Polo-Like Kinase 1 and Smooth Muscle Cells in Coronary Artery Bypass Conduits and Coronary Arteries Following Angioplasty

By

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A DISSERTATION

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Abstract

Objective — An internal mammary artery (IMA) graft is immune to intimal hyperplasia (IH) whereas a saphenous vein (SV) graft is prone to undergo restenosis due to neointima formation. However, the underlying mechanism in vein-graft that makes it prone to stenosis and closure is unknown. In this study, we examined the role of polo-like kinase (PLK)-1 in the PLK1/pPLK1-pCDK1-p-Histone H3 pathway in mediating mitotic progression of smooth muscle cells (SMCs) from human IMA and SV in the development of IH leading to vein-graft disease. We also show in vivo data in support of increased immunopositivity towards PLK1 and pPLK1 in SMCs, following percutaneous transluminal coronary angioplasty (PTCA) and coronary artery bypass grafting that may contribute to greater SMC proliferation in the injured versus uninjured blood vessels in swine.

Approach and Results — At the basal level, higher expression of PLK1 and pPLK1 were noted in SV- than IMA- SMCs. Increased expression and phosphorylation of PLK1 was observed in PDGF-stimulated SV- and IMA-SMCs than their respective controls. Significant increase in PDGF-induced PLK1 mRNA transcript expression was noted in SV- and IMA-SMCs than their respective controls. The PLK1 inhibitor, BI2536, attenuated PDGF-BB-induced proliferation in IMA and SV-SMCs. BI2536 blocked PDGF-induced CDK1 phosphorylation. Silencing the PLK1 gene by siRNA transfection in SV- and IMA-SMCs attenuated the expression of p-Histone H3. Immunofluorescence staining demonstrated an increase in the number of cells showing immunopositivity to PLK1, pPLK1, p-Histone, IFN-
γ and pSTAT-3 in the neointima in post-PTCA coronary arteries and superficial epigastric
vein (SEV) grafts.

Conclusions — We are the first to report PLK1 is expressed in VSMCs and a known VSMC
stimulant increases the phosphorylation of PLK1 in VSMCs leads to increased
phosphorylation of pro-mitotic pCDK1-p-Histone H3 pathway leading to IH. The in vivo
data in swine further confirms significantly higher expression of PLK1 and pPLK1 in VSMC
present in the hyperplastic intima in post PTCA-coronary arteries and SEV-grafts. Therefore,
inhibition of PLK1 activity could be a target in developing better therapeutic approach to
prevent VSMC-mediated fibroproliferative diseases.
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List of Abbreviations

ACE (Angiotensin-Converting Enzyme)
ANOVA (Analysis of Variance)
APC-Cdh1 (Anaphase-Promoting Complex/Cyclosome)
BSA (Bovine Serum Albumin)
BIMA (Bilateral Internal Mammary Artery)
bFGF (Basic Fibroblast Receptors)
CABG (Coronary Artery Bypass Graft)
CAD (Coronary Artery Disease)
CaMKIIδ (Calmodulin-Dependent Protein Kinase II Delta)
CDC2 (Cell Division Cycle 2 Kinase)
CDK1 (Cyclin-Dependent Kinase 1)
CRP (C-Reactive Protein)
DAPI (4’, 6-Diamindino-2-Phenylindole)
DB (Destruction Box)
ECM (Extracellular Matrix)
EGF (Epidermal Growth Factor)
E2F (The E2 Factor)
eNOS (Endothelial Nitric Oxide Synthase)
FBS (Fetal Bovine Serum)
FGF (Fibroblast Growth Factor)
FITC (Fluorescein Isothiocyanate)
GSV (Greater Saphenous Vein)
HDL (High Density Lipoprotein)
H&E (Hematoxylin and Eosin)
ICAM-1 (Intercellular Adhesion Molecule-1)
IFN-γ (Interferon Gamma)
IEL (Internal Elastic Lamina)
IGF-1 (Insulin-Like Growth Factor 1)
IH (Intimal Hyperplasia)
IL-6 (Interleukin-6)
IL-10 (Interleukin-10)
IL-12 (Interleukin-12)
IMA (Internal Elastic Lamina)
KD (Kinase Domain)
KLF4 (Kruppel-Like Factor 4)
LAD (Left Anterior Descending Artery)
LCX (Left Circumflex Artery)
LDL (Low Density Lipoprotein)
LMWH (Low Molecular Weight Heparin)
LSV (Lesser Saphenous Vein)
LIMA (Left Internal Mammary Artery)
LV (Left Ventricular)
MAPK (Mitogen-Activated Protein Kinase)
MCP-1 (Monocyte Chemoattractant Protein-1)
MDM2 (Mouse Double Minute 2)

MMP (Matrix Metalloproteinase)

mRNA (Messenger RNA)

NADPH (Nicotinamide Adenine Dinucleotide Phosphate-Oxidase)

NF-kB (Nuclear Factor Kappa Beta)

NLP (Ninein-Like Protein)

NO (Nitric Oxide)

NOX (NADPH oxidase)

Orc2 (Origin Recognition Complex 2)

PBD (Polo-Box Domain)

PB1 (Polo Boxes)

PBS (Phosphate Buffer Saline)

PCR (Polymerase Chain Reaction)

PCI (Percutaneous Coronary Intervention)

PCNA (Proliferating Cell Nuclear Antigen)

PDGF (Platelet Derived Growth Factor)

PDGFRβ (Platelet-Derived Growth Factor Receptor-beta)

PDK1 (3-Phosphoinositide–Dependent Protein Kinase-1)

PI3K (The Phosphoinositide 3-Kinase)

PKA (Protein Kinase A)

PLK1 (Polo Like Kinase 1)

PTEN (Phosphatase And Tensin Homolog)

PREVENT (Project of Ex Vivo Vein Graft Engineering via Transfection)
PTCA (Percutaneous Transluminal Coronary Angioplasty)
RA (Radial Artery)
Rb (Retinoblastoma)
RCA (Right Coronary Artery)
ROS (Reactive Oxygen Species)
RT-PCR (Reverse –Transcriptase Polymerase Chain Reaction)
RTKs (Tyrosine Kinase Receptors)
SAC (Spindle Assembly Checkpoint)
SEV (Superficial Epigastric Vein)
SKP1 (S Phase Kinase-Associated Protein 1)
Sp1 (Specificity Protein 1)
SM22α (Smooth Muscle 22α)
SMCM (Smooth Muscle Cell Medium)
SMCGS (Smooth Muscle Cell Growth Supplement)
SMemb (Embryonic Form of Smooth Muscle Myosin Heavy Chain)
SLK (STE20-Like Kinase)
SOD (Superoxide Dismutases)
Stat-3 (Signal Transduction and Transcription-3)
SVG (Saphenous Vein Graft)
TF (Tissue Factor)
TGF-β (Transforming Growth Factor-Beta)
TM (Thrombomodulin)
TNF-α (Tumor Necrosis Factor Alpha)
TIMP (Tissue Inhibitor of Metalloproteinases)
tPA (Tissue Plasminogen Activator)
uPA (Urokinase-Type Plasminogen Activator)
uPAR (Urokinase Receptor)
VCAM-1 (Vascular Cell Adhesion Molecule-1)
VDG (Vein-Graft Disease)
VSMC (Vascular Smooth Muscle cell)
Chapter 1

Introduction
1. INTRODUCTION

1.1. Atherosclerosis and Coronary Artery disease
Cardiovascular disease is common in general the population and is responsible for approximately 17.3 million deaths worldwide on an annual basis. Coronary artery disease (CAD) is one of the most common types of heart disease and is the leading cause of death in the United States in both men and women [1-3]. Atherosclerosis or hardening of coronary arteries is responsible for almost all cases of CAD. Histologically the first phase of atherosclerosis is the focal thickening of intima in which an artery thickens as a result of accumulation of lipid-laden macrophages (foam cells), extracellular matrix and smooth muscle cells (SMCs) creating a fatty streak. Over time, this fatty streak develops into atherosclerotic plaques due to the progressive accumulation of SMCs and macrophages [4-10].

1.2. Coronary artery disease (CAD) Treatment
The care of patients with CAD requires an understanding of the severity of the disease, control the means to symptoms, and the therapies to improve survival. Low- and intermediate-risk patients whose symptoms are controlled on medical therapy can be managed without intervention. Commonly used drugs for the management of CAD are beta-blockers, calcium channel blockers, late sodium channel blocker and nitrates. High-risk patients undergo coronary angiography and revascularization with either percutaneous coronary intervention (PCI) or coronary artery bypass graft surgery (CABG). CABG is preferred over PCI for many patients with a significant narrowing of
the proximal left anterior descending (LAD) coronary artery with multivessel disease and a reduced left ventricular ejection fraction or a large area of myocardium at risk [11-17].

1.3. CABG

In obstructive coronary artery disease, restoration of coronary artery blood flow as early as possible is vital to salvaging the myocardium. CABG involves construction of grafts between the arterial and coronary circulations [18-20]. It is the recommended modality of coronary revascularization over PCI in the following conditions: (i) left main coronary artery stenosis of 50% or more; (ii) multi-vessel disease with a proximal LAD coronary artery lesion and abnormal left ventricular (LV) function; (iii) failed PCI with persistent pain or hemodynamic instability in patients with coronary anatomy suitable for surgery; (iv) persistent or recurrent myocardial ischemia refractory to medical therapy in patients who have coronary anatomy suitable for surgery and have substantial myocardium at risk; (v) post-infarction ventricular septal rupture (VSR) or mitral valve insufficiency; (vi) cardiogenic shock; (vii) life-threatening ventricular arrhythmias in the presence of 50% or more stenosis in the left main artery and (or) triple-vessel disease; (viii) patients with previous bypass surgery who have large amounts of myocardium; (ix) patients who have had unsuccessful or complicated PCI; (x) diabetic patients with multi-vessel disease; and (xi) late presentation of the disease or recurrent ischemia [21-26].

Alexis Carrel, who received the Nobel Prize for his research in vascular anastomosis, introduced the surgical method of CABG in 1910 [27]. Later in 1962, Sabiston used SVG for the first time as a bypass conduit. In 1970, Favaloro used SVG and LIMA (left
These early proponents of CABG initiated a search for the ideal bypass conduit.

1.4. Choice of bypass conduit

Different vessels have been studied in the quest for the ideal conduit to revascularize the myocardium. These vessels include greater saphenous vein (GSV), lesser saphenous vein (LSV) [29], cephalic veins [30], basilic veins [31], umbilical vein [31], left internal mammary artery (LIMA), right internal mammary artery (RIMA) [32], right gastroepiploic artery (GEA), inferior epigastric artery [33], radial artery (RA), splenic artery [34], gastro-duodenal artery [33], intercostal artery [35], and bovine IMA [28].

GSV and IMA are the 2 most widely used graft conduits. The unsatisfactory clinical outcomes and high rate of graft failure lead to the introduction of synthetic grafts, mainly because of the ease and flexibility of tailoring their mechanical properties. A few of the synthetic grafts that have been used for experimental purposes are made of dacron and polytetrafluoroethylene [28, 36].

1.4.1. Internal mammary artery (IMA)

1.4.1.1. Advantages

The IMA is the graft of choice for CABG. Both RIMA and LIMA are used to bypass coronary blood flow at either a single site or at multiple arterial branching sites in a sequential manner. IMA can be harvested as a pedicle graft (with surrounding tissue)
or as a skeletonized graft (without surrounding tissue). Owing to denervation, the skeletonized graft expands and helps in side-to-side anastomoses [37-39]. Both, left and right IMAs can be grafted in situ, either as a simple or a sequential graft. It is feasible to reach distal coronary vessels, as both of the IMAs can be used as free grafts [40]. The IMA efficiently withstands the competitive flow between the residual flow through the native coronary artery and the flow provided by the bypass graft, which can otherwise cause graft occlusion [41]. Patients with IMA grafts are found to have a lower rate of MI or recurrent angina and undergo fewer repeated operations compared with those with SVGs. The benefit of using bilateral internal mammary artery (BIMA) on survival has yet to be confirmed. However, a meta-analysis evaluated 15,962 patients and suggested that patients with BIMA grafts have a better survival rate [42].

1.4.1.2. Disadvantages

The IMA cannot be used as a graft in patients with a history of radiation or with aortic narrowing [43]. In patients with stenosis of the ipsilateral subclavian artery or brachiocephalic trunk, the use of IMA in situ is contraindicated [44]. The IMA can undergo stenosis at the site of anastomosis, owing to damage caused to the arterial wall during grafting. This IMA stenosis can occur immediately or years later [45, 46]. The use of BIMA increases the risk of deep sternal infection, intervention time, and morbidity. This is especially true in patients with diabetes, obesity, peripheral vascular disease, chronic renal impairment, or chronic obstructive pulmonary disease. Further, in young patients without diabetes, obesity, or chronic obstructive pulmonary disease the use of BIMA in CABG increases the rate of repeated operation and transfusions [47, 48].
1.4.1.3. Patency

In a report from the Royal Melbourne Hospital, which studied 2127 instances of arterial conduits used in CABG, the target artery and severity of stenosis were the 2 major factors significantly associated with the patency or narrowing of IMA [49]. The patency of IMA is directly related to the degree of stenosis. LIMA, when anastomosed to the LAD, has an early patency rate of 99%, whereas RIMA has a rate of approximately 94% when anastomosed to the major branches of the left circumflex artery [50, 51]. There are few instances of IMA occlusion within 3 months of surgery [52]. However, IMA has a significantly higher long-term patency rate than any other grafts [53-55]. The patency rates of in-situ right and left IMA anastomosis to the LAD are 95% and 97%, respectively. Patency rates with other target vessels were found to be lower [49, 56].

A review from the Clinical Outcomes Assessment Program in the state of Washington demonstrated that LIMA, when used as a bypass conduit, significantly reduces perioperative mortality and morbidity in patients over the age of 75 years, women, and diabetics [57]. A study on 10-year survival of 6000 CABG patients showed that compared with IMA grafts, patients with SVG had an increased (1.61 times greater) risk of death and a 1.41 times greater risk of late MI [53]. Another study supported the above mentioned data by showing that at 15 years, patients with arterial grafts had a higher survival rate of 64% compared with that of 53% in patients with SVG [55]. As a consequence of higher long-term graft patency rate and event free survival, IMA is regarded as the graft of choice in CABG. There are multiple anatomic and physiological factors that make IMA the ideal CABG conduit. Anatomically, IMA has – few
endothelial fenestrations, many intercellular processes with low intercellular junction permeability, well-defined internal and external elastic lamina, no valves, good size match with the grafted native vessel, and high resistance to trauma of harvesting [58]. Physiological factors that make IMA a suitable graft are - high flow reserve, high sheer stress, high NO/prostacyclin production, low vasoconstrictor sensitivity, high vasodilator sensitivity, and slow lipid synthesis and uptake [58].

1.4.2. Saphenous vein (SV)

1.4.2.1. Advantages

In spite of the better patency rate with IMA, the saphenous vein (SV) is still used as a bypass conduit, since it is expendable in most patients, easy to work with, adequate in length for many anastomoses, and most importantly, easy to harvest [55]. Since SVG is a large conduit with negligible resistance to the blood flow, it is not affected by the competitive coronary flow between residual flow through the native coronary artery and the flow provided by the bypass graft [41]. The no-touch technique of harvesting SV with the surrounding fatty tissues has improved its patency, both in the long and short term [59].

1.4.2.2. Disadvantages

The major setback of using SV as a conduit is its low patency rate [60-62]. Veins generally function best in a low-pressure milieu with decreased shear stress and mechanical stretch. Such a structure, when exposed to arterial pressure and pulsatile flow,
undergoes vascular remodeling. This remodeling alters the composition and wall shape of the SV resulting in clinical complications [63, 64]. Moreover, during the harvest, the vein is exposed to a supra-physiologic pressure (300–400 mm Hg; 1 mmHg =133.322 Pa), which damages the vessel wall and structure. When grafted, this leads to premature graft closure [65, 66]. In the first year following SV grafting, angina occurs in 20% of the patients [67]. Vein harvesting methods have to be carefully modified to minimize trauma to the vessel. Factors that predispose patients to SVG disease include a target vessel diameter of less than 2.0 mm, dyslipidemia, hypertension, and smoking [68-74]. The short saphenous vein (SSV) is another viable alternative. Some of its limitations include multiple valves, a cumbersome harvesting technique, and its multiple tributaries [75].

1.4.2.3. Patency
The SVG has a low long-term patency rate of 50% at 10–15 years. At 5 years after the operation, only 65% to 80% of the SVGs remain open, and at 7 to 10 years, only 50% to 60% remain patent [76]. There are several intrinsic and extrinsic causes that lead to early, short term, or long-term SVG failure. Early failure occurs within 0 to 30 days and is generally due to technical failures, such as kinking of the graft and anastomoses [77]. Short-term (30 days to 2 years post CABG) failures are ascribed to the development of intimal hyperplasia. Owing to the development of the intimal hyperplasia, a considerable number of the grafts need to undergo revascularization within 2 years after CABG. The long-term SVG failure (more than 2 years post-CABG) is marked by increased intimal hyperplasia and development of atherosclerotic lesions [78]. Several abnormal
anatomical, histological, and physiological factors contribute to the inferior quality of the vein graft causing serious clinical consequences [77].

1.4.3. Comparative anatomy of IMA and SV grafts

IMA is structured like an elastic artery whose diameter varies from 1.9–2.6 mm and whose wall thickness varies from 180 to 430 µm in adults [79]. The wall of an artery is divided into 3 layers: the intima, the media, and the adventitia. The intima consists of endothelial cells with few endothelial fenestrations. The intima also possesses a well-defined internal elastic lamella with a high content of heparan sulfate. In the medial layer, smooth muscle cells are aligned circumferentially in-between elastic lamellae. This layer also consists of types I, III, IV or V collagen [80]. Depending on the thickness of the medial layer of the IMA, the number of the elastic lamellae varies from 7 to 11. The adventitia forms the thick outer layer and consists of fibroblasts and has few vasa vasorum (tiny blood vessels that nourish the wall of large blood vessels) [81]. The IMA, unlike the SV, is bereft of valves. The IMA size optimally matches that of the epicardial coronary artery (Table 1). IMA is highly resistant to trauma caused during harvesting [79, 81].

The SV is larger than the IMA, with a diameter ranging from 3.1 to 8.5 mm and a wall thickness that varies from 180 to 650 µm [82]. Similar to the IMA, the wall of the vein is also divided into an intima, media, and adventitia. The intima of SV has a thin layer of endothelial cells with many endothelial fenestrations and a subendothelial matrix of glycoproteins and connective tissue elements. The intima does not have a prominent
internal elastic lamella, but it gets its multilayered appearance with the interspersed SMC and collagen. The medial layer is composed of an inner layer of longitudinally arranged smooth muscle cells and outer layer of circumferentially arranged smooth muscle cells. The media of the venous wall is interlaced with collagen and elastic fibrils. The adventitial layer consists of fibroblasts and loose collagen bundles. The vasa vasmorum passes through this layer and provides resistance to ischemic injury. Compared with the IMA, the intimal endothelium of the SV has highly permeable intercellular junctions. The SV has greater amounts of types 1 and III collagen than does the IMA [82]. Unlike the IMA, dermatan sulfate is the dominant glycosaminoglycan in SV [79]. Veins afflicted with pathologies such as venous varicoses are unusable, and when used, have a poor patency rate of almost half of that of non-diseased veins [74, 83-86] (Table 1).

Venous grafts are prone to degeneration and occlusion that continues to be the major challenge for CABG with SV [87]. Perioperative manipulations and poor storage of the venous grafts cause damage to its tissue, leading to endothelial cell injury, denudation, and dysfunction [88]. Endothelial cell injury and dysfunction leads to the development of neointimal hyperplasia and graft failure [89]. Histopathological analyses of early venous graft sections reveal fibrin deposition and endothelial denudation, which are more severe at the peri-anastomotic areas [90]. Higher permeability of the endothelium with acute inflammatory cell infiltration at sites of endothelial denudation was observed after a day of CABG. After 7–14 days, intimal SMCs were observed. A few months after CABG, vein graft shows accumulation of SMCs, collagen, and proteoglycans in the vessel intima causing its thickening. Within 2–3 years, the venous graft undergoes arterialization and
fibrointimal thickening, primarily due to the accumulation of type I and III collagen, proteoglycans, and SMCs in the venous intima [74, 82, 88, 91] (Table 1).

