Forced Overexpression of Translationally Controlled Tumor Protein (TCTP/*TPT1*) Induces a Growth-Dysregulated Phenotype in Endothelial and Smooth Muscle Cells: Role of TCTP Exosomal Export in Paracrine Cell-Cell Signaling Induced by Endothelial Injury

Short Title: TCTP in Regulation of Vascular Cell Growth and Survival

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This thesis is submitted as a partial fulfillment of the M.Sc. program in Cellular and Molecular Medicine

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Authorization

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Abstract

Background: Pulmonary arterial hypertension (PAH) is a lethal disease for which the fundamental molecular mechanisms are only partially understood. Existing therapies, which primarily focus on endothelial dysfunction, have limited effects on improving outcomes. Increases in pulmonary vascular resistance in PAH may be attributed to complex lung arterial remodeling which result in obliterative "plexiform" lesions, a pathological hallmark of this disease. Recent studies have shown that endothelial cell (ECs) apoptosis may be a central trigger for PAH, resulting in the emergence of growth-dysregulated and apoptosis-resistant ECs that contribute to the formation of complex neoplastic-like vascular lesions. However, the mechanism which links ECs apoptosis to dysregulated growth is not yet known. Previous studies in our lab have identified increased expression of translationally controlled tumor protein (TCTP) and its gene (*TPT1*), previously implicated in the transformation of neoplastic cells in cancer, and in blood outgrowth ECs from patients with PAH. Moreover, TCTP expression was found to be elevated in the lungs of patients with PAH, and tightly localized to complex arterial lesions. In addition, it was detected in obliterative intimal lesions of an experimental rat model of severe PAH.

Hypothesis: TCTP represents a central molecular mechanism linking ECs apoptosis to the emergence of growth-dysregulated lung vascular cells and occlusive, complex arterial remodelling in PAH.

Specific Hypotheses:

- Lentiviral overexpression of TCTP in human umbilical vein endothelial cells (HUVECs) and pulmonary artery smooth muscle cells (PASMCs) leads to a hyperproliferative and apoptosis-resistant phenotype.

- Overexpression of TCTP will increase its export into apoptotic extracellular vesicles, thereby augmenting cell-cell signalling between ECs and neighbouring SMCs.

Purpose: My objective was to examine the effects TCTP overexpression on ECs and SMCs survival in terms of proliferation and apoptosis, and TCTP release on the survival of nontransduced ECs and SMCs.

Methods and Results: The effect of TCTP overexpression on ECs growth and survival was studied using *in vitro* models. TCTP was overexpressed via a lentivirus vector in HUVECs and PASMCs. Compared to non-transfected or null transfected cells, TCTP overexpression led to increases in BrdU incorporation, consistent with hyper-proliferation, and decreases in caspase activity, consistent with apoptosis resistance. As well, TCTP was selectively exported into the conditioned media of apoptotic ECs, but not SMCs, despite similar levels of overexpression. In addition, the level of release was greater in serum starved conditioned media in comparison to the exosome fraction. Finally, our data demonstrates a selective effect of conditioned media (CM) from serum-starved ECs on PASMCs, but not ECs, in terms of an increase in proliferation and a decrease in apoptosis.

Conclusions: These support the idea that TCTP overexpression confers an increase in the survival of SMCs and HUVECs. Moreover, TCTP released from apoptotic ECs leads to a growth-dysregulated phenotype within SMCs (but not ECs) and may contribute to the formation of complex lung arterial lesions, leading to arteriolar obliteration in PAH. Finally, an increase in the level of TCTP expression via lentiviral transduction led to an increased TCTP export into the media, but this appeared to be mostly in the soluble portion, and less was associated with exosomes.

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ANOVA	analysis of variance
BMPR2	bone morphogenetic protein receptor type II
BrdU	bromodeoxyuridine
EBM	endothelial basal media
EC	endothelial cell
ET-1	Endothelin-1
HIV	human Immunodeficiency Virus
HPAH	hereditable Pulmonary Arterial Hypertension
HUVECs	human umbilical vein endothelial cells
kDa	kilodalton
Mcl-1	myeloid leukemia cell differentiation protein
MCT	monocrotaline
NO	nitric oxide
PAH	pulmonary Arterial Hypertension
PAP	pulmonary arterial pressure
PASMCs	pulmonary artery smooth muscle cells
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
RVSV	right ventricular systolic volume
SU5416	sugen: 3-(3,5-dimethyl-1H-pyrrol-2-ylmethylene)-1,3-dihydroindol-2-one
TGF-β	transforming growth factor beta
TCTP	translationally controlled tumor protein

TSAP6	tumor suppressor activated pathway-6
VEGFR2	vascular endothelial growth factor receptor-2
WHO	world health organization

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LIST OF ORIGINAL PUBLICATIONS

As a Master's of Science candidate I have had the privilege of collaborating with colleagues from the University of Cambridge to produce the following publication.

Ferrer, E.; Dunmore, B. J.; Hassan, D.; Ormiston, M. L.; Moore, S.; Deighton, J.; Long, L.; Yang, X. D.; Stewart, D. J.; Morrell, N. W. A Potential Role for Exosomal TCTP Export in Vascular Remodeling in Pulmonary Arterial Hypertension. Am. J. Respir. Cell Mol. Biol. 2018;59:467.

1.0 Introduction

1.1 Vascular Structure and Function

Blood vessels are the conduits of the circulatory system. There are three types of blood vessels based on their location in the circulation and function: arteries, capillaries, and veins. Both arteries and veins are composed of three different layers: the inner layer is the tunica intima which is composed of endothelial cells (ECs) and underlying matrix, the second layer is the tunica media which is composed of vascular smooth muscle cells (SMCs), and in larger arteries and the aorta, elastic fibers. Finally, the tunica adventitia is the third layer which consists mainly of fibroblasts and extracellular matrix (ECM) material (Hall, John, 2011). The arteries transport blood away from the heart to either the lungs or the rest of the body. The arterial system consists of the large elastic conduit, the aorta, which branches into muscular arteries that finally give rise to arterioles; the arterioles then branch into smaller capillaries, which then transition into veins that transport blood back towards the heart (Betts, J. Gordon, 2013). The capillaries are characterized as small-diameter vessels consisting of a single layer of ECs (Sakai et. al, 2013). These thin walled microvessels allow for the exchange of gases, solutes, and movement of other biological factors between the blood and the surrounding tissues (Anthea et. al, 1993). It is at the transition between arterioles to capillaries that the greatest change in the velocity of blood flow and blood pressure occurs (Anthea et. al, 1993).

1.2 The Pulmonary Versus the Systemic Circulation

Deoxygenated blood is collected by the systemic venous system of the body and returns to the heart, it is then pumped by the right ventricle into the pulmonary circulation to be oxygenated. Oxygenated blood returns through the pulmonary veins to the left heart, it is then

pumped throughout the systemic arteries to deliver oxygen to the rest of the body (Guyton, 2000). Some key differences exist between the pulmonary and systemic circulation. First, the pulmonary circulation is arranged in series with the systemic circulation, whereas all of the components of the systemic bed (i.e. brain, heart, kidney, etc.) are arranged in parallel (Hine, 2008). Thus, of all organs in the body, only the lungs receive the entire cardiac output while systemic organs receive only a small fraction (Mathew R, 1990). Second, the pulmonary bed operates at very lower pressures, with normal mean pulmonary pressures between 9 and 18 mmHg (Heresi GA, 2013), compared to the systemic bed where mean arterial pressure range between 70 and 100 mmHg. Pulmonary pressures barely change even with peak increases in cardiac output; for example, during exercise (Magder S, 2016). This is accomplished by a very large vascular surface area only a fraction of which is required to accommodate the cardiac output at rest (Magder S, 2016). The lung represents a low pressure, low impedance system, designed to accommodate the entire cardiac output and oxygenate the blood with maximal efficiency; furthermore, during exercise the normal lung can recruit unused vasculature to keep the pulmonary arterial resistance and pressures low (Stamm JA, 2011).

Because of its unique physiology, the lung arterial bed exhibits distinctive structural features compared to systemic arteries. In particular, it is characterized by a thinner media layer containing less SMCs, incomplete muscularization of smaller arterioles, and absence of SMCs in distal lung arterioles (Farber HW, 2004). The absence of musclarization yields for the arterial walls to be fully compliant to blood flow, allowing for the pulmonary circulation to maintain a low resistance system (Rabinovitch M, 2008).

2.0 Pulmonary Hypertension

Pulmonary hypertension (PH) is defined as a resting mean pulmonary artery pressure (PAP) ≥ 25mm Hg or a systolic PAP of ≥35 mm Hg (Badesch DB, 2009; Heresi GA, 2013). The increase in the blood pressure within the pulmonary circulation negatively impacts the function of the heart, ultimately leading to ventricular hypertrophy and right heart failure (Forfia PR, 2013). There are many different causes for elevated PAP and it is vitally important that the etiology for any given patient be accurately defined since the prognosis and treatment are very different.

2.1 Classifications

The World Health Organization (WHO) has defined 5 groups of PH based on the disease etiology (Table 1), which include: Group I - Pulmonary arterial hypertension (PAH), Group II - Pulmonary hypertension owing to left heart disease, Group III - Pulmonary hypertension owing to lung disease and/or hypoxia, Group IV - Chronic thromboembolic pulmonary hypertension, Group V - Pulmonary hypertension with unclear multifactorial mechanisms (National Institute of Health, 2011). In this study, the focus will narrow on Group I PH, or PAH, which is characterized by abnormalities in the precapillary pulmonary arterioles.

Table 1. Updated clinical classification of pulmonary hypertension.

1. Pulmonary arterial hypertension (PAH)
    - 1.1 Idiopathic PAH (IPAH)
    - 1.2 Heritable PAH (HPAH)
        - 1.2.1 BMPR2
        - 1.2.2. ALK-1, ENG, SMAD9, CAV1, KCNK3
        - 1.2.3. Unknown
    - 1.3. Drug and toxin induced
    - 1.4. Associated with:
        - 1.4.1 Connective tissue disease
        - 1.4.2 HIV infection
        - 1.4.3 Portal hypertension
        - 1.4.4 Congenital heart diseases
        - 1.4.5 Schistosomiasis

1'. Pulmonary veno-occlusive disease and/or pulmonary capillary hemangiomastosis

1". Persistent pulmonary hypertension of the newborn

2. Pulmonary hypertension due to left heart disease
    - 2.1 Left ventricular systolic dysfunction
    - 2.2 Left ventricular diastolic dysfunction
    - 2.3 Valvular disease
    - 2.4 Congenital/acquired left heart inflow/outflow tract obstruction and congenital cardiomyopathies

3. Pulmonary hypertension due to lung disease and/or hypoxia
    - 3.1 Chronic obstructive pulmonary disease
    - 3.2 Interstitial lung disease
    - 3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern
    - 3.4 Sleep-disordered breathing
    - 3.5 Alveolar hypoventilation disorders
    - 3.6 Chronic exposure to high altitude
    - 3.7 Developmental lung diseases

4. Chronic thromboembolic pulmonary hypertension (CTEPH)

5. Pulmonary hypertension with unclear multifactorial mechanisms
    - 5.1 Hematologic disorders: chronic hemolytic anemia, myeloproliferative disorders, splenectomy
    - 5.2 Systemic disorders: sarcoidosis, pulmonary histiocytosis, lymphagiomyomatosis
    - 5.3 Metabloic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
    - 5.4 Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure, segmental PH

Adapted from Simonneau G et al., 2013.

2.2 PAH

2.2.1 Clinical Definition

PAH is classified into four subtypes, idiopathic PAH (IPAH), heritable PAH (HPAH), drug and toxin induced, and associated PAH (APAH) which occurs in association with scleroderma, congenital heart disease, and other autoimmune disorders. Patients with a wide range of conditions including those with connective tissue disease, or congenital heart disease, are at a greater risk of developing PAH (Ghamra ZW, 2003; National Institute of Health, 2011).

As PAH progresses, pulmonary vascular resistance (PVR) increases resulting in right ventricular (RV) hypertrophy in response to pressure overload and in the later stages of the disease, RV failure and reduced cardiac output (CO) (Van de Veerdonk MC, 2014; Forfia PR, 2013). PAH often presents with limitation in performing daily activities (Rich S, 1987). Symptoms include, shortness of breath, fatigue, light headedness when performing exertional activity, chest pain, irregular or fast heartbeat, lower extremity swelling or edema, and sometimes increasing abdominal growth where fluid develops around the liver (Ulrich-Axel Bommer, 2004). The probability of survival for patients who are left untreated is 68% at one year, 48% at three years, and 34 % at five years (Wexner Medical Center, 2015); therefore, prompt intervention in PAH is critical. PAH is typically characterized by complex vascular remodeling in the form of dysregulated growth and apoptosis resistance vascular cells, including ECs and SMCs (Ulrich-Axel Bommer, 2006). There is an increasing interest in the mechanism underlying the appearance of these growth-dysregulated cells in PAH.

2.2.2 Pathophysiology of PAH

The pulmonary circulation is a low pressure, low impedance system that accepts the entire CO and functions to provide gas exchange in healthy individuals (Hine, 2008). In patients

with PAH, the ability to maintain a low-pressure system is lost due to the loss of functional microvasculature and the reduction of the cross-sectional area of the arteriolar bed (Jonigk D, 2011). The decrease of arteriolar diameter transpires by a range of mechanisms; for example, some PAH patients are identified by acute vasodilator responsiveness testing to have an increase in pulmonary vascular resistance (PVR) due to chronic vasoconstriction (Sitbon et al., 2005). One treatment for sustained vasoconstriction is the use of calcium channel blockers (CCBs), which relaxes the smooth muscle layer surrounding the arteries and reduces the vascular tonicity (Brozovich, 2016). However, vasoconstriction only plays a minor role in the majority of cases of PAH; it is the vascular rarefaction that is the main contributor to the progression of PAH, leading to vascular remodeling, the decrease of blood flow through minute arteries, and finally the obliteration of the vasculature (Chaudhary et. al, 2017). The loss of functional lung arteriolar microcirculation contributes to increases in PAP and PVR (Campo et al., 2011). These progressive events lead to the inability of the lung to sustain the entire CO, which ultimately leads to mortality of patients with PAH (D'Alonzo et al., 1991). Multiple pathological events have been implicated in contributing to the progression of PAH, which include endothelial dysfunction, proliferation/arterial remodeling, inflammation, and arteriolar degeneration (Giaid A, 1993). While multiple events as described are implicated in the progression of PAH, the precise mechanism remains unclear (Chaudhary et. al, 2017).

2.2.3 Vascular Function and Dysfunction

2.2.3.1 Endothelial Function and Dysfunction in PAH

The endothelium constitutes the inner most layer of the blood vessel (Langille, 1986), responding to inflammatory mediators such as interleukins, prostaglandins, and histamine (Abbas A.B, 2009). The endothelium contributes both to the structure and function of the

vasculature; it contributes to the structure by providing a lining to the lumen of the blood vessels, and it contribute to the function by releasing vasoactive mediators, or biochemical triggers, that interact with the adjacent smooth muscle layer to mediate vasoconstriction or vasodilation (Botting R, 1989). Under normal conditions, there is a balance in the release of vasoactive mediators from the endothelium, however, under abnormal conditions, such as PAH, there is an imbalance of the release of vasoactive factors; namely, an increase in the release of vasoconstrictor, and a decrease in the release of vasodilator factors (Rabinovitch M, 2008). The imbalance of vasoactive mediator release favors vasoconstriction over vasodilation, leading to increased resistance and partially contributes to SMCs proliferation, and narrowing/hyperplasia of the medial layer (Ozkan M, 2001; Montani, 2014). Three pathways have been identified to be involved in PAH within the ECs of the pulmonary vasculature: the endothelin pathway, the nitric oxide pathway, and the prostacyclin pathway (Wideman RF, 2011). These three pathways play instrumental roles in the regulation of vascular tone (Schneider MP, 2007).

The capacity to clear endothelin-1 from the circulation is reduced in PAH, leading to the increase of its abundance (Nazzareno Galié, 2003; Machado RD et al., 2006). Endothelin exerts its effects by activating two distinct receptors, ET-A and ET-B (Marc Humbert, 2004); under normal physiological conditions, ECs only express the ET-B receptors, while SMCs express both receptors (Majed B, 2012). ET-A on SMCs mediate vasoconstriction and cellular proliferation, in PAH excessive stimulation of ET-A receptors is evident (Schneider MP, 2007). ET-B receptors located on ECs mediate endothelial dependent vasodilation and on SMCs mediate vasoconstriction. Under pathologic conditions, SMCs vasoconstriction predominates due to downregulation of ET-B on ECs and upregulation of these receptors on SMCs (Sandoo A, 2010). Blockade of the endothelin-1 receptors (ET-B) on SMCs by a drug such as bosentan has been

shown to limit vascular hypertrophy; however, this dual receptor antagonist is nonspecific and may cause blockade of the ET-B receptors on ECs and reducing vasodilation, hence this could be detrimental to patients suffering from PAH (Joseph EK, 2013; Kowalczyk A, 2015). Blockade of ET-A on SMCs by selective drugs such as sitaxentan and ambrisentan can prevent vasoconstriction, cardiac remodeling, and reduce vascular inflammation (Senthil et al, 2003; Trow, 2009).

Nitric oxide (NO) is a diatomic gas that plays a role in cell signaling by stimulating enzyme activities in the vasculature (Kaneko FT, 1998; Ozkan M, 2001). NO is a vasodilator which can inhibit platelet activation, thrombosis, and inflammation (Wideman RF, 2011). Under normal conditions, NO is produced continuously in the endothelium and diffuses into vascular SMCs where it binds to and activates guanylate cyclase stimulating the synthesis of Cyclic guanosine monophosphate (cGMP); a second messenger for signaling smooth muscle relaxation and inhibiting cellular proliferation (Porta NFM, 2012). Levels of endothelial NO synthase (eNOS), the enzyme responsible for NO production in ECs, are decreased in PAH; resulting in vasoconstriction of blood vessels and proliferation of vascular SMCs (Schneider MP, 2007). The availability of NO is also affected by phosphodiesterase type 5 (PDE-5). PDE-5 degrades cGMP in vascular SMCs and counteracts the vasodilatory effects of NO (Ozkan M, 2001; Schneider MP, 2007). Thereby, inhibition of PDE-5 can block the breakdown of cGMP and mediate the vasodilatory effects of NO.

Prostacyclin is the main arachidonic acid metabolite of vascular ECs and SMCs; it is produced in ECs through the action of prostacyclin synthase (Senthil et al., 2003). Prostacyclin binds to prostaglandin I2 receptors (PGI2) located on ECs and SMCs leading to a cascade that signals adenylate cyclase to produce cyclic adenosine monophosphate (cAMP). cAMP is a

second messenger that inhibits necessary platelet aggregation, and also leads to relaxation of the underlying SMCs (Senthil et al., 2003). In PAH, prostacyclin levels are reduced, leading to vasoconstriction, yet, increased production of thromboxane A2 (TxA2), generated from prostaglandin H2 by thromboxane-A synthase, leads to vasoconstriction, proliferation of SMCs, and platelet activation. In PAH, there is an imbalance between PGI2 and TxA2, with a shift towards an increase of TxA2, leading to the disruption of the signaling pathway and causing a reduction in the vasodilatory and anti-proliferative effects on SMCs (Pardali E, 2012). Administering prostacyclin has been shown to play a pivotal role in the management of PAH via their vasodilatory effects on SMCs (Pardali E, 2012). Vasoactive mediators not only contribute to the vascular tone, but are also involved in structural remodeling of small arteries; thus, the imbalance of endothelial vasoactive mediators contributes to the proliferation of SMCs, possibly playing a role in the emergence of complex arteriolar lesions, and ultimately occlusion of small arteries (Montani D, 2013).

