Reconstitution of Coronary Vasculature by an Active Fraction of *Geum Japonicum* in Ischemic Rat Hearts and the Underlying Mechanisms

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Abstract

of thesis entitled

Reconstitution of Coronary Vasculature by an Active Fraction of *Geum Japonicum* in Ischemic Rat Hearts and the Underlying Mechanisms

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Coronary heart diseases (CHD) remain the most prevalent cause of premature death. Ischemic hearts often result from coronary vasculature occlusion. Significant efforts have been made for the treatment of CHD, including medications and surgical procedures. Currently there are still no effective drugs or therapeutics available for the treatment of the disease. Growing new coronary vessels to naturally bypass narrowed/occluded arteries or forming sufficient collaterals to the ischemic region would lead to substantially improved blood perfusion and correction of ischemia. However, this aim remains a theoretical ideal due to the negligible ability to grow new coronary vessels even with current advances in therapeutic angiogenesis. In the present study, we have isolated and identified an active fraction of *Geum japonicum* (AFGJ) showing significant activity in induction of efficient coronary angiogenesis and heart function improvement.

The therapeutic effect of AFGJ on CHD through reconstitution of partially occluded coronary vessels in CHD animal models was demonstrated with underlying signaling mechanisms identified. Briefly, AFGJ could promote the proliferation of human umbilical vein endothelial cells (HUVECs) *in vitro* and the growth of new blood vessels or coronary collaterals in CHD models after 2-week treatment. The number of newly formed coronary vessels in treated hearts was more than that of vehicle treated hearts, as indicated by both MicroCT and histology analysis. Echocardiography studies demonstrated significant improvement of heart functions 2 weeks after treatment with AFGJ. Furthermore, ECG measurements showed that the altered ST segment in AFGJ treated CHD models almost had full recovery to a normal level while rats in the vehicle group consistently suffered from heart ischemia. Moreover, the results of MicroCT reconstruction directly demonstrated the reconstitution of the damaged coronary vessels with newly formed functional coronary collaterals, as illustrated by more blood vessels density (AFGJ *vs* vehicle [%]: 4.5±0.5 *vs* 2±0.35) and more branching points (AFGJ *vs* vehicle: 0.94±0.07 *vs* 0.65±0.10). These data suggest that AFGJ treatment significantly corrects the ischemia of the affected regions of the heart.

We also explored possible mechanisms underlying the effect of AFGJ. Firstly, AFGJ could induce mesenchymal stem cell (MSC) differentiation into vascular endothelial cells and the differentiated MSCs were involved in

the tube formation. Secondly, Angio-G, the component derived from AFGJ, was able to stimulate significant proliferation of HUVECs in a dose dependent manner. Thirdly, in our tube-like capillary formation test of HUVECs *in vitro*, the length of formed tubes was greatly amplified with increasing concentration of Angio-G. Furthermore, the total length of Angio-G induced tubes was significantly reduced with increasing concentrations of AG490, an inhibitor of JAK/STAT pathways indicating possible involvement of the JAK/STAT signaling pathway.

In addition, proteomics methods were applied to investigate the protein alterations in CHD ischemic hearts and HUVECs. Two dimensional polyacrylamide gel electrophoresis (2-D PAGE) of the heart tissues of CHD rats showed 16 differentially expressed spots compared with sham and vehicle hearts, of which 8 were identified. Furthermore, 11 identified proteins of HUVECs treated with AFGJ or Angio-G at different time points were also observed by 2-D PAGE. The majority of identified proteins was found to be involved in the process of energy metabolisms.

In conclusion, these results have demonstrated therapeutic properties of AFGJ to induce early reconstitution of damaged coronary vasculature through both angiogensis and vasculogenesis. AFGJ treatments may provide a novel therapeutic modality for effective treatment of ischemic heart diseases.