**Table 1:** Differences in anatomical and physiological properties between IMA and SV.

<table>
<thead>
<tr>
<th>Properties</th>
<th>IMA</th>
<th>SV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter</td>
<td>1.9 – 2.6 mm</td>
<td>3.1 – 8.5 mm</td>
</tr>
<tr>
<td>Wall thickness</td>
<td>180 – 430 microns</td>
<td>180 – 650 microns</td>
</tr>
<tr>
<td>Endothelial layer</td>
<td>Thick</td>
<td>Thin</td>
</tr>
<tr>
<td>Endothelial fenestrations</td>
<td>Few</td>
<td>Many</td>
</tr>
<tr>
<td>Internal elastic lamella</td>
<td>Well defined</td>
<td>Poorly defined</td>
</tr>
<tr>
<td>IEL with heparin sulfate</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>SMC in the medial layer</td>
<td>Aligned circumferentially in between elastic lamella</td>
<td>An inner layer of longitudinally arranged &amp; an outer layer aligned circumferentially</td>
</tr>
<tr>
<td>Vasa vasorum</td>
<td>Few</td>
<td>Many</td>
</tr>
<tr>
<td>Intercellular junction in intimal endothelium</td>
<td>Low permeability</td>
<td>High permeability</td>
</tr>
<tr>
<td>Collagen</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Valve</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Size match with grafted vessel</td>
<td>Optimal</td>
<td>Poor</td>
</tr>
<tr>
<td>Trauma during harvesting</td>
<td>Highly resistant</td>
<td>Low resistant</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Competitive coronary flow</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Shear stress</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Vasodilator production such as NO &amp; prostacyclin</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Flow reserve</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Pulsatile blood flow</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Patency</td>
<td>High</td>
<td>Low</td>
</tr>
</tbody>
</table>

1.4.4. Drawback of CABG with venous-graft

Both arterial and venous grafts are used during CABG and long-term graft patency is significantly better using the artery [28]. The saphenous vein is the most common venous graft used for bypassing the coronary circulation [60, 92]. Unlike arterial grafts, SV-grafts have failure rates as high as 25% in the first 12 to 18 months. In approximately 10% of SV-grafts, early occlusion may occur within 30 days of CABG. SVG failure at 12 to 18 months post bypass surgery involves endothelial dysfunction, platelet aggregation, growth factor secretion, inflammation, and significant intimal hyperplasia [58, 93-98]. Injury to the venous graft at the time of transplantation is the initiator of these changes. Mediators of these events including the binding of thrombin to its receptor, induces contraction and proliferation of smooth muscle cells in the saphenous vein [99]. Patency of SVG decreases at the rate of approximately 2% per year from the end of year one, and then 4 to 5% in subsequent years [58, 100-102]. SVG undergoes partial or complete closure by 10 years after the surgical procedure [103]. Vein grafts are used in case of
multiple distal anastomoses and is associated with a significantly higher rate of graft-failure leading to the development of angina, myocardial infarction, or cardiac death [76]. Neointima formation is crucial to vein-graft failure and is predominantly consists of SMCs and extracellular matrix [74, 104-106]. Several approaches have been explored to prevent neointimal formation but to date this has not been achieved.

1.5. Pathophysiology of vein graft failure

The pathophysiology of vein graft disease leading to graft failure consists of 3 interlinked processes: thrombosis, intimal hyperplasia, and atherosclerosis [58] (Table 2) (Figure 1).

Table 2: Schematic representation of the pathology of vein graft diseases.
1.5.1. Thrombosis

Thrombosis occurs at an early stage (first month) after bypass surgery (Table 2) (Figure 1). About 3%–12% of SVG become occluded within the first month. The factors that contribute to vein graft thrombosis are as follows [74, 107-109]:

(i) Exposure of the venous lumen to high pressure resulting in endothelial damage.
(ii) Exposure of the vein to arterial pressure, pulsatile blood flow, mechanical stretch, shear stress, and higher wall tension. Venous walls are less elastic than those of arteries. To accommodate to the hemodynamic characteristics of the arterial milieu, venous graft undergoes vascular remodeling [110, 111].

Endothelial injury attracts platelets and leukocytes from the blood, which triggers the process of thrombosis [112]. Endothelial dysfunction reduces the production of tissue plasminogen activator, nitric oxide (NO), and prostacyclin (PGI2) [106, 113, 114]. Endothelial dysfunction activates tissue factor-mediated coagulation factor [112]. Further, endothelial dysfunction elevates the level of plasma fibrinogen, thus contributing to the prothrombotic activity [115, 116]. The process of vein harvesting significantly reduces the activity of thrombomodulin that contributes to the nonthrombogenic nature of endothelium, thus enhancing the process of thrombosis in SV graft [117]. Venous graft remodeling alters the vessel wall shape [118]. The integral processes of the pathophysiology of graft failure are endothelial denudation and dysfunction accompanied by medial SMC migration and proliferation. Endothelial dysfunction alters local production of protective and harmful vasoactive mediators that disturbs the intra-graft hemodynamics. Moreover, endothelial dysfunction increases the expression of growth
factors and adhesion molecules, and makes the media increasingly sensitive to vasoconstrictors such as endothelin-1. All of these cellular changes result in increased vasoconstriction and attenuate the blood flow [112].

1.5.2. Intimal hyperplasia

Neointimal hyperplasia of a venous graft occurs within 1–12 months after its implantation [104] (Table 2) (Figure 1). Growth factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF), epidermal growth factor and various cytokines such as interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α) are released at the site of vessel injury by the accumulated macrophages, platelets, and denuded endothelial cells [119, 120]. These factors induce proliferation and migration of SMCs from the media to the intima. At the intima, the newly migrated SMCs synthesize and secrete extracellular matrix (ECM) resulting in intimal fibrosis and reduced cellularity [74, 106, 118]. Endothelial cells regenerate over the deposited layer of fibrin and platelets on the thrombogenic basement membrane [118]. In the pig, the enzyme activities of adenylyl cyclase, guanylyl cyclase, and cyclooxygenase are downregulated in the SV – carotid artery interposition grafts one month after surgery [121]. It is observed that post grafting to coronary circulation SV undergoes vasoconstriction in response to vasodilating agents such as acetylcholine [122]. The venous graft loses its nourishment through vasa vasorum, which results in a continuous cycle of ischemia and fibrosis [123]. The ischemic reperfusion reduces the production of endothelial NO and prostacyclin, and induces the production of superoxide [123].
Perivascular fibroblasts can differentiate into myofibroblasts and migrate through the medial layer to the intima [124]. The wall stress that the SVG undergoes when exposed to arterial pressure upregulates growth factor receptors on SMCs such as basic fibroblast receptors (bFGF), which contributes to SMC proliferation and migration [125]. Increased production of platelet-derived growth factor (PDGF-BB) [126], thrombin [99], bFGF [126], insulin-like growth factor 1 (IGF-1) [127], vascular endothelial growth factor (VEGF) [128], superoxide radicals [129], endothelin-1 [130], angiotensin-II [131], serotonin [132], and decreased production of NO [82], prostacyclin [113], and transforming growth factor-beta (TGF-β) [133] disturb the balance, resulting in increased SMC proliferation and migration, and leading to neointimal hyperplasia [74, 93, 106, 125].

1.5.3. Atherosclerosis

Atherosclerosis is the prominent pathological lesion observed first year after CABG (Table 2) (Figure 1), which causes vein occlusion leading to the recurrence of ischemic symptoms [134]. Atherosclerotic lesion in vein grafts are histologically and topographically different from that of the arteries [135]. The major difference between the atheromatous lesion in veins and arteries is the rapid endothelial dysfunction-mediated progression of atherosclerosis in veins [136]. Many foam cells and a high extracellular lipid content at the intima mark advanced atherosclerosis. The majority of the lesions are infiltrated with inflammatory cells such as neutrophils, lymphocytes, monocytes, and macrophages. These macrophages ingest native and modified lipoproteins and become foam cells. Histologically, the venous graft atheroma is infested
with a relatively increased number of foam cells and inflammatory cells compared with an arterial atheroma [137]. Morphologically, atherosclerotic lesions in the venous graft are concentric and diffuse [58]. The graft lesion has no fibrous cap to prevent the blood from coming into contact with the atheroma [72, 112, 138-141]. The factors that lead to venous graft atherosclerosis include the following:

- Poor handling and harvesting of the SVG: heparinization is found to improve the quality of the venous graft and reduces peri-operative trauma [110].
- Elevated expression of adhesion molecules at the site of graft atherosclerosis: monocyte chemoattractant protein (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule (ICAM-1) are just a few of these adhesion molecules. MCP-1 attracts monocytes, VCAM accumulates macrophages, and ICAM-1 causes mononuclear cell adhesion and migration [90, 142, 143].
- Dyslipidemia with increased serum concentration of low density lipoprotein (LDL) cholesterol, apolipoprotein, and low levels of high-density lipoprotein (HDL) predisposes patients to vein-graft lesions [144].
- Other contributing factors include left ventricular dysfunction, smoking, and high mean arterial pressure in the vein [72, 73, 102].

Re-surgery is the only treatment for vein graft atherosclerosis [145].
Figure 1: Sequential events that lead to vein graft failure.
Three phases of vein graft failure: (A) Thrombosis: it occurs within the first month of CABG. Vein graft, when exposed to hemodynamic forces, results in endothelial damage, followed by the adhesion of platelets and leukocytes, which releases a plethora of vasoactive mediators and growth factors. Release of TF initiates the coagulation cascade with the deposition of fibrin. All of these lead to thrombus formation and initiate neointima formation. (B) Intimal hyperplasia: this occurs within 1–12 months after CABG. The expression of MMPs, growth factors, cytokines, and ROS promote the proliferation and migration of medial SMCs towards the intima, resulting in neointima formation. (C) Atherosclerosis: it occurs after 12 months post-CABG. The monocytes from the blood infiltrate the neointima and transform to macrophages that engulf low-density lipoprotein and become foam cells. All of these events lead to occlusion of the vein graft. CABG, coronary artery bypass grafting; TF, tissue factor; GF, growth factor; MMPs, matrix metalloproteinases; ROS, reactive oxygen species; SMCs, smooth muscle cells; VSMS, vascular smooth muscle cells; EC, endothelial cells.

1.6. Smooth Muscle Cells and Intimal Hyperplasia (IH)

SV is widely used as a bypass graft in spite of its poor patency [89]. The underlying pathological process that leads to the occlusion is IH. IH in SVG after CABG is a major clinical problem in the field of vascular biology, whereas IMA is almost resistant to IH-associated fibroproliferative vascular pathology [146]. It is important to note that SMCs from veins and arteries are derived from different embryonic origins and exhibit distinct molecular signatures. Thus, intrinsic functional differences between SV and IMA SMCs contribute to the increased rate of graft stenosis observed in venous grafts than arteries [147]. SMCs and extracellular matrix are the two major constituents of the neointimal lesion. SMCs dedifferentiate from contractile to a synthetic and reparative phenotype, which migrate toward the lumen and proliferate in response to altered environmental cues at the time of surgery [148].
1.6.1. Phenotype switching

“Phenotypic switching” of the SMC is defined as the functional and structural changes exhibited by SMCs in response to increased wall tension, shear stress and endothelial denudation [148]. Mature VSMCs possess a contractile and differentiated phenotype [148, 149]. They have a low rate of proliferation and synthetic activity, and express various unique contractile proteins, ion channels, and signaling molecules required for the cell's contractile function. Upon vessel injury due to angioplasty or bypass surgery, VSMCs dedifferentiate and re-enter the cell cycle [150]. These dedifferentiated SMCs exhibit an increased rate of proliferation, migration, and synthesis of ECM components and have decreased expression of SMC specific contractile markers, such as smooth muscle myosin heavy chains SM-1 and SM-2, smooth muscle α-actin, caldesmon, calponin, and smoothelin [148, 150]. They have an increased expression of SMC synthetic markers including, vimentin, non-muscle myosin heavy chain B (SMemb), topomyosin 4, cellular-retinol binding protein and caldesmon-light chain [148]. This phenotype is known as synthetic or dedifferentiated SMCs. The rhomboid-shaped dedifferentiated SMCs are involved in the pathogenesis of atherosclerotic lesions, restenosis, vein graft arterialization, and several other obstructive vascular disease processes [151]. During normal vessel development, VSMCs undergo de-differentiation but at the same time exhibit contractile capabilities. Once they are completely mature, VSMCs return to a non-proliferative, contractile phenotype [152]. In CABG, SVG undergoes trauma, endothelial denudation, and the subsequent release of multiple growth factors, which play a major role in the phenotype switch [153]. The resident SMCs of the media proliferate and migrate to the neointima, and subsequently secrete ECM with
further SMC proliferation. The plasticity of the VSMC phenotype makes these cells susceptible to several stimuli. Upregulation of VSMC phenotype switching contributes to the development and progression of the intimal hyperplasia, followed by atherosclerosis, resulting in graft failure [154-156]. Several factors contribute to this phenotypic switch (Figure 2), including:

- Environmental factors including cytokines, cell-cell contact, cell adhesion, ECM interaction, trauma, and mechanical forces such as shear and stretch [157]
- Growth factors including PDGF-BB, FGF, and EGF [158, 159]
- The promoters of multiple SMC contractile marker genes including SMA and SM22 have a pair of CArG-box motifs, (CC(A/T)_{6}GG), which are necessary for its expression. Degeneracy of CC(A/T)_{6}GG (CArG) is required for SMC phenotypic switching to the motile form [160, 161]
- Repression of smooth muscle 22α (SM22α) gene expression
- Expression of platelet-derived growth factor receptor-beta (PDGFRβ), Kruppel-like factor 4 (KLF4), and specificity protein 1 (Sp1) [160-162]
- Silencing of SMC-specific marker gene by histone acetyltransferases, deacetylases, and methyltransferases [160-162]
- TGF-β, oxidized phospholipids, and retinoic acid act upon the SMC chromatin structure [162]
- Calmodulin-dependent protein kinase II delta (CaMKIIδ) mediated Ca^{2+} signaling [163, 164]
- Reduced-expression of miR-143 and miR-145 [151, 165]
There are several signaling pathways that are found to regulate VSMC dedifferentiation [151, 155].

Figure 2: Phenotype switching of vascular smooth muscle cells.

Post-CABG, the SVG undergoes trauma followed by endothelial injury exposing the subendothelial matrix and SMCs in the media. Endothelial injury leads to the release of various vasoactive mediators and growth factors that convert “contractile SMCs” to “synthetic SMCs,” and leads to the development and progression of neointima.
1.7. Platelet Derived Growth Factor

Platelet derived growth factor is a serum growth factor for SMCs, glia cells and fibroblasts, which was identified three decades ago [166]. The PDGF family consists of four different polypeptide chains, PDGF-A, PDGF-B, PDGF-C and PDGF-D encoded by four different genes. These four different polypeptide chains exist as homo- or hetero-dimers, PDGF-AA, -AB, -BB, -CC, -DD. The four PDGFs are inactive in their monomeric forms. PDGF-A is 196-211 amino acids long, PDGF-B consists of 241 amino acids, PDGF-C and PDGF-D are 345 and 370 amino acids long [166, 167]. All subtypes of PDGF contain a common growth factor domain of 100 amino acids. \(PDGF-A, PDGF-B, PDGF-C\) and \(PDGF-D\) are the four different genes that code for the four different polypeptide chains and are located on chromosome 7, 22, 4, 11, respectively [168]. \(PDGF-A, B\) and \(D\) have seven exons, whereas PDGF-C has six. However, there is a difference in the size of introns between classical (PDGF-A and B) and novel (PDGF C and D) PDGF polypeptide chain [168, 169]. The highest levels of PDGF-B are found in heart and placenta, while moderate amount of PDGF are found in other organs [170]. The crystallographic structure of PDGF-B revealed that the two subunits in the dimer are arranged in anti-parallel fashion and each subunit is built up of anti-parallel \(\beta\)-strands [171]. PDGF plays a critical role during development and is linked with several diseases and pathological conditions. Overexpression of PDGFs are majorly associated with tumor formation, fibrosis and vascular disease [172].
1.7.1. PDGF Receptor

PDGFR-α and PDGFR-β are tyrosine kinase receptors (RTKs) that bind PDGF [173]. They consist of a common domain structure with five extracellular immunoglobulin loops and an intracellular tyrosine kinase domain. Binding of PDGF to the receptor causes its dimerization followed by autophosphorylation of the tyrosine residues in the intracellular domain, thus initiating signaling. PDGF-AA, PDGF-BB, PDGF-AB and PDGF-CC binds to PDGFR-α and PDGFR-α/β, whereas, only PDGF-BB and PDGF-DD binds to PDGFR-β [167, 174, 175]. In vivo, PDGFR expression pattern is constitutively low but increases during inflammation [176]. PDGFR-α is expressed in mesenchymal cells or their progenitor cells and PDGFR-β is mostly expressed in VSMCs or pericytes [166]. Both of the PDGF receptors induce signaling pathways such as Ras-MAPK, PI3K and PLC-γ [166].

Upon activation of the PDGFR, Grb2/Sos complex binds to the activated PDGFR, thereby allowing interaction with Ras followed by the exchange of GDP for GTP on Ras. Ras interact with Raf-1 and activates MAPK-pathway. MAPK signaling pathway results in gene transcription, cell proliferation, differentiation and migration [177, 178]. PDGFRs activate PI3K pathway and promotes actin reorganization, cell proliferation and inhibition of apoptosis [179]. PLC-γ binds to the activated PDGFR and stimulates various intracellular processes such as cell growth and motility [166]. PDGFR interacts with integrins and other molecules at focal adhesions, where several signaling pathways initiate and cross-talk, which enhances cell proliferation, migration, and survival [180].
Ligand bound PDGFR undergoes endocytosis, and then the internalized receptors/ligand gets degraded in the lysosome limiting the duration of the PDGFR-mediated signaling. c-Cbl ubiquinates PDGFR-β causing its lysosomal degradation and the phosphatase TC-PTP negatively regulates PDGFR-β [166].

1.7.2. Role of Platelet-derived growth factor (PDGF) in vein-graft diseases

Pre- and post-revascularization endothelial injury leads to the accumulation of platelets and macrophages at the site of the injury. Growth factors such as PDGF released from the accumulated platelets, SMCs and monocytes, induces proliferation and migration of SMCs to the site of injury thus compromising the vein graft patency. Mechanical stresses stimulate PDGF production, thereby initiating anti-apoptotic and pro-proliferating and migratory signaling pathways. Surgical or traumatic injury and mechanical forces on the vein graft activates PDGF receptor-α and stimulates vascular SMCs [181].

Upon PDGF-BB stimulation, SV-SMCs proliferate more rapidly than IMA-SMCs [94]. PDGF-BB induced the expression of p42mapk subunit of MAPK and decreased expression of cell cycle inhibitor protein p27Kip1 in SV-SMCs compared to that of IMA-SMCs. 10ng/ml of PDGF induced greater activation of MAPK in SV-SMC than IMA-SMCs. This differential effect of PDGF on vein and arterial SMCs may contribute to the early development of IH in vein-grafts [182]. Further studies are required to define the role of the increased activity of the upstream kinases (MEK-1/2) and phosphatases in the activation of MAPK and the contribution of the activated MAPK on venous graft failure. In organ culture, PDGF-BB produced a relatively profuse IH with increased elastic fibers
in SV compared with IMA [183]. c-Fos and c-Jun are the two heterodimers of the transcription factor activator protein (AP-1), which regulates cell proliferation, differentiation, and apoptosis in response to several stimuli. The expression of c-Fos is greater in SV-SMCs [183].

PDGF and its receptors are found to be upregulated at the site of vascular injury and in the vessel wall in atherosclerotic plaques. Antibody against PDGFRβ reduced neointimal size after one month in a baboon model of arterial injury. Increased expression of PDGFRβ was observed in injured vein graft than in the preimplanted veins [184]. Tyrosine kinase inhibitor Gleevec or blocking the PDGF receptors α and β caused regression of the neointima in the arterial grafts [184]. Plasma C-reactive protein (CRP) was found at an elevated level during stress, trauma, or disease that are characterized by inflammation [185]. Studies showed immunolocalization of CRP in the media and adventitia of failing SV graft [186]. CRP directly stimulates migration and upregulates the expression and activation of PDGFRβ [187]. All these studies suggest a link among SV injury, inflammation, CRP and PDGFRβ [187].