2.2.3.2 The Need for New Therapies

Currently, there is no cure for PAH and continued research is underway to find one. Treatments are available for PAH, which help alleviate the symptoms of the disorder and improve quality of life. These treatments include: PDE-5 inhibitors such as sildenafil, prostacyclin analogue such as epoprostenol, endothelin-1 receptor antagonists such as sitaxentan, and calcium channel blockers, such as diltiazem (Porta NFM, 2012). However, a novel therapeutic target is needed, not merely to slow the progression of PAH, but rather aid in effectively treating or stopping the disorder. Novel therapeutic treatments are needed as the late stage of PAH have moved beyond vasoconstriction and center on dysregulated vascular cell growth and vascular remodeling.

2.2.3.3 Vascular Remodeling in PAH

Vascular remodeling in PAH mainly affects smaller arteries and arterioles; the two types of pulmonary arterial remodeling include, simple vascular remodeling and complex vascular remodeling. Simple vascular remodeling is characterized by medial hypertrophy, muscularization of distal arterioles, which normally have no medial SMCs, and arteriolar wall thickening, which contributes to the narrowing of the arteries (M. J. Mulvany, 2003; G. H. Gibbons 1994). In the lung, the distal arterioles normally lack medial SMCs, and the endothelial layer is supported only by occasional pericytes; however, in PAH there is progressive muscularization of the distal arterioles. Complex arterial remodeling is characterized by dysregulated cell growth that leads to obliterative intimal lesions, which contributes to the narrowing and occlusion of small arterioles (H. Hashimoto, 1987; W. R. Dunn 1997). Simple vascular remodeling may be reversible with the administration of vasoactive agents that aim to restore the imbalance of the vasoactive mediators released from the endothelium (Kaneko FT, 1998). On the other hand, complex vascular remodeling is thought to be irreversible, since the ECs transition from a quiescent state to an anti-apoptotic, hyper-proliferative phenotype.

Hyper-proliferative ECs are thought to play a role in the development of occlusive, intimal, and plexiform lesions that are identified in patients with late stage PAH (Tuder RM, 2007; Jurasz P, 2010). There are progressive histologic changes in the pulmonary arteries and arterioles, as a result of chronically elevated pressure within the pulmonary arteries in patients with congenital heart septal defects (Heath D, Edwards JE, 1958). This progression is classified into 6 grades based on the structural changes; figure 1 demonstrates the progression across the different stages of the disorder utilizing receptor for advanced glycation (RAGE) expression staining. The SU5416/hypoxia rat model of PAH (fig. 2) is an accurate representation of this

type of pulmonary vascular remodeling; here, lung sections from 13- to 14-week SU5416/hypoxia-treatment depict vascular morphological changes within the vasculature of the lung.

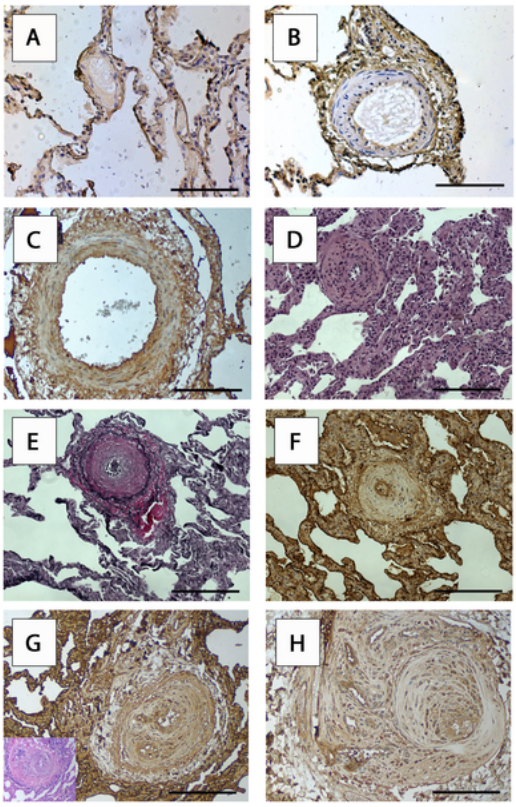

Figure 1: RAGE expression in pulmonary vascular changes of patients with iPAH.
"Representative examples of immunohistochemical analyses of pathognomonic lesions in lung of patients with iPAH according to the modified Heath Edwards classification in PA vessels smaller than 500 µm in diameter are shown (3 patients per every Heath Edwards group were analyzed). RAGE expression in (A) a morphologically regular small PA - stage 0 and (B) a stage 1 histological change in lung of a patient operated for pneumothorax (COPD 0, centriacinar emphysematous changes). RAGE expression in characteristic stage 2 changes in a lung of a patient with iPAH (C). Scale bar in A, B and C: 80 µm. Adjacent sections of H&E (D) and EvG (E) and RAGE staining (F) for stage 3 changes in iPAH. Scale bar in D, E and F: 40 µm. Stage 4, angiomatoid (insert with adjacent H&E section, G), and stage 5, plexiform (H) PA vessel changes are shown. Scale bar in G and H: 80 µm. iPAH idiopathic pulmonary arterial hypertension, PA pulmonary artery, RAGE receptor for advanced glycation endproducts, COPD chronic obstructive pulmonary disease, H&E hematoxylin and eosin staining, EvG Elastica van Gieson staining." Figure and legend (Moser B, 2014).

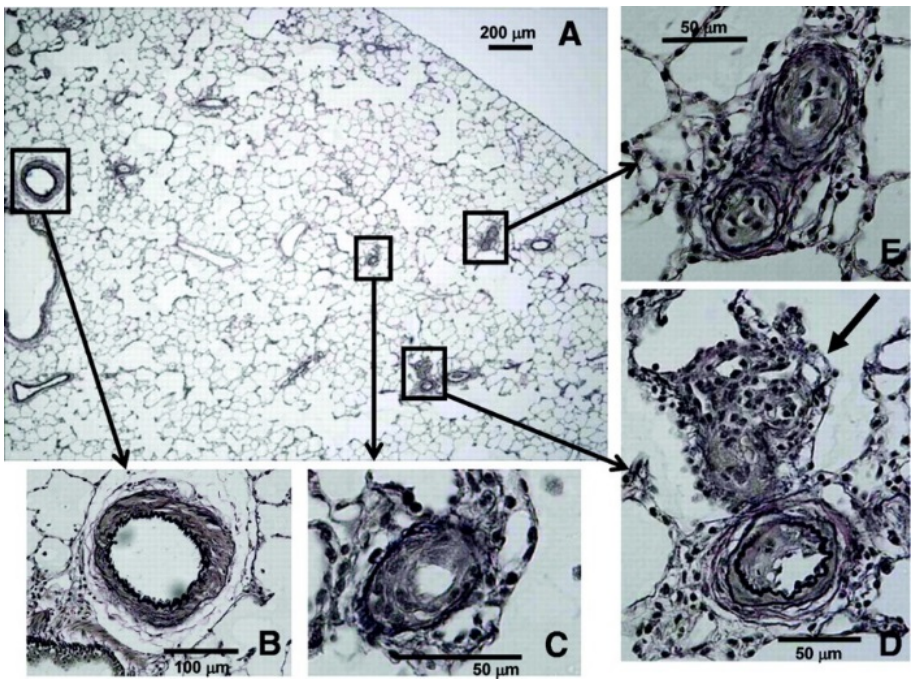

Figure 2. Pulmonary vascular changes within a rat model of PAH "A) representative low-magnification photomicrograph showing various types of pulmonary vascular lesions in a very-late-stage SU5416/hypoxia/normoxia-exposed rat lung. B) through E), Higher-magnification photomicrographs of medial wall thickening (B), concentric cellular laminar neointimal lesion (C), plexiform lesion (arrow) adjacent to a small pulmonary artery with medial wall thickening and eccentric neointimal proliferation (D), and nearly complete occlusion of 2 small pulmonary arteries by concentric neointimal proliferation (E). Verhoeff–van Gieson stained." Figure and legend (Kohtaro Abe, 2010).

2.2.3.4 Animal Models of PAH

2.2.3.4.1 Monocrotaline

In 1967, Kay et al. first reported the use of monocrotaline (MCT) to induce PAH in rats. Rats fed *crotalaria spectabilis* seeds develop medial thickening of the pulmonary arteries, an increased right ventricular systolic pressure, and right ventricle hypertrophy (RVH) (Kay et al., 1967). Specifically, the MCT alkaloid within these seeds is metabolized by the liver enzyme, cyrochrome-p450, converting MCT to pyrrolic derivatives (MCTP). MCTP is capable of inflicting ECs injury and subsequent apoptosis in the lung vasculature and other organs (Shah et al., 2005; Jurasz et al., 2010). This model of PAH is commonly used due to its ease of induction,

and minimal equipment needs (Maarman et al., 2013). This model is established by a single injection of MCT (60-80 mg/kg). Although the rat MCT model exhibit some histological features of human PAH (such as, medial hypertrophy, and intimal hyperplasia) (Maarman et al., 2013), the MCT model lacks other key histological characteristics of the human PAH, such as plexiform lesions and indications of complex arterial remodeling (Voelkel NF et al., 2012). In addition, off target pathological lesions of the liver (centrilobular hemorrhagic necrosis), and the kidneys (swollen glomeruli with thrombosis) post administration lead to limit animal survival and reduce the utility of this model (Hayashi and Lalich, 1967; Schoental and Head, 1955). Furthermore, the MCT rat model can be successfully treated by many therapies, which do not yield similar benefits in treating the human PAH (Maarman et al., 2013; Stenmark et al., 2009). This inconsistency emphasizes an impeding progress to the use of MCT model for translational research in PAH.

2.2.3.4.2 VEGFR2 Inhibition with SU5416

It was first shown that a single subcutaneous injection of sugen (SU5416) in combination with three weeks of chronic hypoxia (8-10% O2) is capable of inducing a severe PAH phenotype in rats (Taraseviciene-Stewart et al., 2001). VEGF and its principle receptor, VEGFR2 (a receptor for VEGF in ECs), have been shown to co-localize with lesions that contribute to the development of human PAH; hence, it was hypothesized that inhibiting VEGFR2 could avert the progression of PAH (Taraseviciene-Stewart et al., 2001). The authors thus utilized a selective inhibitor, SU5416, to inhibit VEGFR2, which was first designed to inhibit angiogenesis in the treatment of solid tumors. Paradoxically, the inhibition of VEGFR2 by SU5416 resulted in a significant exacerbation of the hypoxia-induced increase in both RVH and RVSP in the chronic hypoxia (CH) model of PH (Taraseviciene Stewart et al., 2001). Additionally, ECs apoptosis was

accompanied by the emergence of apoptosis-resistant, hyperproliferative cells which led to complex arterial remodeling with lumen obliteration in small arteries and arterioles in the SU5416/CH rat model (Taraseviciene-Stewart et al. 2001). Indeed, this is one of the first models to reproduce the hallmark pathohistological features of the human disease, in particular the plexiform lesions which are commonly thought to play an important role in the pathogenesis of this disease (Abe et al., 2010). Thus, it has been considered by many investigators in the field to be the model of choice for studying PAH.

2.2.3.5 Genetic Susceptibility to PAH

HPAH is caused by germ-line mutations that are passed down to the offspring (James E Loyd, 2012). More rarely, mutations in ALK-1, ENG, SMAD9, CAV1, and KCNK3 are found in HPAH patients (Deng Z, 2000; James E Loyd, 2012). By far the most common gene in which disease-causing mutations have been identified in PAH is the bone morphogenetic protein receptor, type II (*Bmpr2*), which is a member of the transforming growth factor β signaling family (Van de Veerdonk MC, 2014). BMPRII is ubiquitously expressed in all cells, though in the lung it is expressed predominantly in vascular endothelium (Thiele H, 2000), which permits it to receive and convey signals to its surroundings (Humbert et al., 2002; Abramowicz et al., 2003). The ligands for BMPRII are the bone morphogenetic proteins (BMPs). There are 20 members of the BMP family, six (BMP2 through BMP7) belong to the Transforming growth factor beta superfamily of proteins (Roman BL, 2017). The functional BMP receptor is a heterodimer consisting of R1 and R2 components (Little SC, 2009). There are a number of BMPRI receptors, but the most relevant for PAH in Alk-1, since mutations in this gene while more commonly associated with hereditary hemorrhagic telangiectasia (HHT) (Kjeldsen A D, 2001), have rarely also been found in HPAH families (Chida A, 2012). Interestingly, Alk-1 is

restricted to the endothelium, providing compelling genetic evidence of the central role that dysregulation of BMP signaling in the endothelium contribute to the pathogenesis of this disease (Roman BL, 2017).

Loss of function mutation in BMPR2 are found in 75% of patients with a family history of PAH (Austin and Loyd, 2014). BMPR2 haploinsufficiency, where one copy of the mutation is sufficient to cause the disease, is involved in the pathobiology of PAH (Machado et al., 2001). The risk for PAH development in individuals bearing a BMPR2 mutation is around 20% with a female predominance of around 2.5:1 versus the male counterpart (Austin and Loyd, 2014). The incomplete penetrance of PAH suggests environmental factors in addition to the genetic factors that can affect the disease expression (Ma and Chung, 2014). It has been shown that variations in estrogen metabolism may be liable for the increased predominance of PAH among females (Austin et al., 2009). A study by Austin et al in 2012 has drawn a parallel between the disruption of the transcription factor estrogen receptor α-mediated BMPR2 suppression and the elevation of BMPR2 expression in female patients with PAH (Austin et al., 2012). Moreover, the rate of conversion of estradiol (antiapoptotic, promitogenic) to methoxyextradiol (proapoptotic, antiangiogenic) under conditions of hypoxia, inflammation, or other environmental stressors; this may highlight the role of estrogen metabolites in propagating the disease state of PAH in females (Tofovic, 2010). Unlike in SMCs where BMPR2 induces apoptosis, this pathway has been shown to play a significant role in mediating survival signaling in ECs, preventing apoptosis and thus preserving the integrity of the lung microvessels. This equilibrium of cell death/proliferation normalizes the number of cells in a tissue. Thus, loss of function mutations in this pathway result in increased susceptibility to ECs apoptosis, possibly leading to degeneration of fragile pre-capillary arterioles (See Figure 3) (Nishiharaet al., 2002; Rudarakanchana et al., 2002).

While mutations in Bmpr2 have been shown to be definitively associated with the development of PAH, the mechanism by which haploinsuffiency of these loss-of-function mutations leads to the vascular functional and structural changes of PAH is still unclear (Machado et al.,2001). Previous studies suggest that, in SMCs, a loss-of-function *Bmpr2* mutation result in discrepant response in vascular endothelial and SMCs (James E Loyd, 2012), this may result in growth dysregulation, contributing to medial hyperplasia within the lung's arteries and arterioles leading to inward remodeling and increased vascular resistance (fig. 3). Thus, loss-of-function Bmpr2 mutations predispose to EC apoptosis, which is recognized as a central trigger for PAH. However, these initial consequences of loss of BMP signaling in ECs which ultimately produce complex arterial remodeling, obliteration, and the loss of lung microvasculature is still not entirely clear, and the two main paradigms will be discussed in detail in the next section.

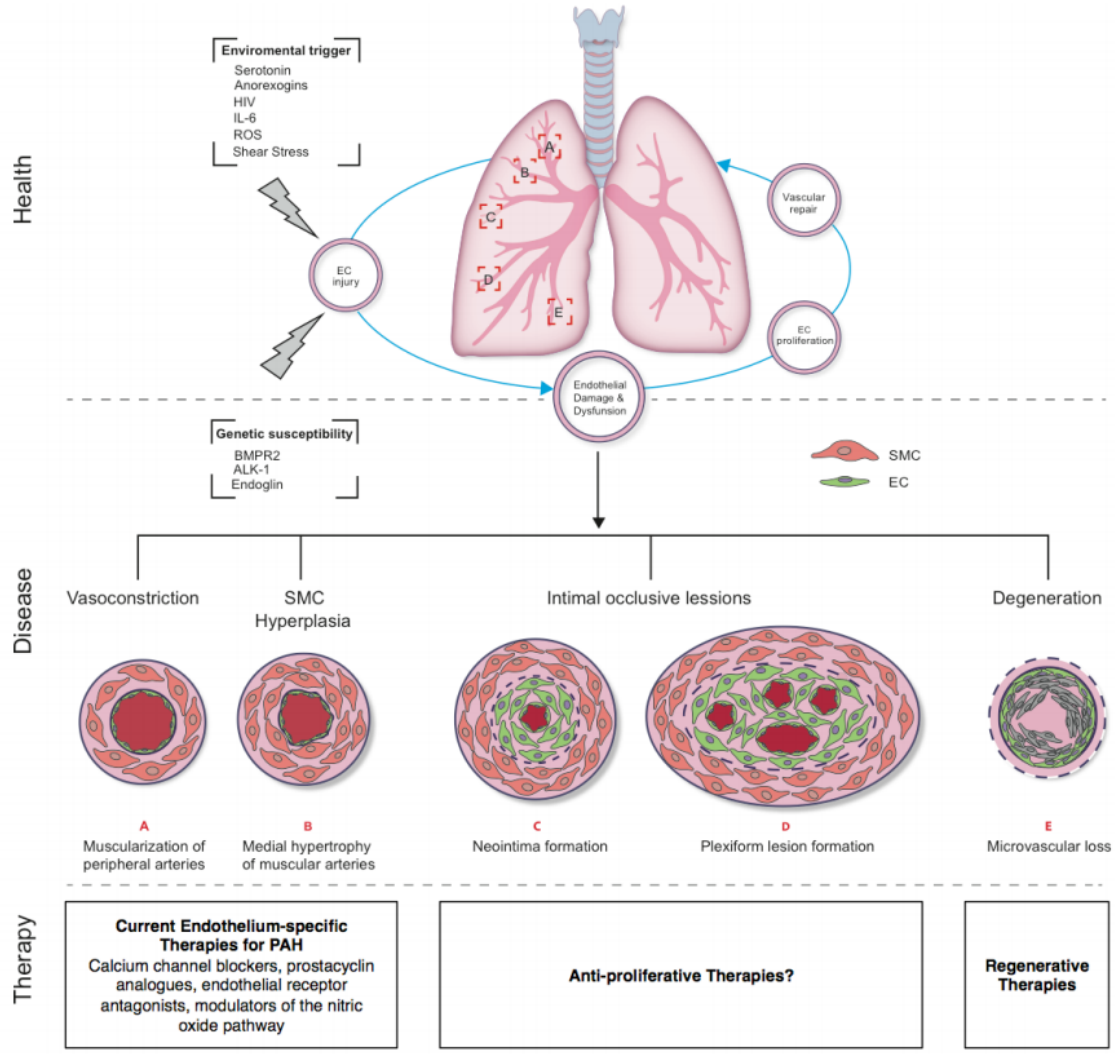

Figure 3. "Pathophysiological overview and modern therapeutic strategies for the treatment of PAH. Exposure to environmental insults such as increased serotonin levels, anorexigens, viruses, increased levels of inflammatory cytokines such as interleukin 6 (IL-6), or sheer stress can contribute to endothelial cell (EC) damage and injury. In healthy individuals, physiological repair processes restore normal lung function via proliferation of nearby ECs and/or the recruitment of circulating endothelial progenitor cells (EPCs). Alternatively, in individuals with PAH, pulmonary vascular cell damage contributes to the degeneration of microvasculature and/or arteriolar remodeling. In patients with hereditary PAH underlying genetic mutations to the genes encoding for bone morphogenetic protein receptor type 2 (BMPR2), activin-like receptor kinase-1 (ALK-1), and endoglin are associated with increased susceptibility to EC damage and injury. Traditional pharmacotherapies aimed at restoring imbalances in vasoactive factors are presented alongside emerging therapies aimed at regenerating the microvasculature. iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stromal/stem cells; PDE-5, phosphodiesterase type 5; ROS, reactive oxygen species; SMCs, smooth muscle cell." Figure and legend (Foster et al., 2014).