中文摘要

冠心病是许多国家常见的严重疾病之一，其病因主要是冠状动脉的阻塞或狭窄所导致的心肌缺血。目前治疗冠心病的手段是通过扩张狭窄或堵塞的冠状动脉血管来改善心肌缺血，主要包括众多的扩血管或降血脂药物，支架手术以及血管搭桥手术来进行治疗,但这些手段仍无法有效的改善心脏的功能。2005年WHO报告显示，全球范围内冠心病的死亡率仍高达13.4%。 本研究从柔毛水杨梅中成功分离并鉴定出其活性成分AFGJ以及后续分离产物Angio-G可以有效的诱导血管的新生并改善心脏功能。

分离出的AFGJ在冠心病大鼠模型中可以有效的重建缺血的冠状动脉。 经AFGJ处理两周后的大鼠心脏中可以促进大量新生血管的再生。心脏超声检查显示AFGJ处理两周后大鼠的心功能与给药前有显著的提高。 心电图的结果显示， 虽然AFGJ处理组在手术后同样ST段有明显的降低,但给药一周后就可以看出ST段降低的程度显著下降，给药两周后基本上恢复到了正常的水平；而未给药组ST没有明显恢复，提示心脏一直处于缺血状态。 MicroCT扫描后的心脏经过三维重建后，AFGJ处理组心脏的血管侧枝循环明显多于对照组。统计结果显示，AFGJ处理组缺血区血管密度以及新生侧枝数量显著高于未给药组。在体外实验中，AFGJ与AFGJ中进一步分离出的活性成分Angio-G均能够刺激内皮细胞的增殖并具剂量依赖性。

在进一步研究中，我们研究了AFGJ可能的作用机制。首先，AFGJ可

在间充质干细胞（MSCs）和人脐静脉内皮细胞的共培养系统同诱导MSCs分化为血管内皮细胞，更重要的是，分化后的间充质干细胞参与了血管形成的过程。其次，在管状毛细血管形成试验中，管状结构的总长度随着Angio-G浓度的增加而增长。此外，JAK/STAT 信号通路特异性抑制剂AG490可以抑制Angio-G诱导的管状结构的形成，表明JAK/STAT信号通路在Angio-G所诱导的血管形成的过程中起重要的用。

为了更好的研究AFGJ及其后续活性成分Angio-G对血管新生的机制，我们用双向聚丙烯酰胺凝胶电泳分别对冠心病大鼠心脏以及体外处理的血管内皮细胞的差异表达蛋白进行了初步的鉴定。结果表明，在AFGJ处理组的心脏组织中有16个蛋白差异点，其中鉴定出8个有效的蛋白。此外，在对内皮细胞的双向电泳中，17个差异表达点被送去质谱测定，并鉴定出11个有效点。在所有鉴定出的19个蛋白当中，8个蛋白参与了细胞的能量代谢和蛋白表达后修饰。

综上所述， 本研究成功分离出具有生物活性的柔毛水杨梅提取物AFGJ,可以通过诱导新生的血管来重建缺血区的血管并改善心脏功能。本研究报道了AFGJ对冠心病的治疗作用，这为治疗缺血性心脏病提供了一种新的有效的候选药物。

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Publication List

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Abbreviations

2-DE	Two-Dimension electrophoresis
ACB	Aortocoronary bypass
ACEI	Angiotensin-converting enzyme inhibitors
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AEEC	Animal Experimentation Ethics Committee
AMI	Acute myocardial infarction
ANG	Angiopoietin
AFGJ	Active fractions from *Geum japonicum*
Angio-G	Angiogenic compound from the adult *Geum japonicum*
AP	Ammonium persulfate
ATCC	American Type Culture Collection
bFGF	Basic fibroblast growth factor
BM-MSC	Bone marrow derived mesenchymal stem cells
BSA	Bovine serum albumin
CABG	Coronary artery bypass surgery
CHD	Coronary heart disease
CPC	Cardiac progenitor cells
CO_2	Carbon Dioxide
CSC	Cardiac stem cells

CT	Computed tomography
CVD	Cardiovascular disease
DAPI	4,6-Damldino-2-phenylindole, dihydrochloride
DES	Drug-eluting stent
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
ECG	Echocardiography
ECGS	Endothelial cell growth supplement
ECM	Extracellular matrix
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EET	Exercise treadmill test
EPCs	Endothelial progenitor cells
ERK1/2	Extracellular signal-regulated kinases 1 and 2
ESC	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCM	Flow cytometric
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
GC-MS	Gas chromatography-mass spectrometry

GFR	Growth factor reduced
HCl	Hydrochloric acid
HE Staining	Haematoxylin & Eosin staining
HMVECs	Human microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
IEF	Isoelectric focusing electrophoresis
JAK	Janus Kinase
LAD	Left descending artery
LMWH	Low molecular heparin
LVEF	Left ventricular ejection fraction
LVFS	Left ventricular fractional shortening
MI	Myocardial infarction
MicroCT	Micro-computed tomography
MIDCAB	Minimally invasive direct coronary artery bypass surgery
MS	Mass spectrometry
MSCs	Mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Thiazolyl Blue Tetrazolium Bromide
n-BuOH	n-Butanol
NCCDPHP	National Center for Chronic Disease Prevention and Health Promotion