The above-mentioned studies suggest that PDGF-BB is one the most potent chemoattractants and mitogen for VSMCs. Numerous biological processes are initiated by PDGF-BB through activation of MAPK. Blocking of PDGF receptors α and β regressed the neointima in the arterial grafts [184]. From these studies we may conclude that PDGF not only play a major role to drive growth of the intima, but also to prevent regression of established thickening of the intima.
1.8. Other Factors Involved in Vein-Graft Failure

Revascularization causes trauma to the vessel lumen, leading to endothelial denudation and dysfunction. Implantation of a venous graft into the arterial system lowers endothelial nitric oxide synthase (eNOS) level [188]. In SVG, NO has vasorelaxant, antithrombotic, anti-inflammatory and antiatherogenic effects [189]. At the basal level, endothelial cells forming the intimal layer of the vein produces lesser NO compared with IMA suggesting less endothelium-dependent relaxation in the SV. Moreover, surgical preparation, pressure dilatation and arterialization of the vein may severely impair the NO-mediated endothelial function of SV, which may contribute to the poor long-term patency of SV coronary graft [190, 191].

Venous grafts have increased expression of angiotensinogen and secretion of several enzymes such as angiotensin-converting enzyme (ACE). Renin catalytically cleave angiotensinogen to Angiotensin I (AngI) [192], subsequently, ACE converts AngI to Angiotensin II (AngII) [193]. This results in an excess production of AngI and AngII in the venous graft. Increased expression of angiotensin II receptor, type 1 (At1R) was noted in response to vessel injury [194, 195]. AngII causes further damage to endothelial cells by initiating several proinflammatory responses, such as cell adhesion molecule expression (VCAM, ICAM, MCP-1) and the production of tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6). AngII augments SMC proliferation by activating PDGF, bFGF, and transforming growth factor-beta (TGF-β). Vascular ACE expression was significantly higher in human vein compared to artery [196-201]. SV produces higher ROS compared to IMA. Human SV produces higher levels of NADPH oxidase.
(NOX1, NOX2, p47phox) -dependent ROS than IMA. AngII induces NOX2 or p47phox, and mediates ROS production [202]. Nox1 and NOX2 are functional in SV SMCs. Therefore, AngII increases NOX2 mediated ROS production in SV-SMC, which contributes to the pathogenesis of intimal hyperplasia [203, 204].

Intravascular injury induces VSMCs phenotypic switching to the dedifferentiated form, which is associated with an increase in the expression of connexins [205]. AngII and insulin-like growth factor 1 (IGF-1) induce differential expression of Cx-43 in SMCs of SV and IMA. The group demonstrated higher expression Cx-43 in SV SMCs compared with IMA SMCs, when exposed to IGF-1 and AngII in a dose- and time-dependent manner. IGF-1 and AngII regulates Cx-43 expression through ERK 1/2 and p38 pathways [206, 207].

Following CABG, endothelial injury let components of the blood stream to come into direct contact with the sub endothelial SMCs leading to the activation of the coagulation cascade, resulting in generation of thrombin [208]. Studies have shown that thrombin is generated, even after thrombus formation, which serves purposes other than coagulation [209-211]. Both SV and IMA SMCs and endothelial cells have functional thrombin receptors [99]. Binding of thrombin to its receptors on endothelial cells cause vasodilation in IMA, but contraction in SV [96]. When thrombin binds to its receptors on SMCs it causes greater proliferation in SMCs of the SV than the IMA. Thrombin was found to be more potent in inducing this growth-signaling pathway in SV SMCs than IMA SMCs [99, 212].
Tissue plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA) are expressed at low levels in the endothelium and medial SMCs of normal SV and IMA [213]. uPAR (urokinase receptor) is found only in the endothelium of a normal vessel. Increased expression of tPA and uPA were found in the intimal and migratory SMCs of stenosed SVGs [212]. Affymetrix microarray compared the gene expression profiles of SMCs cultured from human IMA and SV. The study showed that tissue factor (TF) and tPA, to be differentially expressed between IMA and SV. SV SMCs showed a higher expression of TF, a thrombus-initiating protein, than IMA SMCs. On the other hand, tPA, the regulator of fibrinolysis, was highly expressed in IMA SMCs compared with SV SMCs. These may be a few among many factors that contribute to the characteristic resistance of IMAs to occlusion [212].

IL-1β, TNF-α, interferon-γ (IFN-γ), and bacterial lipopolysaccharide, together induces higher expression of COX-2 protein in human SV-SMCs than IMA-SMCs [214]. This may be due to a relatively more active negative feedback inhibition of COX-2 expression in IMA SMCs compared with SV SMCs. IFNγ stimulates transcriptional activity of pro-proliferative genes through Stat-3 and causes SMC phenotype switching and proliferation leading to IH [215]. Transcription factor Stat-3, which is an oncogene, may undergo phosphorylation/activation in the presence of IFNγ [214].

Leukotrienes are pro-inflammatory mediators released by leukocytes that accumulate at the site of injury [216, 217]. A comparative study on the effect of cysteinyl leukotriene on human SV and IMA showed an increased contractile effect on SV compared with that
of IMA, which varied in a dose-dependent manner [218].

1.9. Cell Cycle

Cell division and the progress of cells through their respective cell cycles are finely regulated and controlled at specific junctures by a complex interplay between kinases and phosphatases [219]. Understanding the critical role of these kinases and phosphatases as major regulators of the cell cycle marked the start of a new era of increased comprehension of cell cycle progression.

Restenosis after percutaneous coronary intervention and vein graft failure post CABG, are chronic pathological conditions that proceed to obstructive vascular lesions over time. SMC phenotype switching and proliferation leading to IH is the primary pathology associated with vessel re-narrowing [148, 220, 221]. In the G1- and G2-phases of cell cycle, the mitogenic and anti-mitogenic signals are combined and the cells exit, pause or continue through the cell cycle [222]. Beyond these two Restriction (R)-points (G1 and G2), cells are committed to further progress through the cell cycle, independent of extracellular stimuli [223]. At the G1 R-point, tumor suppressor proteins such as p27KIP1, Rb, PTEN are down regulated in mitogen stimulated vascular SMCs increasing the transcription and stabilization of cyclin D, which critically contributes to the development of IH [224]. Cyclin D is the first cyclin produced in the cell cycle, in response to extracellular signals such as growth factors. However, the second restriction site in the cell cycle, G2 R-point, may prevent proliferation of the cells, which have lost proper G1/S control [225]. Cyclin B kinases and CDC25 are the major players in G2-
phase that push cells through G2 to M phase [226, 227]. PLK1, activated during G2-M phase, activates CDC25 and controls the activity of CDK1/Cyclin B1 complex thus promoting the G2/M phase transition [228-232]. Injection of anti-PLK1 antibodies in HeLa cells mitotically arrested the cells to divide. PLK1 mediates many mitotic events, including entry into mitosis [233].

1.10. Polo like Kinase 1 (PLK1)

Polo-like kinase 1 (PLK1), one of the polo-like kinase family member, is a serine/threonine kinase that regulates cell cycle progression and mitosis [219]. The expression and activity of PLK1 is elevated in tissues and cells with high mitotic index, such as cancer cells [234-238]. Cancers including breast [234], ovarian [236], endometrial [235], prostate [237], and colorectal [238] are found to have such elevated expression and activity of PLK1. In 2012, Weiwei Shan and colleagues [239], reported increased expression of PLK1 in uterine leiomyosarcomas. In addition, inhibition of the PLK1 upstream kinase, Aurora A kinase decreased the proliferation and induced apoptosis in these malignant cells [239].

Structurally PLK1 has two domains, C-terminal polo box domain (PBD) and amino terminal-kinase domain. PBD targets substrate for sub-cellular localization regulating the functional aspect of PLK1 [240]. On the other hand, the kinase domain is activated upon phosphorylation by other kinases [241-244]. Phosphorylation at Thr210 in the T-loop and Ser137 at the end of the hinge region is required for the activation of PLK1 [245]. Phosphorylation at Ser137 either disrupts the interaction between the kinase and PBD or
leads to the binding of protein aurora borealis (Bora) to the PBD. This binding exposes the Thr210 residue in the T-loop to other activating kinase [246]. A small region called the destruction box (D-box), located distal to the kinase domain is essential for PLK1 degradation [247, 248] (Figure 3). The D-box is not conserved in PLK2, PLK3 and PLK5 [247]. PLK4 is degraded by autophosphorylation of the S phase kinase-associated protein 1 (SKP1)–cullin–F-box Slimb-binding domain [249]. Similar structural architecture was found in all the PLK members, PLK1, 2, 3, 4 and 5 [247]. PLK1-3 has an identical catalytic kinase domain, PLK4 has a divergent primary sequence, and PLK5 has a pseudokinase domain [250, 251]. PLK2, PLK3 and PLK5 are mostly found in non-proliferative tissues and have tumor suppressor function, whereas PLK4 might have a role in mitotic progression, which is yet to be confirmed [252]. PBD regulates PLK1’s localization, substrate binding and its catalytic activity [253]. Proteins such as Bub1 that are already ‘primed’ through phosphorylation by other kinases are optimal for PLK1 binding [254]. However, PBD can also bind to non-phosphorylated proteins or self-primed ones. The catalytic activity of the kinase domain can be inhibited in three ways: PBD binds to the kinase domain and mutually inhibit one another, PBD can also bind to microtubule-associated protein 205 (Map205) thus stabilizing the auto inhibited state of the kinase domain, and the interdomain linker that links the kinase domain with the PBD and sequesters the T-loop can also bind the kinase domain and inhibit its activity [251]. PLK1 to be catalytically active these multiple inhibitions need to be partially or completely released, which may be achieved by either phosphorylation of the Thr210 and Ser137 or by binding of a partner, such as protein aurora borealis (Bora), to the PBD [251, 255]. PLK1 is ubiquitinated and degraded by E3 ubiquitin ligase APC/C [256].
**Figure 3:** The schematic structure of PLK1, which is 603 amino acids (aa) in length.

The serine/threonine kinase domain is at the N-terminal and the Polo-Box domain (PBD) is located at the C-terminal. The PBD is composed of two similar Polo Boxes (PB1 and PB2) separated by a Loop 2 switch region. PC is the Polo Cap that holds the polo boxes in the correct orientation. The arrows indicate the two main activating phosphorylation sites of the kinase domain. The two domains are separated by the Inter-Domain Linker, which contains a Destruction Box (DB).

### 1.10.1. Activation of PLK1

PLK1 undergoes a reversible phosphorylation at multiple sites to become completely catalytically active [241-244]. At G2 phase, Aurora kinase A phosphorylates PLK1 at Thr210 in presence of the cofactor Bora that facilitates the reaction [242] (Figure 3). Studies also showed that CDK 1 phosphorylates Bora and promotes PLK1 phosphorylation by Aurora kinase A [257]. Bora, along with the phosphoinositide 3-kinase (PI3K)–AKT pathway phosphorylates Ser99 in the kinase domain of PLK1 and creates a binding site for 14-3-3γ. This phosphorylation is essential for full activation of the protein and proper progression from metaphase to anaphase [244]. Depletion of 14-3-3γ attenuated mitotic PLK1 activity thus suggesting that PLK1 binding to 14-3-3γ stimulates its catalytic activity [244]. In PI3K–AKT pathway, 3-phosphoinositide–dependent protein kinase-1 (PDK1) is activated upon elevation of PIP3 owing to the loss of PTEN [258]. This activated PDK 1 phosphorylates PLK1, which in turn induces
phosphorylation of MYC [243]. Protein kinase A (PKA) and STE20-like kinase (SLK) phosphorylate PLK1 at Thr210 in vitro [259, 260]. In late mitosis, PLK1 gets phosphorylated at Ser137 in vivo, which might be essential for regulation of the spindle assembly checkpoint (SAC) [261]. p21-activated kinase can induce phosphorylation of PLK1 at Thr210 even in the absence of Aurora A kinase [262]. Another study noted that in response to DNA damage, ATM/ATR-regulated Chk1 and Chk2 kinases might be involved in the inactivation of PLK1 thus stopping the mitotic progression [263].

1.10.2. PTEN-P53-PLK1 Pathway

PTEN regulates the AKT/PKB signaling axis resulting in cell survival and proliferation [127]. A study in prostate cancer demonstrated that PTEN-depleted cells suffer from mitotic stress and PLK1 plays a critical role in these cells to adapt to mitotic stress for survival [264]. Therefore, PLK1 facilitates prostate cancer formation in PTEN-null cell. PTEN positively regulates the activity of anaphase-promoting complex/cyclosome (APC-Cdh1), which in turn can degrade PLK1 protein [264]. Again in cancer cells, PLK1 could cause phosphorylation-mediated inactivation of PTEN and Nedd4-1, an E3 ubiquitin ligase of PTEN protein, thus activating PI3K pathway [265, 266]. Previously we showed that PTEN was significantly more active in IMA-SMCs than SV and PTEN overexpression in SV SMCs induced the transactivation of p53 [127]. PLK1 inactivates p53 in several ways – PLK1 can physically interact with p53 and inhibit its transactivation [267], PLK1 can activate MDM2 and lead to p53 degradation [268], PLK1 can inactivate Topors causing p53 degradation [269], PLK1 can activate GTSE1 and degrade p53 [270] and finally PLK1 can activates CDC25C leading to MDM2-
mediated degradation of p53 [271]. On the other hand, p53 can directly bind to the PLK1 promoter site and inhibit its expression [267]. p53 can also activate microRNA such as miR-143 that in turn inhibits PLK1 expression [272, 273]. p53 inhibit the activity of FoxM1, a transcription factor for PLK1 [274, 275]. Therefore, PLK1, PTEN and p53 interconnect and suppress each other in multiple signaling pathways (Figure 4).

Figure 4: P53-PTEN-PLK1-interacting pathways.

P53, PTEN and PLK1 proteins negatively regulate each other to maintain proliferative control. Inactivation of P53 and PTEN result in mitotically stressed cells that depend on PLK1 to overcome this stress leading to mitotic progression. Cells with high mitotic indexes are sensitized to PLK1 inhibition. PTEN controls the APC/cyclosome (APC-Cdh 1), which is responsible for PLK1 degradation. PTEN directly interacts with CENP-C, and facilitates centromere integrity. Topors is SUMO E3 ligase, and its phosphorylation by PLK1 inhibits sumoylation of p53 while enhancing ubiquitination of p53 and thereby
leading to its degradation. PLK1-mediated phosphorylation of GTSE1 activates and promotes its nuclear accumulation subsequently shuttling p53 to the cytoplasm for its degradation.

1.10.3. Regulation of PLK1 activity

Both Protein phosphatase 1 and Protein phosphatase 2A, can dephosphorylate PLK1 at Thr210 [276]. At the centromeric regions, PP2A dephosphorylate PLK1 and maintains a pool of persistent cohesion. On the other hand, PP1 dephosphorylate PLK1 and suppress premature loss of cohesion [277]. Mypt1 is a regulatory subunit of PP1 that dephosphorylates PLK1 [278]. Optineurin is a phosphatase and interacts with Mypt1 that indirectly regulates PLK1 activity. PLK1 phosphorylates optineurin, which then translocates to the nucleus and activates Mypt1 that in turn dephosphorylate PLK1 [279] (Figure 5). The balance of kinase and phosphatase activities determines chromosomal patterns of cohesion along the metaphase chromosome axis in cell cycle.

1.10.4. Transcription of PLK1

E2F transcription factor 1 (E2F1) [280], phosphorylated-signal transduction and transcription (pStat-3) [281] and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [282] are implicated as the transcription factors of the PLK1 gene [275, 281, 282]. E2F1 coordinates with p53 in regulating the apoptotic and proliferative gene expression thus maintaining homeostasis [283]. Upon DNA damage, p53 directly binds to a p53-responsive element in the promoter of PLK1 and suppresses PLK1 expression [284]. p53 can also form a p53-E2F1 DNA complex suppressing E2F1-dependent PLK1 expression [285]. Expression of PLK 2 and 3 are upregulated upon DNA damage, which activates p53-mediated G2-checkpoint [284]. PLK1 stabilizes β-catenin, which in turn
transcriptionally activates Stat-3. The activated Stat-3 transcribes **PLK1**. This positive feedback loop between PLK1 and Stat-3 leads to uncontrolled proliferation in esophageal cancer cells [281]. p65, a protein involved in the NFκB heterodimer is found to directly binds to the PLK1 promoter and activates it. Resulting in phosphorylation of β-catenin, which inhibits ubiquitination of β-catenin in esophageal squamous cell carcinoma [286] (Figure 5).

**Figure 5**: PLK1 function in mitotic entry.

A complex regulatory network controls the activity of cyclin-dependent kinase 1 (CDK1)–cyclin B and promotes mitotic entry. WEE1-like protein kinase (WEE1) and MYT1 kinases inhibit CDK1 by phosphorylating them at residues Thr14 and Tyr15. Polo-like kinase 1 (PLK1), activated through phosphorylation at its T-loop (Thr210) by several kinases including, Aurora kinase A, Bora, Protein kinase A (PKA), STE20-like kinase (SLK), 3-phosphoinositide–dependent protein kinase-1 (PDK1) and p21-activated kinase. Protein phosphatase 1 can dephosphorylate PLK1 at Thr210. E2F transcription factor 1 (E2F1), phosphorylated-signal transduction and transcription (pStat-3) and
nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) are the transcriptional factors of the PLK1 gene. Dashed arrows indicate non-functional pathways. Gray color indicates inactive form of the protein. P, phosphate.

1.10.5. PLK1 and Mitosis

PLK1 mediates multiple mitotic processes including centrosome maturation [287], assembly of bipolar spindle [288], chromosome segregation [289], activation of anaphase promoting complex (APC/C) [290], and cytokinesis [252, 255, 271, 291-294]. Other than mitotic progression [252], PLK1 also mediates DNA replication [295], G2 DNA damage checkpoint recovery [296], and chromosome and microtubule dynamics [252, 255, 271, 291-294]. Subcellular location of PLK1 is critical for its function. During interphase, PLK1 is located at cytoplasm, microtubules and centrosomes. Throughout mitosis, PLK1 is present in kinetochores, centrosomes, microtubules, central spindle and midbody [297]. PBD of PLK1 has highly conserved residues that mediate significant target specificity [298]. PLK1 phosphorylates Ninein-like protein (NLP) at both Ser237 and Ser1463 sites during mitosis for centrosome maturation [299]. PLK1 also phosphorylates Kizuna at Thr379 to maintain centrosome architecture [300]. CDC25 (Ser198) [301], Myt 1 (Ser426, Thr495) and Wee 1 (Ser53) are phosphorylated by PLK1 and lead the activation of CDK1/Cyclin B1 complex [247, 248, 302]. PLK1 phosphorylates cyclinB1 at Ser133, 147 and triggers the transition of G2/M phase [297]. During M-phase, PLK1 phosphorylates PBD interacting protein (Thr78) [253], inner centromere protein [303], mitotic checkpoint kinase Bub1 [304], Spindle checkpoint component BubR1 [304], NudC (Ser274, 326) [305], and Emi1 (Ser145, 149) [306] to recruit PLK1 at the kinetochore and regulate the activity of APC/C complex [254]. PLK1 might play critical roles in promoting kinetochore attachment or in spindle checkpoint regulation [307].
PLK1 phosphorylates mitotic kinesin-like motor protein (Ser904, 905), Prc1 (Thr602), HsCyk-4 (Ser149, 157, 164,170, 214, 260) and Ect2 (Thr412) to critically regulate various steps in cytokinesis [308-311] (Figure 6).

PLK1 plays an important role in DNA replication and link mitotic events with S-phase [312]. PLK1 phosphorylates histone acetyltransferase binding to the origin recognition complex1 (Hbo) at Ser57 during mitosis. pHbo is required for pre-RC formation and DNA replication during S-phase [313]. Under various stresses, including UV, hydroxyurea, gemcitabine, or aphidicolin treatments, PLK1 phosphorylate origin recognition complex 2 (Orc2) at Ser188 and maintain DNA replication [295]. PLK1 is critical for early embryonic development and chromosomal stability [314]. In normal cells, PLK1 expression and activity is strictly regulated and peaks at the G2–M transition, plateaus throughout mitosis, and has a sharp reduction by proteosomal degradation upon mitotic exit. Deregulated expression and activity of PLK1 leads to uncontrolled proliferation that correlates with carcinogenesis [315] (Figure 6).
Figure 6: Functions of PLK's in multiple stages of cell cycle regulation.

PLK1 directly interacts with several proteins and plays a role in mitotic entry, spindle assembly, anaphase entry and cytokinesis in mitotic phase and DNA replication. In G1 phase, PLK2 and PLK4 promote centriole duplication and in S phase, PLK3 regulates DNA replication.