3.0 Apoptosis

Originating from the ancient Greek language, the term apoptosis signifies “falling off”; it is a process by which the cell undergoes a programed death that only happens in multicellular organisms (Green, 2011). The process encompasses multiple biochemical changes that include cellular blebbing, shrinkage, chromatin condensation, DNA fragmentation, and mRNA decay (Karam, 2009). Unlike necrosis, which is characterized as a cell death resulting from a spontaneous and acute cellular injury, apoptosis is a finely controlled process that bestows advantages to the organism during its lifecycle. For example, during the growth of an embryo, the toes and fingers are separated as the cells between the digits begin to undergo apoptosis (Alberts, 2008). Apoptosis can be triggered by two different pathways, the first is the intrinsic pathway where the cell responds to internal stress and kills itself; conversely, in the extrinsic pathway, the cell responds to external signals from other cells or the environment (Mohan S, 2010). Analogously, both pathways are controlled via the activation of proteases known as caspases, which are enzymes that degrade proteins. The signal begins with initiator caspases and it is then propagated through the activation of other caspases known as executioner caspases which amplify the signal and lead to the degradation of other proteins and ultimately killing the cell (Wajant H, 2002). Since the early 1990, research has revealed that apoptosis is a biological phenomenon that is central in a variety of diseases. Insufficient level of apoptosis leads to uncontrolled cellular proliferation which is evident in many types of cancers; conversely, a high level of apoptosis leads to atrophy and degeneration (Alberts B, 2002). The cell maintains a balanced level of apoptosis by a balance of proteins, such as Fas receptors that promote apoptosis, or other members such as the Bcl-2 family proteins which inhibit apoptosis (Wajant H, 2002).

3.1 Endothelial Cell Apoptosis as a Key Trigger

ECs injury and apoptosis are thought to be triggers for the emergence of anti-apoptotic and hyper-proliferative cells in the development of PAH (Alberts B, 2002; Lavoie J, 2014). It has also been suggested that extensive and persistent pulmonary vascular endothelial apoptosis may favor the selection of apoptosis-resistant and hyper-proliferative ECs (Voelkel et al., 2006). ECs may undergo apoptosis under stressful environmental conditions or triggers (fig.4); however, some ECs may develop resistance, and in turn, those that survive will begin to proliferate within the small arterioles as the body fails to eliminate them via a controlled apoptotic mechanism (fig. 4). Consequently, the proliferation of those 'deviant' cells would lead to narrowing and occlusion of the small arterioles.

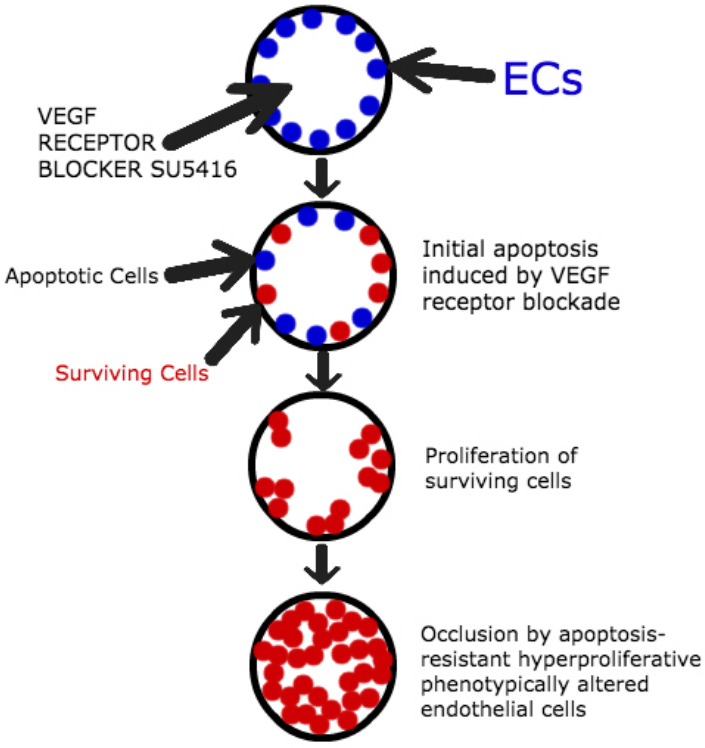

Figure 4. Sequence of events that leads from initial apoptosis to proliferation of apoptosis-resistant ECs. The combination of initial apoptosis induced by VEGF receptor blockade and high fluid shear stress generates apoptosis-resistant proliferative endothelial cells. Definition of abbreviations: ECs: endothelial cells; VEGF: Vascular endothelial growth factor; SU5416: a combined VEDG I and II receptor blocker. Figure and legend (Adapted from: Sakao S, 2009).

Investigating the molecular mechanisms that give rise to the anti-apoptotic phenotype may provide insights into therapeutic strategies for this process, and thus preventing or reversing obliterative pulmonary arterial remodeling. Developing an *in vitro* model of dysregulated ECs with survival characteristics similar to the cells in the disordered state will contribute to our understanding of the mechanisms that act as mediator to the disease and uncovers a possible therapeutic target to reverse the destructive effects of PAH.

3.2 How Does Apoptosis Lead to the Structural and Functional Vascular Changes of PAH?

3.2.1 Degenerative Hypothesis

Data from animal models and samples from patients with PAH support a central role for ECs apoptosis as a "trigger" for the initiation of PAH (Jurasz et al., 2010); however, there is considerable debate about how this is linked to the functional and structural vascular changes of PAH. Our group has previously demonstrated increased ECs apoptosis within distal precapillary arterioles 3 days post injection of MCT (Jurasz et al., 2010); after MCT injection, there was a time-dependent elevation in TUNEL staining in the lung microvasculature which was highly correlated with elevation in RVSP (Zhao et al., 2005). In addition, data obtained from fluorescent microangiography suggest that in the MCT rat model of PAH, there is evidence of discontinuity or "drop-out" of precapillary arterioles resulting in widespread loss of microvascular perfusion, (Zhao et al., 2005). Similarly, in the SU/CH rat model of PAH (Jiang et al., 2015), caspase-3 activation was evident by Western blotting as early as 1 week following SU/CH, which, by immunofluorescent staining was tightly colocalizing with arteriolar endothelium (Jiang et al., 2015). Our lab has championed the view that ECs apoptosis within distal small precapillary arterioles, that consist of little more than endothelial tubes supported by scant extracellular matrix, may result in the degeneration of these fragile vessels and ultimately the loss of

functional lung microcirculation due to a widespread vessel dropout (Jurasz et al., 2010). However, current clinical and experimental evidence suggests an alternate explanation to the emergence of the cancer-like cells, in particular, microvascular rarefaction may be caused by a degenerative process, primarily propelled by chronic ECs apoptosis (Chaudhary et. al, 2017). Therefore, these data suggest that ECs apoptosis within the lung microvasculature causes a loss of integrity of fragile distal precapillary arterioles resulting from a degenerative process, occurring early and is a direct result of ECs death (fig. 5). The implications of the "degenerative" paradigm are central; proposing that proliferative lesions are not the primary cause of microvascular rarefaction, but may potentially be a secondary advent of the disease. Hence, regenerative strategies aimed at restoring the disrupted minute microcirculation is needed.

3.2.2 Proliferation Hypothesis

Initially proposed by Voelkle and Tuder, the proliferative hypothesis of PAH suggests that apoptosis of lung vascular ECs leads to the selection of apoptosis resistant, hyperproliferative cells that are growth dysregulated and do not respond to the regulated apoptosis pathway. These growth-dysregulated ECs lead to the development complex arterial remodeling characterized by intimal hyperplasia and piling up of ECs in the lumen, and ultimately obliterative plexiform lesions that are typical of human PAH pathology (Tuder et al., 1994). Primary ECs derived from patients at late stages of PAH were shown to exhibit an apoptosis-resistant, hyperproliferative phenotype *in vitro* (Masri et al., 2007). The development of these abnormalities within the small precapillary arterioles leads to significant arteriolar narrowing, reduction of blood flow, and ultimately vascular dropout and obliteration (Jonigk D, 2011). ECs harvested from patients with IPAH show a decrease in the expression of pro-apoptotic proteins, such as BAX, BID, and an increase in survival factors, such as survivin

(McMurtry et al., 2005). Due to the role played by the growth-dysregulated ECs in the emergence of the plexiform lesion, a parallel has been drawn between PAH and cancer progression (Rai et al., 2008). This led to the so-called "cancer paradigm" of PAH, suggesting that emergence of apoptosis-resistant cells that collocalize within complex vascular and plexiform lesions exhibit features that are seen in cancer cells, including anti-apoptosis, desensitization to growth signals, and excessive angiogenesis (fig. 5); hence, contributing to narrowing and occlusion of the blood vessels (Rai et al., 2008). The hyperproliferative paradigm has garnered increasing interest within the field; however, the molecular mechanisms that underlie the emergence of growth-dysregulated, cancer-like ECs remain obscure. The elucidation of one potential mechanisms that maybe be common to ECs in PAH and many cancers will be a focus of my thesis as is presented in detail in section 5.0.

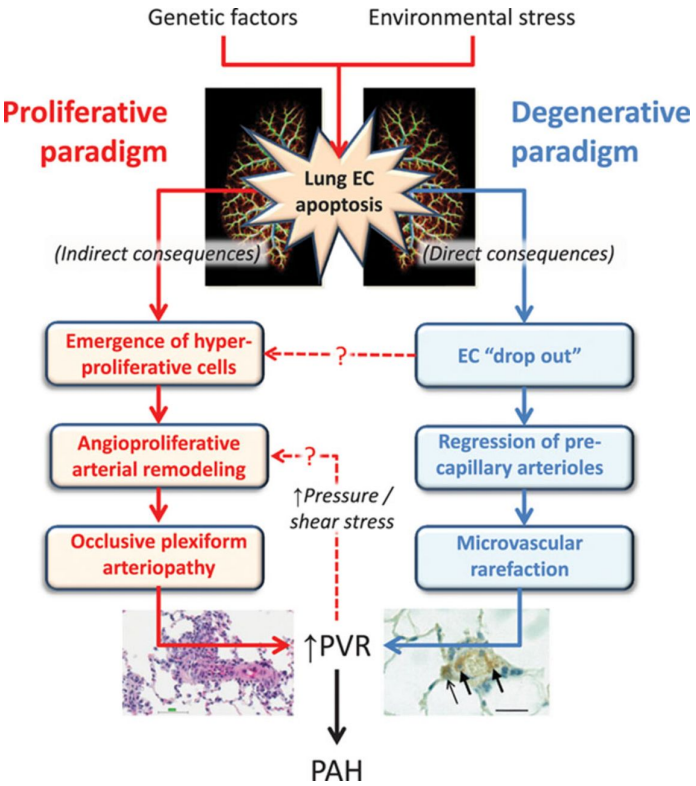

Figure 5. Schematic representation depicting the proliferative vs degenerative paradigms for microvascular rarefaction in PAH. "Genetic and environmental factors lead to endothelial cell (ECs) injury and apoptosis, which has indirect (left hand side) or direct (right hand side) consequences. The first includes the emergence of hyper-proliferative apoptosis-resistant (cancer-like) cells that form occlusive lesions. In the second, EC apoptosis can directly lead to EC drop-out, precapillary regression, and microvascular rarefaction. Both of these mechanisms can lead to increase in vascular resistance and PAH. In this Viewpoint, we are suggesting that proliferative lesions can occur as a consequence of increased pulmonary vascular resistance (PVR), and abnormal hemodynamics caused by microvascular degeneration, and that these lesions may or may not contribute to the progression of PAH. Right lower image represents precapillary arterioles, arrow showing EC apoptosis in the rat monocrotaline model.14 Left lower image represents hyperproliferative lesion in the rat SU hypoxia (SU/HX) model." Figure and legend (Chaudhary, 2017).

4.0 Microvesicles, Exosomes, and Exosome-like Nanovesicles

Exosome studies are a novel avenue that need to be explored further as it is becoming progressively understood that these small extracellular vesicles might be playing a pivotal role in the progression of disorders (Amzallag et al., 2004). Cytoplasmic proteins are incorporated into an endosome, each subset of endosomes bud into a multivesicular body and are now known as intraluminal vesicles, finally, the multivesicular body fuses with the plasma membrane expelling the intraluminal vesicles as exosomes (Keller S, 2006). Exosomes range in size between (20-100nm), contain specific protein surface markers part of the tetraspanin protein family (CD9, CD63, CD81, TSG101) (Amzallag et al., 2004). and studies have shown that they may be playing a role in the progression of neurodegenerative diseases such as Alzheimer's disease (Mattson, 1997), pathological disorders such as cancer (Park et al., 2010), and vascular disorders such as PAH (Amzallag et al., 2004). Exosomes and exosome-like microvesicles contain protein, microRNA and RNA that might substantially affect intercellular signaling between adjacent vascular cells in a paracrine fashion (Keller S, 2006, Thery et al., 2009). In PAH, this might have a remarkable significance, given that the development of complex vascular lesions encompasses the communication of multiple cell types (e.g., SMCs, ECs, stem cell-like and immune cells, and myofibroblasts) (Meng H, 2007). Exosomes are secreted by most cell types in culture and are found to occur naturally in body fluids, suggesting that they have a role as a signaling molecules (Thery, 2006). Extracellular vesicles contain cellular cargo, transporting lipids, nucleic acid, proteins, mRNA and microRNA in their center, and a bilayer surface containing lipid rafts and phosphatidylserine (Thery et al., 2009). Secretion through the non-classical pathway is responsible for transporting some of the well-known proteins that are secreted via exosomes; such as, integrins, major histocompatibility class I and II, and tetraspanins (Urbanelli, 2013).

There are many types of extracellular vesicles that should not be confused with exosomes; this includes microvesicles which are characterized into either apoptotic bodies that are generated from cells undergoing apoptosis, or ectosomes which are generated from plasma membrane shedding (Booth AM, 2006). These different subtypes of extracellular vesicles contain different components of the cytosol, but differences in their components remain to be completely characterized.

As exosomes allow for means of intercellular communication, they may also act to propagate signals that are associated with the spread of disorders. It has been shown that exosomes encompass many disease-associated cargos (Saman S, 2012). To name a few, exosomes carry peptides such as Aβ, and tau that are associated with neurodegenerative disorders such as Alzheimer's disease (Rajendran L, 2006), prions and alpha-synuclein which are associated with Parkinson's disease (Fevrier B, 2004), and also superoxide dismutase 1 which is associated with amyotrophic lateral sclerosis (Emmanouilidou E, 2010; Gomes C, 2007). Thus, exosomes are believed to contribute to the propagation of neurodegenerative protein spread in many disorders, and are central to our investigation in PAH.

5.0 Translationally Controlled Tumor Protein (TCTP)

5.1 General Overview

Translationally controlled tumor protein (TCTP/*TPT1*) is a highly interacting prosurvival protein that is implicated in a wide range of cellular activities (Ulrich-Axel Bommer, 2006). It was initially identified as a proinflammatory factor, called histamine-releasing factor, acting in IgE dependent allergic reactions (Nazzareno Galié, 2003). TCTP expression is thought to be controlled at the translational as well as the transcriptional level (Thiele H, 2000), which led to

one of its commonly used names. The mRNA of TCTP is sequestered in messenger ribonucleoprotein (mRNP) complexes, and the translational repression of ribosome-stalling mRNAs thus allows it to be activated following either endogenous or exogenous signals (Preissler, 2014). TCTP is evolutionary conserved, indicating its vital functions in cellular processes; it interacts with mitotic spindles, F-actin, and controls the cell shape by interacting with the cytoskeleton (Jonigk D, 2011). In addition, TCTP knock-out in mice leads to embryonic lethality (Jonigk D, 2011). TCTP regulates a broad range of cellular processes that are relevant for cell growth and survival (Fig. 6), the regulation of cell cycle, apoptosis and cell survival, and the stress response (Ulrich-Axel Bommer, 2006). Under the control of TSAP6 and p53, TCTP acts as pro-inflammatory regulator, for example, stimulating release of histamine and other cytokines from basophiles (Fig. 6) (Amzallag et al., 2004). TCTP is a potent survival and anti-apoptotic factor, it functions by stabilizing the anti-apoptotic activities of MCL1, BCL-XL, and by inhibiting BAX dimerization to reduce apoptosis. (Nazzareno Galié, 2003).

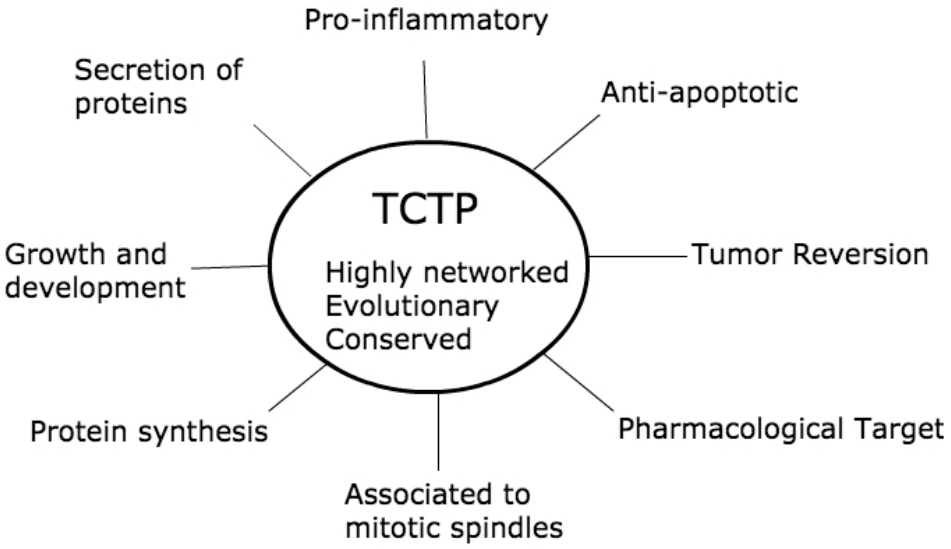

Figure 6. Interactive domains of TCTP. TCTP is a multifunctional protein with intra- and extra-cellular activities (Adapted from: Telerman and Amson, 2009).

5.2 TCTP and Cancer

Numerous studies have highlighted the role of TCTP as an important factor in cancer cell proliferation, survival, and malignant transformation (Telerman & Amson, 2009; Tuynder et al., 2002). On a molecular basis, TCTP is a central regulator of the MDM2-p53 axis and mTOR signaling pathway (Amson et al., 2012). TCTP also participates in a negative feedback loop, acting as a key mediator in the p53 apoptotic pathway (Nazzareno Galié, 2003). Analogously, p53 expression can in turn block the expression of TCTP in a homeostatic manner to keep the levels of the protein in balance. A key role of TCTP is preventing the auto-ubiquintation of MDM2 (murine double minute 2), thereby promoting MDM2-mediated ubiquitination and transcriptional inhibition of p53 (Mouraret N. et al. 2013). MDM2 is an E3 ubiquitin ligase and is auto-ubiquitinated under normal conditions; however, TCTP blocks the auto-ubiquitination of MDM2; thus, fostering an MDM2-mediated ubiquitination of p53, leading to the reduction in the levels of p53 (Amson et al., 2012). Of note, pharmacological inhibition of MDM2 with the anti-cancer drug nutlin3a was recently shown to be an effective treatment for PAH by preventing arterial smooth muscle cell proliferation (M. J. Rigatti, 2012). Under normal conditions, the apoptotic signals typically lead to the dimerization of BAX protein (propapoptotic activity) resulting in the release of cytochrome C (a mitochondrial protein that induces the caspase pathway). Findings have suggested that TCTP inserts within the mitochondrial membrane, interfere with the dimerization of BAX, and delays the initiation of apoptosis (Susini et al., 2008).