NDA	New drug application
NFκB	Nuclear factor kappa B
OD	Optical density
PCNA	Proliferating cell nuclear antigen
PBS	Phosphate-buffered Saline
PCI	Percutaneous coronary intervention
PDGF	Platelet-derived growth factor
PlGF	Placental growth factor
PGS	Prostaglandin synthetase
PFA	Paraformaldehyde
rFGF-2	Recombinant fibroblast growth factor-2
ROI	Region of interest
SD rat	Sprague-Dawley rat
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMC	Smooth muscle cells
SPECT	Single photon emission computed tomography imaging
STAT	Signal transducer and activator of transcription
TCM	Traditional Chinese medicine
TEMED	N,N,N',N'-tetramethylene-diamine
TTP	Thrombotic thrombocytopenic purpura
TXA2	Thromboxane A2

u-PA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor-2
VOI	Volume of the interest
VSMC	Vascular smooth muscle cells
vWF	Von Willebrand Factor
WHO	World Health Organization
DAB	Diaminobenzidine
SDS	Sodium dodecyl sulfate

Chapter 1 Introduction

1.1 The cardiovascular system and ischemic heart diseases

1.1.1 The cardiovascular system

The cardiovascular system is a complete loop system connected by blood vasculature from the heart as the centre to different organs in the body. The heart regularly keeps systolic and diastolic pressure to ensure that blood flows in one direction along blood vessels. There are three different kinds of blood vessels. The arteries are the largest and function to deliver blood away from the heart to distant organs, the only exception being of the pulmonary artery. Veins are smaller and are responsible for sending blood back to the heart. The smallest vasculature, capillaries, is only one cell thick and can form complex networks. According to the different parts and functions in the cardiovascular system, blood circulation can be divided into systemic circulation and pulmonary circulation (Figure 1.1).

Arterial blood containing oxygen is nutrient-rich and flows through the left ventrical into the aorta upon cardiac ventricular contraction. It then reaches the systemic capillaries through different levels of arterial branches. After nutrients consumption and gas exchange by organs, the blood becomes dark red in color because it now contains metabolites and carbon dioxide in abundance. Venous blood flows back to the right atrium through venules and veins to the vena cava and coronary sinuses. This process is defined as systemic circulation or body cycle.

Blood flows into the right ventricle from the right atrium after systemic circulation via the venous return to the heart. When the right ventricle contract, blood flows into the pulmonary trunk from the right ventricle and passes with its branches to the capillary network of alveoli. Carbon dioxide in blood is replaced

with oxygen in alveoli, termed as venous blood change into arterial blood, and then returns to the left atrium through pulmonary venous connection. This process is defined as pulmonary circulation.