1.11. Swine Model of Coronary Restenosis and CABG

The progress made in understanding of the pathophysiology of diseases in human would not have been possible without animal models that replicate the pathology. The species and interventions used to replicate the disease pathology depend on the scientific
question, ethical and economical considerations, accessibility and reproducibility of the model. Several models have been used to provide new approaches to improve the diagnostic and the treatment of these pathologies. Genetic and environmental factors [316] play a critical role in cardiovascular disease making it difficult for a single experimental model to match the complex nature of human pathological condition [317]. However, swine is a good model to study cardiovascular diseases. Both, pig anatomy and coronary artery system are similar to human except the presence of left hemiazygous vein draining into the coronary sinus [318]. In CABG model of swine, the left and right superficial epigastric veins (SEVs) have similar size to branches of the main human saphenous vein, and SEV can be easily harvested [319, 320]. The hemodynamics of pigs including mechanically induced myocardial infarction, reperfusion induced arrhythmogenic activity and wound-healing process are similar to humans [321]. Atherosclerosis can be spontaneously induced in swine by regular chow and by experimental atherogenic diet [322]. Standard diagnostic and interventional equipment used in humans can be used in swine [323]. All these reasons together make swine model widely acceptable as an ideal animal model to study fibroproliferative vascular diseases that involve an interventional procedure or surgery [324, 325].

1.12. Why is PLK1 a novel target to limit IH and restenosis?

Cancer cells are the ideal models of unregulated increased cell proliferation, and upregulated activity/expression of PLK1 has been associated with a wide spectrum of human cancers [230, 234, 236-238, 248, 281]. Hence PLK1 is widely accepted as a proliferation marker leading to oncogenic transformation [326]. Extensive volume of
literature cites the association of PLK1 with G2-M transition and mitosis in numerous cancer conditions [230, 234, 236-238, 248, 281]. Therefore, it is plausible that an association exists between phenotypically altered highly proliferating SMCs and PLK1 in non-neoplastic pathologies such as IH.

Once activated, PLK1 phosphorylates and activates a wide range of substrates including CDC25 [327], Cyclin B1 [232], CDK1 [328] promoting mitotic progression. Therefore, PLK1 acts as a hub, where it receives signals at a specific time and place and in turn reacts by transmitting multiple signals to the effector proteins. Given its well-established functions in regulating cell cycle and promoting proliferation in human cancers, we singled out PLK1 for further study in hyperplastic VSMCs. We focused our initial efforts on examining the role of PLK1 for several reasons: first, PLK1 functions in activating the major kinases [241-244], phosphatases [276, 279] and cyclins [232] that promotes mitotic entry and progression through the G2-M cell cycle check point, second, PLK1 overexpression has been well documented in cells with high mitotic index including the SMC tumor, leiomyosarcoma [239], third, a number of small ATP-competitive inhibitors such as BI 2536 [329, 330] and BI 6727 [331], highly selective for PLK1 are currently in different phases of clinical trial against other common human cancers and show promising therapeutic values. Essential role played by PLK1 in cell cycle progression is evident, but upregulation of it may lead to a diseased condition. Understanding how and when to use PLK1 as a target for the development of therapy to inhibit IH and restenosis is crucial.
1.13. Current approaches for neointimal hyperplasia and restenosis

As discussed earlier, closure of the vein graft post CABG occurs as a result of IH within a time period of months to years. Vein graft intimal thickening and remodeling [332] occurs as an adaptation to increased wall tension and arterial flow, as a result of persistent inflammation [333] and IH [334]. The interaction between the VSMCs and surrounding extracellular matrix (ECM) maintains the SMC in quiescent state. Along with the growth factors secreted at the site of the vein-graft injury, ECM remodeling by extracellular proteases, including plasminogen activators, heparanases and matrix metalloproteinases (MMPs) is also required for SMC proliferation and migration [335, 336]. MMPs, the extracellular protease, is critical for SMC migration from the medial layer towards the intima and proliferation in the neointima [337]. Under physiological condition proteolytic activity of MMPs are controlled by tissue inhibitors of metalloproteinases (TIMPs) [338]. Increased expression of MMP and reduced expression of TIMPs is associated with the development of VGD [339]. Different animal models are used to investigate the effect of treatments to prevent vein graft disease [340]. Many therapies showed promising result in reducing VGD but were found to be less successful when tested clinically [341]. A few of the potential limitations of the therapies targeted to VGD are as follows:

I. Rodent, particularly rats and mouse, is frequently used as an animal model for VGD. However, rodents are not proven reliable at predicting the outcome of all studies in humans [342]. We cannot replace all animal experiments in the immediate future, the highest standards of welfare are upheld [343]. The use of a
species that have similar anatomic and physiologic characteristics with humans will eliminate less appropriate therapies from progressing to the clinic [344-347].

II. In animal model easily accessible arteries, which may not have a problem clinically is used to examine the response to a treatment. This does not always give the right idea of the effect of the treatment to an injury [348].

III. The doses of drugs that are effective in animal models are often not used clinically. This may be because of the compliance issues or due to the undesirable side effect in humans [349].

IV. Proper timing for the administration of the drug is crucial to treat a disease. At times pretreatment with the drug is important to get the desired effect. Many a times a drug is administered later than its optimal dosing time [350].

In order to diminish undesired side effects the agent should be administered locally rather than systemically. There are many ways an agent can be locally delivered, including the use of drug-eluting stents [351], catheters coated with a gel containing the agent [352], perivascular delivery where the agent is placed around the engrafted vein [353], drug-linked antibodies [354], and the use of nanoparticles [355]. There is a considerable interest in the \textit{ex vivo} treatment of the vein after it is harvested and in the use of gene therapy [341, 356]. This section will review the drugs that have been tested in vitro or in vivo, and pre-clinical and clinical studies of gene therapy for VGD.

Anticoagulant heparin was covalently coated on the stent, which was placed in the pig coronary artery and were found to be effective in limiting SMC proliferation [357].
However, other researchers using a similar model failed to find a difference in neointimal development compared to bare-metal stent [358]. Clinical trials of the heparin-eluting stent [358] showed it was effective in reducing thrombotic events and the degree of restenosis but another trial using the low dose of heparin was inconclusive [358]. Thus this heparin site-delivery studies were labeled as “conflicting” and further research was stopped in this area [359-361]. Low molecular weight heparin (LMWH) inhibited proliferation of cultured human SMC [362]. In vivo, ten times the clinical dose of LMWH was needed to maintain the lumen diameter in a rabbit models [363]. Clinical studies found that high dose LMWHs may improve patency in peripheral bypass grafts, but cannot prevent restenosis [364]. Further modifications showed that LMWH when conjugated with nanoparticle and delivered, decreased neointima formation and increased patency compared with the control agents in rats [365]. Hirudin-coated stents reduced thrombus formation and the neointimal area in pig arteries [366]. But systemic delivery of therapeutic dose of antithrombotic agent such as hirudin may lead to uncontrolled bleeding or rebound thrombosis [367]. SMCs at the site of VGD have impaired tissue plasminogen activator (tPA) production, and local delivery of tPA gene to the vein graft in pig limited the acute thrombus formation following implantation of the graft into pig carotid arteries [368]. Major limitation was the dose of tPA, as the higher tPA may result in early extracellular matrix degradation and increased neointimal formation [368]. Agents that block the platelet GPIIb/IIIa receptor such as aspirin, ticlopidine, roxifiban, and clopidogrel clinically reduce the incidence of cardiac events but are not effective in reducing restenosis [369]. Stent-delivered abciximab, antibody against the GP IIb/IIIa receptor, in pigs showed reduction in neointimal formation and inflammation levels [370-
Vasodilator and antithrombic, Iloprost when given systemically, it improved flow through artificial venous replacement grafts. However, this effect did not last for long [373]. Cilostazol-eluting stents reduced neointimal area in pigs compared with bare metal stents and cilostazol-rich pluronic gels reduced neointimal formation in injured rat arteries [374].

Stents coated with both dexamethasone and rapamycin prevented in-stent restenosis in pig and dog models [375]. Early results from clinical trials of dexamethasone-bound stents had favorable results that led to further trials using catheter to site-deliver dexamethasone to the adventitia via the lumen [376-378]. Prevention of REStenosis with Tranilast and its Outcomes (PRESTO) study showed that anti-inflammatory drug, tranilast had no effect on the restenosis but targeted delivery of tranilast may have an effect on the factors that initiate the restenotic response [379]. Trapidil (a PDGF antagonist), tyrphostin (a SMC proliferation inhibitor via GF receptor activity), angiopeptin (a somatostatin analog) and ACE-inhibitors are designed to limit SMC proliferation. All these have been used successfully in culture system and in animal models, but are not currently suitable for clinical use [380-385]. In culture, Cholchicine reduced SMC proliferation but lacked clinical effect and had adverse side effects [386]. In pigs, antiproliferative paclitaxel when administered locally in vein graft resulted in reduced neointima formation [387]. However, this benefit did not last in the long term. Clinical studies are underway to show the benefit of paclitaxel-eluting-stents in coronary vasculature. Just like paclitaxel, rapamycin has a positive short-term effect only if the drug is administered early [387]. Ex vivo treatment of pig SV with rapamycin prior to its
Engraftment into the carotid artery had a smaller neointimal area at 4 weeks. But 12 weeks post engraftment; there was no difference between control and experimental grafts [372]. Short to medium term clinical trials with rapamycin showed that it prevents the recovery of endothelium and increase fibrin deposition [372].

Statin therapy showed promising results in reducing the incidence of restenosis and also reduces the incidence of in-stent thrombosis in patients with an inflammatory profile [388]. Stent-delivered cerivastatin in pig was effective in improving endothelial function and reducing inflammation and neointima formation [389]. A few studies also showed that statin did not alter the restenosis rate. Vein grafts are found to have increased superoxide levels upon exposure to the arterial environment and an ex vivo experiment indicated that aspirin may prevent thrombosis, spasms and neointima formation in vein grafts [390]. Stent-directed gene therapy with extracellular superoxide dismutase may improve endothelial cell recovery and can benefit in vein grafts [391].

As we have highlighted under section 1, a significant amount of work is done in animal models that demonstrates the efficacy of various gene therapy approaches. In this section we will briefly discuss the Prevention of Recurrent Venous Thromboembolism (PREVENT) trials. Gene therapy against E2F family of transcription factor is the only strategy that has been evaluated in clinical trial. A 14-base pair double-stranded oligodeoxynucleotide was delivered to cells or tissues. It sequesters E2F from genomic DNA and prevents the expression of the target gene that inhibits the cellular proliferation [392]. This study showed promising result in inhibiting SMC proliferation,
IH, and also preserved endothelial function in rabbit model and in human SV [393, 394]. This led to the development of PREVENT clinical trial. In the phase I of this trial, 41 patients undergoing lower extremity bypass surgery received E2F decoy gene. The result of this phase I trial demonstrated safety and biologic efficiency of this gene therapy. In the phase II, 200 patients undergoing CABG received the gene therapy against E2F. Patients, who received E2F decoy had 30% reduction in critical stenosis without any adverse side effect [395]. Promising result from phase II trial paved the path for phase III and IV. In phase III, gene therapy targeting E2F was performed in lower extremity bypass surgery. In phase IV, gene therapy targeting E2F was performed in CABG. Results from phase III and IV did not show significant difference in primary graft patency and graft stenosis at the first year. Hence, this study failed to translate the pre-clinical success into humans [392-396].

The PREVENT trials did not lead to a clinical treatment but they established the fact that gene therapy for VGD is safe and feasible. Although numerous gene therapy approaches have effectively reduced IH and other aspects of VGD in animal models, the development of more sensitive and clinically meaningful endpoints will allow efficient translation of pre-clinical studies into successful therapies. Therefore, continued effort to characterize the molecular mechanisms of VGD in animal models and humans are needed. As discussed before, vein graft disease is a multifaceted disease and a single drug targeted to a single aspect of treatment may not be efficient. Therefore, administration of a single multi-functional drug against most of these pathophysiologies will be more efficient alternative to targeting single aspect of treatment. Hence, additional concerns
must be addressed before moving promising treatment strategies into human trials of VGD.

1.13.1. Targeted Treatments for Vein Graft Disease

Pathogenesis of vein-graft disease is a complex process and still not fully understood. Studies have elaborated on the various factors that might contribute to the increased susceptibility of the SVG to IH and occlusion [397]. Basic science and clinical research outlining the factors responsible for SVG failure have helped surgeons to adopt better harvesting and risk factor management techniques [398]. This has resulted in improvements in the patency rates of SVG over the years [59]. Some of the changes to the pre-, peri-, and post-operative management of CABG patients undergoing SVG implantation are the result of the synergistic efforts of basic science and clinical research [399]. The “no-touch” (nor stripped or distended and harvested on a pedicle) technique for harvesting the SV produces grafts with improved patency than “endoscopic vein-graft harvesting”, which may result in poor graft quality [400]. The primary cellular targets in the development and progression of vein graft disease have been endothelial cells and SMCs [398, 401]. Endothelial damage results in platelet adhesion and activation, leukocyte recruitment, activation of the coagulation cascade, inflammation, phenotypic change, proliferation and migration of SMCs in the vessel wall [402-408]. All of these events may act as a target for molecular intervention. Drugs that could effectively prevent some of the factors leading to graft failure and are discussed as follows:

- Antithrombotic and Antiplatelet Agents - heparin, hirudin, aspirin, ticlopidine, roxifiban, clopidogrel, ridogrel, Ventavis, Pletal [364, 371, 373, 409-414];
- Anti-Inflammatory agent - dexamethasone, bisphosphonates, tranilast [91, 415-419];
- Anti-proliferative and anti-migrating agents – trapidil, tryphostin, angiopeptin, ACE inhibitors [373, 420-422];
- Cytostatic and Immunosuppressive Agents – colchicine, paclitaxel, rapamycin [386, 410, 423-426];
- Antioxidants – probucol, resveratrol, quercetin, aspirin [427-430];
- Regulators of Lipid Metabolism – statins [431-433].

Along with the pharmacological approaches, gene therapy may also be considered as a promising area in preventing vein-graft failure. Studies reported that over expression of endothelial nitric oxide [434], cyclooxygenase-1 [435], superoxide dismutases (SOD) [436], thrombomodulin (TM) have been targeted using gene therapy approaches to improve the patency of the vein-graft [434-444]. Over-expression of TIMPs such as TIMP-1 and TIMP-3 decreased SMC migration and neointimal formation in SVG [445-448]. Over-expression of PTEN can be a potential target for gene therapy to increase the longevity of SVG [449-451]. Experimental studies reported the successful blockage of the expression of proliferating cell nuclear antigen (PCNA), cell division cycle 2 kinase (CDC2), MCP-1, IL-1, IL-6, IL-8, TNF-α, PDGF, TGF-β, NF-κB, Gβγ signaling, and PI3K pathway may prevent vein-graft IH [90, 452-458]. tPA gene therapy in vein-graft limited acute thrombus formation following implantation of the graft into pig carotid arteries [368, 459]. Targeted gene therapy might be the path that leads us to what once seemed an unachievable goal to increase the patency of SVG. However, there is still a
need for well-designed, coordinated, and multi-centered research to focus on the various candidate proteins and genes that have been demonstrated to play a critical role in SVG failure.

Vein-graft disease (VGD) is a multifaceted disease [460] and refinement of treatments to prevent this disease is an active area of medical research. Instead of delivering a drug directed at a single aspect of treatment, it is necessary to administer compounds directed against the various aspects of restenosis. This can be in the form of a single multi-functional drug against many or all of the features that causes VGD. Development of such multiple-drug therapies is the challenge. In order to find that ideal drug or ideal treatment to inhibit VGD, we need a thorough understanding of the molecular mechanisms that underlie IH, which is complex and incompletely understood. Hence, more studies are required to find the “panacea” for SVG failure.

1.14. Research Goals

Given the well-established functions of PLK1 in regulating cell cycle and promoting proliferation in neoplastic cells, it could be a novel target for highly proliferating VSMC in VGD. As we know that the major contributing factor for vein-graft failure is excessive proliferation of VSMC. The role of PLK1 in these hyperplastic VSMCs has never been studied. Therefore, the aim of this study is to understand the role of PLK1 protein in VSMC proliferation (Figure 7). We studied the expression and role of PLK1 in SMCs isolated from human saphenous vein and internal mammary artery. We also investigated the effect of inhibition of PLK1 activity and expression on its downstream proteins, such
as CDC2/CDK1, the master mitosis kinase and histone H3, an established mitotic marker for SMCs. In this study we hypothesized that human SV-SMCs have greater PLK1 expression and phosphorylation than IMA, which in part could explain the increased propensity of the SV conduits to undergo intimal hyperplasia than IMA following CABG. Understanding the role of PLK1 in VSMC proliferation and an in depth comparison of the underlying mechanisms of the PLK1/pPLK1/pCDK1/pHistone H3 axis in the pathophysiology of intimal hyperplasia in human SV and IMA could provide an opportunity to develop better therapeutic strategies to prolong vein-graft lifespan.

**Figure 7:** Central question of the present research.
1.15. **Hypothesis and Aims:**

The central hypothesis is that human SV SMCs have greater PLK1 expression and phosphorylation than IMA, which in part could explain the increased propensity of the SV conduits to undergo intimal hyperplasia following CABG than the IMA (Figure 7).

*Specific Aim 1:* To investigate the expression of PLK1 and pPLK1 in human SV and IMA-SMCs.

*Specific Aim 2:* To determine the downstream mediators of PLK1 in human SV and IMA-SMCs and its role in VSMC proliferation.

*Specific Aim 3:* To assess the presence of PLK1 and pPLK1 in the injured blood vessels following coronary angioplasty and bypass surgery to re-vascularize occluded coronary arteries of swine.

1.16. **Translational Value:**

No one has studied the role of PLK1 in highly proliferating VSMCs. If our hypothesis is tested right, then we will be the first to show the crucial role played by PLK1 in the development of VGD; and inhibition of PLK1 activity can inhibit VSMC proliferation and hence prevent neointima formation/vein graft failure. This may pave the way to use the PLK1 inhibitors, which are currently in clinical trials and are showing promising results against highly proliferating tumor cells, for the management of VSMC-mediated IH (Figure 8).
Figure 8: Clinical significance of the present study.
Chapter 2

Materials and Methods
2. MATERIALS AND METHODS

2.1. Human Tissue Collection and Cell Culture

The Institutional Review Board of Creighton University permitted the research protocol as an exempt status since all tissue samples were collected in an anonymous manner. None of the research investigators could recognize the patients from whom the SV and IMA tissues were obtained (Figure 9). The Institutional Review Board of Creighton University waived the need for consent. Investigators were provided the specimens with the information of age, sex and ethnicity only. Post-surgery, the tissues were collected in University of Wisconsin (UW) (Bridge to Life, Columbia, SC) solution and brought to the laboratory for the isolation of SMCs. The UW solution is commonly used for organ collection and transport. Studies showed that the viability and the function of the organs are well-maintained for at least 24 hrs [461] post-collection. Aseptic techniques were followed for subsequent processing of tissues.
Figure 9: The left over human SV and IMA from a CABG surgery.

The adventitia and the endothelial layer were removed from the tissues by gentle blunt dissection and washed with Dulbecco’s modified Eagle’s medium [DMEM] (Sigma, St. Louis, MO USA) containing 1% penicillin/streptomycin/ amphotericin B solution (P/S/A) (Sigma, USA). Then the tissues were minced with a sterile scalpel in DMEM containing 1% P/S/A, followed by incubation in 0.25% trypsin (1x) solution (Thermo Scientific, Waltham, MA USA) for 30 min at 37°C and then washed with DMEM. Tissues were then subjected to enzymatic digestion with 0.2% collagenase (Sigma Chemical Co., St. Louis, MO, USA) for 3 hrs at 37°C. The cell digest was neutralized with Smooth Muscle Cell Medium (SMCM) containing 10% fetal bovine serum (FBS) and centrifuged at 1500 rpm for 5 min. The cell pellet was suspended in SMCM
supplemented with 20% heat-inactivated fetal bovine solution (FBS) and 1% P/S/A and plated in 25 cm² corning culture flasks (Figure 11). Flasks were maintained at 37°C and 5% CO₂. SMCM was changed every two days and the cells were passaged once they were confluent.

The sub cultured human SMCs, between passages 3–5, were used for the in vitro experiments. The purity of isolated cells was confirmed by staining for smooth muscle α-actin and myosin heavy chain and showed the characteristic hill-and-valley pattern (Figure 10). Over 95% of cells were positive for smooth muscle α-actin and smooth muscle myosin heavy chain.
Figure 10: Protocol for isolation and culture of human saphenous vein and internal mammary artery smooth muscle cells.