TCTP expression is linked to anti-apoptotic phenotype in cancerous cells (Amson et al., 2012; Li et al., 2001; Liu et al., 2005; Rho et al., 2011). It has been shown that a complete suppression of TCTP in breast cancer cell line (MCF-7), human osteosarcoma, and cervical

tumor cell lines lead to a widespread apoptosis (Li et al., 2001). Another proposed mechanism suggests that TCTP functions to stabilize the myeloid cell leukemia Mcl-1 protein (antiapoptotic activity) thus acting to confer an anti-apoptotic phenotype within the cell (Liu et al., 2005; Susini et al., 2008). Thus, TCTP inhibits cell apoptosis by a number of different and complementary pathways and therefore can be consider as a key regular of cell survival.

The small GTPase Rheb (RAS homolog enriched in brain: GTP-binding protein) is ubiquitously expressed in humans and other mammals. In its GTP-bound state, it is a necessary and potent stimulator of mTORC1 kinase activity. The mTOR pathway is a master growth regulator that senses and integrates diverse nutritional and environmental cues, including growth factors, energy levels, cellular stress, and amino acids (Dowling RJ, 2010). It couples these signals to the promotion of the cell cycle and cellular growth by phosphorylating substrates that potentiate anabolic processes (Dowling RJ, 2010). TCTP also associates with mitotic spindle and can be phosphorylated by poll like kinases (PLK) to mediate the transition from metaphase to anaphase. In addition, it has been shown to bind to elongation factors EF1B and EF1A to promote protein synthesis. Thus, TCTP is important in the growth and development of the cell, it determines organ size, cell number, and it has been shown that a mouse knockout model of TCTP dies in utero (Telerman and Amson, 2009) (fig. 6).

Elevated levels of TCTP have been shown in multiple types of tumors, including those originating in the liver, breast, and lung (Jung Eun et al., 2018). TCTP plays a profound role in the process by which cancer cells acquire their malignant phenotype (i.e. malignant transformation). Additionally, the inhibition of TCTP was shown to restore a regulated, nonmalignant phenotype, a process known as tumor reversion (Arcuri et al., 2004; Chan et al., 2012; Kim et al., 2008; Tuynder et al., 2002; Wu et al., 2012). Tuynder et al. and colleagues have

compared both malignant cancer cells and the revertant cells, showing that rarely does a malignant cell revert to a nonmalignant phenotype. Inhibition of TCTP expression using small interfering RNA molecules or anti-sense cDNA resulted in the suppression of the malignant phenotype (Tuynder et al., 2002). This suggested that diminishing TCTP within tumor cells was a potential therapeutic approach for the treatment of cancer (Tuynder et al., 2002). Indeed, clinical trials in phase I/II using sertraline as a blocker of TCTP expression was undertaken in an end-stage myeloid leukemia to reverse the progression of cancer cells (Telerman and Amson, 2009). Small molecules sertraline and thioridazine have been shown to directly interact with TCTP *in vitro* and neutralize its activation of the MDM2-p53 axis thus promoting p53 stabilization (Mouraret N. et al. 2013).

5.3 Possible role of Translationally Controlled Tumor Protein (TCTP) in PAH

From a pathobiological perspective, identifying the molecular mechanism linking EC apoptosis to the emergence of hyperproliferative and apoptosis resistant vascular cells that lead to the development of occlusive and complex vascular remodeling could lead to better understanding of the progression of PAH and assist in identifying early disease markers. From a therapeutic point of view, understanding the mechanism leading to the emergence of those complex vascular lesions would pave the way to novel therapeutic targets that aim at reversing the progression of PAH. However, it remains difficult to study the molecular mechanisms involved in the initiation of human disease since there is little access lung tissues from patients with early stage PAH. To overcome this obstacle, our group utilized late outgrowth endothelial progenitor cells, also known as blood outgrowth ECs (BOECs) derived from patients with PAH compared to control cells from normal subjects. This allowed us to investigate the molecular mechanisms of dysregulation in ECs growth and survival that may contribute to early

development of PAH. BOECs are derived from prolonged culture of peripheral blood mononuclear cells which can be conveniently harvested from whole blood (Asahara et al., 1997; Ingram et al., 2004). They have been comprehensively characterized to display a mature ECs phenotype and they exhibit uniform endothelial cell characteristics *in vitro* (Ingram et al., 2004). Utilizing two-dimensional polyacrylamide gel, in combination with high-throughput mass spectrometry, Dr. Jessie Lavoie, a former doctoral candidate in our lab, performed proteomic profiling of BOECs derived from patients with HPAH with known *Bmpr2* mutations or IPAH patients with no identifiable mutations compared to cells from healthy controls (Lavoie et al., 2014). TCTP was among the upregulated proteins and was chosen for additional characterization due to the fact that it has been previously implicated in the transformation of malignant cells and the promotion of cell proliferation, inflammation, and conferring a strong anti-apoptosis phenotype (Amson et al., 2013; MacDonald et al., 1995; Tuynder et al., 2004; Tuynder et al., 2002). Furthermore, BOECs from PAH patients exhibited increased proliferation and knockdown of TCTP in these cells using a small interfering RNA (siRNA) resulted in an increase of apoptosis and a decrease of proliferation. Importantly, in the SU/CH model of severe PAH, immunofluorescence staining revealed that high levels of TCTP expression were colocalized with the CD31 positive ECs (an ECs marker) mainly within complex obliterative arterial lesions, associated with actively proliferating cells, suggesting a possible role of TCTP in mediating the progression of PAH.

Immunohistochemical staining analysis of TCTP expression in lung sections from patients with HPAH and IPAH revealed a marked increase of TCTP expression in comparison to the control lung tissues. Lung tissues of HPAH and IPAH patients showed an increase of TCTP immunoreactivity in the cell lining of the luminal surface of remodeled vessels. In addition,

adjacent hematoxylin Immunofluorescent staining revealed that TCTP-positive intimal cells co-expressed CD31, in comparison, TCTP staining was not seen in the intimal surface of vessels from control lungs. TCTP is strongly expressed in adventitial regions neighboring remodeled vessels in PAH tissues; this is consistent with the known expression pattern of TCTP in immune cells which are typically copious in human tissues from patients with end-stage disease.

5.3.1 TCTP is Released in EC Apoptotic Nanovesicles (Exosomes)

TCTP release in exosomes was first identified by Amzallag et al. while characterizing the key role of TSAP6 (Tumor Suppressor Activated Pathway 6) in exporting TCTP (Amzallag et al., 2004). Sirois and colleagues have shown the presence of TCTP on the surface and within exosomes which were purified from serum starved media conditioned apoptotic human umbilical ECs (HUVECs) (Sirois et al., 2011). They showed that caspase activation within HUVECs leads an increased release of exosome-like nanovesicles from HUVECs. Using a proteomic approach, they identified that TCTP was one of the most abundant proteins localized with exosomes at a concentration 20x greater than in the ECs cytoplasm (Sirois et al., 2011). Interestingly, inhibiting caspase resulted in the inhibition of exosome release from HUVECs. They further demonstrated that the TCTP-containing nanovesicles (exosomes) promoted survival of SMCs after serum withdrawal (Sirois et al., 2011). In addition, inhibiting TCTP resulted in the inhibition of prosurvival effects on vascular SMCs. These findings suggest a novel role for TCTP as a paracrine survival factor mediating cell-cell signaling and promoting increased SMCs growth in response to endothelial injury.

5.3.2 Is TCTP the "Missing Link" Between EC Apoptosis and Dysregulated Vascular Cell Growth in PAH?

TCTP has many of the hallmarks that suggest it may play a role in mediating the link between ECs injury and apoptosis and emergence of growth dysregulated "cancer-like" cells, which include: 1) release from apoptotic ECs by a caspase-dependent mechanism; 2) potent anti-apoptotic factor that has been implicated in transformation of benign cells to a growth-dysregulated malignant phenotype; 3) it is overexpressed in ECs derived from HPAH patients and 4) increased TCTP expression is largely localized to hyperproliferative cells within intimal occlusive plexiform lesions in experimental and human PAH. Hence, TCTP release appears to be a novel factor that warrants further study in terms of its role in mediating growth dysregulation of lung vascular cells which may ultimately result in the development of irreversible arteriolar complex and obliterative lesions.

6.0 Objectives and Hypotheses

6.1 Research Objective

Pulmonary arterial hypertension (PAH) is a multifaceted disease with a poorly understood pathobiology. It is hypothesized that pulmonary ECs damage/injury leading to widespread EC apoptosis represents an initiating event in the development of PAH (Jurasz P, 2010). It has been suggested that this results in selection of a population of apoptosis-resistance and hyper-proliferative ECs which contribute to the development of intimal occlusion and vessel obliteration (Voelkel NF, 2012). TCTP is a prospective novel target for innovative therapeutic interventions in PAH. Previous work in our lab demonstrated that TCTP is one of 22 dysregulated proteins in BOECs harvested from patients suffering from HPAH with a known BMPR2 mutation (Lavoie et al., 2014). Further investigations of TCTP in the progression of

PAH has been the focal point of this research project due to the protein's implication in vascular cell survival; namely, anti-apoptosis, pro-proliferation, malignant transformation, and chronic non-resolving inflammation (Tuynder et al., 2002; Nazzareno Galié, 2003; Ulrich-Axel Bommer, 2006). We hypothesize that TCTP plays a role in the regulation of ECs and SMCs apoptosis and proliferation, leading to the emergence of dysfunctional growth-dysregulated vascular cells. The first aim of my thesis project was to investigate whether the overexpression of TCTP would induce an apoptosis-resistant and hyperproliferative phenotype in ECs and SMCs. The second aim was to then understand if this overexpression of TCTP lead to higher levels of TCTP release from ECs and SMCs. Finally, we sought to investigate whether secreted TCTP confers a pro-survival effect on adjacent ECs and SMCs by reducing apoptosis or increasing proliferation. Developing an *in vitro* model of dysregulated ECs with survival characteristics similar to the cells in the disordered state will contribute to our understanding of the mechanisms that act as mediator to the disease and uncovers a possible therapeutic target to reverse the destructive effects of PAH.

6.2 Hypothesis

6.2.1 General Hypothesis:

TCTP represents a central molecular mechanism linking EC apoptosis to the emergence of growth-dysregulated lung vascular cells and occlusive, complex arterial remodelling in PAH.

6.2.2 Specific Hypotheses:

- Lentiviral overexpression of TCTP in HUVECs and PASMCs will result in a growth-dysregulated phenotype, characterized by hyperproliferation and resistance to apoptosis

- Overexpression of TCTP will increase its export into apoptotic nanovesicles, thereby augmenting cell-cell signalling between ECs and neighbouring SMCs.

7.0 METHODS & MATERIALS

7.1 Cell Culture

Commercially available HUVECs (Cell Biologics) and PASMCs (ATCC) were grown and transduced at a passage number ranging from 2-4 and utilized for *in vitro* experiments. HUVECs were cultured in EGM2 media with 10 % FBS (Lonza scientific), while PASMCs were cultured in SmGM-2 media with 5% FBS (Lonza scientific). Cells were grown under regular media conditions, unless otherwise indicated, at 37°C in 5% CO2

7.2 Lentiviral Plasmid:

The pLVX-IRES-tdTomato plasmid is an HIV-1-based, lentiviral expression vector that allows for the expression of a cloned protein and the simultaneous expression of a fluorescent protein, tdTomato, in most mammalian cells types including primary cells (fig. X). Expression of the bicistronic transcript is driven by the constitutively active human cytomegalovirus immediate early (PCMV IE) promoter located just upstream of the multiple cloning site (MCS). An encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), positioned between the MCS and tdTomato (fig. Y), facilitates cap-independent translation of tdTomato from an internal start site at the IRES/tdTomato junction.

7.3 Lentiviral Production:

Lentiviral particles were created by cloning *TPT1* gene (Origene scientific; human: RC201664, or rat: RN213660) into pLVX-IRES-tdTomato plasmid (Clonetech scientific). *TPT1* cloning was performed using the restriction and ligation method utilizing the restriction enzymes ECOR1 and BAMH1 (fig. 7), the sequence was then confirmed by direct sequencing (Illumina Next Generation Sequencing Facility, OHRI). Lentiviral particles were generated from

HEK293T cells (ATCC scientific) using the cloned vector along with packaging enzymes (fig. 7) at the following proportions of reagents, cloned vector: 2.5 µg, pCMV-ΔR8.91: 2.25 µg, pMD.G: 0.25 µg, and OPTI-MEM was added to total volume 250 µl. An identical vector, but without the *TPT1* insert, was used to generate sham viral particles and used as a control for *TPT1* overexpression. The lentiviral particles were concentrated using the PEG-it virus precipitation solution (FroggaBio, SBI). Quantification of the lentiviral particles was performed via flow cytometry analysis using the Attune® Auto Sampler (Life Technologies). The resulting flow cytometry data were analyzed using FlowJo (version 10.0.7, FlowJo LLC).

7.4 Lentiviral Transduction:

Following the production and harvesting of Lentiviral particles, virus-containing supernatants were titrated to determine the amount needed to transduce >95% of the cells. 300,000 target cells (HUVECs/PASMCs) were seeded per T75 flask. The lentiviral particles were added at a multiplicity of infection (MOI) of 25 particles/cell. The cells were allowed to grow and the media was changed every 48 hours for both cell types.

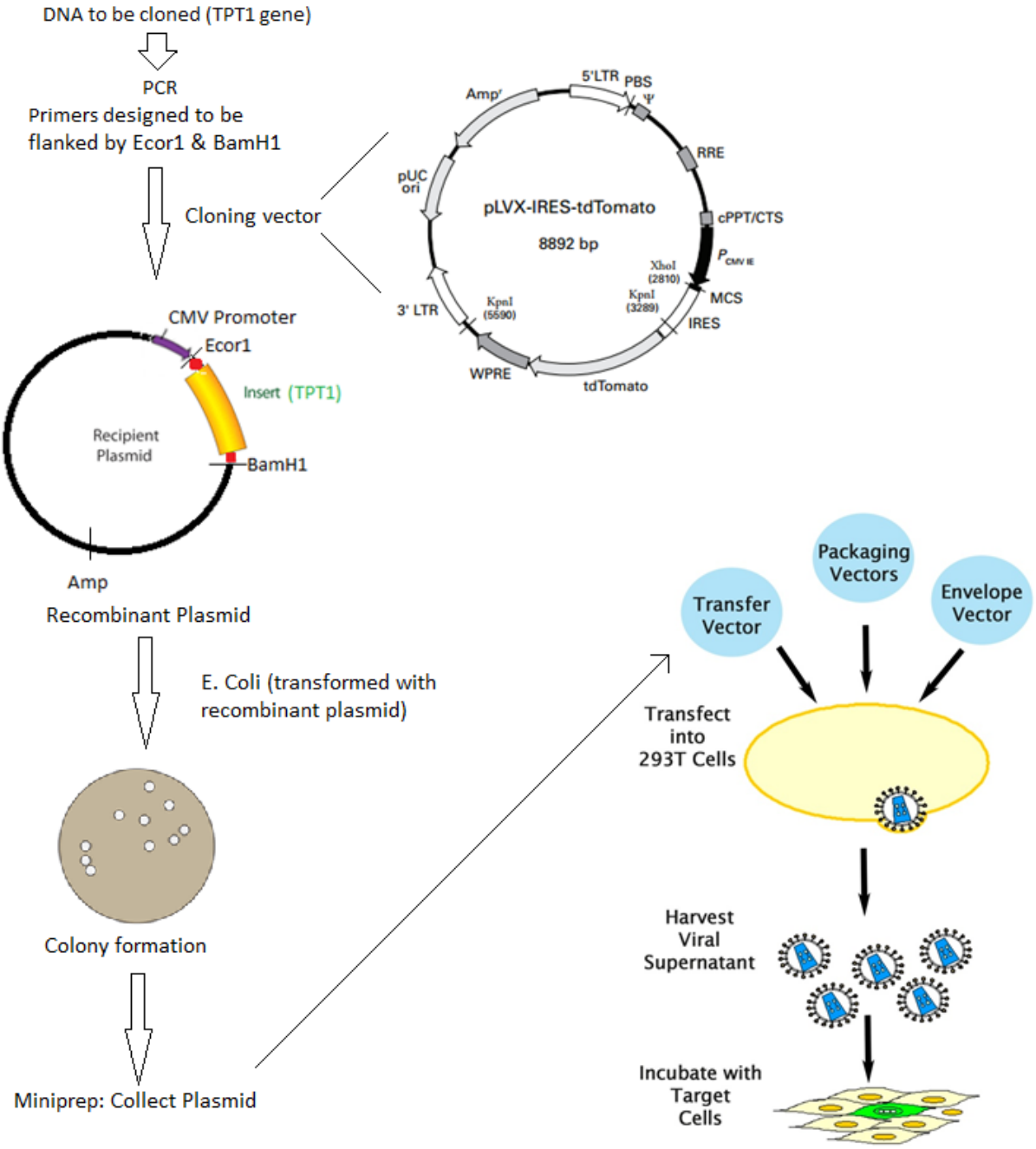

Figure 7. Lentiviral production sequence. Cloning of the *TPT1* gene into a plasmid containing tdTomato and an IRES domain under the control of a CMV promoter. TPT1: tumor protein translationally controlled – 1, IRES: internal ribosome entry site, CMV: cytomegalovirus promoter.

7.5 Flow Cytometry Analysis

3 days post lentiviral transduction (described above), a sample of the cells was harvested with TrypLE (Invitrogen, Burlington, ON), centrifuged to collect the cell pellet, and analysis was done by flow cytometry (Beckman Coulter, SC Quanta, Mississauga, ON) to assess the percentage of cells that are expressing tdTomato. A minimum of 2.0×10^4 events were analyzed per sample; with the control being non-transduced cells gates based on electronic volume and side scatter were set to eliminate cellular debris and cell clusters. The resulting flow cytometry data was analyzed using FlowJo (version 10.0.7, FlowJo LLC).

7.6 Fluorescence Activated Cell Sorting

The presence of tdTomato (excitation maximum of 554 nm and an emission maximum of 581 nm) allows transductants to be visualized by fluorescence microscopy and sorted. Cells were sorted by fluorescence activated cell sorting (FACXS) flow cytometry with standard FITC filter sets (BeckmanCoulter MoFlo XDP, Ottawa Hospital Sprott Centre for Stem Cell research).

7.7 Cell Counting Assay

Cells were seeded at 50,000 cells per T25 flask and allowed to adhere and proliferate. Media was changed every 48 hours and cells were collected at around 80-90% confluency. To lift the cells, TrypLE Express (12604013, Life Technologies) was added and the plates were incubated at 37°C for 5 minutes. Cells were pelleted at 400xG for 5 minutes, re-suspended in 1ml of media, and counted by 0.4% trypan blue exclusion using a cell countess (Countess®, Life Technologies).

