROS in endothelial cells, the phenomenon has also been reported in other cell types, such as smooth muscle cells (Krauskopf et al. 2005) and renal glomerular cells (O'Connell et al. 2012), suggesting it may be a common effect. Tumor-derived ROS may affect angiogenesis in two ways. First, tumor derived ROS may directly enter endothelial cells to promote angiogenic processes. . CsA treatment could elevate ROS levels in multiple cells types, including tumor cells, which can diffuse out into the surrounding environment, enter endothelial cells, and induce an angiogenic response. This notion could be tested by measurement of intra-tumor and serum H_2O_2, a stable and membrane permanent ROS,. .

Secondly, tumor-derived ROS has been shown to induce the transcription of pro-angiogenic factors such as VEGF (Xia et al. 2007). The angiogenic secretome in

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conditioned media from tumor cells treated with CsA, antioxidant, or combination of both could be assessed using an antibody array to determine whether CsA affects angiogenic factors in a ROS-dependent manner.

To determine the source of the ROS responsible for angiogenesis, overexpression of antioxidant genes such as superoxide dismutase or catalase specifically in tumor cells to quench tumor-derived ROS in a xenograft tumor model with CsA treatment would help separate the source of ROS responsible for angiogenesis; whether it is tumor or host derived.

Effect of CsA on angiogenesis: contribution of smooth muscle cells

In addition to the direct pro-proliferative and migratory effect of CsA on endothelial cells, CsA may also promote tumor angiogenesis through its effect on other cell types in the tumor microenvironment. In vascular smooth muscle cells, CsA has been demonstrated to block proliferation and modulate the vascular smooth muscle cell phenotype through a NFAT-independent mechanism (Garvey et al. 2010). The CsA binding partner cyclophilin A is secreted by smooth muscle cells and is linked to proliferation, vascular remodeling, and recruitment of inflammatory cells (Nigro et al. 2011). Vascular smooth muscle cells modulate tumor angiogenesis through regulation of vessel diameter, permeability, and contractility, as well as by regulating endothelial cell sprouting (Raza et al. 2010). CsA present in the tumor microenvironment may act upon smooth muscle cells to contribute to the increased angiogenesis observed. Comparison of pericyte markers such as α–SMA, NG2, desmin between tumors from CsA or vehicle

treated mice, followed by *in vitro* characterization of vascular smooth muscle cells

treated with CsA would contribute to a more complete understanding of the contribution

of CsA-microenvironment effects towards tumor angiogenesis.

Differential effects of CsA on the vascular system: established vessels vs. new growth

Oral CsA treatment in transplant patients also results in other vascular side effects,

the most notable being systemic hypertension. As discussed previously, CsA-induced

hypertension is the consequence of several pathological changes including elevated

endotehlin-1, decreased nitric oxide, and activation of the renal angiotensin system

(Olyaei et al. 2001). Of note, the primary effect of CsA in the established adult

vasculature is an increase in vascular tone; excessive vessel sprouting or angiogenesis has

not been described. Physiologic adult vasculature is composed of mature and quiescent

endothelial cells, which are long-living with low proliferative potential (Bergers &

Benjamin 2003).

There appears to be a context-dependent response of endothelial cells to CsA

treatment. Our studies on proliferating endothelial cells and tumors demonstrate CsA

treatment further increases endothelial cell proliferation and angiogenesis, whereas in the

established quiescent vasculature, CsA treatment elevates vasculature tone leading to

hypertension. These different outcomes are dictated by unclear mechanisms, but may

allude to the differential responses between quiescent or activated endothelial cells to

ROS (Yung et al. 2006). In established mature vessels, redox signaling can cause

endothelial dysfunction, promote secretion of inflammatory cytokines, hinder

vasorelaxation, or induce apoptosis (Yung et al. 2006). Furthermore, elevated ROS oppose vasodilation by converting the vasodilatory nitric oxide into the inactive peroxynitrite, diminishing the vasoactive capacity in the vascular wall (Yung et al. 2006). In the context of pathologic angiogenesis, the pro-angiogenic regulator VEGF-A has been shown to steer the direction of ROS signaling towards cell division and migration (Yung et al. 2006; Ushio-Fukai 2006; Xia et al. 2007). Therefore, the tumor microenvironment may provide a pro-angiogenic environment for CsA signaling whereas the same CsA treatment in established vasculature results in hypertensive responses.

Calcineurin-independent effects of CsA

Untangling the pathways: small molecule inhibition of calcineurin and cyclophilins
Calcineurin Inhibitors: FK506 vs CsA

While the best studied target of CsA is calcineurin, CsA also interacts with other molecules. Therefore, to determine whether calcineurin inhibition is responsible for CsA-induced proliferation and migration in endothelial cells, we compared the effects of several other calcineurin inhibitors to CsA. Previous studies have shown that overexpression of the endogenous inhibitor Down Syndrome Candidate Region-1 (DSCR1) suppresses VEGF induced angiogenesis by inhibiting the calcineurin pathway (Baek et al. 2009; Hesser et al. 2004; Chan et al. 2005; Yao & Duh 2004; Iizuka et al. 2004). We showed that treatment with CsA in endothelial cells overexpressing DSCR1

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increased proliferation, suggesting CsA acts in a dissimilar manner to DSCR1. We also compared the effects of CsA with the independent pharmacological calcineurin inhibitor, FK506. Also known as tacrolimus, FK506 is a small molecule which binds to the intracellular proteins FK Binding Proteins (FKBPs) to form a calcineurin-inhibitory complex (Rusnak & Mertz 2000) that is distinct from the CsA-cyclophilin complex. The only similarity between CsA and FK506 is the ability to inhibit calcineurin. Therefore, FK506 should theoretically act as an excellent control to determine the calcineurin-dependence of the endothelial cell effects seen with CsA treatment.

We determined the calcineurin-dependence of the effects of CsA on endothelial cells by comparing it to FK506. Treatment with the clinically relevant dose 0.15mg/kg/day of FK506 *in vivo* did not increase B16-F10 tumor growth compared to control treatment, while treatment with CsA promoted tumor growth. FK506 treatment decreased VEGF-induced migration, in direct contrast to CsA, which increased endothelial cell migration. CsA, but not FK506, treatment increased endothelial cell proliferation. Interestingly, treatment with FK506 inhibited endothelial cell growth, but to a lesser magnitude than calcineurin inhibition via DSCR1 (Baek et al. 2009).

These data are consistent with our hypothesis that CsA promotes an endothelial cell phenotype in a calcineurin independent-manner. As a calcineurin inhibitor, FK506 is expected to vastly decrease endothelial cell proliferation compared to vehicle treated, but the magnitude of its effect in these experiments, while significant, is paltry. This effect may reflect an off-target effect of FK506 on FKBPs, which fall under the immunophilin family of proteins (Barik 2006). FK506 binds to multiple FKBP family members, which

has cellular roles that include calcium regulation(MacMillan 2013), and inhibition of TGF-β signaling (Spiekerkoetter et al. 2013). The FKBPs are highly expressed in endothelial cells (Higgins et al. 2003) and loss or inhibition of FKBPs in the vascular compartment results in decreased nitric oxide production and increased TGF-β and BMP signaling (Spiekerkoetter et al. 2013). The outcomes of TGF-β signaling in endothelial cells can be variable depending on context, but have been shown to promote angiogenesis, wound healing responses, and tube formation. Treatment with FK506, through sequestering FKBPs, a negative regulator of TGF-β, could potentially enhance TGF-β signaling in endothelial cells, opposing calcineurin inhibition, resulting in only a slight depression in endothelial cell proliferation, as seen in our studies.

FK506 may also have a direct negative effect on tumor progression independent of endothelial cells as a previous study found topical applications of FK506 inhibited chemically-induced skin tumorigenesis and was associated with decreased IL-1α levels (Yamamoto & Jiang 1994). These studies, along with our data, indicate that while FK506 and CsA are often considered comparable drugs clinically and experimentally, they are indeed very different molecules which can have a myriad of unrelated calcineurin-independent effects. We show they have different effects on tumorigenesis, but this may extend to other physiological effects as well. Therefore, the clinical side effect profiles of these two drugs should be evaluated independently and their use in experimental studies should take into consideration the dissimilar off-target effects.