2.2. Treatment of Human SV- and IMA-SMCs

When flasks were 70-80% confluent, the cultures were serum starved with DMEM containing 1% heat-inactivated FBS and 1% P/S/A for 24 hrs. The serum-starved cells were then treated with the test substance prepared in DMEM for the required time points. For our experiments we have used 10ng/ml of PDGF-BB (PeproTech, Rocky Hill, NJ) or 100nM BI2536 (Selleckchem, Houston, TX). Post-stimulation, the cells were trypsinized with 0.25% trypsin at 37°C for 5min, then neutralized with SMCM containing 20% heat-inactivated SMCM. Cells were then centrifuged at 1500 rpm for 5 min at 4°C and the supernatant were discarded. The cell pellet was used for cell count, RNA and protein
isolation as per the following lab standardized protocol [224].

2.3. RNA Isolation

Total RNA isolation from the cells was done with RNeasy Mini Kit (Qiagen, Valencia, CA). RNeasy Lysis Buffer (RLT) (guanidine thiocyanate buffer), with 10 µl/ml of β-mercapto ethanol was added to the cell pellet to disrupt the cells in 15ml tubes then vortexed for a min. One volume of 70% ethanol was added to the homogenized lysate and mixed thoroughly by pipetting. 700 µl of the mixture was transferred to the RNeasy spin column. The column was centrifuged at 8000x g for 15 sec at room temperature. Next, 700 µl Buffer RW1 was added to the column and the column was centrifuged at 8000x g for 15 sec at room temperature. The column was then washed with 500 µl of buffer RPE at 8000x g for 15 sec at room temperature. In the end, 30-50 µl of RNase free water was added directly to the spin column membrane and centrifuged for 15 s at 8000x g and the RNA eluted. The yield of RNA was quantified by using a Nanodrop instrument, GE Genequant 1300 Spectrophotometer (GE Healthcare Life Sciences Little Chalfont, Buckinghamshire, UK).

2.3.1. Reverse Transcription

The isolated RNA was used to synthesize cDNA using ImProm-II™ reverse Transcription System (Promega, Madison, WI). 2µl of Oligo dT (0.5µg/l) was added to 1µg of isolated RNA and incubated at 70° C using a Veriti® Thermal Cycler (Applied Biosystems, Grand Island, NY) for 5 minutes and then chilled on ice. In the mean time, a reverse transcription mix of 20 µl total volume was prepared containing: 1 µg of the total
RNA with 13.3 µl of ImProm-II™ 5X reaction buffer, 5 µl of magnesium chloride, 3.4 µl of dNTP mix, 1.75 µl RNasin® ribonuclease Inhibitor, 3.25 µl of ImProm-II™ reverse transcriptase to make a total volume of 20 µl. Next, the chilled reaction mixture was incubated with the reverse transcription reaction mix as per manufacturer’s instructions in the Improm II reverse transcription kit (Promega, Madison, WI) and was placed in the Veriti® Thermal Cycler. Cycling conditions for reverse transcription procedure were: annealing at 25°C for 5 min., extension at 42°C for 60 min., inactivation of the reverse transcriptase at 72°C for 15 min. A sample with no reverse transcriptase was prepared for every sample of the RNA isolated using the same cDNA preparation protocol to detect the presence of genomic DNA in the RNA samples isolated from the cells. The cDNAs was stored at -20°C for future use.

2.3.2. Real Time PCR

Real time PCR was conducted using a CFX 96 - real-time PCR system (Bio-Rad, Hercules, CA). The real time PCR mix was prepared by using 3.2 ng of cDNA, 10 µl SYBR Green PCR master mix (BioRad Laboratories, Hercules, CA) and 1 µl of forward and reverse primers (10 pmol/µl) (Integrated DNA Technologies, Coralville, IA). The primer specificity was analyzed by running a melting curve. The PCR cycling conditions used were 2 min at 95 °C for initial denaturation, then 44 cycles of 30 sec at 95° C, 30 sec at 61° C and 30 sec at 72° C. Final extension was executed for 5 min at 72°C. The primer sequences used are mentioned in the following Table 3. Each sample was carried out in triplicate and the threshold cycle values were averaged. Real time PCR for both SV and IMA were run together in the same 96-well plate. The PCR cycling conditions were
automatically calculated by a real time PCR system (CFX96, BioRad Laboratories, Hercules, CA) using the protocol-AutowriterTM (Bio-Rad) based on the annealing temperature of the primers and amplicon length [462]. The calculation of relative gene expression was based on the differences in the threshold cycles. The fold change in expression in each samples was calculated by fold change = $2^{-ΔΔCt}$ method [463]. The results were normalized to 18S rRNA expression.

Table 3: List of primers used.

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Primer Sequence</th>
</tr>
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| PLK1             | Forward Primer: 5’-ACA AAC ACC AGA CTC AGA CG-3’  
|                  | Reverse Primer: 5’-CTG TGA ATA GCT GAC CTA CGG-3’ |
| 18S (18S ribosomal RNA) | Forward Primer: 5’-TCA ACT TTC GAT GGT AGT CGC CGT-3’  
|                  | Reverse Primer: 5’- TCC TTG GAT GTG GTA GCC GTT TC-3’ |

2.4. Protein Isolation and Quantification

One hundred ml of Radio-Immunoprecipitation Assay (RIPA) buffer containing a protease inhibitor cocktail (20µl/ml of RIPA) (P8340, Sigma, St. Louis, MO), and phosphatase inhibitor cocktails 1 and 2 (20µl/ml of RIPA) (Sigma-Aldrich, St. Louis, MO) were added to the cell pellet isolated from one T25 flask. In some instances cells were grown in a 6-well plate and we added 50µl of RIPA containing all the inhibitors. The samples with RIPA lysis buffer were incubated on ice for an hour with intermittent
mixing every 10 min. The samples were then centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was then aliquoted into fresh eppendorf tube and flash frozen with dry ice and stored at -80°C, until further use.

The concentration of protein in each sample was determined using the Bicinchoninic acid (BCA) protein assay [464]. Albumin ranging in concentration from 0.2 to 1.0 mg/ml was used to generate a standard curve. Ten µl of each standard was placed in duplicate in 96-well microtiter plate. Five µl of sample was prepared with 5 µl of nanopure water were placed in each well in duplicates. 200 µl BC/ copper sulfate solution (1:50 dilution of 4% copper sulfate in BC solution) was then added to each well. The 96 well microtiter plate was then incubated at 37°C for 15 min. Absorbance was measured at 550 nm on a microplate reader (Perkin Elmer, Waltham, MA).

2.4.1. Western Blot using Sodium Dodecyl Sulfate - Polyacrylamide Gel (SDS PAGE) Electrophoresis

Thirty µg of protein from each sample was mixed with 2x Laemmli loading buffer (Biorad, Hercules, CA) containing 10% mercaptoethanol (Sigma, St. Louis, MO) and separated by electrophoresis using 4-20% polyacrylamide gels (Biorad, Hercules, CA) using the 10x tris-glycine SDS (sodium dodecyl sulfate) running buffer (Biorad). After electrophoresis the proteins were transferred from the gel onto a nitrocellulose membrane (Biorad, Hercules, CA) in ice-cold 10x tris-glycine transfer buffer (Biorad). Immobilon® PVDF transfer membrane (EMD Millipore Corporation, Billerica, MA) of 0.45 µm pore size was used if the molecular weight of the protein to be detected was
below 30 KDa. For antibody detection of the specific proteins, the membrane was blocked in 5% non-fat dry milk prepared in phosphate-buffered saline with tween-20 (PBST) buffer for 1 hour. To detect phosphorylated-protein, we used 5% BSA in tris-buffered saline with tween-20 (TBST) for blocking. After blocking, the membrane was incubated overnight with a diluted solution of the primary antibody in 5% milk or BSA under gentle agitation at 4°C. The primary antibodies used are polo-like kinase1 (PLK1) (Abcam, ab17056), rabbit PLK1 phosphorylated at Thr210 (pPLK1) (Abcam, ab155095), rabbit CDC2 phosphorylated at Ser198 (pCDC2) (Cell Signalling Technology, #9529), rabbit histone H3 phosphorylated at Ser-10 (p-Histone H3) (Abcam, ab32107). Then the membrane was washed 4 times (15 minutes each) with washing buffer (0.1% Tween-20 with PBS or TBS) to remove unbound primary antibody. Tris Base Saline (TBS) is preferred when detecting phospho-proteins as PBS may interfere with anti-phosphate antibody binding. Next, the membrane was exposed to the secondary antibody conjugated to horseradish peroxidase (1:2000, Novus Biological, Littleton, CO); directed at species-specific epitopes of the primary antibody. The membrane was incubated for 1hr at room temperature, with gentle agitation. The membrane was washed 4 times (15 minutes each) with washing buffer (0.1% Tween-20 with PBS or TBS) to remove unbound secondary antibody. The immunoblot was developed with ECL chemiluminescence detection reagent (Bio-Rad, Hercules, CA). The chemiluminescence of the protein bands was detected, and images were captured using the ChemiDoc™ MP Imaging system (Biorad, Hercules, CA). Results were normalized to the levels of the housekeeping protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Novus Biologicals, NB300-221). Densitometric measurement for quantification of the relative protein expression was
performed using ImageJ software by NIH or the software provided by the ChemiDoc™ MP Imaging system (Biorad, Hercules, CA). This software automatically quantitates protein bands using relative quantity tool. A reference band needs to be selected from an image, and then the relative quantity tool quantitates all other bands relative to the reference band in the image. We have used control band of an image as the reference band to quantitate all other bands of the same image. The relative quantity is the ratio of the background-adjusted volume divided by the background-adjusted reference volume. Values above 1.0 indicate that the volume is greater than the reference volume (control). Values below 1.0 indicate the volume is less than the reference volume.

2.5. Silencing RNA Knockdown

Small interfering RNA (siRNA), an efficient tool to downregulate the expression of target genes in cultured cells, was used to knockdown PLK1. Both SV and IMA-SMCs were grown to 65 to 70% confluency in SMCM containing 10% FBS and 1% P/S/A. Then the cells were exposed to DMEM with 1% FBS for 3hrs. After being exposed to DMEM, the cells were then transfected with 100nM of control (Cell Signalling technology, #6568) or PLK1 (Cell Signalling technology, #6292) siRNAs mixed with Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA) for 22 hrs. Then the medium was changed and the cells were treated with DMEM with 1% FBS and P/S/A for 6hrs. The cells were then treated with 10ng/ml of PDGF-BB for 6 and 36 hrs. The knockdown efficiency was analyzed by western blot analysis, which demonstrated efficient downregulation of PLK1 protein.
2.6. Cell Proliferation Assay

2.6.1. Cell count:
SV- and IMA-SMCs (50,000 cells/well) were seeded in each well of a 24-well plate with 1 ml SMCM + 10% FBS + 1% P/S/A. Cells were incubated at 37° C till 65-70% confluency. Then the cells were rinsed with DMEM+1% FBS+1% P/S/A and later incubated in 1ml of the DMEM for 24hrs for cell synchronization. Cells were then stimulated with 10ng/ml of PDGF-BB in DMEM+1% FBS+1% P/S/A for 48 hrs. At the end of the 48 hours incubation, cells were removed with trypsin and quantified with trypan blue dye exclusion method. The Countess™ automated cell counter (Invitrogen, Carlsbad, CA, #C10227) was used to count the number of live, dead, and total cells per sample. 0.4% Trypan blue stain was used, which is taken up by the dead cells with uniform blue color throughout the cell whereas, live cells have bright centers and dark edges.

2.7. Immunocytochemistry
SV- and IMA-SMCs were cultured in Lab-Tek chamber slides (Thermo Fischer Scientific, Rochester, NY) overnight. Once the cells are 60 to 70% confluent, they were fixed with ice-cold acetone or 4% (wt/vol) paraformaldehyde for 15 minutes at room temperature. Next, the slides were washed with PBS for 5 min followed by blocking with 1 % BSA for 1 hr. The SMCs were then incubated with primary antibody mouse smooth muscle alpha actin (α-SMA) (Abcam, ab7817) for 2 hrs then rinsed in PBS. After this the cells were incubated with cyanine3-conjugated secondary antibody (Jackson ImmunoResarch, Westgrove, PA) for 2 hrs then again rinsed in PBS. Slides were
mounted with Vectashield mounting medium (Vector Laboratories Burlingame, CA) and examined using fluorescence microscope.

2.8. Animal

The swine model of coronary artery IH followed by PTCA and CABG were implemented, as previously established by our group [465-468]. Tissues from these swine models were sectioned and stored, and these tissue sections from the storage were used for my experiments. This experimental protocol has obtained formal approval from Creighton University Institutional Animal Care and Use Committee. The animals were housed in the Animal Resource Facility of Creighton University, Omaha, NE and cared for as per the guide for the care and use of laboratory animals.

Yucatan™ miniature and microswine, weighing 20–45 kg, were purchased from Sinclair Bio-resources (Windham, MA USA). The swine were fed high cholesterol diet. High cholesterol diet (HC) consisting of 37.2% corn (8.5% protein), 23.5% soybean meal (44% protein), 20% chocolate mix, 5% alfalfa, 4% cholesterol, 4% peanut oil, 1.5% sodium cholate, and 1% lard; with 52.8% of the kilocalories from carbohydrates and 23.1% of the kilocalories from fat. After 5 to 14 months of the high cholesterol diet the animals were subjected to percutaneous transluminal balloon angioplasty (PTCA) in left anterior descending artery (LAD) or left circumflex artery (LCX). The control group of Yucatan™ (2 swine) and domestic swine (3 swine) were fed the normal diet but were excluded from undergoing PTCA. Five to eight months post-PTCA, the swine were euthanized and tissues were collected for experimental purpose.
Domestic swine were obtained from the UNL Swine Research Unit (Mead, NE) [465, 468]. The domestic swine were fed a normal diet (Teklad Miniswine diet 8753, Harlan Laboratories) composed of wheat middlings, ground corn, soybean hulls, dehulled soybean meal, dehydrated alfalfa meal, dicalcium phosphate, and soybean oil. Within the diet, 57% of the kilocalories were from carbohydrates, 28% of the kilocalories from protein, and 15% of the kilocalories from fat. After 1 to 2 months of acclimatization, the animals were subjected to off-pump CABG; the right SEV was used as a vein graft and the left was used as a control. Two months post-CABG, the swine were euthanized and tissues were collected for research purpose.

As I have mentioned earlier, specimens from the in vivo study with swine were used in different studies and I have used these tissue sections from the storage for my research project [465-468]. For CABG we have used domestic pigs, however, for our PTCA study we have used domestic, Yucatan™ miniature and microswine. The purpose of this part of my study was to understand if PLK1 and pPLK1 are expressed in vein and artery of a clinically relevant model such as swine. Also, to determine any difference in the expression of PLK1 and pPLK1 in swine with or without surgery/intervention.

2.8.1. PTCA Procedure
A pre-trained group in the lab that includes an interventional cardiologist at the Creighton medical center performed PTCA in swine [325, 467]. With the help of an introducer needle, access in the femoral artery in the leg of the swine was created. Then to keep the artery open and control bleeding 6F sheath introducer was placed. Followed by a guiding
catheter to the left coronary artery on a guide wire under fluoroscopic guidance. Through the guiding catheter a guide wire is inserted into the coronary artery. Coronary angiography was done using nonionic contrast media. In the heart, LCX or LAD balloon angioplasties were done followed by OCT (optical coherence tomography) to verify the placement and to get a baseline measurement of the surface are of both the LAD and LCX. Then the catheter was removed and the femoral artery sutured followed by closure of leg wound. The pigs were allowed to recover under a thermal blanket. The i.v. fluid was continued until pigs are able to drink water and the pigs were subsequently moved back to the animal care facility.

2.8.2. CABG Procedure

A pre-trained group in the lab that includes surgeon at the Creighton medical center performed CABG in swine [465, 468]. The off-pump CABG method was used. The SEV was harvested using the no touch technique through a longitudinal incision parallel to the mammary line. The chest was then opened through a sternotomy incision using the electric sternotomy saw. The heart was then exposed after incising the pericardium. The ascending aorta was exposed and prepared for the anastomosis using an aortic side clamp and a circular opening was created using an aortic punch. Then the proximal ascending aorta and the distal end of the SEV were anastomosed. A cardiac surface stabilizer device was used to stabilize the area of the intended anastomosis with the LAD. The LAD was then dissected and incised; a temporary coronary shunt was used to maintain the coronary blood flow during the anastomosis. Then the LAD was anastomosed to the proximal end of the SEV. The temporary coronary shunt was removed before putting in the last stitch.
The blood flow in the bypass graft was verified before ligating the LAD proximal to the bypass to allow exclusive blood flow in the vein graft. The sternotomy was then closed using stainless wire and the animal was allowed to recover under thermal blanket. The i.v. fluid was continued until pigs were able to drink water and the pigs were subsequently moved back to the animal care facility.

Tissue Harvest and Processing

The heart was removed and placed in DMEM from both the swine models post PTCA and CABG. Coronary vessels (LAD, LCX, RCA), SEV-graft (right SEV) and left SEV were dissected, removed and fixed in 10% formalin for 24 hours at room temperature. Tissues were embedded in paraffin and thin sections (5µm) were obtained using microtome (Leica, Germany). Sections were stained with hematoxylin and eosin (H&E) for histomorphometric studies. The noninjured vessels from swine with normal diet were used as controls.

2.8.2.1. Deparaffinization of the Sections

These sectioned slides were then used in my study and were stored for future investigation. In order to perform staining and IF of the sections, first we submerged the slides with the sections in xylene to remove paraffin and shake at low speed for 15min. This step was repeated twice. Then submerge slides in etanol:xylene (1:1) and shake at low speed for 15min. Place the slides respectively in 100%, 95%, 80%, and 70% EtOH for 5 min each. Finally rehydrate the slides by dipping a couple of times in distilled water.
2.8.2.2. H&E Staining

The slides were placed in Harris hematoxylin solution for 30 sec. They were rinsed in running tap water for 5 min. Then the slides were dipped in acid ethanol for 10 sec to destain. Briefly rinsed the slides in distilled water. Placed the slides in counterstain eosin solution for 22 sec. Next, the slides were dipped in 95% EtOH for 10 times. The slides were further dipped in 100% EtOH, xylene/ethanol mix (1:1), and xylene respectively for 5 times each. Finally, the slides were mounted using xylene-based mounting medium.

2.8.2.3. Immunohistochemistry

Paraffin embedded samples, after deparaffinization and rehydration, were treated by steam heating in DAKO antigen retrieval solution (DAKO, Carpenteria, CA) for antigen retrieval (20–30 min). Slides were washed using Tris buffered saline and 0.1% tween (TBS) twice 5 min each. Sections were incubated for 2 hr in block/permeabilizing solutions containing TBS, 0.25 % Triton X-100, and 5% (v/v) goat serum at room temperature. The slides were subsequently incubated with primary antibody solutions including mouse polo like kinase1 (PLK1) (Abcam, ab17056), rabbit PLK1 phosphorylated at Thr210 (pPLK1) (Abcam, ab155095), mouse/rabbit non-muscle myosin heavy chain B (SMemb) (Abcam, ab684/ab24761), mouse smooth muscle alpha actin (α-SMA) (Abcam, ab7817), rabbit histone H3 phosphorylated at Ser-10 (pHis H3) (Abcam, ab32107), rabbit interferon gamma (IFN-γ) (Abcam, ab9657) and rabbit STAT3 phosphorylated at Tyr-705 (pSTAT3) (Bioss, bs-1658R) at 4°C overnight. On the following day, slides were rinsed in TBST three times for 5 minutes each, and the secondary antibody was incubated on the slides for 2 hours at room temp. In case of
polyclonal primary antibodies, we used Alexa Fluor® 594-conjugated AffiniPure Goat Anti-Rabbit or Goat Anti-Mouse; Alexa Fluor 488-conjugated AffiniPure Goat Anti-mouse or Goat Anti-Rabbit secondary antibody, and for the monoclonal primary antibodies, we used Alexa Fluor 488 Goat Anti Mouse IgG2b secondary antibody, 1:200 (Jackson ImmunoResearch, Westgrove, PA). Negative controls with complete omission of primary antibody were run in parallel with normal host IgG. Sections were washed with TBST three times for 5 min. Nuclei were counterstained with 4’,6- diamidino-2-phenylindole (DAPI) using Vector laboratories DAPI mounting medium. Tissue sections were viewed with an Olympus BX-51 epi-fluorescent microscope and images were photographed with an Olympus DP71 camera. Number of cells showing immunopositivity in the stained slides with either control or treatment tissues were visually quantified by double blind technique.