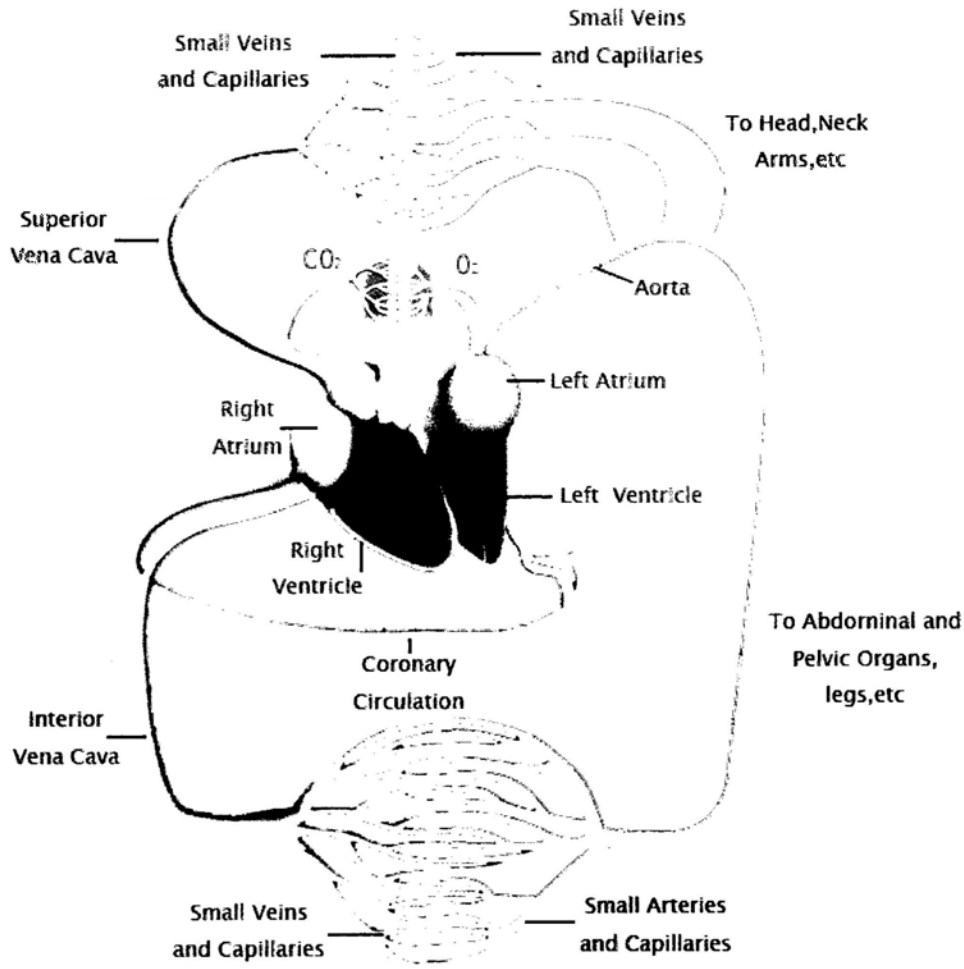

Figure 1.1 Diagram of cardiovascular system. (Adopt and modified from Chung and Rich, 1990)

1.1.2 Cardiovascular diseases and chronic coronary heart disease

Cardiovascular diseases (CVDs) include CHD, aortic aneurysm and dissection, peripheral arterial disease, rheumatic heart disease, congenital heart disease, heart failure, hypertension, and stroke. CVD is still the number one killer, which contributes to nearly 17.5 million deaths every year, estimated as 30% of all global deaths in 2005 according to the report by World Health Organization (WHO). Of these deaths, 7.6 million were due to CHD (heart attacks). Approximately 80% of global CVD deaths occur in low and middle-income countries. Furthermore, more than 80 million Americans currently live with one or more type of cardiovascular disease. More than 870 thousands of Americans die of heart disease and stroke every year. CHD contributed to more than 50% of deaths from CVDs according to Centers for Disease Control and Prevention National Center for Chronic Disease Prevention and Health Promotion (NCCDPHP) in USA.

CHD is the narrowing of the blood vessels that supply blood and oxygen to the heart and surrounding tissues (coronary arteries). Coronary disease usually results from the buildup of calcium, fatty material and cholesterol plaque (atherosclerosis). As the coronary arteries narrow, the flow of blood to the heart can slow or stop. The narrowing of the coronary arteries is the first step towards a heart attack. Fat packs up the walls of the arteries and forms a plaque. When one is under physical or other stress, the plaque cracks or tears and the body's emergency repair system rallies. Platelets stick to the fat that forms a clot. If arteries are already narrowed, the clot can completely block the blood supply downstream to the heart. Within a few minutes, muscle cells in the heart are damaged and begin to die. The disease can cause chest pain (stable angina),

shortness of breath, irregular and rapid heartbeat (heart palpitations) and/or heart attack (myocardial infarction).

Up to now, CHD is still a heavy burden on the economies of countries. In China, the incidence of CHD grows rapidly as well. It is reported that over one millions persons are killed by CHD each year, and more than 1.5 million by the complication of hypertension. Over 45% of the total population is estimated to be on the edge of being diagnosed with CHD in the coming decade. According to the prospect of WHO, China will lose 558 billion RMB in foregone national income due to the combination of heart disease, stroke and diabetes in the next 10 years (2006-2015). In addition, lower socioeconomic groups in high-income countries generally have a greater prevalence of risks factors, diseases and mortality. A similar pattern is emerging as the CVD epidemic evolves in low- and middle-income countries.

1.2 Ways to treat chronic coronary heart disease

1.2.1 Drugs and clinical therapeutics in chronic coronary heart disease

Although significant efforts have been made to treat heart diseases, it remains a growing risk of public health around the world. A lot of drugs were developed to reduce the threat of CHD. The current therapeutic treatments for CHD mainly focus on the following prospects.