Cyclophilin inhibitors: NIM811 vs CsA

Due to the calcineurin-independent effects of FK506 and the possible confounding factor of FKBP signaling, FK506 may not be an ideal control for determining the calcineurin-dependency of the effects seen with CsA. Therefore, we then used NIM811, a non-immunosuppressive cyclosporin analog. NIM811, along with Alisporivir (DEB025), was originally developed as a potential therapy for hepatitis C, which binds to cyclophilins as part of its viral life cycle (Hopkins & Gallay 2012). NIM811 is identical in chemical structure to CsA with the exception of an extra methyl group on amino acid 4 (Rosenwirth et al. 1994), rendering it unable to bind to calcineurin and an ideal control to test the calcineurin-dependence of the effect of CsA on endothelial cells. The binding of NIM811 to cyclophilin proteins are unchanged compared to CsA and unaffected by this side group difference (Rosenwirth et al. 1994). When treated with NIM811, endothelial cell proliferation increased in a manner comparable to when treated with CsA. Both NIM811 and CsA treatment induced a similar bell-shaped growth curve, showing both cyclosporin analogs exert the same dose-dependent effect on proliferation. Additionally, NIM811 also promotes endothelial cell migration across a transwell filter. These data are in accordance with the hypothesis that CsA promotes endothelial cell proliferation and migration in a calcineurin-independent manner.

Cyclophilin inhibitors: clinical implications

These results clearly identify calcineurin-independent effects of CsA on endothelial cells, but also raise questions regarding the clinical usage of CsA analogs.

Non-immunosuppressive cyclosporin analogs are currently in development for treatment

of hepatilis C (hep C). Inhibition of cyclophilins with cyclosporins inhibits viral

replication. Three cyclosporin derived non-immunosuppressive cyclophilin inhibitors:

Alisporivir (Debio-025), SCY-635, and NIM811, are currently in investigation as

potential therapy for hep C (Hopkins & Gallay 2012). Our studies showing that the

cyclosporin analog NIM811 promotes endothelial cell proliferation highlights a potential

oncogenic risk with cyclophilin inhibitors similar to the effect seen with CsA. The

completed clinical trials of these cyclophilin inhibitors have not found an increase in

cancer risk or aberrant angiogenic events (Gunter et al. 2010). However, this may reflect

the duration of drug use in these trials, usually lasting 4 weeks or less. A Phase IIa trial

with Alisporivir lasting 48 weeks also did not identify increases in cancer risk or vascular

events (Alberti et al. 2012; JM et al. 2012). While its similarities to CsA label this

cyclophilin inhibitor as a potentially pro-tumorigenic drug, to the best of our knowledge,

we are the only group to study the effect of NIM811 on endothelial cells. So far no

clinical data has emerged which would cement an association between NIM811, or other

cyclophilin inhibitors, and increased cancer risk. As these cyclophilin inhibitors are still

investigational therapies, they have not yet been thoroughly tested in patient populations.

Additionally, transplanted associated cancers generally appear after years of continuous

treatment, a scenario which cyclophilin inhibitors have not yet been subjected to. The

cancer risk found with CsA treatment in transplant patients is dose dependent. Therefore,

given the nature of hep C treatment, which is shorter in duration than transplant

immunosuppression, the cumulative dose of cyclophilin inhibitor accrued over the

duration of treatment may not be sufficient to affect cancer risk. Taken together, these cyclophilin inhibitors, in their current clinical niche, do not appear to pose a significant cancer risk. Nonetheless, our studies comparing CsA and NIM811 suggests additional investigations into the safety of the cyclophilin inhibitors maybe warranted, especially in regards to potential cancer risk.

An interesting finding from our studies was that both CsA and NIM811 treatment induced a bell-shaped dose-dependent effect on endothelial cell growth *in vitro*. Compared to vehicle treated controls, CsA and NIM811 caused an increase in endothelial cell growth starting at 10nM and increased with drug concentration until a maximum of 250nM, then causes cytotoxicity at $>1\mu M$. This data demonstrates the complexity of the cellular response to these drugs, and possibly reflects the combined effect of more than one affected pathway. At least two distinct and opposing effects on proliferation have been ascribed to CsA in endothelial cells through two different pathways. We show CsA, at low levels, promotes endothelial cell proliferation through a calcineurin-independent and ROS dependent pathway. At high levels CsA inhibits endothelial cell migration, tube formation, and corneal angiogenesis through calcineurin inhibition (Hernández et al. 2001). Additionally, ROS signaling can be either pro-mitogenic or pro-apoptotic depending on its levels (Yung et al. 2006). The delicate balance in which CsA induces multiple and sometimes opposing effects due to drug concentrations and cellular context may explain the difficulty of managing its therapeutic window. While CsA has revolutionized the transplant field by preventing graft rejection, its use is riddled with

multiple severe side effects. Taking into consideration the literature as well as our studies, clinical indications for CsA should instead consider other, more specific and safer drugs.

Calcineurin loss and cell migration

To determine whether CsA promotes angiogenesis in a calcineurin-dependent or independent manner, we used the lox-stop-lox technology to create cells in which the calcineurin B subunit can be genetically deleted, leading to the degradation of calcineurin A and loss of calcineurin activity. Loss of calcineurin nearly completely abolished endothelial cell migration across a transwell filter when compared to calcineurin-competent cells. While unexpected, this is not surprising given that calcineurin-NFAT signaling promotes tumor cell migration and tumor invasion through integrin signaling (Liu et al. 2010; Jauliac et al. 2002). Additionally, inhibition of calcineurin through overexpression of DSCR1 (Iizuka et al. 2004) or treatment with FK506 decreased migration *in vitro*. In endothelial cells, NFAT promotes endothelial cell migration through upregulation of Cox-2 (Hernández et al. 2001). These studies indicate that calcineurin activity is important for endothelial cell migration, a critical aspect of angiogenesis. Therefore, the net migratory effect of CsA seen here is the sum of a large pro-migratory effect of CsA-induced ROS negating the anti-migratory effect of calcineurin inhibition. This further supports the hypothesis that CsA is affecting endothelial cell biology in a calcineurin-independent manner. Based on our results and these studies, current experiments in our laboratory now focus on the involvement of

calcineurin in fibroblast activation, in which cell migration and adhesion plays an integral

role.

Calcineurin-independent effects of CsA: why Cyclophilin D and the mitochondria?

Despite the importance of calcineurin signaling in endothelial cell biology and

angiogenesis, we have demonstrated the calcineurin inhibitor CsA is able to promote

angiogenesis in a calcineurin-independent manner. Binding of CsA to cyclophilin A

forms a calcineurin-inhibitory complex. In addition to cyclophilin A, CsA also binds to

other cyclophilin family members, resulting in calcineurin-independent effects.

Cyclophilins are a family of proteins with peptidyl-propyl isomerase functions, able to

catalyze the cis-to-trans reaction. Binding of CsA to cyclophilins disrupt their catalytic

function (Nigro et al. 2013). While conserved from yeast to humans, very little is known

about the physiological role of the cyclophilin proteins. Knockout of all cyclophilins in *S.*

cervasie did not yield any phenotypes (Dolinski et al. 1997), suggesting they may be

important only during pathological or stressed circumstances. In mammals, cyclophilins

have been linked to a number of pathological conditions. Cyclophilin A, in particular, has

been associated with atherosclerosis, cancers, and inflammatory diseases (Nigro et al.

2013). As CsA-induced angiogenesis occurs in a calcineurin-independent manner, , we

theorize that it results from CsA binding to cyclophilin proteins. The most pronounced

and consistent phenotype of pan-cyclophilin inhibition is mediated by cyclophilin D

inhibition. Loss of cyclophilin D activity through pharmacological inhibitors or genetic

deletion results in a 'stiff' pore that is resistant to opening in response to stimulus, such as

cell death signals. Despite being a pan-cyclophilin inhibitor, the use of CsA in multiple

cell types is remarkably specific in its ability to decrease cell death in response to

apoptotic stimulus, underlying the importance of cyclophilin D to cellular homeostasis.