7.8 Cell Lysate Preparation and Western Blotting

Cells were lifted as described above. Cell viability was assessed for all cells by 0.4% trypan blue exclusion and the cells were then pelleted by centrifugation at 400XG for 5 minutes at 4^oC. The supernatant was aspirated and the remaining cell pellet was re-suspended in RIPA buffer

containing protease inhibitors (Roche Scientifics). RIPA lysis buffer was added (100ul/1million cells) and the sample was placed on ice for 30 min. Protein lysates were sonicated (10-second pulse followed by 10 seconds off, for 3 cycles) for a total of 1min at 4°C, then centrifuged at 14,000 g for 15 minutes at 4^{o}C, subsequently the supernatant was collected. Protein lysates were separated on NuPAGE 4-12 % bis-tris mini gels (Life technologies, Burlington, ON, Canada), transferred on a membrane and incubated in 5 % non-fat dry milk in tween/TBS for 1 hour, then probed with antibodies against TCTP 1:500 (rabbit polyclonal #ab37506, Abcam, Toronto, ON, Canada), β-actin 1:20,000 (mouse monoclonal #A5441; Sigma-Aldrich, Oakville, ON, Canada). For the duration of probing with antibodies against TCTP, the membrane was incubated overnight (~18 hours) at 4°C. The following day, membranes were washed free of primary antibody and incubated with HRP conjugated secondary anti-rabbit (anti-TCTP 1:15,000) /anti-mouse (1:20,000) antibodies from Mandel Scientific (IRDye, Li-Cor) for 1 hour at room-temperature. Detection of the above-mentioned proteins was done via imaging (Li-COR Odyssey Infrared Imaging System, Li-Cor Biosciences, Guelph, ON, Canada). β-actin was chosen for the housekeeping gene in order to normalize the level of our protein expression within the cells.

7.9 Caspase 3/7 Activity Assay

The Apo-ONE®Caspase-3/7 Assay is a fluorescent assay that measures caspase-3/7 activities. The assay was performed by seeding the cells into 96-well cell culture clear-bottom black plates (3000 per well) with 0.1 ml of normal medium (PASMCs: EGM2 with 5 % FBS or HUVECs: EBM2 with 10% FBS) for 48 hours. PASMCs or HUVECs were washed and maintained in a serum-free basal medium (EGM2 or EBM-2 respectively; 0% FBS) for 4 hours. caspase 3/7 activity was determined using the Apo-ONE® Homogeneous Caspase-3/7 Assay (G7792, Promega). In accordance with the manufacturer's instructions, pro-fluorescent Apo-ONE® Caspase-3/7 reagent was prepared by combining Z-DEVD-R110 [rhodamine 110, bis-(N-

CBZL-aspartyl-Lglutamyl-L-valyl-L-aspartic acid amide] substrate with permeabilization buffer. The profluorescent reagent was then added to each well and incubated at room temperature for 1 hour. During the incubation period cells with active caspase 3/7 sequentially cleave DEVD peptides from the Z-DEVD-R100 substrate producing highly fluorescent rhodamine 110 (emission maximum of 521nm). Following incubation, rhodamine 100 fluorescence was assayed using the BioTek Synergy HT plate reader (BioTek) in accordance with the manufacturer's instructions.

7.10 BrdU Incorporation Assay

Cells were seeded into 96-well cell culture clear-bottom black plates (3000 per well) with 0.1 ml of regular medium for 24 hours. Bromodeoxyuridine (BrdU) (100 μM) was added after 24 hours of seeding, and cells were incubated for an additional 24 hours. In BrdU, the U is labelled, so when it's incorporated into a newly synthesized strand of DNA it can be detected. The higher rate of incorporation, the more DNA that is being freshly transcribed. The BrdU incorporation was measured according to the manufacturer's instructions (Roche Diagnostics, Laval, QC, Canada). Absorbance was assayed using the BioTek Synergy HT plate reader (BioTek), the magnitude of absorbance at 450nm is proportional to the quantity of BrdU incorporated into cells.

7.11 Harvesting Apoptotic HUVECs-Conditioned Media

Cells were seeded into 96-well clear-bottom black plates with 0.1 ml of normal medium for 48hours (fig. 8). The cells were allowed to reach confluency and then serum starved for 4 hours in 0.2 ml serum free basal media to produce a serum starved conditioned media (endothelial cells conditioned media: ECs-CM). The ECs-CM was collected and subsequently added onto PASMCs or HUVECs seeded to confluency in a 96 well plate, as aforementioned. The PASMCs and HUVECs were then either incubated for 4 hours with serum-free CM

followed by a caspase assay, or incubated for 24 hours with BrdU reagent (100 µM) (fig. 8); assays were performed as aforementioned.

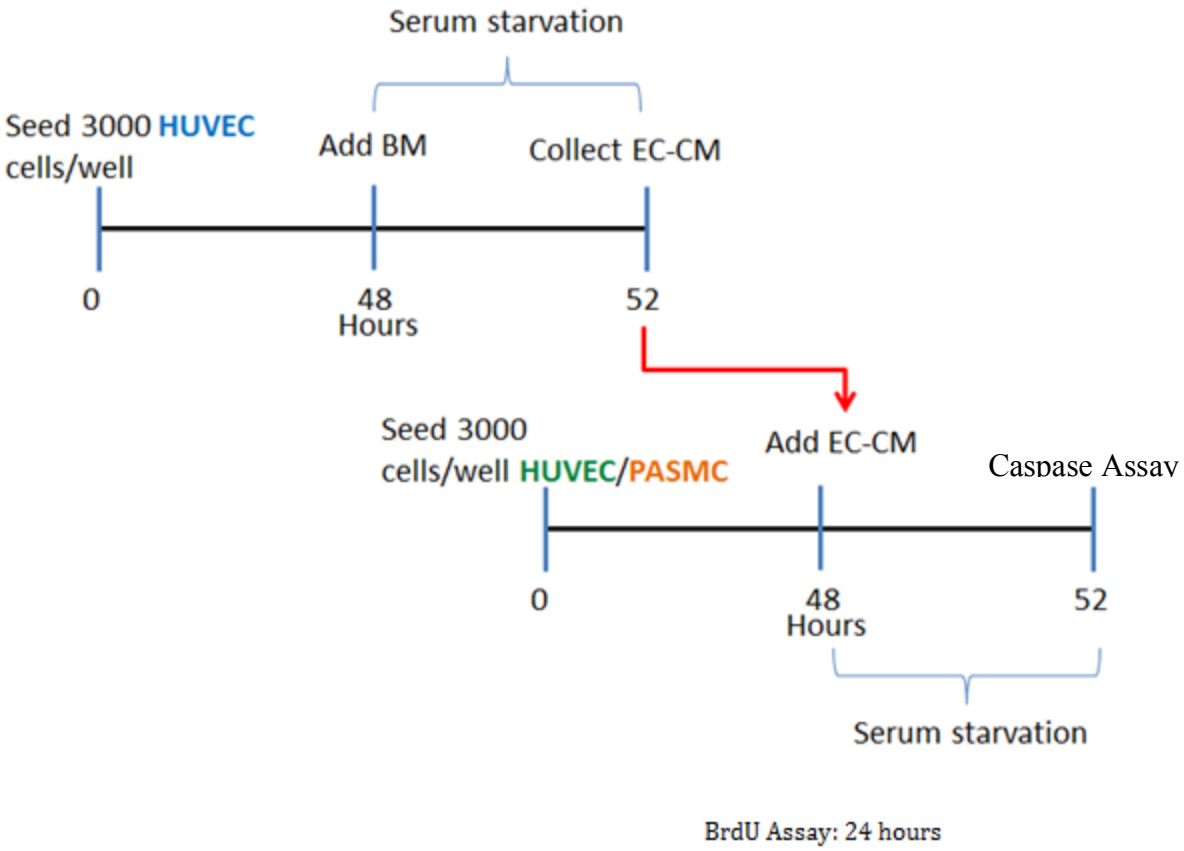

Figure 8. Sequence of producing serum-free CM and cellular incubation. Time-line of serum starvation of HUVECs, followed by the collection of the serum starved conditioned media by the apoptotic endothelial cells at 4 hours. Subsequently adding the serum-free CM to either HUVECs or PASMCs prior to performing either the caspase or BrdU assay. CM: conditioned media, HUVECs: human umbilical vein endothelial cells, PASMCs: pulmonary arterial smooth muscle cells.

7.12 Differential Ultracentrifugation

Cells were plated in T-175 flasks at a density of 10,000 cells/cm^2. Upon reaching 90% confluency, the HUVECs and PASMCs were serum starved with basal media (EBM-2 or EGM2 respectively; 0% FBS) for 4 hours. The serum starved conditioned media was collected and centrifuged (Beckman Coulter, Inc. CA; 70.1 Ti rotor) at 300g for 10min at 4^{o}C to remove cell debris, including whole cells, large membrane fragments, and other cellular remains. The

supernatant was then centrifuged at 2500g for 10min at 4^{o}C to remove large apoptotic bodies and debris. The supernatant was collected and centrifuged at 20,000g for 20min at 4^{o}C to isolate microparticles. The supernatant was collected and centrifuged at 100,000g for 90min at 4^{o}C to isolate exosomes from the supernatant, and the remaining supernatant was collected for further analysis.

7.13 Exosome Isolation Kit

Cells were plated in T-175 flasks at a density of 10,000 cells/cm^{2}. Upon reaching 90% confluency, the HUVECs and PASMCs were serum starved with basal media (EBM-2 or EGM2 respectively; 0% FBS) for 4 hours. The serum starved conditioned media was collected and centrifuged (Beckman Coulter, Inc. CA; 70.1 Ti rotor) at 300g for 10min at 4^{o}C to remove cell debris, including whole cells, large membrane fragments, and other cellular remains. The supernatant was then centrifuged at 2500g for 10min at 4^{o}C to remove large apoptotic bodies and debris. The supernatant was collected. 500ul of ExoQuick™ (SBI systembio, Palo Alto, CA 94303) magnetic capture beads coupled with antibodies that recognize exosome surface antigens CD81 was added to 1 ml of the media in a microfuge tube. This was followed by adding 1ml treatment buffer and 1 ml sample buffer as per the manufacturer's protocol. The tube was then placed on a magnetic stand to collect the beads-exosome complex, then elution buffer was added in accordance with the manufacturer's protocol to separate the exosomes.

7.14 Statistical Analysis

Results are presented as mean ± SEM. Statistical analysis was performed using the GraphPad Prism software, version 5.1. The significance between multiple means were determined by one-way analysis of variance (ANOVA) in addition to multiple comparisons, and when overall differences were detected, the Tukey's post-hoc analysis was used to determine significant differences between individual means.

Figure X. Nucleotide Sequence for Clonetech's pLVX-IRES-tdTomato Vector

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accgctgttgagatccagttcgatgtaacccactcgtgcacccaactgatcttcagcatcttttactttcaccagcgtttctgggtgagcaaaaa
caggaaggcaaaatgccgcaaaaaagggaataagggcgacacggaaatgttgaatactcatactcttccttttcaatattattgaagcattta
tcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaataaacaaatagggttccgcgcacatttccccgaaaagtgccac
ctgacgtcgacggatcgggagatcaacttgtttattgcagcttataatggttacaaataaagcaatagcatcacaaatttcacaaataaagcatt
tttttcactgcattctagttgtggtttgtccaaactcatcaatgtatcttatcatgtctggatcaactggataactcaagctaaccaaaatcatccca
aacttcccaccccataccctattaccactgccaattacctgtggtttcatttactctaaacctgtgattcctctgaattattttcattttaaagaaattg
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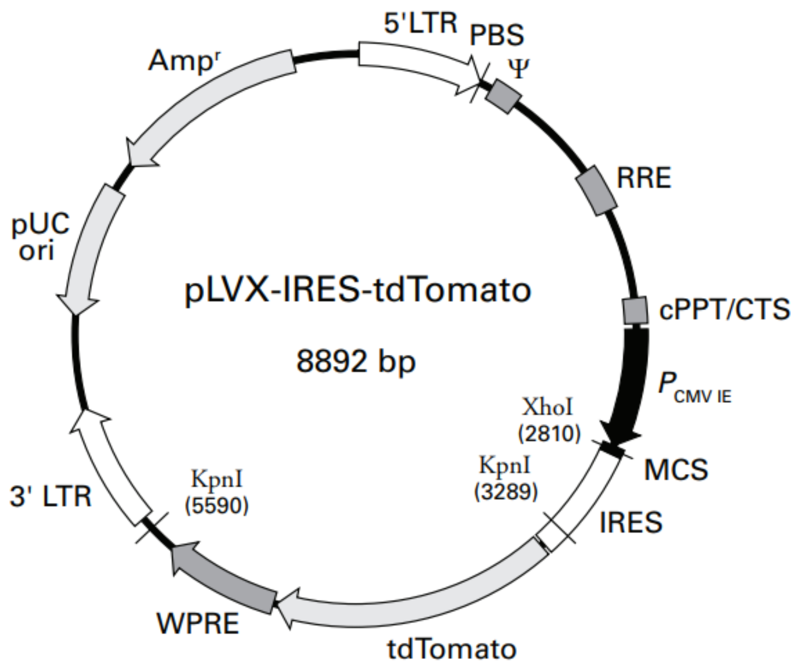
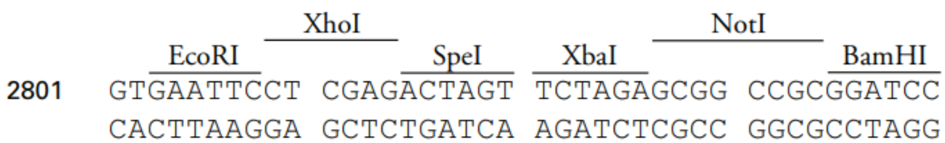
Figure Y. pLVX-IRES-tdTomato Vector Map and Multiple Cloning Site (MCS)





8.0 Results

8.1 Effects of *in vitro* TCTP Overexpression

To study the effects of TCTP over-expression (OE), we utilized a lentivirus vector OE system. Three vectors were generated, each containing a Td-Tomato red fluorescent tag (RFP), with or without the *TPT1* gene. The CMV was chosen as the promoter in order to allow constitutive expression in a variety of cell types. We utilized flow-cytometry to quantify the transduction efficiency prior to sorting, by assessing the percentage of RFP positive cells in HUVECs. The RFP positive cells were 80% and 74% for *H-TPT1* and *R-TPT1* respectively, both significantly higher than the RFP positive cells within the sham control (empty vector transduction) (Fig. 9a). After transduction, cells were sorted by FACS based on the RFP tag; only sorted cells were used for all future experiments. In order to confirm that TCTP was in fact overexpressed in these cells, western blot analysis was performed and it revealed a 6-fold and 3-fold increase in TCTP levels (green bands) in both *H-TPT1* / *R-TPT1* transfected cells in HUVECs and PASMCs respectively (Fig. 9b). Further confirmation of constitutive expression was performed to ensure that the cells continued to translate the protein; fluorescent microscopy confirmed that almost all of the RFP+ selected cells were expressing tdTomato over time and freeze thaw cycles, indicating that TPT1 expression was still intact (Fig. 9c).

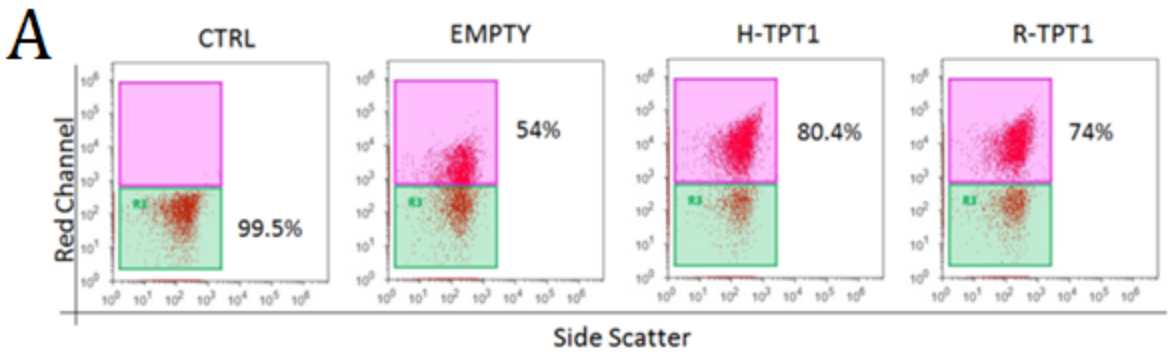
8.2 Functional Assessment of TCTP Overexpression on Proliferation and Apoptosis in HUVECs and PASMCs

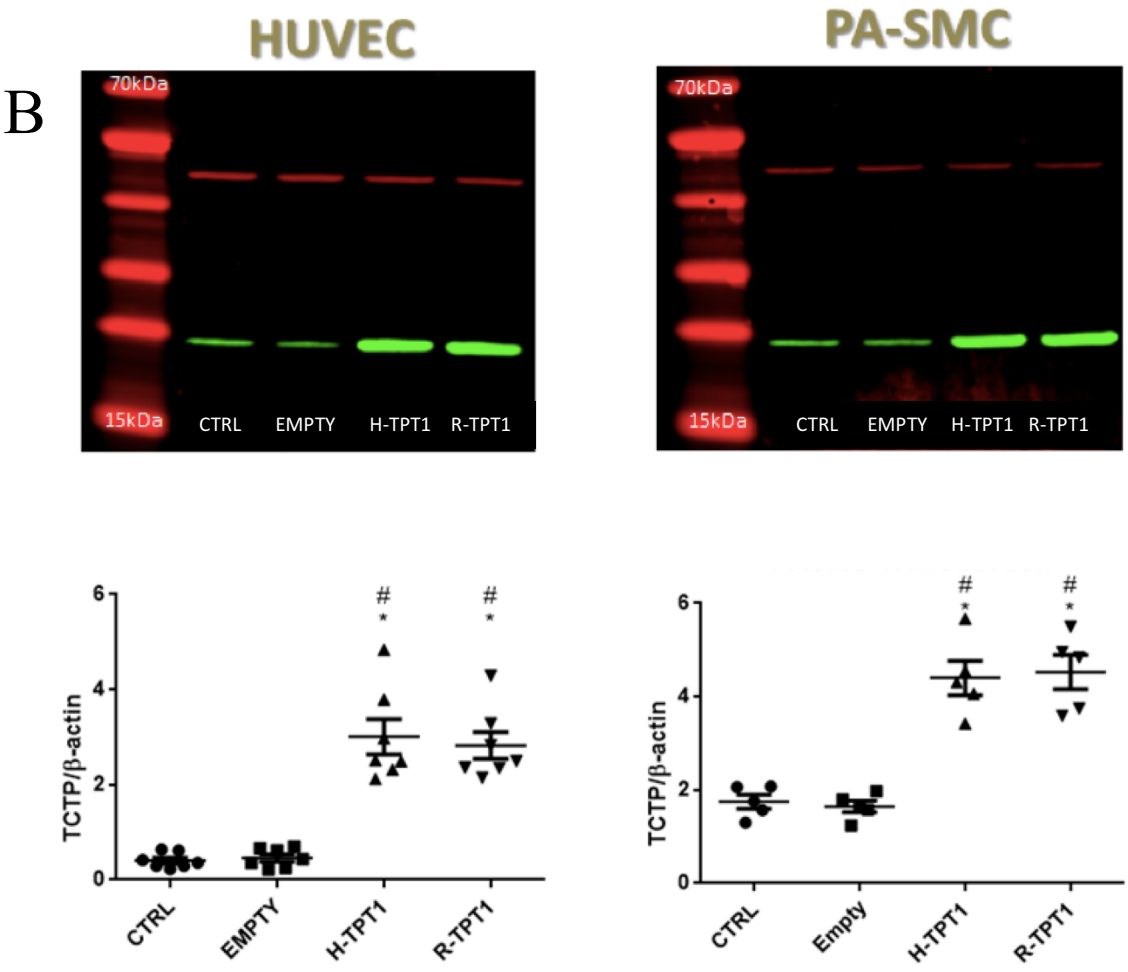
To address the role of TCTP overexpression on cell proliferation and survival, we assessed cell number, proliferation by BrdU incorporation, and apoptosis by caspase 3/7 activity in *TPT1* transduced cells compared to controls. TCTP overexpression led to an increase in proliferation of both HUVECs and PASMCs as evident by a significant increase in cell number 5 days post-seeding (Fig. 10a) and BrdU incorporation 24 hours post-incubation with BrdU (Fig.