The first method is by angiogenesis using growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) (Kranz et al., 2000, Ware and Simons, 1997). An initial series of VEGF intravenous administration for therapeutic myocardial

angiogenesis resulted in evidence of enhanced collateral blood flow in dogs with ischemic hearts (Banai et al., 1994). FGF was also introduced to apply in the ischemic heart disease treatment. It was reported that intracoronary administration of bFGF resulted in heart function improvement, decreased infarct size and increased capillary density in canine models (Yanagisawa-Miwa et al., 1992). Another study also confirmed the effect of bFGF on increasing microvessel numbers but no myocardial function change in microembolization infarcted swine (Battler et al., 1993). The uses of angiogenic factors were considered to be a useful strategy for treating patients with ischemic heart disease at that time. In clinical trials, Udelson, together with others, evaluated the effects of the recombinant fibroblast growth factor-2 (rFGF-2) protein on myocardial perfusion for patients with severe symptomatic CHD using single photon emission computed tomography imaging (SPECT). Fifty-nine patients unsuitable for traditional treatments received rFGF-2 instead. The resting perfusion score of 37 patients were improved, indicating that applying rFGF-2 to patients with severe symptomatic coronary heart diseases could improve resting myocardial perfusion and attenuate the stress-induced ischemia (Udelson et al., 2000).

However, the limitations are obvious since the effect of these factors is transient and some side effects in reconstitution of coronary blood vessels have appeared. VEGF and FGF are likely to speed up the plaque formation in vessels, leading to the expansion and the break-outs of the plaque or promoting the development of coronary artery stenosis by stimulating fibroblast and the smooth muscle cell proliferation in the mid-layer of blood vessels (Ferreira, 2002). Evermore, high dose of FGF may induce anemia, thrombocytopenia and the membranous nephropathy. Meanwhile, the symptoms of tumor and diabetic

retinopathy would be worsen by long administration of VEGF (Baumgartner, 2000). Besides, VEGF could induce fetal cardiovascular malformations and embryonic death, owing to the fact that the marked activity of blood vessels may lead to systemic hypotension (Feucht et al., 1997).

The second approach is the application of drugs that lower the symptoms of cholesterol. Over the past decade, many anti atherogenic drugs and the genetic variations contributing to CHD have been identified, such as ApoE which is suggested to possibly be able to block the development of atherosclerosis (Desurmont et al., 2000). Troponin I (TnI), the Ca^{2+} dependent inhibitory subunit of the troponin complex, was shown to improve sarcomeric Ca^{2+} activation and myocardial performance in the ischemic and failing heart of transgenic mice (Day et al., 2006). In clinical drug application, the mainly used drugs are divided into four types: 1) cholesterol-modifying medications; 2) antithrombotic drugs; 3) angiotensin-converting enzyme inhibitors (ACEI) and 4) the β-blocker and calcium channel blockers. Although these drugs have some promising effects on CHD and may reduce the mortality of CVD, these efforts lack the more powerful agents in clinical setting where plaque ruptures and thrombosis can pose a problem. Furthermore, altering blood flow does not affect the rate of atherosclerosis. Therefore these drugs only delay inadequate coronary circulation. Once the occlusion of the arteries becomes worse, severe blockage may demand surgery.

There are two pathological characteristics of CHD which is suitable to do the operations: 1) the narrowness and block are formed by atherosis plaque in the coronary artery; 2) the blood vessel with lesion is under the epicardium, the lesion