Other cyclophilins: possible contributions to angiogenesis

  While cyclophilin D remains the most obvious modulator of CsA pro-proliferative

and migratory effects in endothelial cells, we did not rule out the contribution of other

cyclophilins, or cyclophilin-independent effects of CsA. To this date, no cyclophilin-

independent effects of CsA have been described, but the absence of its knowledge does

not necessary rule out its existence. Additionally, while inhibition of cyclophilin D is the

most pronounced cellular effect of pan-cyclophilin inhibitors, CsA still binds to other

cyclophilin family members, the effects of which are unknown.

Cyclophilin A

  Cyclophilin A, which binds CsA to form the calcineurin inhibitory complex, is the

most abundant cyclophilin family member, constituting about 0.1-0.6% of total cellular

protein and is characterized with diverse contextual and cell-specific functions. It has

been implicated in pathological conditions such as abdominal aortic aneurysm,

atherosclerosis, diabetes, cancer, neurodegeneration, and several inflammatory conditions

(Nigro et al. 2013). In response to inflammatory stimulus, Ang II, or other stressors,

cyclophilin A can be secreted from macrophages and vascular smooth muscle cells as an

inflammatory cytokine, and can act on vascular smooth muscle cells to increase matrix

metalloprotease production, promote growth and decrease apoptosis, which contributes to vascular remodeling (Nigro et al. 2011). Cyclophilin A signaling in endothelial cells has also been shown to increase adhesion molecules, decrease eNOS, and promote apoptosis (Nigro et al. 2013). Exogenously administered cyclophilin A has a biphasic effect on endothelial cell activation where low levels promoted angiogenesis but high levels decreased endothelial cell viability (Kim et al. 2004).

These studies indicate an important but complex role of cyclophilin A in pathological vascular conditions. It is unclear whether cyclophilin A has a role in tumor angiogenesis, despite being strongly linked to cancer progression and metastasis in a cell-autonomous manner (Lee & Kim 2010).

Previous studies have shown knockdown of cyclophilin A protects against TNF-α induced endothelial cell apoptosis (Nigro et al. 2011). While we did show increased cell numbers with CsA treatment, it was due to increased proliferation without any change in apoptosis. As a cyclophilin inhibitor, CsA sequesters and inhibits cyclophilin A activity in endothelial cells, a phenotype which should resemble cyclophilin A deletion mutants. However, the phenotypes we observed in endothelial cells after treatment with CsA, and to a lesser extent, NIM811, do not appear to phenocopy cyclophilin A loss described elsewhere. Therefore, cyclophilin A inhibition by CsA is unlikely to be responsible for the pro-angiogenic effects of CsA.

Cyclophilin B

Cyclophilin B is another cyclophilin that is both secreted and retained in the endoplasmic reticulum, and is important for migration and adhesion of immune cells and protein folding (Mckeon 1994; Allain et al. 2002). Cyclophilin B expression and activity has also been found to be aberrant in several tumor types in a cell autonomous manner. For example, cyclophilin B is overexpressed in 80% of hepatocellular carcinomas and colon cancers, where it is transcriptionally regulated by HIF-1α and protects against cisplatin and hypoxia-induced apoptosis. In the same study, cyclophilin B was also shown to regulate HIF-1α associated angiogenesis by inducing VEGF production (Kim et al. 2011). Furthermore, cyclophilin B was also found to support glioblastoma multiform tumor cell survival by stabilizing the oncogenic proteins myc and p53, likely through its chaperone activity (Choi et al. 2014). In harsh oxidative conditions, cyclophilin B may also indirectly protect against oxidative damage by stabilizing myc, leading to increased transcription of UCP2, a mitochondrial uncoupling protein (Choi et al. 2014). These studies indicate a broad potential role of cyclophilin B in a cancer cell-autonomous manner. In the vasculature, however, cyclophilin B is not known to be expressed or have a physiological role. While cyclophilin B has been shown to be expressed in endothelial cells after shock wave stress (Holfeld et al. 2014), it has not been otherwise described to be present or functional in endothelial cells or angiogenesis.

Inhibition of cyclophilin B by CsA in tumor cells could potentially decrease hypoxia-induced HIF-1α responses such as angiogenesis and also destabilize mutant p53 and oncogenic c-myc, which could lead to decreased protection against oxidative stress. This may explain the increased ROS-dependent tumor angiogenesis *in vivo* seen in the

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setting of CsA treatment. However, it does not account for the effects of CsA on endothelial cells *in vitro*, which are untransformed primary cells cultured in normoxic conditions.

Cyclophilins C & E

Also discovered as a binding partner to cyclosporin A, cyclophilin C is expressed at much lower levels than cyclophilin A and has lower binding affinity to CsA than either cyclophilin A or B (Schneider et al. 1994). Little is known about the physiological function of cyclophilin C. Similarly, cyclophilin E, a nuclear and RNA binding cyclophilin, was recently described to bind to influenza viral proteins to retard their life cycle but is otherwise poorly understood (Wang et al. 2011). These proteins have not been described to be important in tumorigenesis, and none have been described to participate in angiogenesis. The outcomes of inhibition of these cyclophilins by CsA are unclear.

Unbiased screen vs. candidate approach for CsA interactors

While other cyclophilin family members, in particular, cyclophilin A, may be involved in pathological endothelial cell biology, cyclophilin D still remains the most attractive candidate to explain the proliferative and migratory phenotype seen with CsA treatment. Nevertheless, future studies are needed to definitively rule out the contribution of other CsA interactors, either cyclophilins or other molecules, to the pro-proliferative phenotype observed with CsA.

In this study, I have taken a candidate approach where I identified a likely target protein through a literature analysis. Subsequent testing of the phenotypical output such as the mitochondrial ROS was consistent with my hypothesis. To fully confirm this hypothesis, genetic manipulation should be used.

Transgenic mice lacking cyclophilin D are viable and fertile and are protected from ischemia-reperfusion injury *in vivo* (Nakagawa et al. 2005) but display increased anxiety and brain white adipose tissue (Luvisetto et al. 2008), indicating a role for cyclophilin D in mitochondria-associated cell death and metabolism. Primary endothelial cells isolated from mice lacking cyclophilin D is expected to behave similarly to wild-type endothelial cells treated with CsA or NIM811, which inhibits cyclophilin D. In fact, mitochondria isolated from cyclophilin D null cells have MPTP that are resistant to calcium-induced opening, in a manner similar to inhibition of cyclophilin D by CsA (Nakagawa et al. 2005). Given my hypothesis, I expect endothelial cells isolated from cyclophilin D null mice to have increased proliferation and migration *in vitro* compared to wild-type endothelial cells, similar to CsA treatment. Additionally, cyclophilin D null endothelial cells are expected to display increased mitochondrial membrane potential and mitochondrial ROS production compared to wild-type endothelial cells. Xenografted tumors on cyclophilin D null mice are expected to have increased tumor growth and angiogenesis. To test whether endothelial cell cyclophilin D is important for CsA-induced tumor angiogenesis *in vivo,* one can conditionally delete cyclophilin D specifically in endothelial cells, rather than complete elimination of cyclophilin in all host cells. Breeding the transgenic VE-cadherin Cre recombinase mouse with the transgenic

cyclophilin D$^{loxP/loxP}$ mouse (Schinzel et al. 2005) should yield transgenic mice with deletion of cyclophilin D only in endothelial cells. If endothelial cells are responsible for tumor angiogenesis and growth in CsA-treated animals, then loss of cyclophilin D specifically in the endothelial cells should mimic CsA treatment in wild-type animals when subjected to a tumor model. Any deviations from these expected outcomes, either partially or completely, would indicate that CsA is either not binding to cyclophilin D or is affecting multiple pathways in addition to cyclophilin D to result in pro-angiogenic effects.

If cyclophilin D loss does not phenotypically mimic CsA treatment *in vitro* and *in vivo* on endothelial cells and tumor growth, it is possible that CsA is exerting an effect through another cyclophilin protein, or a yet unidentified non-cyclophilin pathway. If we assume CsA is exerting its effects through another cyclophilin protein, then systematic siRNA knockdown of all 7 mammalian cyclophilin genes (16 unique proteins) should identify the CsA interactor(s) responsible for increased proliferation, migration, and mitochondrial ROS in endothelial cells.