2.9. Statistical and sample size analysis

Data was analyzed using GraphPad Prism 7.0 biochemical statistical package (GraphPad Software, Inc., San Diego, CA). Statistical analysis was performed using paired Student’s t-test to compare two groups. Multiple group comparison was performed using repeated one-way analysis of variance (ANOVA) with a Dunnett or Tukey post–hoc test. Values of all measurements are expressed as mean ± SEM. Differences at p<0.05 were considered significant.
Chapter 3

Results
3. RESULTS

3.1. Immunocytochemical determination of the cell type isolated from human SV- and IMA

3.1.1. Isolated cells from SV and IMA stained positive for smooth muscle alpha actin (α-SMA) and smooth muscle myosin heavy chain (SM-MHC), contractile SMC markers.

To determine the phenotype of cells isolated, we performed immunofluorescent staining on the cells isolated from the IMA and SV. The presence of α-SMA and SM-MHC in the isolated cells were examined using a monoclonal anti-α-SMA and SM-MHC antibody, whose specificity has been previously reported. α-SMA and SM-MHC are contractile markers specific for vascular SMCs [148]. These are highly conserved proteins that play an important role in SMC shape and movement [469]. In this present experiment, the cells were immunopositive for contractile SMC-markers, α-SMA (Figure 11 A-B) and SM-MHC (Figure 11 C-D). These result suggests that the isolated cultured cells are VSMCs with an overall purity of > 95%. These VSMCs were then used for in-vitro experiments for further investigations to understand the pathophysiology of vein-graft disease.
**Figure 11:** Immunocytochemical determination of the cell type isolated from human SV and IMA.

Representative immunopositive image demonstrating that the isolated vascular smooth muscle cells (VSMCs) from SV (A) and IMA (B) exhibit positive staining for contractile smooth muscle alpha actin (α-SMA) (red). DAPI (blue), a fluorescent stain used to stain the nucleus of the cells. Representative immunopositive images demonstrating that the isolated VSMCS from SV (C) and IMA (D) exhibit positive staining for contractile smooth muscle alpha actin (α-SMA) (red). DAPI (blue), a fluorescent stain used to stain the nucleus of the cells.
smooth muscle myosin heavy chain (SM-MHC) (red). DAPI (blue), a fluorescent stain used to stain the nucleus of the cells. Scale bar = 200 µm.

3.2. Basal level expression of PLK1 and pPLK1 in isolated SV-SMCs and IMA-SMCs

3.2.1. At the basal level SV-SMCs have greater expression and phosphorylation of PLK1 than IMA-SMCs.

Protein was isolated from the cultured SV- and IMA-SMCs. The vessels used in this experiment were from the same patients. 35 µg of the isolated protein was used for immunoblotting using antibodies specific to PLK1 (Figure 12 A) and pPLK1 (Figure 12 B). Data from this study showed that VSMCs expressed PLK1 and pPLK1. However, at the basal level SV-SMCs have greater expression of PLK1 and pPLK1 than IMA-SMCs.

![Figure 12: Expression and phosphorylation of PLK1 at the basal level in SV and IMA-SMCs.](image-url)
Isolated protein from SV- and IMA-SMCs were subjected to immunoblotting for PLK1 and pPLK1. A) A representative blot and densitometric quantification of PLK1 expression in SV- and IMA-SMCs. B) A representative blot and densitometric quantification of pPLK1 level in SV- and IMA-SMCs. GAPDH was used as the loading control. The bar charts depict relative optical density (normalized to GAPDH) of PLK1 or pPLK1 protein bands. The densitometry was done using ImageJ software. Data shown are mean ± SEM from four to five independent experiments. *P <0.05 versus control. Paired t-test was used to analyze the data.

3.3. Effect of PDGF-BB on PLK1 Expression and Phosphorylation

3.3.1. PDGF-BB induced the expression of PLK1 and its phosphorylation in SV- and IMA-SMCs.

Cultured SMCs isolated from human SV and IMA was serum-starved with 1% heat-inactivated serum for 24 hrs followed by PDGF stimulation and protein was then isolated for immunoblotting using antibodies specific to PLK1 and pPLK1. PDGF-BB (10 ng/ml) significantly increased the expression of PLK1 in SV- and IMA- SMCs at 6 and 10 hrs (Figure 13). Similarly, exposure of the SMCs to PDGF promotes phosphorylation of PLK1 at 10 hrs in SV- and IMA-SMCs (Figure 14 A and B). Since the activity of PLK1 is dependent on the state of its phosphorylation, our results indicate that in SV- and IMA-SMCs, the PDGF-induced expression and phosphorylation of PLK1 (Figures 13 and 14).
Figure 13: Effect of PDGF-BB on PLK1 expression in SV and IMA-SMCs.

SMCs were serum starved with 1% heat-inactivated serum for 24 hrs and treated with PDGF-BB (10ng/ml) for an additional 6, 10 and 12 hrs. Protein was isolated from cell lysates and subjected to immunoblotting using total PLK1 antibody. A) A representative blot and densitometric quantification of PLK1 expression in SV-SMCs. B) A representative blot and densitometric quantification of PLK1 expression in IMA-SMCs. Protein expression levels were normalized to the housekeeping gene GAPDH and calculated as percentage level of induction in comparison to control. Data is mean ± SEM from six independent experiments. *P <0.05 versus control. Repeated measures ANOVA was used to analyze the data.
**Figure 14:** Effect of PDGF-BB on phosphorylation of PLK1 (pPLK1) in SV and IMA-SMCs.

SMCs were serum starved with 1% heat-inactivated serum for 24 hrs and treated with PDGF-BB (10ng/ml) for an additional 6, 10 and 12 hrs. Protein was isolated from cell lysates for Immunoblotting using antibody to phosphorylated PLK1. GAPDH was used as the loading control. A) A representative blot and densitometric quantification of pPLK1 levels in SV-SMCs. B) A representative blot and densitometric quantification of pPLK1 levels in IMA-SMCs. The graphs represent densitometric analysis of the bands. Protein expression levels were normalized to the housekeeping gene GAPDH and calculated as percentage level of induction in comparison to control. Data is mean ± SEM from six independent experiments. *P <0.05 versus control. Repeated measures ANOVA was used to analyze the data.
3.4. Effect of PDGF-BB on the expression of PLK1 mRNA transcript in SV- and IMA-SMCs

3.4.1. PDGF-BB induced expression of the PLK1 mRNA transcript over time in SV- and IMA-SMCs

Cultured SMCs isolated from human SV and IMA were serum starved with 1% heat-inactivated serum for 24 hrs and then stimulated with 10ng/ml of PDGF-BB for 1, 4 and 8 hrs. PLK1 mRNA expression was quantified by real time qPCR and normalized against the housekeeping gene 18S ribosomal RNA. PLK1 mRNA transcript expression increased in SV- and IMA-SMCs at 1 hr stimulation with PDGF (Figure 15 A and B). This was followed by decreased expression of PLK1 at 4 hrs in both, IMA- and SV-SMCs. Repeated measures ANOVA was used to analyze the data.

![Graphs showing PLK1 mRNA expression in SV and IMA SMCs](image)

**Figure 15:** PDGF-BB induced the expression of PLK1 mRNA transcripts in SV and IMA-SMCs

SV and IMA-SMCs were serum starved with 1% heat-inactivated serum for 24 hrs and then treated with PDGF-BB (10ng/ml) for an additional 1, 4 and 8 hrs. (A) PDGF-BB
81

(10ng/ml) stimulated the mRNA expression in SV-SMC. (B) PDGF-BB (10ng/ml) stimulated PLK1 mRNA expression in IMA-SMC. The results were normalized against 18S ribosomal RNA. Data is mean ± SEM. Note n = 4. *P <0.05 versus control. Repeated measures ANOVA was used to analyze the data.

3.5. Inhibition of PLK1 with a Clinically Relevant ATP-Competitive PLK1 Inhibitor Attenuates PDGF-BB Induced Phosphorylation of PLK1 in VSMCs

3.5.1. ATP-competitive PLK1 inhibitor, BI2536 inhibits PDGF-BB induced phosphorylation of PLK1 in VSMCs.

Quiescent SMCs of both SV and IMA were treated with 100nM of BI2536 for 2 hrs followed by an additional treatment with 10ng/ml of PDGF-BB for 10 hrs. Total protein was isolated from cell lysates and subjected to immunoblotting using pPLK1 antibody. As shown in Figure 16A and B, treatment with BI2536 blocked the PDGF-induced phosphorylation of PLK1 in both SV and IMA-SMCs. This confirms that BI2536 is a potent PLK1 inhibitor in VSMCs.
**Figure 16:** In VSMCs, 2 hrs pre-treatment with 100nM of BI2536 blocked PDGF-induced phosphorylation of PLK1 at 10hrs.

(A) A representative blot and densitometric quantification of pPLK1 level with and without BI2536 in SV-SMCs. (B) A representative blot and densitometric quantification of pPLK1 level with and without BI2536 in IMA-SMCs. GAPDH was used to show equal loading. The graphs represent densitometric analysis of the bands. Protein levels were normalized to the housekeeping gene GAPDH and calculated as percentage level of phosphorylated protein in comparison to control. Data is mean ± SEM from five independent experiments. *P <0.05 versus control. Repeated measures ANOVA was used to analyze the data.

3.6. PDGF-BB induced proliferation in SV- and IMA-SMCs

3.6.1. PDGF-BB induced phosphorylation of a highly conserved serine residue in the histone H3 tail, which is an established mitotic marker in VSMCs, marking the onset of mitosis in VSMCs.

In this experiment, we wanted to identify the time at which PDGF-induces VSMCs to undergo mitosis. Since phosphorylation of histone is an established mitotic marker, we wanted to study the time at which histone H3 undergoes phosphorylation at Ser10 in VSMCs [470-472]. Cultured SMCs isolated from human SV and IMA were serum
starved with 1% heat-inactivated serum for 24 hrs and stimulated with 10ng/ml of PDGF-BB for 24, 36 and 48 hrs. Histone H3 is phosphorylated during mitosis at Ser 10 that relates to the onset of early prophase in mitotic cells [470]. In SV-SMCs, PDGF-induced an 8-fold increase in phosphorylation of histone H3 at 36 hrs followed by return to the control level of phosphorylation at 48 hrs (Figure 17A). In IMA-SMCs, there was increased PDGF-stimulated phosphorylation of histone H3 either at 36 hrs or at 48 hrs in IMA-SMCs (Figure 17B). The representative immunoblot shows an increased phosphorylation of histone H3 only at 48 hrs. Histone H3 phosphorylation at Ser-10 is required for mitotic chromosome condensation [470, 471]. Therefore, histone H3 phosphorylation at Ser-10 correlates directly with the number of cells that are undergoing mitosis at a given time.

**Figure 17:** PDGF-BB time dependently stimulated phosphorylation of mitotic marker histone H3 in SV and IMA-SMCs.
Quiescent VSMCs when treated with 10ng/ml of PDGF-BB for 24, 36 and 48 hrs, induced phosphorylation of histone H3 thus marking the number of cells undergoing mitosis. GAPDH was used to show equal loading. (A) A representative blot and densitometric quantification of p-Histone H3 level in SV-SMCs. (B) A representative blot and densitometric quantification of p-Histone H3 level in IMA-SMCs. GAPDH was used to show equal loading. The graphs represent densitometric analysis of the bands. Protein expression levels were normalized to the housekeeping gene GAPDH and calculated as percentage level of induction in comparison to control. Data is mean ± SEM from seven independent experiments. *P <0.05 versus control. Repeated measures ANOVA was used to analyze the data.

3.7. PLK1 Inhibition Blocked PDGF-BB Induced Phosphorylation Of Histone H3 In VSMCs

3.7.1. BI2536, a PLK1 blocker, significantly inhibited PDGF-induced phosphorylation of mitotic marker histone H3 in SV- and IMA-SMCs. Since phosphorylation of histone is an established mitotic marker, we wanted to study if inhibiting the activity of PLK1 has any effect on phosphorylation of histone H3 in VSMCs [470-472]. The quiescent SMCs of both SV and IMA were treated with 100nM of BI2536 for 2 hrs followed by an additional treatment with 10ng/ml of PDGF-BB for 36 hrs. Protein was isolated and immunoblotting was performed using the p-Histone H3 antibody. As shown in Figure 18A and B, treatment with BI2536 blocked the PDGF-induced phosphorylation of p-Histone H3 in both SV and IMA-SMCs.

3.7.2. PLK1 Blocker Attenuated the PDGF-BB-Induced Proliferation of SV And IMA-SMCs

Proliferative properties of SMCs with or without BI2536 were analyzed after 48 hrs of PDGF-BB stimulation. ATP-competitive blocker, PLK1 attenuated SMC proliferation in
both SV and IMA (Figure 19). Using cell counting by the trypan blue dye exclusion method, SMCs of both SV and IMA were counted pre- and post-treatment. As shown in Figure 19, stimulation with PDGF-BB (10ng/ml) significantly increased proliferation in SMCs isolated from both the vessels but co-treatment with BI2536 (100nM) reduced PDGF-BB-induced proliferation. This demonstrates the impact of inhibition of PLK1 function on the proliferative properties of SMCs.

![Image of Figure 19 showing the effects of PDGF-BB and BI2536 on SMC proliferation in SV and IMA.]

**Figure 18:** Effect of ATP-competitive PLK1 blocker, BI2536 on PDGF-induced phosphorylation of histone H3 in SV and IMA-SMCs.

Quiescent SMCs were treated with 100nM of BI2536 for 2 hrs followed by stimulation with 10ng/ml of PDGF-BB for 36 hrs. Protein was isolated and subjected to immunoblotting using phosphorylated histone H3 (Ser10) antibody. (A) A representative blot and densitometric quantification of p-Histone H3 level in SV-SMCs. (B) A representative blot and densitometric quantification of p-Histone H3 level in IMA-SMCs. The graphs represent densitometric analysis of the bands. GAPDH was used to show equal loading. Protein expression levels were normalized to the housekeeping gene GAPDH and calculated as percentage level of induction in comparison to control. Data is mean ± SEM from four independent experiments. *P < 0.05 versus control. Repeated measures ANOVA was used to analyze the data.
**Figure 19:** Effect of PLK1 inhibition on the proliferation of SMCs in SV and IMA-SMCs.

BI2536 treated and untreated SMCs were incubated with PDGF-BB (10 ng/ml) for 48 hrs. (A) The graphs represent cell number in SV-SMCs with or without BI2536 and/or PDGF. (B) The graphs represent cell number in IMA-SMCs with or without the treatment of BI2536 and/or PDGF. Proliferation of SMCs is significantly attenuated when treated with BI2536 in the presence of PDGF than the group treated with PDGF but not with PLK1 inhibitor. Data shown are mean ± SEM from four different samples. *P <0.05 versus control. Repeated measures ANOVA was used to analyze the data.

3.8. Effect of PDGF-BB on phosphorylation of Master Mitotic Kinase

**CDC2/CDK1 in SV- and IMA-SMCs.**

3.8.1. PDGF-induced phosphorylation of a highly conserved threonine residue of CDC2/CDK1 for the progression of mitosis in cell cycle in VSMCs.

We wanted to step things up and study the downstream mediators of PLK1. Hence we chose CDK1 as it is the master mitotic kinase and marks the beginning of mitosis [473]. In this experiment we are trying to identify the time-point at which CDK1 gets
phosphorylated and hence activated in VSMCs [474, 475]. Cultured SMCs isolated from human SV and IMA were serum starved with 1% heat-inactivated serum for 24 hrs and then stimulated with 10ng/ml of PDGF-BB for 20 and 22 hrs. Protein was isolated for immunoblotting using antibody to phosphorylated CDC2/CDK1 (Ser198) (Figure 20). SV- and IMA-SMCs stimulated with PDGF-BB showed significantly increased phosphorylation of CDK1 at Ser198 (Figure 20 A and B).

**Figure 20:** PDGF stimulated phosphorylation of cell cycle controller CDK1 in SV and IMA-SMCs at 20 and 22 hrs.

SV and IMA-SMCs were serum starved with 1% heat-inactivated serum for 24 hrs and then stimulated with PDGF-BB (10ng/ml) for an additional 20 and 22 hrs. Protein was isolated for immunoblotting using phosphorylated CDK1 (Ser198) antibody. GAPDH was used to normalize the data. (A) A representative blot and densitometric quantification of pCDK1 level in SV-SMCs. (B) A representative blot and densitometric quantification of pCDK1 level in IMA-SMCs. Protein expression levels were normalized to the housekeeping gene GAPDH and calculated as percentage level of induction in comparison to control. Data is mean ± SEM from five to six independent experiments. *$P$ <0.05 versus control. Repeated measures ANOVA was used to analyze the data.
3.9. PLK1 Inhibitor, BI2536 Attenuated PDGF-BB Induced Phosphorylation of CDK1 in SV- and IMA-SMCs

3.9.1. PLK1 blocker BI2536 significantly inhibited PDGF-induced phosphorylation of CDK1 in SV and IMA-SMCs.

We anticipated that inhibition of PLK1 activity would in turn decrease the ability of PDGF to induce phosphorylation of CDK1. To test this, the quiescent SMCs of both SV and IMA were treated with or without 100nM of BI2536 for 2 hrs followed by an additional treatment with 10ng/ml of PDGF-BB for 22 hrs. Protein was isolated for immunoblotting using pCDK1 antibody. As shown in Figure 21, PLK1 inactivation reduced PDGF induced elevation of pCDK1 levels in SV and IMA-SMCs. Taken together, this result shows that ATP-competitive inhibition of PLK1 attenuates PDGF-induced phosphorylation of CDK1, which is a master mitotic kinase.
Figure 21: BI2536 inhibited the PDGF-induced phosphorylation of CDK1 in SV and IMA-SMCs.

Quiescent VSMCs when treated with 100nM of BI2536 for 2 hrs followed by an additional treatment with 10ng/ml of PDGF-BB for 22 hrs, inhibited the phosphorylation of CDK1 at 22 hrs in SV and IMA-SMCs. GAPDH was used as a loading control. A) A representative blot and densitometric quantification of pCDK1 level in SV-SMCs. B) A representative blot and densitometric quantification of pCDK1 level in IMA-SMCs. The graphs represent densitometric analysis of the bands. Protein expression levels were normalized to the housekeeping gene GAPDH and calculated as percentage level of induction in comparison to control. Data is mean ± SEM from four to five independent experiments. *P <0.05 versus control. Repeated measures ANOVA was used to analyze the data.

3.10. Effect Of PLK1 Gene Silencing On Proliferation in SV- and IMA-SMCs

3.10.1. PLK1 gene silencing attenuates the phosphorylation of mitotic marker histone H3 at Ser-10

So far all our experiments were done with ATP-competitive PLK1 blocker and we wanted to confirm the data by silencing PLK1 gene. First, we wanted to confirm the
potency of the PLK1 specific siRNA and then to study the effect of PLK1 gene silencing on the proliferation of VSMCs. SV- and IMA-SMCs were transfected with PLK1 specific siRNA or with scrambled siRNA and then stimulated with PDGF for 6 hrs. Protein was isolated for immunoblotting using antibody to PLK1. The result confirmed silencing with PLK1-specific siRNA reduced the protein expression (Figure 22A and B). We then examined the effect of silencing PLK1 expression on the phosphorylation of mitotic marker histone H3 in SV and IMA-SMCs (Figure 22C and D). SV and IMA-SMCs transfected with PLK1-specific siRNA showed markedly reduced phosphorylation of histone H3 compared to non-transfected cells. This demonstrates the impact of PLK1 activity on the phosphorylation of histone H3, hence on the proliferation of SMCs. This further confirms the result obtained previously with the PLK1 inhibitor, BI2536.
**Figure 22:** Effect of PLK1 gene silencing on PLK1 expression and phosphorylation of histone H3 at Ser10 in SV and IMA-SMCs.
(A-B) PLK1-specific and scrambled siRNA transfected SMCs were incubated with PDGF-BB (10 ng/ml) for 6 hrs. PLK1 expression was assessed by Western blot and the results are shown as densitometric quantification. PLK1-specific siRNA transfection abolished the PDGF-induced expression of PLK1 in SV (A) and IMA-SMCs (B). In order to observe the effects of PLK1 gene silencing on proliferation, we assessed the presence of p-Histone H3 in PLK1-specific and scrambled siRNA transfected SMCs after stimulation with PDGF for 36 hrs. (C-D), p-Histone H3 level in SV (C) and IMA-SMCs (D) was determined by Western blotting. Phosphorylation of histone H3 in SMCs is considerably decreased in PLK1-silenced PDGF-BB- stimulated transfected SMCs as compared to the scrambled siRNA transfected cells. Protein expression levels were normalized to the housekeeping gene GAPDH and calculated as percentage level of induction in comparison to control. Data is mean ± SEM from four to six independent experiments. *P <0.05 versus control. Repeated measures ANOVA was used to analyze the data.