10b). TCTP overexpression led to a significant reduction in caspase 3/7 activation of both HUVECs and PASMCs after a 4 hour incubation period in serum-free media (Fig. 10c). Similar effects were seen in *H-TPTI* and *R-TPT1* transfected cells, suggesting that overexpression of TCTP both increases the proliferation of HUVECs and PASMCs and decreases their apoptotic activity.

8.3 Assessment of TCTP Overexpression on the Levels of Export from Apoptotic HUVECs

To address the role of endogenous TCTP overexpression on the levels of TCTP export from the cells, we performed a western blot assay of cell lysate and conditioned media; either complete regular media with serum (RM) or serum-free basal media (BM), from both HUVECs and PASMCs. We assessed protein levels in cell lysates of both *TPT1* transduced cells and controls. TCTP overexpression led to a significant increase in TCTP export from HUVECs after 4 hours of serum-free basal media and to a lesser extent when cultured in regular media as indicated by the brightness of the bands on the membrane (Fig 11). In contrast, PASMCs showed no secretion of TCTP regardless of serum content, suggesting that export of TCTP by cells undergoing apoptosis was specific for HUVECs. The levels of TCTP export were similar between both *H-TPT1* and *R-TPT1* transduced cells. The data demonstrates that TCTP overexpression with either the human or rat sequence enhances TCTP export selectively from HUVECs under conditions of apoptosis, while PASMCs do not exhibit the ability to secrete TCTP even after serum starvation.


A
CTRL
EMPTY
H-TPT1
R-TPT1
Red Channel
99.5%
54%
80.4%
74%
Side Scatter


B
HUVEC
PA-SMC
70kDa
15kDa
CTRL
EMPTY
H-TPT1
R-TPT1
TCTP/β-actin
Empty

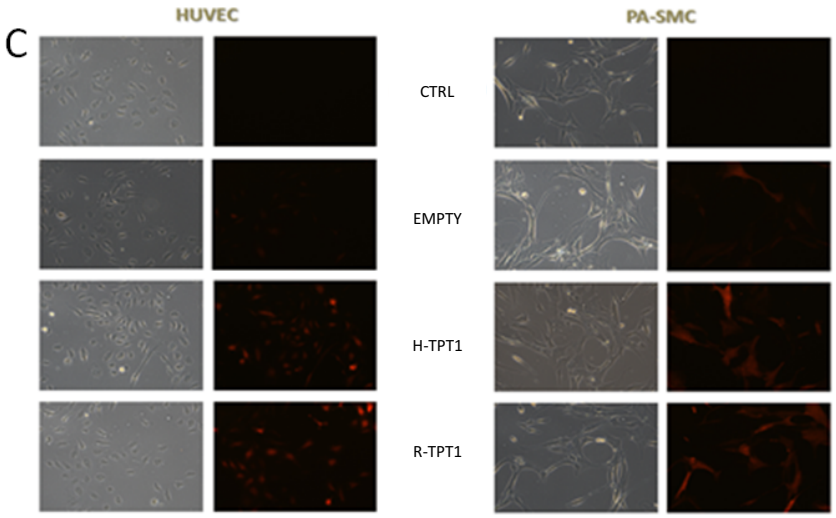


Figure 9. Fluorescent microscope images of HUVECs and PASMCs post sorting. Validation of *in vitro* TCTP over-expression levels in HUVECs and PASMCs in nontransduced (CTRL), cell transduced with an empty lentiviral vector (EMPTY) or cells transduced with either human (H) or rat (R) *R-TPT1*. A) Flow cytometry analysis of tdTomato (red-tag) expression of HUVECs post transduction. B) Representative western blot and summary data (mean±SEM) of TCTP levels (green) normalized to β-actin (red) in transduced and nontransduced HUVECs. C) Representative fluorescent microscopy images of the red tag expression. Expression levels post sorting based on Td-Tomato expression in HUVECs and PASMCs.. Statistical differences were assessed by 1-way ANOVA followed by multiple comparisons. # symbol represents the statistics of comparison with the CTRL group, * symbol represents the statistics of comparisons with the EMPTY group *,# $P<0.0001$.

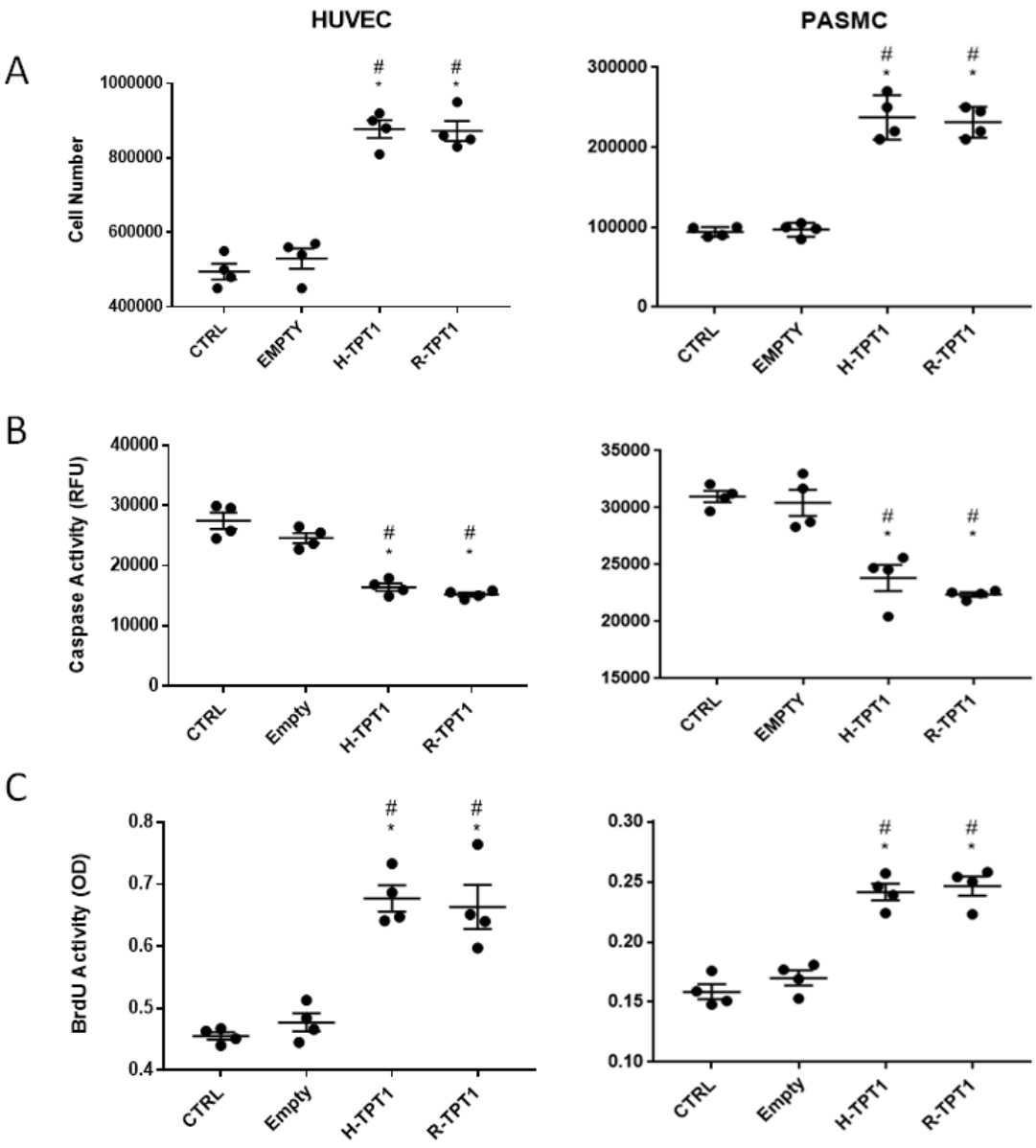


Figure 10. Functional assessment of the role of TCTP on proliferation and apoptosis of HUVECs and PASMCs. Comparison between CTRL, EMPTY, *H-TPT1*, *R-TPT1*. # symbol represents the statistics of comparison with the CTRL group, * symbol represents the statistics of comparisons with the EMPTY group. (mean±SEM) Statistical differences were assessed by 1-way ANOVA followed by multiple comparisons. A) Quantification of cell number: *,# P<0.0001. B) Caspase 3/7 activation: *,# (*H-TPT1*), * (*R-TPT1*) P<0.001. # (*R-TPT1*) P<0.0001. C) BrdU incorporation. Statistical differences. (mean±SEM) were assessed by 1-way ANOVA followed by multiple comparisons, *,# P<0.0001.

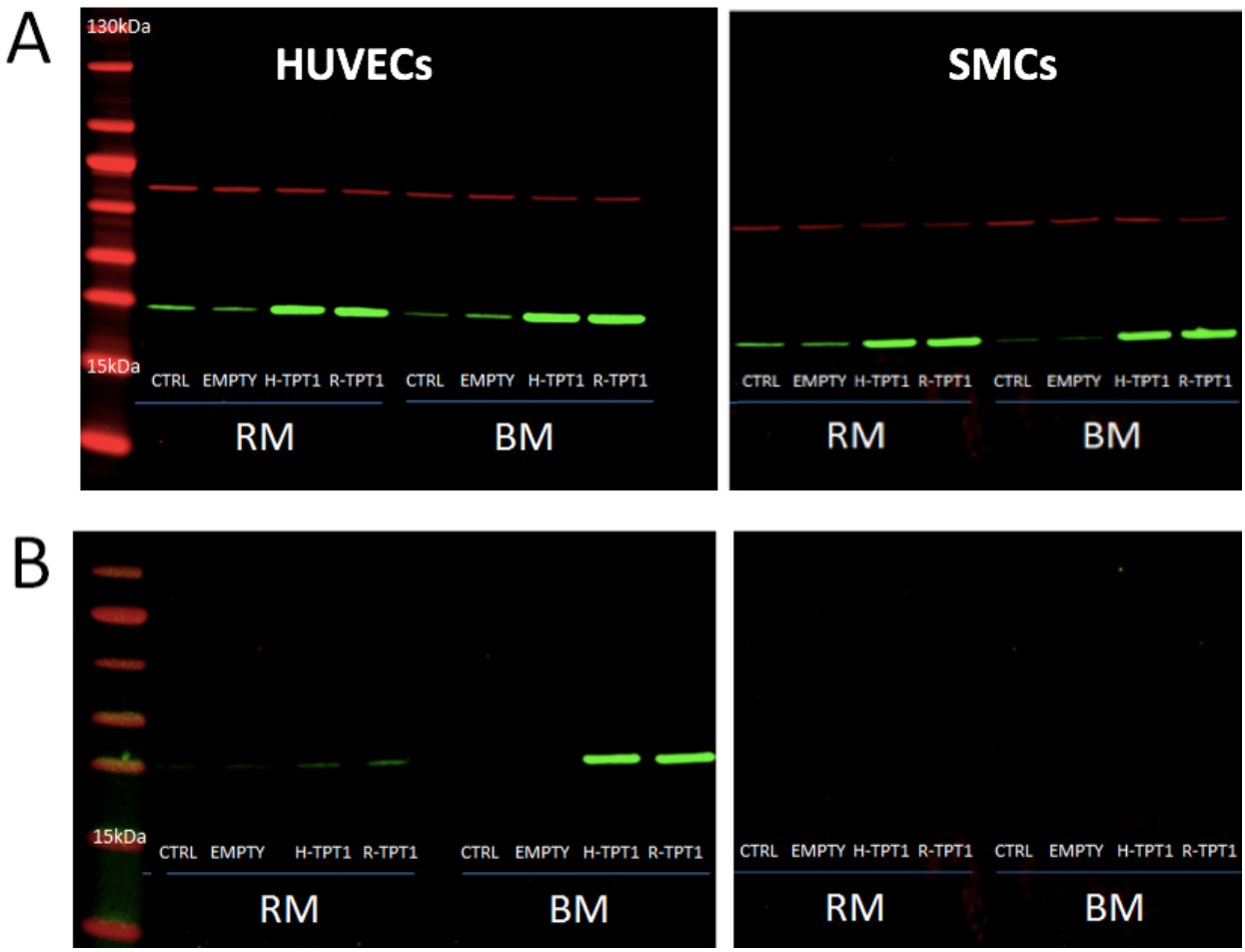


Figure 11. Western blot analysis of cell lysate and serum-free CM. Western blot depicting the levels of TCTP in; A) cell lysate or B) serum starved conditioned media, in both HUVECs and PASMCs, under serum-free basal media (BM) or serum containing regular media (RM). Comparison between CTRL, EMPTY, *H-TPT1*, *R-TPT1*. Red bands represent b-actin, while green bands represent TCTP.

8.4 Functional Assessment of the Role of Exported TCTP on Proliferation and Apoptosis of HUVECs and PASMCs

The effects of exported TCTP on cell growth and survival in terms of proliferation and apoptosis was assessed by exposing non-transduced HUVECs and PASMCs to conditioned media (CM) harvested from apoptosis-induced HUVECs overexpressing either rat or human TCTP. We wanted to address whether TCTP release from HUVECs confers pro-survival effects via a 'paracrine-like' mechanism. Therefore, we assessed the effect of serum-free CM from apoptotic endothelial cells (HUVECs), both from nontransduced cells or cells overexpressing the human/rat TPT1 sequence, on the growth and survival of HUVECs and PASMCs in the absence of serum. serum-free CM from apoptotic HUVECs overexpressing the rat/human TCTP led to a significant decrease in caspase activity in PASMCs, but interestingly not HUVECs (Fig 12a). Similarly, incubating PASMCs with CM from HUVECs overexpressing the rat/human TCTP led to a significant increase in BrdU incorporation within PASMCs, but again not HUVECs (Fig 12b). The effects of the CM harvested from either the human or rat TPT1 transduced cell were similar on PASMCs, suggesting similar roles in control of BrdU incorporation and caspase 3/7 activation. The data demonstrate that not only is TCTP selectively secreted from HUVECs (but not PASMCs) undergoing apoptosis, but that it also has a selective effect promoting PASMCs growth, suggesting that this may represent a unique, unidirectional mechanism of cell-cell signaling, potentially contributing to vascular remodeling after ECs injury.

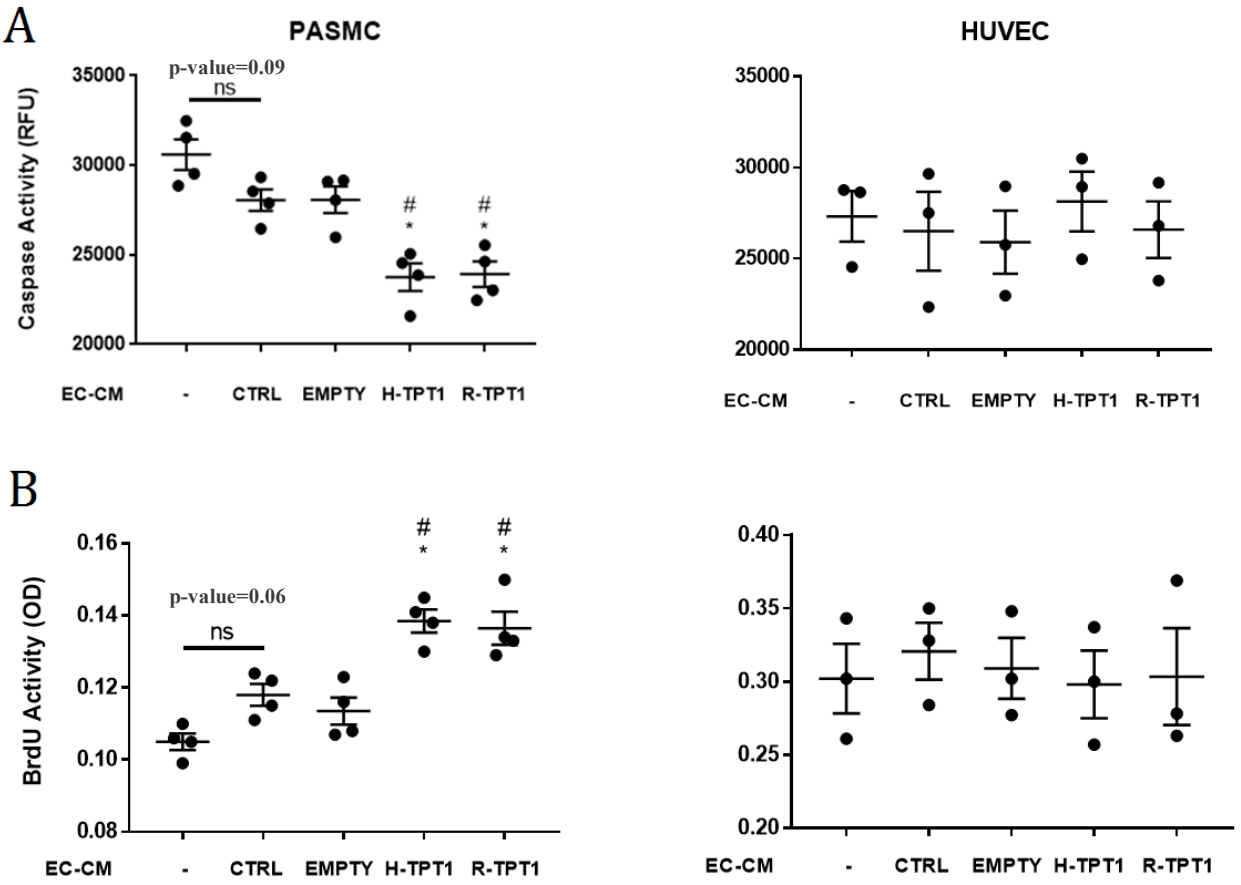


Figure 12. Functional assessment of the role of TCTP on proliferation and apoptosis of HUVECs and PASMCs. Apoptotic endothelial cell conditioned media (EC-CM): Effect of 4 hours exposure to fresh media (-) or media conditioned by serum-starved endothelial cells (EC-CM) on A) Caspase 3/7 activation measurements or B) BrdU incorporation in PASMCs (left panel) or HUVECs (right panel): EC-CM was harvested from HUVECs that are nontransduced cells (CTRL), or transduced with an empty vector (EMPTY), or with human (H-) or rat (R-) *TPT1*. # P<0.01 vs. CTRL; * P<0.01 vs. EMPTY. Statistical differences (mean±SEM) were assessed by 1-way ANOVA followed by multiple comparisons.

8.5 Assessment of Role of Exosomes in TCTP Export from Apoptotic HUVECs

To understand where TCTP is exported, we assessed protein levels in different extracellular fractions, specifically, microvesicles, exosomes, and supernatant obtained from TCTP overexpression cell models. We isolated the fractions based on ultracentrifugation method (described in the method section) and performed a western blot assay by loading equal amounts of protein of each fraction harvested from serum free media conditioned by apoptotic HUVECs.