To more broadly understand the effects of CsA and identify potential interacting proteins responsible for the effects observed, including non-cyclophilin proteins, it would be pertinent to use an unbiased screen. Unlike a targeted candidate approach, an unbiased screen systematically manipulates a much larger number of potential targets. Using a broader approach, such as an unbiased library of RNA interference (RNAi), to knockdown single genes in endothelial cells with or without CsA treatment, would allow for identification of genes that are immediately downstream of CsA-induced readout such

as cell proliferation and ROS generation. In this case, RNAi knockdown of the putative

CsA interactor would then negate the pro-proliferative and pro-ROS effects of CsA

whereas RNAi knockdown of unrelated transcripts should not affect CsA's effects on

proliferation and ROS (when compared to vehicle-treated). Potential targets would then

be identified and direct binding to CsA would be confirmed through immunoprecipitation.

Further characterization studies using FK506 and NIM811 would be expected to agree

with existing experiments. The RNAi approach has been used extensively but is not

without flaws as RNA interference only partially suppresses target gene expression and

may have off-target effects on unrelated transcripts.

A more recent screen uses the newly developed clustered regularly interspaced

short palindromic repeat (CRISPR)-Cas system, which was conceived from a RNA-based

prokaryotic immune system (Wang et al. 2014). In this system, lentiviral vectors carry

small segments of RNA, corresponding to the gene of interest, engineered as single-guide

RNA (sgRNA), and direct Cas nucleases (usually Cas9, also expressed from the same

viral vector) to cause double-stranded cleavage of matching targeted genomic DNA

sequence (Shalem et al. 2014; Zhou et al. 2014; Wang et al. 2014). This allows for

specific genomic deletion of a target gene. When used in combination with a library,

either an unbiased or focused library, it is possible to individually delete target genes and

then perform functional studies after subjecting to experimental treatments. Using this

technology, one can identify proteins that either interact with CsA or are downstream of

CsA interactors by screening for loss of CsA-induced cell proliferation and elevated ROS

in cells. The advantage of the CRISPR-Cas9 screening system over the RNAi screening

system is that suppression of the target gene is complete due to its excision, whereas the RNAi system only partially suppress the target gene. A limitation of both systems is that in addition to identifying direct CsA interactors, they would also identify genes downstream of those proteins. Therefore, once target genes are identified, direct CsA interaction would need to be confirmed with immunoprecipitation studies.

Genetic screens, either the RNAi or CRISPR-Cas9 system, would provide an unbiased means to identify CsA interactor(s) or confirm the current selected candidate that is/are responsible for the pro-angiogenic effects seen. Future studies using either one of these systems would greatly strength my hypothesis and may provide additional targets to understanding the tumorigenic effects of CsA.

Relationship between ROS and CsA

Mitochondria and ROS: evidence for the actions of CsA

Treatment with CsA or its non-immunosuppressive analog NIM811 results in increased mitochondrial ROS and cell proliferation, which was abolished with antioxidant co-treatment. Moderate increases in ROS have been shown to promote mitogenic signaling pathways (Thannickal & Fanburg 2000), consistent with our data.

CsA is also known to inhibit calcium-induced irreversible MPTP opening in the setting of mitophagy or cell death. Inhibition of cyclophilin D during cell death raises the stimulus threshold required for the collapse of the mitochondrial membrane potential, ultimately decreasing apoptosis (Halestrap 2009; Kim et al. 2003). This property of CsA

is currently exploited to manipulate ischemia-reperfusion injury in cardiomyocytes (Halestrap & Richardson 2014).

In the absence of cell death signals, treatment with CsA increases mitochondrial ROS production, in agreement with previous studies (Krauskopf et al. 2005; Redondo-Horcajo et al. 2010; Akool et al. 2012). This process originates from an alternative mitochondrial pore state termed 'physiological flickering'. In this reversible state, the transient opening of the MPTP is brief, isolated, stochastic, reversible, and does not result in membrane depolarization or cell death (Wang et al. 2012). Instead, individual reversible MPTP opening events are thought to act as a pressure release valve to regulate membrane potential, ultimately affecting mitochondria metabolism and the mitochondria-cytoplasm crosstalk (Wang et al. 2012). Changes in mitochondrial membrane potential can alter the production of ROS (Korshunov et al. 1997), a byproduct of the electron transport chain during the process of oxidative phosphorylation. We hypothesize that inhibition of the reversible MPTP opening by CsA increases mitochondrial membrane potential, leading to increased ROS production. In order to fully appreciate the effects of CsA on mitochondrial biology, a thorough summary of mitochondrial bioenergetics leading to ROS production will be presented in the following section, followed by a discussion on the effects with CsA on this system.

Oxidative-phosphylorative pathway

The mitochondrion is a major source of reactive oxygen species, produced as a byproduct of the electron transport chain. The electron transport chain is the final portion

of cellular respiration in which high energy electrons from energy-rich substrates such as

NADH and $FADH_2$ are transferred, in a sequential and stepwise manner, to various

energy carriers along the chain, until it becomes accepted by oxygen to form water. The

fall of electrons down its energy gradient results in the generation of an electrochemical

gradient across the mitochondria inner membrane which serves as the driving force for

the regeneration of ATP. Comprehension of mitochondrial bioenergetics, especially

oxidative phosphorylation, is critical to understanding how regulating MPTP activity can

affect mitochondrial reactive oxygen species production.

The mitochondrion is separated into two compartments (the intermembrane space

and the matrix) by two membranes (outer and inner mitochondrial membrane) through

which electrochemical gradients can be generated and exploited for energy production.

The outer mitochondrial membrane defines the mitochondria as an organelle. In between

the outer mitochondrial membrane and the inner mitochondrial membrane is the

intermembrane space, which has a similar electrolyte and small solute composition as the

cytosol. The inner mitochondrial membrane is highly convoluted and folded, thereby

presenting a large surface area through which metabolic reactions can take place. The

inner mitochondrial membrane encloses the mitochondria matrix, which is highly basic

and negatively-charged (Rich & Marechal 2010).

In eukaryotes, the electron transport chain is composed of five major complexes

and two electron carriers. High energy substrates such as NADH (or FADH2) are

generated in the mitochondrial matrix by the citric acid cycle. NADH in the matrix is

taken up by complex I (also known as the NADH dehydrogenase), a large flavoprotein

complex which contains iron sulfur redox centers and spans the inner mitochondrial membrane. Complex I then acts to remove two electrons from NADH and deposits them onto Coenzyme Q (CoQ), reducing the oxidized CoQ (ubiquinone) to reduced CoQ (ubiquinol). This electron transfer from NADH to CoQ also drives the movement of 4 protons (H^+) from the mitochondrial matrix out into the intermembrane space, contributing to the electrochemical gradient. CoQ is a lipid-soluble mobile electron carrier that can then move from the membrane-spanning complex I to complex III (Rich & Marechal 2010).

Complex II (also known as succinate dehydrogenase) also supplies reduced CoQ to Complex III, but does so by converting succinate to fumarate, using $FADH_2$ as the high energy intermediate. Unlike complex I, III, IV, or V, complex II does not pump H^+ (Rich & Marechal 2010).

Reduced CoQ generated from Complex I (or II) is taken up by Complex III (also known as cytochrome c reductase) it is bound to cytochrome b and the electrons are transferred onto cytochrome c at the Rieske 2Fe/2S center. One electron from the reduced CoQ is placed onto cytochrome c during each cycle while the other electron is passed from $cyt.b_L$ onto $cyt.b_H$ to regenerate the oxidized CoQ in a process called the Q cycle. While the Q cycle does not affect the final output of complex III, where two electrons are transferred from one CoQ onto two cytochrome c (each cytochrome c can only carry one electron), the Q cycle is a potential source of ROS production and will be addressed later. For each CoQ that is oxidized, two H^+ are pumped from the mitochondrial matrix into the intermembrane space (Rich & Marechal 2010).