is superficial epicardial coronary, so the artery in the cardiac muscle is nearly not affected by the atherosis at all. Vascular stenting and angioplasty that may be effective treatment in improving blood supply, but does not reduce the degree of blockage in coronary vasculature. These procedures, including the most commonly performed operation, coronary artery bypass grafting (CABG), are invasive, and while generally successful, have many accompanied cardiovascular complications (Hamm et al., 1994, Halon et al., 2002, Hollman et al., 1992, Kimmel et al., 1997). In addition, some clinical studies using combinition of surgery with vascular growth factors to treat CHD. Experimental results were reported initially by Schumacher *et al* that they conducted normal bypass surgeries on twenty patients with multivessel coronary artery diseases, injected bFGF by 0.01mg/kg into cardiac muscle close to LAD and away from the anastomose place, after matching the internal mammary artery and LAD. 12 weeks later, intra-arterial digital subtraction angiography demonstrated that the capillary network could be seen growing around the site of injection and bypassed the stenoses and rejoined the distal parts of the vessel (Schumacher et al., 1998). Another trial by Losordo *et al* implanted the naked plasmid DNA encoding VEGF through the mini left anterior thoracotomy into myocardial ischemia of five patients who had angina with coronary heart diseases. The injection treatment reduced the symptoms of angina represented by nitroglycerin usage (Nitroglycerin dosage before the injection is 53.9±10.0/week, the dosage after injection is 9.8±6.9/week) (Losordo et al., 1998). It provided an optional method to treat the selected patients with chronic myocardial ischemia, which could reduce the symptoms of angina and rest myocardial perfusion. However, all of the above are

tentative experiments in clinical setting to cure coronary heart disease. Larger scale and longer-term research is required.

In summary, comparing with all the different kinds of surgery for coronary diseases, including stenting, angioplasty and coronary artery bypass grafting (It may be more effective in preventing vessels from narrowing, but again does not affect the degree of blockage in coronary vasculature) shows that these procedures are invasive, and while generally successful, have many accompanied cardiovascular complications (Hamm et al., 1994, Halon et al., 2002, Hollman et al., 1992, Kimmel et al., 1997). Furthermore, these treatments are still limited to application of occluded capillary vessels.

1.2.2 Stem cell therapy for cardiovascular diseases and chronic coronary heart disease

Cell transplantation as a regenerative therapy for heart diseases has some exciting findings in the past decade. Soonpaa *et al* was the first to confirm the possibility of mouse embryonic cell transplantation for myocardium (Soonpaa et al., 1994). Two years later, Li's group successfully transplanted the embryonic myocardial cells into rat hearts 4 weeks after cryoinjury. The results showed that transplanted cells formed cardiac tissue in the myocardial scar and improved heart function (Li et al., 1996). However, embryonic cardiac myocytes were very sensitive to ischemia and culture environment *in vitro* and less capable of proliferation (Reinecke et al., 1999). In addition, the most severe problem was that embryonic myocardial cells were not readily available and are still surrounded by ethical controversy. These challenges limit the application of transplantation of embryonic myocardial cells. Since Soonpaa *et al* and Li's

group, several different sources of cells have been explored to examine their effects on healing coronary heart disease. The advantages and disadvantages of cells examined are listed in table 1. This subtopic mainly focuses on stem cell transplantation.

Table 1 Potential cell sources for cell-based therapies for CHD

Cell type	Advantages	Disadvantages	Animal model	Ref.
Fetal/neonatal cardiomyocytes	Ability to establish electromechanical coupling	Less capable of proliferation; Ethically controversial; Immunologic rejection; Sensitive to ischemic injury; Donor availability dependency;	Rat	(Soonpaa et al., 1994, Li et al., 1996, Leor et al., 1996, Reinecke et al., 1999)
Skeletal myoblasts	Autologous source; Easy to isolate and culture scalability; Ischemic resistance; High proliferation potential	Uncontrolled proliferation; Hard to establish electromechanical coupling; Potential arrhythmias; Poor survival of engrafted cells	Rat, Dog and Pig	(Leobon et al., 2003, Al Attar et al., 2003, Zhong et al., 2003, Dib et al., 2002)
Smooth muscle cells (SMC)	Autologous source; Easy to isolate and culture scalability; Secret angiogenic factors	Hard to establish electromechanical coupling; Potential arrhythmias; Poor survival of engrafted cells	Rat	(Li et al., 1999, Stavri et al., 1995)
Embryonic stem	Pluripotent;	Ethically controversial;	Rat and Mouse	(Kehat et al.,

cells (ESC)	Ability to establish electromechanical coupling;	Immunologic rejection; Lacking characterization; Tumorigenesis; Donor availability dependency; Poor survival of engrafted cells		2001, Yamashita et al., 2000, Singla et al., 2006, Min et al., 2002)
Bone marrow derived mesenchymal stem cells (BM-MSC)	Pluripotent; Easy to isolate and culture scalability; Autologous source; Ability to establish electromechanical coupling	Lacking characterization; Poor survival of engrafted cells	Rat and Mouse	(Miyahara et al., 2006, Day et al., 2006, Amado et al., 2005)
Cardiac/endothelial progenitor cells (CPC/EPC)	Easy to isolate from endomyocardial biopsy; Autologous source	Lacking characterization; Poor survival of engrafted cells	Rat and Mouse	(Yang et al., 2008, Tillmanns et al., 2008, Rota et al., 2008, Miyahara et al., 2006, Kattman et al., 2006)