TCTP overexpression led to a significant increase in TCTP export in comparison to non-overexpressing cells after 4 hours of incubation in serum-free media (Fig. 13). TCTP was detected in both the exosome fraction and the cell supernatant based on western blot analysis, however, it was not detected within the microvesicle fraction. We further assessed protein levels under non-reduced conditions in both the exosome and supernatant fractions isolated from serum-free CM from HUVECs that are overexpressing TCTP (fig. 14 A). The data demonstrates that TCTP is secreted within exosomes as evident by the positive staining of known exosome surface markers CD81 (Green band; 23 kDa) and CD63 (green smear; ~35-130 kDa). In order to visualize any possible TCTP bands (red) within the exosome fraction that may have been overshadows by the CD63 staining (smear), the instrument was set to only detect the red signal (TCTP) (Fig. 14 B). Figure 14 B depicts the non- reduced bands of TCTP at ~55 kDa within the exosome fraction. Although TCTP staining appears to be positive within the exosome fraction, it appears to be more abundant in the supernatant. As previously reported, different conformations of TCTP were observed under the non-reduced conditions as evident by the observation of multiple bands indicative of various degrees of oligomerization (Sirois et al., 2011). To further confirm our findings, we isolated exosomes from the supernatant utilizing an exosome isolation kit as an alternative means to ultracentrifugation (Fig. 15). The data suggests that TCTP (red band) is present to a higher degree in the supernatant (middle panel), absent from the exosome fraction (CD63 was utilized to stain for the exosome surface markers; left panel), and stained positive at multiple bands under non-reduced conditions (right panel).

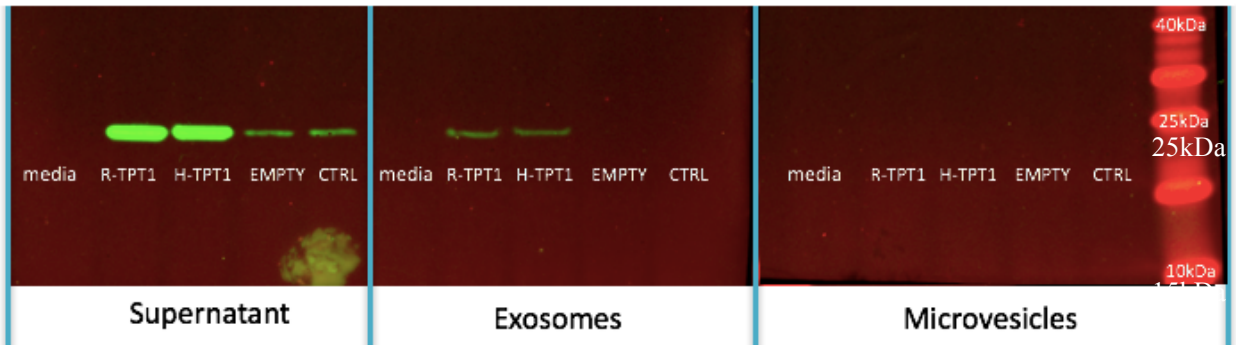


Figure 13. Western blot analysis of different fractions isolated from the serum-free CM. Western blot depicting the levels of TCTP in the supernatant, exosomes, or microvesicles within the conditioned media harvested from apoptotic HUVECs after 4 hours of incubation in serum-free media. 12ug protein loaded in each well.

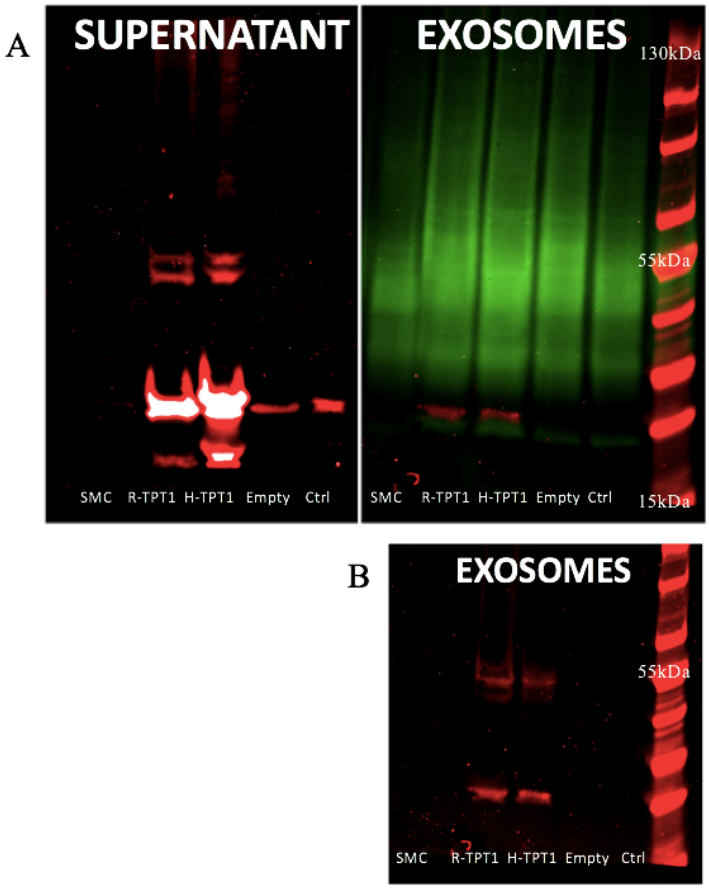


Figure 14. Western blot analysis of the exosome rich fraction and the exosome poor fraction isolated from the serum-free CM following ultracentrifugation. The western blot

depicts the levels of TCTP (red) in exosome-depleted supernatant or exosomes from the conditioned media harvested from apoptotic HUVECs after 4 hours of incubation in serum- free media. CD63 (smear; ~35-130kDa) and CD81 (23kDa) (green). 25ug protein loaded. 6B is the same blot as 6A right panel, it depicts a western blot gel exposed for the red signal alone in order to show the non- reduced bands of TCTP at ~55 kDa.

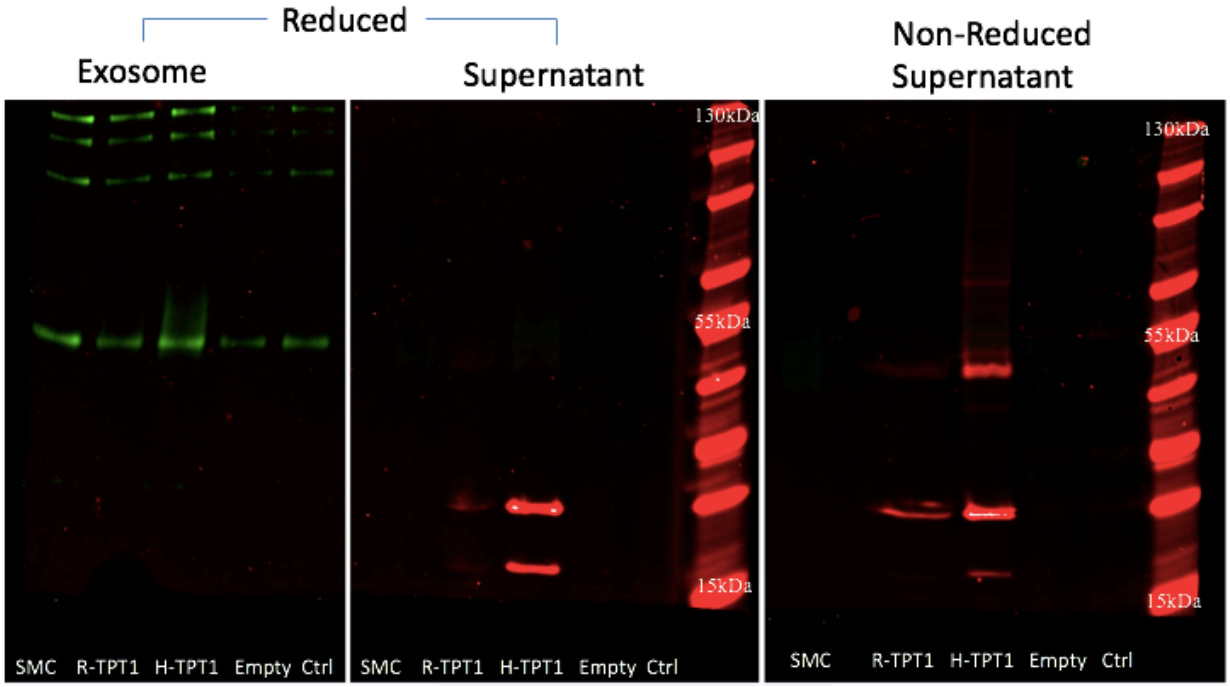


Figure 15. Western blot analysis of the exosome rich fraction and the exosome poor fraction isolated from the serum-free CM following an exosome isolation kit. Levels of TCTP (red) in exosomes or exosome-depleted supernatant from the conditioned media harvested from apoptotic HUVECs after 4 hours of incubation in serum- free media. CD63 was used to visualize the exosome surface markers (green). 25ug of protein was loaded. The right panel depicts a western blot under non-reduced conditions showing the TCTP bands (red) within the exosome- depleted supernatant

9.0 DISCUSSION

9.1 General Discussion

Currently, patients with PAH have few options for treatment, which chiefly focus on alleviating the symptoms of the disorder. Therefore, there is a need to look for additional pharmaceutical targets that may be capable of more effectively stopping the progression of PAH, and even reversing the disease. In the later stages, the disorder moves beyond vasoconstriction and centers on dysregulated vascular cell growth and vascular remodeling. Endothelial cells are important because they contribute both to the structure and function of the small pulmonary arteries. They contribute to the function by releasing vasoactive mediators, or biochemical triggers, that interact with the adjacent smooth muscle layer to mediate vasoconstriction or vasodilation; and they contribute to the structure not only by providing a lining for the lumen of blood vessels, but also by controlling the growth and survival of medial cells again largely by paracrine mechanisms (Botting R, 1989). Indeed, the endothelium dysfunction is the central feature of PAH, contributing to increased arterial tone and remodeling, and ECs injury and apoptosis is a critical trigger for the emergence of hyperproliferative, apoptotic-resistance cells within the vasculature. Thus, there is an urgent need for further investigation into the mechanisms that link lung endothelial cell apoptosis and complex arterial remodeling with the hope that this could lead to a novel therapeutic strategy for treating PAH.

TCTP is a novel prospective target for a therapeutic intervention in PAH. Previous work in our lab shows TCTP to be one of 22 dysregulated proteins in BOECs harvested from patients suffering from HPAH with a known BMPR2 mutation (Lavoie et al., 2014). Furthering our understanding of TCTP as it pertains to the progression of PAH has been the overarching goal of this research project. Certainly, the biological profile of its activities suggests a potential role of

TCTP in mediating vascular cell survival and growth; namely, anti-apoptosis, pro-proliferation, malignant transformation, and chronic non-resolving inflammation (Tuynder et al., 2002; Nazzareno Galié, 2003; Ulrich-Axel Bommer, 2006). The first aim of this project was to investigate whether the overexpression of TCTP would induce an apoptosis-resistant and hyperproliferative phenotype in HUVECs and PASMCs. The second aim was to then understand if this overexpression of TCTP leads to higher levels of TCTP release from apoptosing HUVECs compared with PASMCs. Finally, we sought to investigate whether secreted TCTP confers a pro-survival effect on adjacent HUVECs and PASMCs by reducing apoptosis or increasing proliferation. HUVECs were chosen for this study given that they are easy to grow, have an optimal high passage number capacity ranging between 9-15, and have been shown to be efficiently transduced via lentiviral particles for protein overexpression (Dekker RJ, 2006; Hergenreider E, 2012; Dishart et al., 2003). As well, experiments were performed on PASMCs given the low basal level expression of TCTP, in addition to their importance for lung arterial remodeling, in particular medial hypertrophy that leads to luminal narrowing.

Lentiviruses are a class of retroviruses that are derived from the human immunodeficiency virus (HIV) (Mátrai, et al., 2010; Sakuma, et al., 2012). This viral system allows for the integration of a transgene into the genome of the target cell, thus enabling stable protein expression in both dividing and non-dividing cells (Dull, *et al.* 1998). In addition, lentiviruses are capable of infecting almost all mammalian cell types, making it a suitable vector to use in both our *in vitro* and *in vivo* studies (Sakuma, et al., 2012). We therefore created two lentiviral plasmids, one with the rat sequence of the *TPT1* gene (*R-TPT1*) and one with the human sequence (*H-TPT1*) which encode for TCTP. This would ultimately allow us to use these vectors to later conduct our studies in a rat animal model. The TCTP transgenes were expressed

under the control of a CMV promoter, and the constructs also contained an internal ribosome entry site (IRES) domain for tandem expression of a tdTomato fluorescent protein. The CMV promoter was chosen because it allows for a constitutive expression in a variety of cell types (Qin JY, Zhang L, 2010). The IRES domain mediates the simultaneous expression of TCTP and tdTomato, hence enabling us to visualize our *in vitro* expression under a fluorescent microscope. This allowed us to assess the transduction efficiency and provided the possibility of sorting the cells. We generated three kinds of lentiviral particles; the first contained an empty lentiviral vector lacking the *TPT1* insert, the second contained a recombinant plasmid housing *H-TPT1* gene, and the third contained *R-TPT1* gene. Since *H-TPT1* and *R-TPT1* genes are 93% homologous, we wanted to test their integration and overexpression in both human and rat cells. Given the slight variation in the genetic sequence, any phenotypical measurable differences observed between the translation of either the human or rat proteins would further be assessed to highlight the functional domains within the TPT1 genes.

9.2 Confirmation of Transgene Expression

We observed a lower tdTomato expression in the sham-transduced cells when compared to its expression in cells transduced with the *H-TPT1 and R-TPT1* together with the reporter gene (fig. 9c). This could be explained by the fact that the IRES domain is too close to the CMV promoter within the empty vector; however, within the *H-TPT1* or *R-TPT1* lentiviral vector, the *TPT1* insert separates the CMV promoter from the IRES domain. In a previous study, it has been shown that multiple promoters within a lentiviral vector result in promoter interference during protein expression (Curtin JA, 2008). The interference was demonstrated by the presence of an IRES, acting in a bidirectional manner, where both promoters reduced the activity of each other resulting in a decrease in the overall expression of the adjacent transcription units (Curtin JA,

2008). In this case, the IRES may have interfered with the activity of the promoter and ultimately negatively influenced the downstream expression of tdTomato within the sham vector. Nonetheless, the cells were sorted based on tdTomato fluorescence to avoid the confounding effect of any differences in the overall expression in the assays and analysis. TCTP overexpression in both HUVECs and PASMCs was then demonstrated via western blot analysis (Fig. 9b). The cells overexpressing either the human or rat TCTP exhibited a many-fold increase of TCTP expression in comparison to the non-transduced cells. Similar levels of TCTP expression were observed between the *H-TPT1* to the *R-TPT1* transduced cells in both HUVECs and PASMCs. This indicates that the minor differences in sequence between the human and rat sequences did not translate into differences of protein expression between the sequences.

9.3 TCTP Overexpression: Proliferation and Apoptosis

Previous studies from our lab showed that TCTP is upregulated in BOECs harvested from patients suffering from HPAH (Lavoie et al., 2014). In addition, the colocalization of TCTP was notable in plexiform, intimal, complex occlusive lesions, and within vascular cells located within the occluded arterial lumen of a severe PAH model, as well as in lung sections from patients suffering from HPAH (Machado RD et al., 2006; Lavoie et al., 2014). Our lab has previously shown that administering a single dose of SU5416, a tyrosine kinase receptor inhibitor, in a SU hyper-responder colony of Sprague-Dawley was sufficient to create a progressive and severe PAH model (Jiang, 2012). This rat model also developed complex pulmonary arterial obliterative lesions, similar to those also seen in humans (Abe et al., 2010, Nicolls MR, 2012, Lavoie et al., 2014); however, whether TCTP plays a causal role in the development of these lesions is still unknown. In a rat MCT model of PAH, the angioproliferative occlusive lesions are not present; instead, the arterial remodeling is

characterized by SMCs proliferation and marked medial hypertrophy (Tuder RM, 2013). The TCTP expression in this model was not significantly increased, consistent with the idea that TCTP might be a key mediator in the formation of vascular lesions in PAH.

Our results demonstrate that 5 days post-seeding of either PASMCs or HUVECs, cells that are overexpressing TCTP had a higher cell count in comparison to cells expressing TCTP at the basal level (fig. 10 A). This is consistent with a role for TCTP in increasing proliferation and/or survival of cells, and is in agreement with previous findings highlighting the role of TCTP in cell proliferation and growth (Nazzareno Galié, 2003 Ulrich-Axel Bommer, 2006; Jonigk D, 2011). Moreover, previous studies have highlighted the role of TCTP in cancer cell proliferation, survival, and malignant transformation (Li et al., 2001; Telerman & Amson, 2009; Tuynder et al., 2002). The results from the BrdU incorporation assay suggest that overexpression of TCTP in HUVECs and PASMCs resulted in a higher rate of proliferation compared to the cells that are expressing TCTP at the basal level (fig. 10 C). In previous studies, TCTP immunoreactivity was confined to cells also expressing the proliferating cell nuclear antigen (PCNA) (Machado RD et al., 2006; Baylot V, 2012; Kobayashi D, 2014). TCTP was among the upregulated proteins and was chosen for additional characterization due to the fact that it has been previously implicated in the transformation of malignant cells and the promotion of cell proliferation, inflammation, and conferring a strong anti-apoptosis phenotype. (Amson et al., 2013; MacDonald et al., 1995; Tuynder et al., 2004; Tuynder et al., 2002). TCTP plays a profound role in the process by which cancer cells acquire their malignant phenotype (i.e. malignant transformation). Additionally, the inhibition of TCTP was shown to restore a regulated, nonmalignant phenotype, a process known as tumor reversion (Arcuri et al., 2004; Chan et al., 2012; Kim et al., 2008; Tuynder et al., 2002; Wu et al., 2013). Inhibition of TCTP expression using small interfering RNA molecules or anti-

sense cDNA resulted in the suppression of the malignant phenotype (Tuynder et al., 2002). This growth dysregulation and hyperproliferation of these vascular endothelial cells is thought to play a critical role in the development of occlusive, intimal, and plexiform lesions that are identified in patients with late stage PAH (Tuder RM, 2007; Jurasz P, 2010). This suggests that TCTP may be linked to cellular proliferation and may very well be playing an active role in facilitating the emergence of dysregulated cells within vascular lesions.

TCTP has been shown to inhibit the amplification of mitochondrial-mediated apoptotic signaling in HeLa cells by interacting with the caspase recruitment domain of Apaf-1 in the apoptosome (Jung et al., 2014). Experimental evidence also suggests that structural similarities with the BAX domain allow for TCTP to antagonize apoptosis by inserting into the mitochondrial membrane and inhibiting BAX dimerization, thus delaying the initiation of apoptosis (Susini et al., 2008). Our results show that the overexpression of TCTP induced an apoptotic-resistant phenotype as evident by the reduction in caspase activation in both HUVECs and PASMCs (fig. 10 B). TCTP is also involved in a negative feedback loop acting as a key mediator in the p53 apoptotic pathway (Nazzareno Galié, 2003; Amson et al., 2012). Hence, increasing the level of TCTP may have interfered with the activity of p53 in regulating apoptosis within the vascular cells in our model. Another proposed mechanism suggests that TCTP functions to stabilize the myeloid cell leukemia Mcl-1 protein, which has anti-apoptotic activity, in turn delaying caspase activation (Liu et al., 2005; Susini et al., 2008; Nagano-Ito M, 2009). Based on this evidence, it is clear that TCTP inhibits cell apoptosis by a number of different and complementary pathways and therefore can be considered a master regulator of cell survival.