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Cytochrome c is a mobile electron carrier in the intermembrane space. Each reduced cytochrome c carries one electron from Complex III to Complex IV (also known as cytochrome c oxidase) where it is passed through a sequence of heme and metal groups, generating energy to pump 2 H^+ into the intermembrane space. The terminal electron acceptor in Complex IV is O_2, which requires four electrons to become reduced to two molecules of water (4 H^+ are added to maintain the charge neutrality) (Rich & Marechal 2010). While cytochrome c participates primarily in oxidative phosphorylation as an electron carrier, it also has a pro-apoptotic role when released into the cytosol by facilitating the formation of an apoptosome (Tait & Green 2010).

Complex V is the final unit in the oxidative phosphylorative pathway. More commonly known as the ATP F_O/F_1 synthetase, complex V does not move electrons, but instead it utilizes the proton gradient created by Complex I, III, and IV to regenerate ATP from ADP. This large structure is composed of two portions: F_O-a ring shaped complex containing the ATP synthase F_O subunit 6 (encoded by *ATP6*) which forms a proton channel, and F_1-a central stalk ending in a ball complex. The F_O ring is embedded in the inner membrane and through its subunit 6/ATP6, encompasses a channel to allow for the passage of H^+ to move from the intermembrane space into the matrix down its gradient. The movement of H^+ causes the F_O ring to rotate, which causes rotation of the central stalk (part of the F_1 portion). The matrix end of the central stalk has a small hook and is connected to the ball-shaped complex of the F_1 portion. Rotation of the central stalk (due to proton movement through the F_O ring) causes the hook at the end of the stalk to disturb

the ball-shaped complex in the F_1 portion, providing energy for the conformational

changes leading to ATP synthesis (Rich & Marechal 2010).

Source of ROS generation in the mitochondria

The electron transport chain is very tightly coupled and the electrical efficiency of

each complex in individual mitochondria must be perfectly balanced to the other

complexes. Deviations or changes in any of the complexes can short the electrical circuit

across the inner membrane. Therefore, the genes transcribing critical electrical

components in these complexes must be inherited together. Assorted inheritance of these

genes through sexual recombination would off-set the coupling coefficiency of these

complexes and the ensuing results would be disastrous for the organism. Therefore, the

mitochondrial genome (mtDNA), which is vertically inherited from mother to child,

encodes for the core electrical components of Complex I, III, IV, and V, effectively

avoiding the combination of incompatible circuit elements (Wallace 2005).

The inheritance pattern of the core mitochondrial complexes demonstrates the

importance of maintaining the electrochemical gradient in the mitochondria. Alterations

in the gradient not only affect ATP generation, but also alter the efficiency of the electron

movement in the electron transport chain. The movement of electrons through the

electron transport chain is tightly regulated and controlled, but as they are high energy,

unstable atomic particles, electrons may occasionally react directly with oxygen instead

of passing on the respiratory chain. The addition of an electron directly to O_2 results in

the formation of superoxide anion ($O_2^{\cdot-}$), a type of reactive oxygen species, which can be

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converted to other reactive oxygen species by various chemical reactions. Even under optimal conditions, there is always a small electron leak from the electron transport chain which results in ROS formation (Adam-Vizi & Chinopoulos 2006). Under non-physiologic (excessively low or high) metabolic states, the electron leak may become greater, leading to greater ROS production (Selivanov et al. 2011).

The main sites of electron leak are at complex I and complex III. When succinate is in abundance, it leads to a high pool of reduced CoQ, which can drive the reverse electron transport in Complex I (Lambert & Brand 2009). The mobile electrons in the accumulated reduced CoQ proteins in the iron sulfur centers and FMN in Complex I can react with O_2, leading to superoxide generation. Superoxide production from complex I has been implicated in the pathogenesis of Parkinson's disease, fatal infantile lactic acidosis, and Leigh disease (Hirst et al. 2008). ROS produced from Complex I appears to be delivered exclusively into the mitochondrial matrix (Lambert & Brand 2009). Another site of ROS production on the electron transport chain is Complex III. Like Complex I, Complex III can also produce ROS during reverse electron transport, although the rate of ROS production in those conditions is thought to be relatively low in comparison (Lambert & Brand 2009). Inhibition of the Q-cycle (which recycles oxidized CoQ within Complex III) between cyt b_H and Q_i site by antimycin causes the formation of the unstable semiquinone at the Q_0 site, which readily transfers its single electron to O_2 (Cape et al. 2007). ROS produced from Complex III is delivered into both the mitochondrial matrix and the intermembrane space (Lambert & Brand 2009).

The addition of electrons directly to oxygen produces superoxide, a highly

reactive non-diffusible reactive oxygen species. Although superoxide is charged and

incapable of diffusing across membranes, it is converted to hydrogen peroxide by either

manganese superoxide dismutase (in the mitochondrial matrix) or copper zinc superoxide

dismutase (in the intermembrane space). The resulting hydrogen peroxide is more stable

and freely diffusible, and is able to leave the mitochondria to enter the cytoplasm, and

further into extracellular space, affecting redox metabolism to beyond the mitochondria

(Giorgio et al. 2007).

Factors affecting ROS production in the mitochondria: effect of membrane potential

While particular uncoupling drugs can selectively affect ROS production

specifically in either Complex I or III, the entire electron transport chain is sensitive to

changes in the mitochondrial electrochemical gradient. The electrochemical gradient is

the combined effect of the pH gradient and the mitochondrial membrane potential ($\Delta\psi$).

Changes in either mitochondrial matrix pH (Lambert & Brand 2004) or $\Delta\psi$ (Selivanov et

al. 2011; Nicholls 2004) have been shown to affect the mitochondrial ROS production.

When the mitochondrial membrane potential increases (denoted by a more

hyperpolarized, or negative $\Delta\psi$), the mitochondrial matrix is more reduced, making it

energetically more difficult for the negatively charged electrons to move to the matrix-

side of the complexes. This result in stalling of the electron transport chain, and the

reduction of alternative acceptors, such as O_2, become more favorable. Therefore, the

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mitochondrial membrane potential is tightly controlled to optimize ATP generation and minimize ROS generation.

MPTP: regulation of mitochondrial membrane potential

The MPTP was historically discovered in relation to its participation in cell death (Crompton 1999). However, the MPTP has also been observed to be active in non-apoptotic conditions. In the absence of continued calcium stimulation, the MPTP can stochastically flicker open and close (Wang et al. 2008). The function of these stochastic openings has not been clearly demonstrated, but is thought to regulate mitochondria-cytosolic crosstalk or mitochondrial metabolism (Wang et al. 2012). One of the immediate effects of MPTP flickering is that the transient opening between the mitochondrial matrix and cytosol allows for a brief movement of charged solutes (<300Da) between these two compartments, causing a dip in the mitochondrial membrane potential (Wang et al. 2012; Brenner & Moulin 2012). As fluctuations in the mitochondrial membrane potential can modulate ATP generation and ROS production, the transient opening of the MPTP is thought be a regulatory means for maintaining the mitochondrial membrane potential at approximately -180mV (Huser & Blatter 1999; Wang et al. 2012). These transient flickering of the MPTP has also been proposed to regulate mitochondrial matrix pH (Ichas & Mazat 1998).

Like the irreversible mode of opening, this transient form of MPTP opening is also sensitive to inhibition by CsA. Similarly, CsA treatment does not completely render the MPTP refractory from opening, but instead it raises the stimulus threshold required

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for it to open. Considering the transient MPTP flickering may be responsible for maintaining the mitochondrial membrane potential by acting as a release valve, decreasing pore opening through treatment with CsA would theoretically lead to increased mitochondrial membrane potential, which has been independently established to increase ROS production in the electron transport chain (Korshunov et al. 1997; Hirst et al. 2008; Miwa & Brand 2003).

Our data is consistent with this hypothesis and shows CsA treatment potently increases mitochondrial membrane potential, demonstrating that it is a feasible means through which CsA could increase mitochondrial ROS. One caveat of our experiments is that it only provides an associative link between the increased mitochondrial membrane potential and mitochondrial ROS production in the setting of CsA treatment, and future studies are need to show a causal effect. One potential means of addressing the issue of mitochondrial ROS is the genetic overexpression of mitochondrial antioxidants such as manganese dismutase or copper zinc dismutase, which would neutralize any elevated mitochondrial ROS and should abolish CsA-induced effects. Additionally, cells isolated from cyclophilin D null mice should behave similarly to CsA treated wild-type cells with a basal increase in mitochondrial membrane potential and increased mitochondrial ROS production.