3.11. In Vivo Studies

3.11.1. Vessel damage and development of intimal hyperplasia post-PTCA and CABG

Percutaneous transluminal coronary angioplasty (PTCA) caused vessel damage and led to the development of IH post PTCA in swine model. We used five Yucatan™ miniature and microswine to undergo PTCA. These Yucatan™ swine and were fed high cholesterol diet for 5 to 14 months. The animals were then subjected to PTCA in left anterior descending artery (LAD) or left circumflex artery (LCX). Five to eight months post-PTCA, the swine were euthanized and tissues were collected. Balloon injury resulted in medial rupture in all PTCA. Significant neointima proliferation was observed both in LAD and LCX following PTCA. The control group had five swine out of which two were Yucatan™ and three were domestic swine. The control group was fed the normal diet and was excluded from undergoing PTCA. Significant restenosis was observed in post PTCA-coronary arteries compared to control coronary artery (Figure 23 A-D) [325, 467].
Coronary artery bypass graft (CABG) surgery exposed the vein to the arterial pressure that induced the development of IH in our swine model of CABG-study. We used two domestic swine for our CABG-study, where the right superficial epigastric vein (SEV) was used as a graft and the left SEV that was not used as a graft served as control. Domestic swine were fed normal diet. After 1 to 2 months of acclimatization, the animals were subjected to off-pump CABG; the right SEV was used as a graft and the left SEV that was not used as a graft served as control. Two months post-CABG, swine were euthanized and tissues were collected. CAGB procedure exposes the lumen of SEV-graft to high arterial pressure resulting in endothelial damage and dysfunction accompanied with medial SMC migration and proliferation. Significant development of IH in the SEV graft was observed compared to the left SEV that was not used as a graft (Figure 23E-H) [465, 466, 468].
Figure 23: Histology of swine coronary artery and SEV tissue sections.

The H&E staining of uninjured coronary artery (A, B) shows normal histology, whereas five to eight months after balloon angioplasty neointimal hyperplasia (arrows) is noted in post PTCA-coronary arteries (C, D). H&E staining of the uninjured or control SEV showed normal histology (E, F). SEV that was used as a graft (G, H) developed neointimal hyperplasia (arrows). All images were taken in 4x and 20x objective. This is a representative of tissue histology in 2-5 individual swine in each experimental group. L: lumen, M: medial layer, NI: neointima. Scale bar = 200 µm.

3.11.2. Vascular injury and PLK1 expression

Arteries post balloon angioplasty and SEV-graft post bypass surgery were examined for PLK1 and non-muscle myosin heavy chain B (SMemb) expression by immunofluorescent staining. Immunopositivity to SMemb establishes the presence of highly proliferating synthetic-SMCs. In order to be able to examine the co-distribution of
both PLK1 and SMemb in the tissue, a double immunofluorescent staining was performed. Expression of PLK1 and SMemb increased in the post PTCA-coronaries and SEV-grafts compared to their respective uninjured counterparts (Figure 24). Few SMCs in the neointima expressed both PLK1 and SMemb. These results suggest a strong association between post-therapeutic interventional vascular injury, VSMC proliferation and PLK1 expression.

**Figure 24:** Double immunofluorescent staining showing immunopositivity towards PLK1 and SMemb in post-PTCA coronary arteries and SEV-grafts than their respective uninjured counterparts in porcine model.

Significantly increased immunopositivity towards SMemb and PLK1 in the neointima of the post-PTCA restenotic coronary artery (arrows) was noted compared to uninjured coronary artery. SEV-graft had significantly increased immunopositivity towards SMemb
and PLK1 in the neointima compared to uninjured SEV. There is less immunopositivity to PLK1 (red) and SMemb (green) expression in uninjured coronary artery at low (20x) (A) and high (40x) magnification (B, C, D). The immunostaining of PLK1 (red) and SMemb (green) is noted in restenotic post PTCA- coronary arteries (arrows) at low (20x) (E) and high (40x) magnification (F, G, H). Similarly, less immunostaining of PLK1 (red) and SMemb (green) expression is noted in the uninjured SEV at low (20x) (I) and high (40x) magnification (J, K, L) compared to SEV post CABG, where many cells showed immunopositivity to PLK1 (red) and SMemb (green) at low (20x) (M) and high (40x) (N, O, P) magnification. This is a representative of tissue histology in 2-5 individual swine in each experimental group. L: lumen, M: medial layer, NI: neointima. Scale bar = 200 µm.

3.11.3. Vascular injury and phosphorylation of PLK1

Post PTCA-arteries and SEV used as a coronary bypass grafts were examined for pPLK1 and synthetic SMC marker SMemb expression by immunofluorescent staining. Phosphorylation of PLK1 at Thr-210 by other kinases is required for its catalytic activity [241-243, 245, 257, 302]. Restenotic coronary artery post PTCA showed pPLK1 immunopositivity, which was limited to the neointima. Cells in the neointima and also in the medial layer were immunopositive for SMemb. Since SMemb is a marker for proliferating SMCs, expression of SMemb confirms SMC-proliferation. Few cells in the neointima were found to be immunopositive for both, pPLK1 and SMemb (Figure 25 A-H). Moreover, significantly increased immunopositivity of SMemb and pPLK1 were noted in the neointima of the SEV-graft compared to uninjured left SEV (Figure 25 I-P). In the SEV-graft, a group of cells in the neointima expressed either SMemb and pPLK1 or a combination of both. These results suggest a strong association between post-therapeutic interventions or post-bypass surgery mediated vascular injury and pPLK1 expression.
Figure 25: Double immunofluorescent staining showing immunopositivity towards pPLK1 and SMemb in post-PTCA coronary arteries and SEV-grafts than their respective uninjured counterparts in swine.

Post-PTCA restenotic coronary arteries were immunopositive for SMemb and pPLK1. SEV-graft had increased immunopositivity to SMemb and pPLK1 in the neointima. There is less immunopositivity to pPLK1 (green) and SMemb (red) expression in uninjured coronary artery at low (20x) (A) and high (40x) magnification (B, C, D). The immunostaining of pPLK1 (green) and SMemb (red) is noted in restenotic post PTCA-coronary arteries (arrows) at low (20x) (E) and high (40x) magnification (F, G, H). Similarly, less immunostaining of pPLK1 (green) and SMemb (red) are noted in the uninjured SEV shown at low (20x) (I) and high (40x) magnification (J, K, L) compared to SEV post CABG, where many cells showed immunopositivity to pPLK1 (green) and SMemb (red) at low (20x) (M) and high (40x) (N, O, P) magnification. This is a representative of tissue histology in 2-5 individual swine in each experimental group. L: lumen, M: medial layer, NI: neointima. Scale bar = 200 µm.
3.11.4. Vascular injury, p-Histone H3 and SMemb

As mentioned earlier, to study the number of cells undergoing mitosis, we immunostained the tissue sections of swine with or without angioplasty and bypass surgery towards phospho-histone H3 (p-Histone H3) at Ser-10. Phosphorylation at a highly conserved serine residue (Ser-10) in the histone H3 tail is considered to be a crucial event for the onset of mitosis. There was significantly more immunopositivity towards p-Histone H3 and SMemb in the neointima of post-PTCA coronaries and SEV-grafts (Figure 26). Many cells in the neointima of the injured vessels were immunopositive for both p-Histone H3 at Ser-10 and SMemb. This shows that synthetic SMCs in the neointima are undergoing mitosis.
Figure 26: Double immunofluorescent staining showing immunopositivity to p-Histone H3 at Ser-10 and SMemb in thin sections of post-PTCA coronary arteries and SEV-grafts than their respective controls.

Strong immunopositivity to p-Histone H3 was found in the neointima of the post-PTCA coronary arteries and in SEV-graft. Many cells in the neointima of the injured vessels were immunopositive for both p-Histone H3 and SMemb, especially around the pseudo lumen in SEV-graft. There is less immunopositivity to p-Histone (green) and SMemb (red) expression in uninjured coronary artery shown at low (20x) (A) and high (40x) magnification (B, C, D). The immunostaining of p-Histone (green) and SMemb (red) is noted in restenotic post-PTCA coronary arteries (arrows) at low (20x) (E) and high (40x) magnification (F, G, H). Similarly, less immunoreactivity to p-Histone (green) and SMemb (red) is noted in the uninjured SEV shown at low (20x) (I) and high (40x) magnification (J, K, L) compared to SEV post-CABG, where many cells showed immunopositivity to p-Histone (green) and SMemb (red) at low (20x) (M) and high (40x) (N, O, P) magnification. This is a representative of tissue histology in 2-5 individual swine in each experimental group. L: lumen, M: medial layer, NI: neointima. Scale bar = 200 µm.
3.11.5. Vascular injury, SMemb and smooth muscle α-actin

Immunopositive for SMemb in both post PTCA-coronaries and SEV-graft establishes the presence of highly proliferating synthetic-SMCs (Figure 27). High expression of smooth muscle α-actin (α-SMA) was found in all the vessels. Immunopositivity to α-SMA and SMemb in these lesions confirmed that vascular SMCs were the main cellular component of neointimal proliferative lesions. There is higher immunopositivity of SMemb at the neointima in post PTCA-coronaries and SEV-grafts than to their uninjured counterparts. In PTCA-coronaries, the majority of the cells in the neointima were stained positive for SMemb; some of these SMCs also stained positive for α-SMA. However, in the SEV-grafts, two distinct sets of SMCs were observed, one was immunopositive for SMemb and the other was immunopositive for α-SMA. This would mean that not all the SMCs are synthetic yet as they expressed their specific contractile marker. A few SMCs around the pseudo-lumen showed co-localization of both the markers.
**Figure 27:** Double immunofluorescent staining showing positivity to SM alpha-actin and SMemb in tissue sections of uninjured coronary arteries, post-PTCA coronary arteries, uninjured SEV and SEV-grafts in swine.

Higher density of SMemb-immunopositive cells are found at the neointima in post PTCA- coronary arteries and SEV-grafts than normal uninjured coronary artery and vein, respectively. Strong expression of α-SMA is noted in all the vessels, injured or uninjured. Immunoreactivity to α-SMA (green) and SMemb (red) are noted in uninjured coronary artery at low (20x) (A) and high (40x) magnification (B, C, D). Less immunoreactivity to α-SMA (green) and increased immunoreactivity to SMemb (red) are found in the hyperplastic region post PTCA- coronary arteries (arrows) at low (20x) (E) and high (40x) magnification (F, G, H). Similarly, immunopositivity to α-SMA (green) and SMemb (red) are observed in the uninjured SEV at low (20x) (I) and high (40x) magnification (J, K, L) compared to SEV post CABG, where few cells are immunopositive to α-SMA (green) and many cells showed immunopositivity to SMemb (red) at low (20x) (M) and high (40x) (N, O, P) magnification. This is a representative of tissue histology in 2-5 individual swine in each experimental group. L: lumen, M: medial layer, NI: neointima. Scale bar = 200 µm.
3.11.6. Vascular injury, IFN$\gamma$ and SMemb

A double immunofluorescent staining was performed to examine the immunoreactivity towards IFN$\gamma$ and SMemb. There was more immunopositivity to IFN$\gamma$ and SMemb in post PTCA-coronaries and SEV-grafts (Figure 28). The immunopositivity to IFN$\gamma$ was limited to the neointima of the injured vessel. Most of the SMCs in the neointima of post PTCA-coronaries and SEV-grafts co-expressed IFN$\gamma$ and SMemb.

![Image of double immunofluorescent staining showing immunoreactivity to IFN$\gamma$ and SMemb in tissue sections of post-PTCA coronary arteries and SEV-grafts than their respective controls.](image)

**Figure 28:** Double immunofluorescent staining showing immunoreactivity to IFN-gamma and SMemb in tissue sections of post-PTCA coronary arteries and SEV-grafts than their respective controls.

Many cells in the neointima of the injured vessels were immunopositive for both, IFN$\gamma$ and SMemb, especially around the pseudo lumen in SEV-graft. Less immunopositivity to IFN$\gamma$ (green) and SMemb (red) are noted in uninjured coronary artery at low (20x) (A)
and high (40x) magnification (B, C, D). Strong immunoreactivity to IFNγ (green) and SMemb (red) are found in the hyperplastic region post PTCA- coronary arteries (arrows) at low (20x) (E) and high (40x) magnification (F, G, H). Similarly, less immunostaining of IFNγ (green) and SMemb (red) is noted in the uninjured SEV at low (20x) (I) and high (40x) magnification (J, K, L) compared to SEV post-CABG, where many cells showed immunopositivity to IFNγ (green) and SMemb (red) at low (20x) (M) and high (40x) (N,O,P) magnification. This is a representative of tissue histology in 2-5 individual swine in each experimental group. L: lumen, M: medial layer, NI: neointima. Scale bar = 200 μm.

3.11.7. Vascular injury, pStat-3 and PLK1

There was higher expression of pStat-3 and PLK1 in post PTCA-coronary arteries and SEV-grafts than the uninjured counterparts (Figure 29). Many SMCs in the neointima of the injured vessels showed immunopositivity towards both pStat-3 and PLK1.
Figure 29: Double immunofluorescent staining showing immunopositivity to pStat-3 and PLK1 in tissue sections of post-PTCA coronary arteries and SEV-grafts than their uninjured controls in swine.

There was more immunopositivity towards pStat-3 and PLK1 in the neointimal region of the injured vessels compared to the uninjured vessels. Less immunopositivity to pStat-3 (green) and PLK1 (red) are noted in uninjured coronary artery at low (20x) (A) and high (40x) magnification (B, C, D). Strong immunopositivity to pStat-3 (green) and PLK1 (red) are found in the hyperplastic region post-PTCA coronary arteries (arrows) at low (20x) (E) and high (40x) magnification (F, G, H). Similarly, less immunostaining of pStat-3 (green) and PLK1 (red) expression is noted in the uninjured SEV at low (20x) (I) and high (40x) magnification (J, K, L) compared to SEV post CABG, where many cells showed immunopositivity to pStat-3 (green) and PLK1 (red) at low (20x) (M) and high (40x) (N, O, P) magnification. This is a representative of tissue histology in 2-5 individual swine in each experimental group. L: lumen, M: medial layer, NI: neointima. Scale bar = 200 µm.
Chapter 4

Discussion
4. DISCUSSION:

The frequently performed surgical method CABG was demonstrated to improve event-free survival compared to PTCA based on early-, mid-, and long-term results [476]. In the beginning of 1964, Vasilii Kolesov was the first to anastomose IMA to LAD. Later that year Michael DeBakey performed a saphenous vein aorta-coronary bypass [477, 478]. Over the years, this surgical procedure constantly improved with low perioperative mortality rates and relatively low complication rates [479]. As mentioned earlier in the introduction the commonly used SVG has lower patency rate than the IMA graft [480]. Data from coronary artery surgery study (CASS) showed that after undergoing CABG, the patency of SVG from 89% in the first year drops to 61% after 10 years unlike IMA, which still have 85% patency after 10 years [481-483]. Vein graft failure rate is influenced by thrombosis (early failure) and intimal hyperplasia followed by atherosclerosis (late failure) [400]. Endothelial dysfunction and oxidative stress damage followed by CABG contributes to persisting systemic inflammatory response [484, 485]. Elaboration of multiple mitogens at the site of the vein-graft injury stimulates proliferation and migration of matured and non-proliferative medial SMCs [486]. We are the first to document a possible pro-proliferative role of PLK1 in the pathology of IH development resulting in vein graft failure post CABG. Inhibition of PLK1 expression or phosphorylation in SMCs attenuated SMC proliferation in vein and artery cell culture.

In this study, we compared the role of mitotic kinase PLK1 in SV-SMCs and IMA-SMCs and focused on its relationship with major cell cycle regulators such as CDK1 and
Histone H3 that play a critical role in the development of IH in coronary arteries following intervention and in vein graft post-CABG.

An extensive volume of literature cites the association of PLK1 with G2-M transition and mitosis in numerous cancer conditions [230, 234, 236-238, 248, 281]. In 2012, Shan and colleagues [239] reported that inhibition of the PLK1 upstream kinase, Aurora A, in leiomyosarcoma, a neoplastic lesion involving SMCs of the uterine myometrium, decreased the proliferation and induced apoptosis in these malignant cells. Therefore, it is plausible that an association between phenotypically altered VSMCs and PLK1 also exists in hyperplastic cells found in non-neoplastic pathologies such as IH. In this study, first I isolated the SMCs from human SV- and IMA then examined the expression of α-SMA and SM-MHC (Figure 11). Once these cells were stained positive for VSMCs phenotypic markers, they were used for in vitro experiments to understand the pathophysiology of vein-graft disease. First, we conducted an experiment to study the basal level expression of PLK1 and pPLK1 in isolated SV- and IMA-SMCs. SMCs isolated from SV and IMA of the same patients undergoing CABG were immunoblotted using antibody to PLK1 and pPLK1. Result from this experiment proved that PLK1 and pPLK1 are present in VSMCs, and at the basal level the expression and phosphorylation of PLK1 is higher in vein than artery (Figure 12 A and B). To the best of our knowledge, this is the first study to detect the presence of total and phosphorylated PLK1 protein in VSMCs (Figure 12 A and B). It is possible that higher phosphorylation and expression of PLK1 at basal level in venous SMCs may contribute towards the increased propensity of SV-SMCs to undergo greater proliferation than IMA-SMCs in the presence of multiple
growth factors at the site of injury due to surgery. To further understand the role of PLK1 in pathophysiology of vein-graft failure post CABG, we used the VSMC proliferation stimulant PDGF-BB to study if it has any effect on PLK1 expression and phosphorylation in SV- and IMA-SMCs [181]. From here, all the experiments were performed with SV- and IMA-SMCs isolated from different patients with their respective control. Results from our study showed that 10 ng/ml of PDGF-BB induced the expression of PLK1 protein at 6 and 10 hrs in SV- and IMA-SMCs (Figure 13). This documented the effect of PDGF on the expression of PLK1 in SV- and IMA-SMCs. We were then curious to study the effect of PDGF-stimulation on activation of PLK1 in vein and artery. Catalytic activation of PLK1 requires phosphorylation of its conserved threonine residue (Thr 210) in its T-loop [487]. In SV- and IMA-SMCs, PDGF-stimulated phosphorylation of PLK1 at 10 hrs (Figure 14). Phosphorylation on Thr 210 is a prerequisite for PLK1 to promote mitotic entry; taken together this data suggests – 1) PLK1 and pPLK1 are present in VSMC, 2) at basal level, SV-SMCs have greater expression and phosphorylation of PLK1 than IMA-SMCs and 3) PDGF-induced expression and phosphorylation of PLK1 in VSMCs. From these results it is conceivable that PLK1 may play a crucial role in excessive proliferation of VSMCs and may contribute in the pathophysiology of vein-graft disease (Figures 12, 13 and 14).

Since PDGF-induced the expression of PLK1 protein in VSMCs, next we wanted to detect PDGF-induced mRNA transcript level. A gene is transcriptionally regulated to produce mRNA [488], which then undergoes posttranscriptional [489], posttranslational [490] and degradative regulation [491] to finally express proteins at a preferred level.
Transcription, translation and degradation are mostly coupled and may often regulate each other through feedback loops. It is important to understand such interactions, as they are essential to comprehend the level of a protein. Real-time PCR was conducted with SV and IMA-SMCs after stimulation with PDGF-BB for 1, 4 and 8 hrs. An increase in PLK1 mRNA expression in SV-SMCs was observed only at 1 hr, which steadily falls as the time of stimulation increases. Similarly, increased PLK1 mRNA expression in IMA-SMCs was detected at 1 hr followed by return to control level. Taken together PDGF-induces PLK1 mRNA transcript expression both in SV- and IMA-SMCs (Figure 15).