Stem cells, equipped with the potential to differentiate into multiple cell types, were used in an attempt to achieve cell-based therapy for cardiac heart disease. Classification of stem cells is based on their ability to differentiate. Stem cells are either totipotent, pluripotent or multipotent and the order signifies decreased potential. There are two categories of cells: embryonic stem cells and adult stem cells. Adult stem cells are classified with bone marrow stem cells, mesenchymal stem cells (MSCs) and cardiac stem cells (CSCs).

Theoretically embryonic stem cells are able to differentiate into various types of cells. Doetschman *et al.* reported mouse embryonic stem cells (mESCs) were able to differentiate into the visceral yolk sac, blood islands and myocardium, providing the first evidence for potential cardiogenensis from mESCs (Doetschman et al., 1985). Since then, human embryonic stem cells (hESCs) that had the capacity of differentiating into cardiomyocytes that are positive for cardiomyocytes markers such as desmin, myosin heavy chain and α-actinin were approved (Kehat et al., 2001, Kehat et al., 2004, Westfall et al., 1997). Meanwhile, transplantation attempts of ESCs in myocardial infarct animal models have been achieved. Hodgson and coworkers reported that α-actin-derived ECFP-mESCs were detected in the infarcted myocardium. There was less scar formation and decreased the myocardial necrosis (Hodgson et al., 2004). Singla *et al* reported that mESCs implanted in infarct area and differentiated into endothelial cells and cardiomyocytes after 2 weeks injection. Furthermore, heart function was improved without apparent tumor formation (Singla et al., 2006). Nelson *et al.* also supported that engraftment of mESCs in the infarct region could increase the heart function such as ejection fraction (EF) and peak mitral blood flow velocity. However, 21% of the engrafted hearts were found to have mESC-derived tumors in the pericardial space. It is a critical safety issue that needs

to be resolved before consideration for human application (Nelson et al., 2006). Furthermore, this application is also limited by inefficient differentiation (<1% of ESCs), and most of the cells that successfully differentiate into cardiomyocytes die after transplantation. A recent report described an attempt to overcome these limitations (Laflamme et al., 2007). The cardiomycytes derived from hESCs were treated with activin A and BMP4 to improve the cells survival and differentiation efficiency were transplanted into myocardial infarcted hearts of athymic nude rats. The results showed hESCs could partially remuscularize myocardial infarction and the heart function improved compared with the control. However, in this case, most transplanted cells initially died same as in previously described researches above.

Adult stem cells have some advantages compared to ESCs. It is easily collected from autologous, avoids facing ethical disputes over ESC transplantation, and there is less immunorejection after cell transplantation, thus allowing them to be more suitable for clinical applications. Therefore, adult stem cells for cardiac heart disease treatments have become the highlight in recent years.

As an effort to get ESC cell-based replacement therapy, MSCs and cardiac stem cells (CSCs) have been inspected. MSCs also have multi-differentiation potential and no immunorejections because they lacks MHC class II and B7 costimulatory molecule expression (Ryan et al., 2005). Additionally, the identification of CSCs recently suggests that the heart is not the terminal differentiated organ, in which the heart is regulated by stem cells. MSC and CSC transplantation were widely investigated in animal models of ischemic heart disease and carried out with some promising results. Miyahara and coworkers transplanted a monolayer of MSCs derived from adipose tissue onto rat infarcted hearts. Wall thinning reversal in the infarcted region and improved heart function were detected but few MSCs of the

engrafted sheet differentiated into cardiomyocytes (Miyahara et al., 2006). Research by Amado *et al.* in a porcine MI model demonstrated that injected allogeneic MSCs would promote myocardium regeneration, improve heart function and reduce infarct size (Amado et al., 2005, Amado et al., 2006). Mangi *et al* claimed that the transplantation of bone marrow stem cells was able to increase the expression of cell protective protein and promote functional recovery in acute ischemic hearts (Mangi et al., 2003). Some researchers also believe that bone marrow stem cells can enhance angiogenesis to support cells, which secret vascular growth factors and promote proliferation and differentiation of host endothelial cells and smooth muscle cells (Kinnaird et al., 2004).