Global effector molecule: CsA side effects and ROS signaling

While we show that CsA-induced ROS in endothelial cells promotes cell proliferation, other groups have found it to confer toxicity (Redondo-Horcajo et al. 2010).
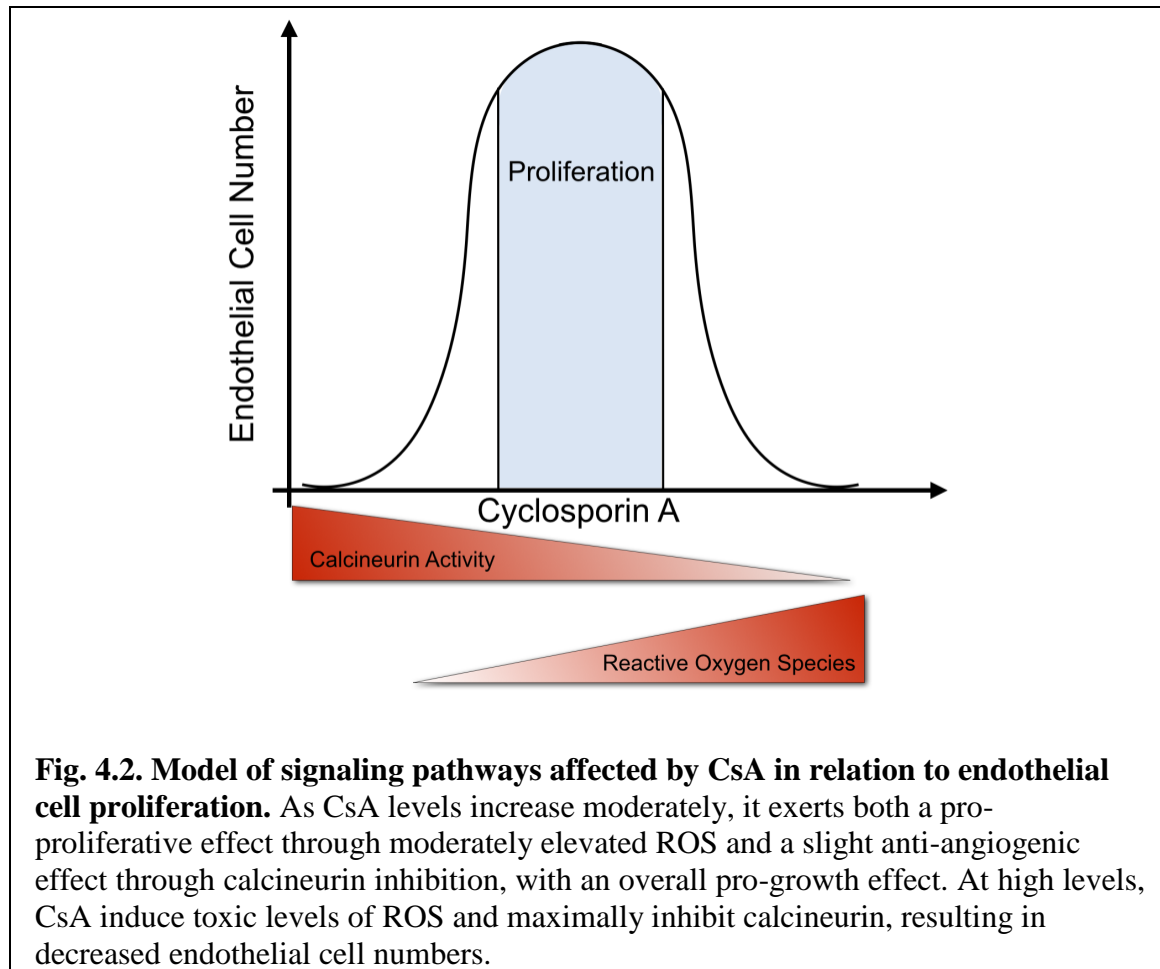
The diverse effects associated with CsA-induced ROS may be due to the promiscuous nature of ROS signaling, especially in different cell types. Moreover, the level of ROS may be dependent on CsA concentrations.

Dose-dependent induction of ROS by CsA would have broad clinical significance, as variable tissue accumulation of CsA in combination with cell-specific responses to CsA-induced ROS may further explain the numerous multi-organ side effects seen with CsA treatment. Indeed, CsA-induced ROS can contribute to CsA-associated nephrotoxicity. *In vivo* studies have found CsA treatment increases the glomerular synthesis of ROS, thromboxane, and lipid peroxidation products in the kidney (Parra Cid et al. 2003). Furthermore, increased ROS has been linked to hypertension pathogenesis, as superoxide can react with nitric oxide to generate peroxynitrate, decreasing the pool of the vasodilator nitric oxide (Yung et al. 2006). Additionally ROS has been identified as a mediator between CsA and the activation of the renal-angiotensin system in the setting of transplant associated hypertension (Nishiyama et al. 2003). Taken together, CsA-induced ROS may be a viable target for the development of therapies to mitigate adverse effects of CsA use.

CsA effects in endothelial cells: aggregation of calcineurin and ROS signaling.

As is evident from the literature and our studies, CsA treatment has multiple effects. This stems partly from the cell-specific role of calcineurin and the dose-dependent effects of ROS. Depending on the scale of inhibition or activation of these

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pathways, the final effect of CsA on cell biology is variable. In endothelial cells

specifically, CsA negatively regulates the VEGF-calcineurin axis, where maximal

Fig. 4.2. Model of signaling pathways affected by CsA in relation to endothelial cell proliferation. As CsA levels increase moderately, it exerts both a pro-proliferative effect through moderately elevated ROS and a slight anti-angiogenic effect through calcineurin inhibition, with an overall pro-growth effect. At high levels, CsA induce toxic levels of ROS and maximally inhibit calcineurin, resulting in decreased endothelial cell numbers.

calcineurin inhibition blocks angiogenesis (Hernández et al. 2001). Furthermore, CsA-

induced ROS has a gradient effect on proliferation and apoptosis. It is important to

recognize that CsA treatment in endothelial cells has a dose-dependent effect on both the

calcineurin and ROS signaling pathways, which results in a bell-shaped effect on

proliferation and cell numbers (Fig. 4.2.). This model allows for the pro-proliferative

outcome seen in our studies, but also explains the cytotoxic effects seen at other CsA

doses.

Clinical Implications

Antioxidant therapy

   We are the first to demonstrate that antioxidant therapy decreases CsA-induced tumor growth in an *in vivo* model. This result raises obvious clinical implication of using antioxidants therapeutically for patients on CsA containing drug regiments. Antioxidants are a class of drugs with a large therapeutic window, mild side effects, few major drug interactions, and can be obtained over-the-counter as health supplements. While large clinical trials have failed to show the efficacy of antioxidants for preventing cardiovascular disease (Vivekananthan et al. 2003), it nonetheless has been shown to be a safe and well tolerated drug. More studies are necessary to determine the exact antioxidant, optimal dose, and its effect on outcome.

   One potential point of concern for antioxidant treatment in the setting of cancer is highlighted by a recent study which showed increased mortality and decreased survival in a mouse model of cancer with antioxidant treatment. In this study, N-acetyl cysteine (NAC) and vitamin E treatment increased progression of B-raf and K-ras induced lung tumors and reduced the survival of these tumor-bearing mice compared to vehicle treated (Sayin et al. 2014). This was thought to be due to disruption of the ROS-p53 axis by antioxidants, which would otherwise limit tumor growth. It is important to take into consideration that the role of reactive oxygen species, and therefore, antioxidant treatment is complex and may have adverse unintended effects.

NAC used in our studies is a FDA approved antioxidant, used clinically as an antidote for acetaminophen overdose (Atkuri et al. 2007). Other antioxidants such as Vitamin C and E may be more applicable for long term use. Given the potential pro-tumor risk associated with NAC and vitamin E (Sayin et al. 2014), mitochondrially targeted antioxidants may be more specific for targeting mitochondrially generated ROS. A diet high in antioxidant-rich foods such as fruit and vegetable are recommended for a healthy lifestyle, and may be even more important for transplant patients.