9.4 TCTP Export: Exosomes and Secretion

Protein export is an important paracrine mechanism for cell-cell communication in disorders such as Alzheimer's disease (Rajendran L, 2006), Parkinson's disease (Fevrier B, 2004), and amyotrophic lateral sclerosis (Emmanouilidou E, 2010; Gomes C, 2007). Cells communicate by transporting cellular proteins in a paracrine fashion to carry information that may halt the progression of the disease or create harm by helping the disease spread and allowing it to progress (Park et al., 2010). Exosomes are one way that cells use to package this information; they are small membrane vesicles ranging in size between 30–150 nm and contain RNA and protein cargos. They are secreted by most cell types in culture and are found to occur naturally in body fluids (Amzallag et al., 2004). Exosomes are now viewed as secretory vesicles containing protein and microRNA cargo that enable intercellular communication and have become the focus of growing interest (Keller S, 2006; Meng H, 2007). We know that the vascular remodelling seen in PAH patients involves the communication of many different cell types such as ECs, SMCs, myofibroblasts, stem cell-like cells, and immune cells; therefore, it is logical to explore the role of exosomes as a pillar of this disorder.

Previously published data from Sirois and colleagues suggested that caspase activation and in the context of ECs apoptosis under serum-starved conditions led to the release of a subset of exosome-like nanovesicles that were enriched in TCTP (Booth AM, 2006; Sirois et al., 2011). Therefore, we wanted to investigate whether TCTP is released from both apoptotic HUVECs and PASMCs in the exosome fraction, or if the release is ECs-specific. Our results suggest that only HUVECs demonstrated TCTP release into the conditioned media when undergoing apoptosis induced by serum withdrawal, whereas this was not detectable in PASMCs (Fig. 11). Interestingly, some TCTP release was seen when HUVECs were cultured in complete media

conditions, but this was only evident in the *TPT1* transduced cells, that expressed supra-physiological levels, and was not in the control or sham-transduced ECs. Serum starved conditions are known to activate the caspase cascade pathway, triggering the release of TCTP (Higuchi A, 2006; Braun F, 2011). Amzallag et al have shown that the overexpression of TSAP6 leads to an enhanced secretion of TCTP under the serum-starved conditions that are known to induce apoptosis (Amzallag et al., 2004; Ulrich-Axel Bommer, 2006). TSAP6 is a glycosylated protein present in the trans-Golgi network, endosomal-vesicular compartment and cytoplasmic membrane (Passer BJ, 2003; Amzallag et al., 2004). Lespagnol et al has shown that in TSAP6-deficient mice have dysregulated transferrin receptor downregulation, the exosome production is severely compromised, and the DNA damage-induced p53-dependent nonclassical exosomal secretory pathway is perturbed. The results suggest that the exosome formation is a tightly controlled biological process dependent of TSAP6 (Lespagnol et al, 2008). Hence, TSAP6 appears to have a role in the export of TCTP and could have a general role in the vesicular trafficking regulation and secretion (Passer BJ, 2003; Amzallag et al., 2004; Lespagnol et al, 2008; Wan C, 2012)

It is possible TCTP may be confering its effects on adjacent cells via a paracrine mechanism, acting as a survival signaling protein; specifically, secreted under apoptotic conditions to interact with adjacent vascular cells. The fact that TCTP is only released from HUVECs, and not PASMCs, may suggest that this is unique to endothelial cells, and plays a specific role in mediating cell-cell signaling with adjacent PASMCs within the vasculature. This idea was further investigated in the next section, in which we discuss whether the TCTP released from HUVECs would exhibit a pro-survival effect on both HUVECs and PASMCs.

9.5 TCTP Export: Proliferation and Apoptosis

TCTP release from apoptotic HUVECs has been shown to confer an anti-apoptotic phenotype on adjacent SMCs (Sirois et al., 2011). To assess the potential paracrine effects of TCTP export on adjacent vascular cells, HUVECs or PASMCs were incubated with serum-starved media conditioned by apoptotic HUVECs. Interestingly, the media conditioned by serum-free CM from ECs provided a survival advantage to PASMCs, but not HUVECs themselves (fig. 12 a, b). Why HUVECs do not show the same response to the TCTP released in by apoptotic ECs is unclear. It cannot be an inability to respond to TCTP since, according to our results, they showed an equally strong proliferative and anti-apoptotic response to the forced overexpression of TCTP as PASMCs. Thus, it is likely that this is may be explained by the possible lack of ability to take up TCTP-loaded exosomes, suggesting the intriguing possibility that there may be cell-specific mechanism for the recognition and uptake of exosomes. We speculate that a cell-specific exosome recognition and uptake mechanisms could be possible to test in future directions; One idea being that this may possibly be related to early membrane blebbing of HUVECs in response to serum starvation, hence, preventing ECs from taking up TCTP.

We observed a unique ability of apoptotic ECs (but not SMCs) to release TCTP. This suggests that not only is TCTP released only in response in cells undergoing apoptosis in a caspase-dependent manner, but this also specific to ECs. Therefore, we suggest that under environmental triggers (i.e. serum starvation), HUVECs may be releasing TCTP as a signaling molecule that is transported to and taken up specifically by PASMCs, providing a survival advantage against the harsh environmental triggers that caused the initial apoptosis of ECs (fig. 16). Our data suggests a possible unique unidirectional, cell-cell signaling mechanism regulating

vascular response to injury following ECs apoptosis. Such a possible mechanism has been highlighted in a study by Eduard and colleagues where they demonstrated the endothelial-protective effects of ECs on SMCs. They highlighted the communication between endothelial cells and smooth muscle cells; in addition, illustrating how endothelial cells can regulate SMC gene expression in co-culture systems (Hergenreider E, 2012).

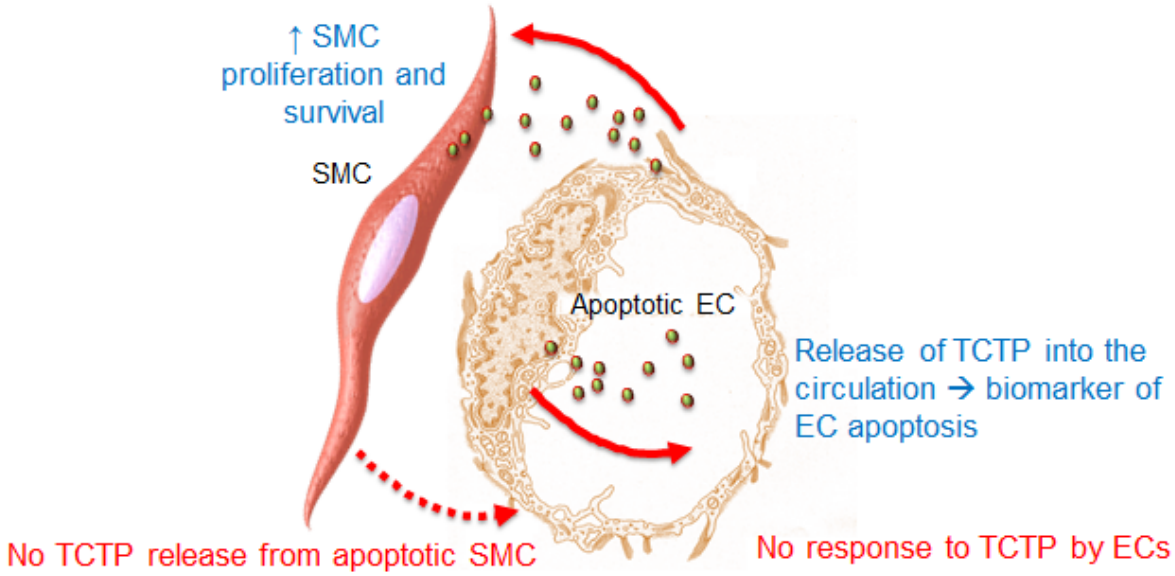


Figure 16: Apoptosis of endothelial cells lead to the release of TCTP into the circulation, leading to the proliferation and survival of SMCs. TCTP was not shown to be released from apoptotic SMC, and endothelial cells do not appear to respond to TCTP rel2ease in a paracrine fashion.

To determine in what form TCTP is released in the media, we separated the microvesicular and exosomal fractions of the media harvested from serum starved HUVECs (fig. 13). However, we were surprised that by far the greatest amount of TCTP was found in the supernatant, presumably as freely released protein, but much less was seen associated with exosomes (fig. 13). Quantitative western blots demonstrated an increase in export of TCTP in the CD63/CD81-positive exosome fraction from HUVECs overexpressing TCTP in comparison to the control group (fig. 14). Further investigation of the exosome and media fraction confirmed

that TCTP export from HUVECs was present in exosomes, but to a lesser degree than that of the supernatant in comparison (fig. 14). It is possible that this could be explained by the disruption of exosomes during the harsh ultracentrifugation process, which has previously been reported to damage and destroy exosomes during isolation, resulting in the release of their contents into solution (Turchinovich A, 2011; Zhang H-G, 2014). Hence, we used an alternative, and much gentler procedure to isolate exosomes from the endothelial serum-free CM using an exosome isolation kit based on CD81 antibody binding and precipitation (fig. 15). The data confirmed our previous findings, suggesting that TCTP was indeed present within the supernatant to a higher degree when compared to the exosome rich fraction (fig. 15). Even though TCTP levels are mainly in the supernatant, it is possible that only TCTP in exosomes get preferentially taken up and therefore has a greater role in cell-cell signaling than freely released protein.

Through immunogold staining combined with ultracentrifugation, Sirois and colleagues have demonstrated the presence of TCTP on the surface of exosomes; however, they did not quantify the amount of TCTP in the supernatant (Sirois et al., 2011). Failure to quantity the amount of TCTP within the exosomes is a huge confounding variable as the staining within the exosomes could either be non-specific or the presence of TCTP on the surface of exosomes could have been a result of the aggregation of the protein on the surface following ultracentrifugation at high speeds. Deciphering the mechanism at play that confers a survival advantage on PASMCs will provide further insight into the mechanism of intracellular communication in PAH which will help to understand the progression of vascular remodeling and the role of TCTP on other cells within the vasculature.

9.6 TCTP as the Possible Molecular Link Between EC Apoptosis and Growth Dysregulation

Previous studies in our lab demonstrated that TCTP knockdown using small interfering RNA in BOECs harvested from patients with HPAH led to a decrease in proliferation and a significant increase in apoptosis (Lavoie et al., 2014). This demonstrates that endogenous TCTP expression in these cells plays a key cell-autonomous role in the regulation of growth and survival in BOECs. Similar findings on gene-silencing studies supported the role of TCTP in apoptosis and cell proliferation in various cancer cell types (Tuynder et al., 2002; Gnanasekar et al., 2009). Our research group, among others, have shown that apoptosis is a central trigger linking vascular injury to vascular remodeling and the progression of PAH (Jeffery, 2001; Jurasz et al., 2010; Tuder et al., 2001). TCTP may represent the missing molecular link between lung vascular EC death (an initial trigger) and the subsequent emergence of lung vascular cell proliferation which causes the progression of PAH in patients to the irreversible stages. This is demonstrated by the SU/CH model of severe PAH, in which EC apoptosis is triggered by inhibiting VEGFR2 survival signaling, resulting in the initiation of a widespread EC apoptosis within the arterioles of the lung microcirculation (Taraseviciene-Stewart et al., 2001). Since TSAP 6 is coordinated with p53 to increase TCTP export, and TCTP expression is increased by apoptosis, we suggest that chronic elevated levels of TCTP in the presence of cellular injury, mutations, or apoptosis-inducing chemicals may contribute to cell survival as indicated by apoptosis resistance and later hyperproliferation. In other words, an increased expression is an adaptive response to stress in order to mitigate the increase in apoptosis, this may result in growth dysregulation if sustained. Ultimately, these dysregulated hyperproliferating apoptosis-resistant cells may result in obliterative changes to the lung's microvasculature leading to worsening symptoms and advancement of PAH. Overall, we suggest that TCTP may represent a

key molecular mechanism resulting in the transition of normal ECs and SMCs into growth dysregulated, apoptotic-resistant cells, that have been previously shown to participate in proliferative and occlusive arterial remodeling in PAH. Based on my findings, TCTP is a potential therapeutic target, and inhibitors could be used as a possible treatment. However, while it may reduce proliferative arterial remodeling, it may also increase apoptosis particularly in earlier stages of disease, therefore, potentially exacerbating the trigger. Thus, we suggest that TCTP inhibition may be effective in the late stages of the disorder as this may be vital in reversing complex remodeling once PAH is established. However, it could be detrimental in the early stages, as the decrease of TCTP levels would contribute to increasing EC apoptosis.

9.7 Limitations

This study is limited by the type endothelial cells used, as HUVECs are harvested from the umbilical vein which carries oxygenated blood to the fetus, and thus is part of the systemic circulation. These ECs are easy to grow and has been shown to be efficiently transduced for protein overexpression; therefore, had a number of advantages for the work that was performed. The data acquired from our experiments would have had more relevance for the human PAH if we had utilized pulmonary artery ECs that are found within the lung's vasculature. Experiments using a more relevant EC are need to confirm the generalizability of our data and may have provided more relevant data pertaining to the effects of TCTP on the cells within the lung vasculature and the phenotypical characterization of cells that participate in the emergence of complex vascular remodeling in the human model of PAH.

Second, the presence of a moderate baseline level of TCTP expression within HUVECs and PASMCs might be considered a limitation since this may reduce the apparent effects of forced overexpression depending on the magnitude of effect attributable to endogenous TCTP.

Previous work from our lab has indeed shown a modest cell autonomous effect of endogenous TCTP expression using siRNA knockdown approaches.

Third, a confounding variable pertaining to the third chapter of this project on TCTP export revolves around the use of lentivirus as an overexpression model. To our knowledge, this is the first research paper investigating the release of TCTP under serum free conditions subsequent to the overexpression of the protein via lentiviral particles. Hence, we speculate that the lentiviral system may have potentially interfered with the export capacity of TCTP; this may have occurred as the lentiviral system could have potentially interfered with the cell's capacity to package and export exosomes. To answer this speculation, repeating the experiments with a non-transduced cell, specifically, a cancerous cell known to have a high expression of TCTP at basal levels would eliminate the need for recreating an overexpression model and may allow for TCTP export *in vitro*.

Fourth, some problems exist within the methods for isolating exosomes. Exosome isolation remains a young field, and the golden standards of methodology is continuously being calibrated. As aforementioned, studies have illustrated the disruption of exosomes during the separation process; a proportion of exosomes were destroyed during isolation, resulting in the release of their contents into solution (Turchinovich A, 2011; Zhang H-G, 2014).

Finally, forced over expression might not replicate faithfully the effects of endogenous TCTP expression in its naïve form. This could be overcome by isolation of growth-dysregulated cells from the lungs of patients with PAH. This way, we may be able to obtain a naturally occurring overexpression model of TCTP from the disordered state that is a resultant from PAH. Here, we may be able to repeat our experiments in an *ex vivo* model.

9.8 Future Studies

The data I have generated during my thesis work support a number of future avenues of research studies to better define the role we have described for TCTP in mediated lung vascular cell growth dysregulation in the pathogenesis of complex arterial remodeling in PAH; in addition to some studies, such as the use human-derived pulmonary artery microvascular ECs that are more relevant to the vasculature of human PAH patients, which have been mentioned above.

Further investigation is warranted to determine whether is TCTP released in the free form or within exosomes using state of the art isolation technologies such tangential flow filtration (TFF) (Giulia Corso et al., 2017) which increases throughput and yields of exosomes while yielding a superior quality product. Moreover, since TCTP is selectively released by apoptotic ECs, it could provide a unique biomarker of the degree of ongoing ECs damage in PAH which could correlate with the severity of disease. At least one report has suggested that TCTP plasma levels can be used as a sensitive and rapid marker of apoptosis in the context of cancer chemotherapy (Kim et al., 2008). Future studies focusing on the role of TCTP as a biomarker for ECs apoptosis could pave the way to early diagnosis of PAH in patients if the secretion of TCTP correlates with earlier signs of the disorder. As noted by Sirois and colleagues when studying the role of caspase mediated TCTP release, TCTP is exported in association with exosome-like nanovesicles from apoptotic ECs under serum starved conditions which later mediates the enhanced survival of adjacent PASMCs (Sirois et al., 2011). They have highlighted in their study that TCTP is associated with exosomes; specifically, present on the surface of the exosome-like nanovesicles. Therefore, the TCTP found on the surface of exosomes could potentially act as a signaling molecule; investigating the mechanism of action via a receptor-like interaction seems to be a promising study. According to our recent findings, it appears that TCTP is freely secreted

as well; hence, additional studies would be key in assessing the primary mechanism by which TCTP functions as a survival factor as it aids in the progression of complex vascular remodeling as highlighted by the apoptotic resistant phenotype and hyperproliferative characteristics it mediates.

In addition, there is literature suggesting that TSAP6 mediates both exosome and TCTP release *in vitro* (Amzallag et al., 2004); hence, overexpressing TSAP6 may have mediated a better study design in creating TCTP enriched exosomes for *in vitro* or future *in vivo* injection studies. This would eliminate many confounding variables within the lentiviral overexpression system that may have influenced the survival mechanisms within our target cells.

Moreover, our data suggest, for the first time, that TCTP may mediate a highly selective, one-way mechanism of cell-cell signaling as evident from our data which show the prosurvival phenotype that serum starved conditioned media harvested from HUVECs confer onto PASMCs but not HUVECs themselves. In order to test this, we can label exosomes in order to assess their uptake by SMCs and not ECs. Investigating the mechanism of TCTP uptake, found within exosomes or the freely secreted, by PASMCs will contribute to a better understanding of the role of exosomes in cellular communication, and survival.

Finally, we need to assess the levels of TCTP-loaded exosomes in the circulation of rats after experimentally-inducing PAH via the SU/CH model. This model is the golden standards of reproducing PAH model in an animal. If the levels of TCTP release are in fact increased, perhaps there is a biological mechanism at play that mediates the export of TCTP to confer an increased survival of adjacent vascular cells. Investigating the effects of exported TCTP within the plasma on vascular cells *in vitro* would further solidify our current data on the role of TCTP in cell

survival. Another intriguing possibility is that the administration of TCTP containing exosomes could induce changes of PAH in a normal rat, providing strong evidence to support their central role in mediate complex arterial remodeling. The availability of cells strongly overexpressing TCTP provides a rich source of TCTP exosomes that could be used for this purpose. As well, the TFF isolation system mentioned above, could also allow the scale up of exosome isolation to levels that would be needed for such *in vivo* rat experiments.

9.9 Conclusions

Overall, we observed that TCTP overexpression using lentiviral transduction confers an increase in the survival of PASMCs and HUVECs. Second, TCTP is released from HUVECs but not PASMCs, and to a higher degree under apoptotic serum starved conditions, Third, TCTP released from apoptotic HUVECs leads to a growth-dysregulated phenotype within SMCs but not HUVECs; suggesting the release of TCTP in PAH to be more specific and profound. Finally, TCTP overexpression led to an increased TCTP export into the media, mostly in the freely secreted form not associated with exosomes. This contributes to our understanding of TCTP as a survival mediator, and may contribute to the formation of occlusive pulmonary vascular remodeling, leading to arteriolar obliteration and the progression of PAH.

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