A growing body of research suggests that higher PLK1 expression and activity in various cancer patients correlates with significantly poorer rate of survival [492]. This suggests that the level of PLK1 expression or activity serves as a prognostic marker for many types of malignancies [237]. Since PLK1 activity is correlated with cell proliferation, we wanted to examine the effect of inhibition of PLK1 activity on VSMC proliferation. We chose a potent and highly selective ATP-competitive PLK1 inhibitor, BI2536 with a half-maximal inhibitory concentration (IC50) of 0.83 nM for our next set of experiments [330]. A narrow concentration range of 10–100 nM of BI2536 was found to be sufficient for PLK1 inhibition, further supports the specificity of this inhibitor [330]. Studies confirmed that a concentration of 100 nM of BI 2536 was typically sufficient for inducing a complete mitotic arrest in HeLa cells [493]. Currently the effects of BI2536 as a monotherapy or combination therapy in cancer patients are been evaluated in clinical trials [494]. In line with this notion, we initially extended the usage of 100 nM of BI2536 to confirm the inhibition of phosphorylation of PLK1 in VSMCs. We observed that 2 hrs
treatments with 100nM BI2536 blocked PDGF-induced phosphorylation of PLK1 at 10 hrs in both SV- and IMA-SMCs (Figure 16). Next, we tested the effect of PLK1 inactivation on PDGF-induced VSMC proliferation by cell count. We found that PDGF induced the proliferation of SMCs in SV and in IMA at 48 hrs. But, BI2536 inhibited the effect of PDGF-induced SMC proliferation in SV and IMA [330, 495, 496] (Figure 19). Although in this cell count method to quantify PDGF-induced proliferation of SMCs, the proliferation upon stimulation with the mitogen in SV and IMA was similar. Therefore, mitogens may elicit the activation of PLK1, and anti-mitotic kinase drugs could be useful in the treatment of intimal hyperplasia and restenosis following interventional procedures.

PLK1 is a multifunctional protein in cancer biology that is involved in various mitotic processes across eukaryotic species [497]. In normal cells, PLK1 expression and activity is strictly regulated and peaks at G2–M transition, plateaus throughout mitosis, and has a sharp reduction by proteosomal degradation upon mitotic exit [498]. We tested the effect of PDGF on p-Histone H3 with or without the PLK1 inhibitor and siRNA in SV- and IMA-SMCs focusing on the regulatory activity of PLK1 (Figures 18 and 22 C, D). Phosphorylation at a highly conserved serine residue (Ser-10) in the histone H3 tail is considered to be a crucial event for the onset of mitosis [470-472]. Phosphorylation of histone molecules are an absolute requirement for the conversion of relaxed interphase chromatin to mitotic condensed chromosomes, which is essential for the subsequent cell division [470-472]. Phosphorylation of histone H3 has been characterized as a marker for mitotic cells in cell cycle progression [470, 471]. We wanted to study the time at which
PDGF-stimulates the SMCs from the vein and artery to undergo mitosis, which could be established by increased phosphorylation of histone H3. We demonstrated that treatment with 10 ng/ml of PDGF-BB induced phosphorylation of histone H3 at 36 hrs in SV-SMCs. In case of IMA-SMCs, the same concentration of PDGF induced phosphorylation of histone H3 at 36 and 48 hrs (Figure 17). Next, we investigated the effect of PLK1 inhibitor BI2536 on PDGF-induced phosphorylation of histone H3 in SV- and IMA-SMCs (Figure 18). We showed that PLK1 inhibitor abolished PDGF-induced phosphorylation of histone H3 in SMCs from SV and IMA (Figures 18 A and B). This demonstrates that mitotic marker p-Histone H3 is positively regulated by PLK1 and inhibition of PLK1 inhibits the phosphorylation of histone thus inhibiting VSMCs from undergoing mitosis upon PDGF stimulation. This result co-relates with our cell proliferation data and states that inhibition of PLK1 inhibits VSMCs from undergoing mitosis or proliferation.

So far for all our experiments, we have used ATP-competitive PLK1 inhibitor, next we wanted to interfere with expression of PLK1 protein. In next couple of experiments we have used PLK1-specific siRNA and scrambled siRNA. In order to confirm the efficiency of PLK1-specific siRNA, we studied the effect of PDGF on PLK1 expression in both SMCs treated with PLK1-specific and scrambled siRNA. We found gene silencing of PLK1 resulted in the abolition of mitogen-mediated expression of PLK1 protein in VSMCs (Figure 22 A and B). We then examined the effect of PLK1-gene silencing on the mitotic marker p-Histone H3 in VSMCs. SMCs transfected with PLK1-specific siRNA showed reduced phosphorylation of mitotic marker histone H3 (Figures
22 C and D). These findings further confirm potent anti-proliferative effect of clinically relevant PLK1 inhibitor, BI2536 in PDGF-induced mitotic progression in VSMCs. These observations support the concept that PLK1 is a critical regulator of cell cycle and hence widen our understanding of the role of PLK1 in VSMCs.

The process of DNA doubling (S phase) and distribution (mitosis) are separated by G1 and G2 phases, respectively [499]. Beyond the two R-points (G1 and G2), cells are committed to further progress through the cell cycle, independent of extracellular stimuli [500-502]. At G1 R-point, p27KIP1, Rb, PTEN proteins are down regulated in mitogen stimulated VSMCs increasing the transcription and stabilization of cyclin D that contributes to the development of IH [127, 224, 503]. However, the second restriction site in the cell cycle, G2 R-point can also prevent proliferation of the cells [225]. In excessively proliferating cancer cells, pPLK1 controls the G2/M phase transition activity of CDK1/Cyclin B1 complex by phosphorylating and activating CDK1 at Ser198 [228, 257]. Injection of anti-PLK1 antibodies in HeLa cells mitotically arrested the cells [228-233]. To further understand the role of PLK1 in regulating mitotic progression in VSMCs, we studied the effect of PLK1 inhibition on the regulatory activity of its downstream cell-cycle controller and master mitotic kinase CDC2/CDK1 in the progression of G2-M phase in VSMCs. First in this study, we established the time at which PDGF-BB stimulates phosphorylation of a highly conserved threonine residue of CDC2/CDK1 (Figure 20). For promoting mitosis CDK1 needs to be activated by undergoing phosphorylation at Ser198, a residue on its T-loop. In excessively proliferating cancer cells, PLK1 positively regulates CDK1-activity through direct
phosphorylation and inhibition of Wee1, which inactivates CDK1. We observed that PDGF stimulated phosphorylation of CDK1 at 20 hrs and 22 hrs, respectively in SV- and IMA-SMCs (Figure 20 A and B). We then were curious to know if blocking of PLK1 would also inhibit phosphorylation of CDK1 at Ser198. We found that in VSMCs, PLK1 blocker BI2536, completely blocked the phosphorylation of CDK1 at Ser198 when stimulated with PDGF (Figure 21 A and B). This suggests that there is a temporally increased PDGF-BB-induced activation of the pPLK1-pCDK1 pathway in both venous and arterial SMCs (Figure 21 A and B). Therefore, in SMCs, PLK1 positively regulate CDK1-activity by directly phosphorylating it at Ser198, which then plays a key role in regulating the mitotic progression of VSMCs. It is apparent that pPLK1-pCDK1-phistone H3 axis plays an important role in the proliferation of SMCs. Further studies are warranted to examine the effect of other inflammatory cytokines on PLK1 mediated proliferation.

We are the first to report PLK1 expression and phosphorylation in SV- and IMA-SMCs. We are the first to demonstrate the positive regulatory role of PLK1 on phosphorylation of CDK at Ser198 in VSMCs. To the best of our knowledge, this is the first report to connect PLK1 and highly proliferating SMC in SV- and IMA. Results from this study showed clinically relevant ATP-competitive PLK1 inhibitor BI2536 and PLK1-specific siRNA attenuated PLK1 phosphorylation and expression in VSMC thus inhibiting cell proliferation. In short, we showed that PLK1 positively regulates the phosphorylation of CDK1 and histone H3 and that is how it regulates the proliferation of VSMCs. Hence, we have established PLK1 may play a critical role in VSMC proliferation. Further studies are
warranted to elucidate and dissect cellular and molecular mechanisms underlying expression and phosphorylation of PLK1 in SV and IMA. Kinases and phosphatases might play a critical role in regulating the expression and phosphorylation of PLK1 in SV and IMA. Based on the information derived from this study one could hypothesize that inhibition of PLK1 activity could inhibit VSMC mitosis and hence delay neointima formation/venous graft failure. Thus the G2-R-point in the cell cycle may provide a promising target for therapeutic intervention should the G1-R-point mechanism fail.

Next, we wanted to know if PLK1 is expressed and phosphorylated in a clinically relevant model. So we used swine model to study its expression and phosphorylation in the injured blood vessels following PTCA and CABG. Swine have similar anatomic and physiologic characteristics to humans, which makes swine a suitable model to study translational research and surgical procedure. The swine heart is almost identical to human [324]. Swine have 90% similarity in the blood supply to the coronary arteries and conduction system to that of the humans. All these reasons together make swine model widely acceptable as an ideal animal model to study fibroproliferative vascular diseases that involves an interventional procedure or surgery [504, 505]. In this part of the study we have demonstrated for the first time an increased number of cells showing immunopositivity to PLK1 and pPLK1 in SMCs present in the hyperplastic zone in vessels exposed to post-therapeutic intervention (Figure 24 and 25). Restenosis after percutaneous coronary intervention, and vein graft failure post CABG, are chronic pathological conditions that proceed to obstructive vascular lesions with time. Mitogenic
stimuli released at the site of the injury elicit different signaling pathways, but all finally converge at the cell cycle of the proliferating medial smooth muscle cells.

First, we wanted to know if the swine that underwent surgery or intervention have developed intimal thickening. Neointimal thickening was observed in arteries post-PTCA and SEV-grafts (Figure 23). We were then curious to know if these cells in the neointima of the injured vessels are immunopositive for PLK1 and pPLK1. We performed double immunofluorescent staining of the tissue sections for PLK1 or pPLK1 and proliferative-SMC marker SMemb. We documented an increase in the number of PLK1-positive (+) cells in the neointima of restenotic coronary arteries, post balloon angioplasty and in SEV grafts post CABG. Many of the SMCs in the neointimal region were positive for PLK1, were also immunopositive for SMC synthetic marker SMemb (Figure 24). PLK1 undergoes phosphorylation at Thr-210 and becomes catalytically active. We showed the co-distribution of both pPLK1 and SMemb in the hyperplastic region of coronary arteries and SEVs post intervention or surgery (Figure 25). Neointimal-SMCs in the injured vessels were immunopositive for pPLK1. Increased expression of SMemb was not only noted in the neointima but also in the medial layer of the occluded vessel. These results indicate that the proliferative SMCs in the neointima were over expressing PLK1. In the post CABG SEV-graft tissue-sections, we found a group of cells in the hyperplastic zone that showed immunopositivity for SMemb, PLK1 and pPLK1 (Figures 24 and 25). These results reveal a strong association between vascular injury and pro-proliferative mitotic kinase PLK1 expression and phosphorylation in post PTCA coronary arteries and SEV-grafts. The potential ability of SMCs to replicate under mitogenic stimuli could occur
through the loss of negative growth regulators and/or to the overexpression of pro-proliferative proteins [146].

In the neointimal region, we observed immunopositivity to p-Histone H3 at Ser-10 that correlates with the cells undergoing mitosis. Increased in the number of the p-Histone-positive SMCs were noted in balloon-injured coronaries and SEV grafts. The expression of p-Histone was limited only to the neointima and many of these cells also co-expressed SMC-synthetic marker SMemb (Figure 26). This may be due to the high proliferative nature of the synthetic SMCs. As mentioned above many of these highly proliferative cells in the neointima were also positive for PLK1 and pPLK1 (Figures 24 and 25). To the best of our knowledge, this is the first study to demonstrate increased immunopositivity to PLK1, pPLK1, p-Histone H3 and SMC-synthetic marker SMemb in the neointima after vascular injury (Figures 24, 25 and 26).

Next, we wanted to know if these are contractile SMCs expressing the contractile SMC marker, α-SMA. Strong immunopositivity to α-SMA was observed in all the vessels, injured or uninjured, but cells in the neointima were mostly positive for SMemb and expressed less α-SMA. In post PTCA-coronary arteries, many cells in the neointima were immunopositive to both, α-SMA and SMemb, however, in the SEV grafts two distinct sets of cells were found to be positive to α-SMA and SMemb. The difference in α-SMA and SMemb expression in post-PTCA coronary and SEV-graft may be due to the higher rate of phenotypic transformation of medial VSMCs in post-CABG venous grafts, than the post-PTCA coronary arteries. Venous grafts are more sensitive to shear stress than
coronary arteries and this would explain the more pronounced IH in the venous grafts than in the post-PTCA coronary arteries (Figure 27).

Increased number of immunopositive cells to IFN-γ and pStat-3 were observed in the neointima of both, post-PTCA coronary arteries and SEV-grafts (Figures 28 and 29). IFN-γ was expressed along with SMemb in the neointima of the vessels (Figure 28). This might indicate that synthetic SMCs are one of the cellular sources of IFN-γ. Many phosphorylated Stat-3 positive cells were also immunopositive for PLK1. IFN-γ can induce phosphorylation of stat-3 which in-turn can initiate transcription of protein PLK1 [148, 220, 221, 506, 507] (Figure 29). We found elevated SMemb, p-Histone H3, PLK1, pPLK1, IFN-γ and pStat-3 in the neointima of post-PTCA coronary arteries and SEV-grafts. This suggests that each of these markers/proteins may have played a critical role in the formation of the intimal hyperplasia post vascular injury.

Cardiovascular disease is the leading global cause of death, claiming 17.3 million deaths per year. Of these, 7.4 million people died of CAD [1, 508]. Existing treatment approaches and surgical intervention are still unable to effectively manage this CAD. CABG is recommended over medical therapy or PCI for a long-term event free survival in obstructive CAD patients [509]. However, the most commonly used venous graft has as high as up to 25% failure rate in the first 12 to 18 months. Therefore, efforts are ongoing to explore novel targets and strategies for the management of long term venous graft patency [510]. An in-depth understanding of the key pathways that may lead to
excessive cellular proliferation resulting in the development of intimal hyperplasia may provide the basis for novel therapeutic strategies against vein graft failure [146, 511].

With this in mind we teased out PLK1, an established proliferation marker in highly proliferating cancer cells, to understand its role in proliferation of VSMCs. In this study we showed that targeting PLK1 may have therapeutic potential for the prevention of excessive proliferation of VSMCs. Our in vivo data supports our in vitro data and suggests that PLK1 may play a critical role in pathogenic remodeling of vein-graft and in post-PTCA restenosis. Our in vivo data showed PTCA and CABG injures the vascular wall causing VSMC metaplasia. Bypass surgery or angioplasty increases the number of SMCs expressing PLK1 and pPLK1. This suggests that CABG or PCI associated injury might itself be a stimulus to the upregulation and phosphorylation of PLK1 resulting in high proliferation in the grafts or in post-PTCA coronary arteries [465].

Inflammatory response and VSMC proliferation are the two major underlying processes in restenosis development. After coronary intervention or grafting, there is an inflammatory response in injured vessels associated with increased expression of synthetic or proliferative SMC-marker. IFN-γ, an inflammatory cytokine is a key regulator of the development of restenosis. IFN-γ was mostly associated with SMemb in the neointima of the injured vessels in swine. α-SMA and SMemb were found in the injured vessels, confirming that vascular SMCs were the main cellular component of neointimal proliferative lesions. However, synthetic SMC-marker SMemb occurred only in the injured vessels and not in the uninjured counterparts, suggesting a phenotypic shift
of SMCs to the synthetic phenotype due to the injury during intervention or surgery leading to restenosis development. Immunopositivity to p-Histone H3 in the neointimal proliferative lesions in injured vessels confirms that most of the SMCs are in mitotic phase of the cell cycle. The cells are also positive for PLK, pPLK and IFN-γ, whereas in the uninjured vessels, contractile SMCs showed no immunopositivity to p-Histone H3, PLK, pPLK and IFN-γ, suggesting that those SMCs are not proliferating.

In accordance with this in vivo data, in vitro we found that when stimulated with PDGF-BB, VSMCs have increased phosphorylation of histone H3. Clinically relevant ATP competitive inhibitor of PLK1 not only completely blocked its phosphorylation but also inhibited the phosphorylation of histone H3 indicating that PLK1 activity may be an important pathway for the progression of mitosis in VSMCs. To the best of our knowledge, ours is the first study to show that PLK1 plays a critical role in the SMC-proliferation in the venous graft post CABG and in coronary artery post PTCA [465].

My study showed increased pStat-3 after balloon injury to coronary artery and in venous graft post CABG. Other studies found that IFN-γ can mediate phosphorylation of the transcription factor Stat-3, which can activate the transcription of PLK1 in cancer cells [281, 507]. Hence, increased expression of IFN-γ may also lead to phosphorylation of Stat-3 and activate the transcription of PLK1 in VSMCs post injury. My in vitro data in VSMCs indicates that inhibition of PLK1 also blocks the phosphorylation of master mitotic kinase CDK1 in VSMC stimulated with PDGF-BB. This suggests that PLK1 activity is important for the onset of mitosis in the model.
Based on the information derived from this study one could hypothesize that PLK1 can be a new drug target for developing clinical therapies against neointimal hyperplasia characterized by VSMCs proliferation. Many PLK1 inhibitors are currently in different phases of clinical trials against cancer. A few of them, such as BI2516, BI6727 and TKM080301, have reached second phase of the trial and are showing promising therapeutic value. These inhibitors can also be used in clinical trials for the management of VSMC-mediated IH that restricts the blood flow to the heart. Gene therapy in clinical trials is another worthwhile option. Gene therapy aimed at PLK1 or the pharmacological inhibitors of PLK1 may be developed for the treatment/management of IH in vein-graft disease.

**4.1. CONCLUSION**

In summary, early occlusion of the venous graft is a major clinical problem. Venous graft has a lower rate of patency than arterial grafts due to intimal hyperplasia. SV-graft when exposed to increased hemodynamic stress undergoes arterialization that upregulates the expression of growth factors and cytokines resulting in cell cycle progression leading to SMC proliferation. Therefore, it is important to understand the pathophysiology of the process of intimal hyperplasia and the key molecules involved. In our *in vitro* study, at basal level we demonstrated greater expression and phosphorylation of PLK1 in SV-SMCs than IMA-SMCs. PDGF induced the PLK1 expression and phosphorylation in SV and IMA-SMCs. Inhibition of PLK1 either with BI2536 or siRNA in SV- and IMA-SMCs blocked the phosphorylation of mitotic marker histone H3 and master mitotic kinase CDK1 thus resulting in inhibiting proliferation of VSMCs. Therefore, pPLK1-
pCDK1-p-Histone H3 could be one of the mechanisms for the development of intimal hyperplasia in SV bypass grafts. These results demonstrated PLK1 is a pro-proliferative protein, inhibition of which blocks mitotic progression of VSMCs. Again in our in vivo study we found an increased number of cells in the neointima showing immunopositivity to PLK1 and pPLK1 in coronary artery post-PTCA and in SEV-graft post-CABG. Moreover, these cells in the neointima expressed synthetic-SMC marker SMemb. These findings suggest that highly proliferative SMCs in the IH express PLK, which is activated by phosphorylation. Therefore, we can say that injury caused by therapeutic interventions such as PTCA or CABG induces SMCs to proliferate resulting in the development of neointima. These highly proliferating SMCs have higher expression of PLK1 and pPLK1. In vivo data in swine model supports my in vitro data showing that PLK1 is expressed by VSMCs present at the site of hyperplastic intima of the injured vessels. These findings suggest that inhibition of PLK1 activity in SV-SMCs might help in reducing restenotic lesion development post-CABG. Further in vivo studies are needed to confirm that the ability of synthetic SMCs to replicate under mitogenic stimuli is due to the activity of PLK1, a positive growth regulator. In our present study, we identified PLK1 as a potential contributory factor in VSMC proliferation post-surgery or intervention.

Understanding the underlying molecular mechanism involving the regulatory enzyme PLK1 at the second restriction site G2 R-point in the cell cycle may prevent proliferation of the cells, which have lost proper G1/S control [225]. Based on the above discussion it is conceivable that PLK1 may be a new drug target for developing clinical therapies against neointimal hyperplasia characterized by VSMCs proliferation. Gene therapeutic
approaches targeting PLK1 or the pharmacological inhibitors of PLK1 may be used for the management of VSMCs-mediated early-occlusion of vein-graft. Moreover, inhibition of PLK1 diminishes the viability of highly proliferating cancer cells without affecting the viability of normal cells.

### 4.2. Future Direction

To the best of our knowledge, this is the first study to demonstrate the presence of PLK1 and pPLK1 in VSMCs. This is the first report to connect PLK1 and highly proliferating SMCs in vein and artery. This study should pave the way for further in vivo studies involving targeting PLK1 to inhibit stenosis in arteries and vein. It will be interesting to study the various kinases and phosphatases that regulate the function of PLK1 in VSMCs.
Chapter 5

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