Surgical excision is the mainstay for cutaneous squamous cell carcinoma, but chemoradiation is often utilized in setting of transplant-associated skin cancers. Many current chemotherapeutic drugs have high toxicity, and some are also carcinogenic or mutagenic. Therefore, in transplant patients, it is important to weigh the potential dangers of antioxidant treatment with the risks of cancer, and also compare its benefits and toxicities with that of the current standard treatments.

Prophylactic use:

Transplant associated cancers typically occurs years to decades after the onset of immunosuppressive treatment. This variable latent period opens up the possibility of taking preventive measures to decrease the oncogenic risk. Drugs used for prophylaxis must balance the risks and benefits of use, especially for long term use for an adverse event which materializes in only 50% of patients (Euvrard et al. 2003). Systemic retinoid have shown efficacy as chemopreventative treatments for decreasing the incidence of squamous cell carcinoma in transplant patients (Lien et al. 2012; Bettoli et al. 2013), but

incurs multiple side effects including a high risk of teratogenicity. While antioxidant usage generally results in less severe side effects than systemic retinoid, more studies are needed to determine its safety, especially in the light of potential tumor-promoting properties discussed earlier. In our experiments, we show that antioxidant treatment in a mouse model of melanoma decreases tumor growth, but the ideal goal of prophylactic treatment is to decrease both the incidence of disease in addition to disease burden. Future studies using spontaneous animal models of skin cancer such as the DMBA-TPA chemical carcinogenesis model or UV induced SCC model would be useful to further characterize the effect antioxidant prophylaxis on CsA-associated tumors.

Therapeutic use:

In addition to preventative use, antioxidants may also be useful as therapeutic treatment, in combination with standard therapy for transplant associated cancers. Transplant associated skin cancers are much more aggressive and metastatic than their spontaneous counterparts (Euvrard et al. 2003). In the case of spontaneous squamous and basal cell carcinomas, the tumors remain relatively indolent for long periods of time, and carries excellent prognosis if surgically removed. In the case of CsA-associated skin cancers, however, invasion occurs much faster, and multi-organ metastasis is not uncommon. Moreover, prognosis for CsA-associated skin cancers is much poorer than spontaneous skin cancers and current treatments recommended for skin cancers, surgical and occasional chemoradiation therapies (Jennings & Schmults 2010) are not always sufficient. Therefore, there exists a niche for better treatment options. Here we show

CsA-associated tumor growth can be slowed with antioxidant treatment, suggesting antioxidants may be utilized as adjuvant therapy, in addition to current standard-of-care, to treat transplant associated cancers.

Anti-angiogenic therapy to target CsA-associated cancers

In our study, we have discerned that CsA treatment increases tumor angiogenesis through increased ROS, and antioxidants may be both a prophylactic and therapeutic treatment option. Alternatively, there are many existing anti-angiogenic therapies that may have a beneficial application for transplant associated cancers. Although invasiveness in skin cancers is associated with greater vascularization (Staibano et al. 1996), anti-angiogenic therapies are not routinely used to treat skin cancers. Anti-angiogenic therapies for other cancer types have shown mixed results (Bergers & Hanahan 2008), reflecting the complexity of the tumor vessel contribution to both disease progression and treatment efficacy. Nonetheless, anti-angiogenic drugs are FDA approved and well characterized, and may offer treatment benefit for transplant associated skin cancers.

Anti-angiogenic target: calcineurin pathway

One of the questions leading to this study was the paradox of how CsA, a calcineurin inhibitor, increased tumor growth. According to the previously defined role of calcineurin in endothelial cells, inhibition of calcineurin interferes with VEGF signaling in endothelial cells, resulting in decreased tumor angiogenesis and tumor growth *in vivo*

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(Baek et al. 2009). The development of calcineurin inhibitors as anti-angiogenic drugs was hampered by the clinical experience with CsA usage, which clearly demonstrates pro-tumor activities. As we have dissected out the angiogenic component of CsA's pro-tumorigenic effect to be calcineurin-independent, it allows calcineurin inhibition to be once again considered as an anti-angiogenic target. There are a number of calcineurin inhibitors, both small molecule drugs and endogenous inhibitors, which can be employed. CsA is a poor choice as it promotes angiogenesis through an independent mechanism. From our studies, it would appear FK506 is also an inferior choice as it did not decrease tumor growth compared to control treatment. Pimecrolimus, a macrolide antibiotic similar to FK506 remains a possibility, but needs to be investigated. While not clinically used, other molecules such as DSCR1, autoinhibitory domain (AID) (Perrino 1999), cabin-1/cain (Sun et al. 1998), AKAP79 (Kashishian et al. 1998), and the A238L protein from the African swine virus (Miskin 1998) are known to inhibit calcineurin directly. Additionally, other molecules such as Dyrk-1A (Arron et al. 2006; Gwack et al. 2006) can target NFAT phosphorylation, the main effector of calcineurin activation, to block calcineurin activity further downstream. VIVIT, a small molecule developed based on the NFAT docking sequence, inhibits the calcineurin/NFAT interaction (Yu et al. 2007), and is an attractive candidate to selectively inhibit calcineurin activity in endothelial cells. Significant work, however, is needed for the development of any of these small molecule inhibitors to clinical use in order to address the issues of permeability, stability, and pharmacokinetics. Nonetheless, they remain promising novel therapeutic agents for selective inhibition of calcineurin-induced tumor angiogenesis.

CHAPTER 5: MATERIALS AND METHODS

Primary endothelial cell isolation

Primary lung endothelial cells were isolated from 3-4 week old adult mice. Briefly, whole lungs were minced and digested in Hanks Balanced Salt Solution (HBSS; Invitrogen) containing 10mg/mL type II collagenase (Worthington) and 20µg/mL DNase I (Sigma-Aldrich) for 30-45 minutes at 37°C with shaking. The collagenase was quenched with equivolume FBS, then filtered through progressively smaller cell strainers (100 and 40 µm) and centrifuged. Red blood cells were lysed with ACK Lysing Buffer (Invitrogen), then centrifuged and washed once in HBSS with 0.5% BSA (Invitrogen). Cells were incubated first with mouse Fc blocking reagent (Miltenyi Biotech) for 5 minutes on ice, then anti-CD31 biotinylated antibodies (1:100; ebiosciences, Clone 390) for 30 minutes at 4°C, rotating, washed once in MACs buffer, and then resuspended in MACs buffer with anti-biotin microbeads (Miltenyi Biotech) for 15 minutes before being applied to a magnetic column (Miltenyi Biotech). CD31 positive cells were seeded on 0.1% gelatin coated plates and cultured in endothelial cell media, Advanced DMEM (Invitrogen) supplemented with 15% FBS, 2mM glutamine, 100units/mL penicillin, and 100µg/mL streptomycin, 25mM Hepes (Invitrogen), 100µg/mL heparin (Sigma-Aldrich), and 100µg/mL ECGS (Biomedical Technologies, Stoughton MA). After 7 days, a second CD31 selection was performed. Endothelial cell purity was determined by immunostaining with anti-CD31 rat antibody (BD Pharmingen).

Reagents

NIM811 was a gift from Novartis. CsA and FK506 (Sigma-Aldrich) for in vitro experiments were reconstituted in ethanol at 8.3mM for a stock solution and further diluted in sterile water for working solutions and used at 0.25µM and 0.1µM respectively, unless indicated otherwise. N-acetyl cysteine (Sigma-Aldrich) was reconstituted in sterile water and used at 5mM for *in vitro* experiments or 40mM for *in vivo* treatment. Mn(III)tetrakis(4-benzoic acid)porphyrin Chloride (Millipore) was diluted in sterile water and used at 10µM. Angiotensin II (Sigma-Aldrich) was reconstituted in sterile water and used at 100nM.

Animals and Tumor Studies

WT C57Bl/6 mice were from The Jackson Laboratory. *Dscr1* transgenic mice with a third copy of *Dscr1* targeted into the hypoxanthine phosphor-ribosyltransferase locus were generated as previously described (Baek et al., 2009). *Calcineurin B*$^{f/f}$ mice with loxP sites flanking the *Calcineurin B* gene were originally from The Jackson Laboratory. For CsA or FK506 drug treatments, mice were orally gavaged daily with 10mg/kg of CsA oral solution USP modified (Hospital of University of Pennsylvania (HUP) Pharmacy) diluted in peanut oil or 0.15mg/kg of FK506 (HUP Pharmacy) diluted in simple syrup, for the duration of the experiment. Mice were 8-12 weeks old. All animal experiments were performed according to protocols approved by the University of Pennsylvania institutional animal care and use committee.

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Chemical carcinogenesis skin tumor model

Mice were treated with a single cutaneous application of 25μg of 7,12-dimethylbenz(a)anthracene (DMBA; Sigma-Aldrich) in 200μL of acetone on the shaved dorsal surface, followed by twice weekly cutaneous applications of 12.5μg 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich) in 200μL of acetone for 40 weeks. Tumor incidence was palpated by hand weekly.

Melanoma tumor model

B16-F10 melanoma cells (ATCC) were cultured in DMEM (Invitrogen) containing 10% FBS, 2mM glutamine, 100units/mL penicillin, and 100μg/mL streptomycin. For injection, cells were trypsinized, washed in PBS, counted and resuspended in sterile PBS. Each mouse received $3x10^5$ cells subcutaneously on the dorsal side. Tumor growth was measured by calipers daily. Mice were euthanized if they became moribund or tumors became ulcerated prior to experimental endpoint.

Immunofluorescence and CD31 quantification

Tumors were harvest from mice after euthanasia and frozen in OCT freezing medium (Tissue-Tek), then sectioned for staining. Slides were fixed and permeabilized in ice cold acetone for ten minutes, blocked with 5% normal goat serum and 1% BSA. Then the slides were incubated overnight at 4° C with rat anti-mouse CD31 antibody (1:50, BD Biosciences). Secondary antibody was Alexa 594 goat anti-rat (1:2000, Invitrogen) and nuclei were identified with Hoechst33342 (1:1000, Invitrogen). Five random 10X

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magnification pictures were taken of each slide. Images were taken at RT with a 10× or 20× magnification objective lens and with a digital camera AxioCAM HRc (Zeiss, Thornwood, CT) mounted on Zeiss Imager M1 Axio using Zeiss AxioVision Acquisition software (version 4.5). The area of CD31$^+$ structures, visible lumens, total vessels, and vessels >100μm were counted. Vessel structures for all tumors within a treatment group were averaged to determine average and standard deviation.

Miles Assay of vessel permeability

200μL of 0.5% sterile solution of Evans blue in PBS was injected tail vein into tumor-bearing mice and allowed to distribute into tissues for 30 minutes, followed by animal sacrificing and tumor removal. Excised tumors were weighed and 500μL formamide was added to each sample and incubated at 55ºC for 24-48 hours to extract the Evans blue. The tumor/formamide mixture was pelleted and the dye concentration in the supernatant was measured with absorbance at 610nm and calculated as ng Evans blue extravasated per mg tissue.

Proliferation and cell growth

Endothelial cells were plated in triplicates at 5 x 10^3 cells per well in 0.1% gelatin coated 24-well tissue culture plates. Cells were counted daily or every other day. Media was changed every 3 days.

BrdU incorporation

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2.5 x 10^3 endothelial cells were plated onto 0.1% gelatin coated glass coverslips.

10µM BrdU (BD Biosciences) was added to cells for 1.5 hours at 37°C. Cells were fixed

with 4% paraformaldehyde (PFA) for 15 minutes, permeabilized with 0.1% TBST for 15

minutes, and DNA was denatured with 2N HCL for 30 min. Endothelial cells were

stained with anti-BrdU mouse antibody (1:50, Dako) for 1 hour at room temperature.

Secondary antibody was Alexa 594 goat anti-rat (1:2000, Invitrogen) and nuclei were

identified with Hoechst33342 (1:1000, Invitrogen). Seven random 10X magnification

pictures were taken of each slide and BrdU positive cells and total cell number were

counted.

Tunel apoptosis assay

To measure apoptosis, in situ terminal deoxynucleotidyltransferase-mediated

dUTP nick end labeling (TUNEL) labeling was performed using the DeadEnd

Fluorometric TUNEL System (Promega, Madison, WI) according to manufacturer's

instructions. Briefly, endothelial cells were washed with PBS and trypsinized, then fixed

with 4% PFA for 20 minutes on ice. Cells were permeabilized by 70% EtOH for 4 hours

at -20C, washed with PBS, then incubated in equilibration buffer for 5 minutes. Cells

were then incubated with TdT reaction mix for 60 minutes, washed, and resuspended in

20mM EDTA to stop the reaction. Cells were then incubated with 5µg/mL propidium

iodide (Sigma-Aldrich) with 1mg/mL RNase for 20 minutes at room temperature. Flow

cytometry was performed on a FACS Canto flow cytometer (BD Biosciences) and

analyzed with FlowJo Software (TreeStar, Ashland, OR).

Migration assay

Basal endothelial cell media, Advanced DMEM (Invitrogen) supplemented with 0.5% FBS, 2mM glutamine, 100units/mL penicillin, and 100µg/mL streptomycin, 25mM Hepes (Invitrogen), and 100µg/mL heparin (Sigma-Aldrich) was placed in the lower chamber of a modified Boyden chamber (Corning) separated by a filter with 8µm pores. Endothelial cells were serum starved overnight with basal endothelial cell media, treated with experimental reagents for 2 hours, then trypsinized and resuspended in basal media at 2×10^5 cells/mL. 2×10^4 cells were plated in the upper chamber and allowed to migrate for 3 hours at 37ºC. The filters were stained with Diff-Quick solution (Baxter, Miami, FL) and images were taken at RT with a $10\times$ or $20\times$ magnification objective lens and with a digital camera AxioCAM HRc (Zeiss, Thornwood, CT) mounted on Zeiss Imager M1 Axio using Zeiss AxioVision Acquisition software (version 4.5). 5 random images were taken of the filters and the number of cells that migrated across the filter was counted.

Matrigel tube formation assay

3×10^5 primary lung endothelial cells in 600µL optiMEM with 1% FBS were plated on top of 600µL of polymerized matrigel in a 12 well plate. Cells were incubated at 37ºC and 5% CO_2 to allow for tube formation. Images were taken at indicated time points.

AdCre infection

For *in vitro* experiments, primary lung endothelial cells from $CnB^{f/f}$ mice were infected with Cre recombinase adenovirus (Ad5CMVCre; University of Iowa, Gene Transfer Vector Core) at 500 MOI overnight, followed by PBS wash and media change. After two passages, cells were assessed for *Calcineurin B* deletion by immunoblotting for calcineurin A protein, which degrades in absence of calcineurin B.

DCFDA staining

DCFDA (Abcam) staining was performed following manufacturer's instructions. Briefly, cells were treated with drug for 24 hours, then washed and stained with 25µM DCFDA for 45 minutes at 37ºC. Signal was read at 535nm.

Mitosox and TMRE Staining

Mitosox (Invitrogen) and TMRE (Invitrogen) staining of endothelial cells *in vitro* were done following manufacturer's instructions. Briefly, endothelial cells were washed with PBS and trypsinized, and then resuspended in calcium and magnesium containing, phenol red free HBSS (Gibco) supplemented with 0.1µM sodium pyruvate (Sigma) and 5µM Mitosox or 100nM TMRE. Cells were incubated for 10 min (Mitosox) or 20 min (TMRE) at 37°C in the dark, then flow cytometry was performed using a FACS Canto flow cytometry machine reading at 610 or 613nm (Mitosox) or 575 nm (TMRE). The results were analyzed with FlowJo software (TreeStar, Ashland, OR).

Western blotting

Endothelial cells were scraped from tissue culture dishes and lysed with RIPA buffer supplemented with a protease inhibitor cocktail (Roche). Whole cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with the following antibodies: p-ERK, total ERK, p-AKT, total AKT (Cell Signaling Technologies), Calcineurin A (Santa Cruz), β-actin (Sigma). To measure relative gel densities, band densities were quantified with densitometry analysis using ImageJ software (NIH) and then normalized to β-actin, or if blotting for a phospho-protein, the total-protein.

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