CSCs were identified as c-kit$^+$ cells with the properties of stem cells that were self-renewing, clonogenic, and multipotent derived from embryos (Wu et al., 2006) or adult hearts (Beltrami et al., 2003). These cells were reported to have the capacity of differentiating into cardiomyoctes, endothelial cells and smooth muscle cells *in vitro* as well as *in vivo* (Kattman et al., 2006, Fazel et al., 2006). A series of papers were published to confirm the functions of these cells in MI models. Bearzi *et al* transplanted the c-kit$^+$ hCSCs into immunodeficient mice and immunosuppressed rat MI model. The results demonstrated that the engraftment of hCSCs generated a chimeric heart and contributed to the performance of infarcted heart (Bearzi et al., 2007). More recent studies by this group also reported that c-kit$^+$ CSCs activated by IGF and HGF implanted in scarred myocardium generated the myocytes and coronary vessels improving the functions of infarct hearts (Tillmanns et al., 2008, Rota et al., 2008). Another study by Wu *et al.* found that c-kit$^+$Nkx2.5$^+$ cells isolated from mouse embryos were able to spontaneously differentiate into both smooth muscle cells and myocardial cells (Wu et al., 2006). In addition, Yang *et al.*

generated KDR^{low}/c-kit$^-$ cells derived from hESCs. These cells were induced with activin A; BMP4; bFGF, VEGF and DKK1 in serum-free media. These KDR^{low}/c-kit$^-$ cells displayed cardiac myocytes, endothelial and vascular smooth muscle potential *in vitro* and *in vivo* (Yang et al., 2008).

The applications of stem cell transplantation provide an opportunity to treat heart diseases. However, there are still many problems that prevent this technology from becoming a therapeutic alternative. Firstly, the long-term fate of transplanted stem cells is still unclear. It is difficult to determine the capacity of transplanted stem cells in finding their optimum "niche" within existing surrounding myocytes. Additionally, the best time for application of stem cell grafts and the efficiency of incorporated cardiomyogenic differentiation of transplanted cells is hard to determine. Such complications limit its practical therapeutic significance. Secondly, although a study (Mangi et al., 2003) has suggested that it is able to enhance cardiac repair through improvement of the survivability of engrafted cells by overexpressing Akt, transplanted cells still cannot survive long enough in the infarcted area without blood supply. Furthermore, all attempts involved treatment of cells *in vitro* before transplantation. Treatment will increase opportunities to yield mutant cells or create an alternative to limit the application in humans. Therefore, stem cells therapy on heart diseases is still surrounded by controversies and ethical debates concerning its clinical application.

1.2.3 Traditional Chinese medicine therapy in CHD

The history of herbs applications for humans dates back 5000 years ago. The initial theoretical system of traditional Chinese medicine was formed during the Han Dynasty (200AD), in which practitioners followed "pattern-differentiated" strategies proposed by Zhang Zhongjing (Ping, 1999). Recently, an increasing number of researchers have tried to develop drugs from traditional medical herbs, identify the active ingredients and their underlying mechanisms. In regard to the cardiovascular system (Shi et al., 2006), there has also been a lot of herbal medicine applications such as Shuxinning Pian, Guanxin II formula, Qiangxinyin, Guanxinsuhe Wan, ShexiangBaoxin Pills and Xiaoxintong. Although treatments with traditional Chinese medicines are different, their major effects to treat CHD are as follows: 1) decrease blood viscosity and improve the volume of myocardium; 2) decrease infarct area and remission of heart ischemia induced by vasopressin; 3) increase concentration of 6-kefo-PGFa; 4) inhibition of platelet adhesion and aggregation and 5) improve the function of the left atrium.

Honokiol and Magnolol isolated from *Magnolia officinalis*, were used to treat MI rats. Four different doses were used which could reduce the infarct zone and suppress arrhythmia during ischemia (Tsai et al., 1996). Further studies demonstrated that Magnolol has an anti-apoptotic effect by enhancing the activation of ERK1/2 and modulation of Bcl-xl proteins (Jin et al., 2008). Danshen reagents were introduced into the clinical setting for ischemic stroke and ischemic heart disease from the 1970's in China. A series of studies also reported Danshen and its active compounds could suppress the aggregation of platelets, remove blood stasis, protect against ischemic reperfusion injury, and enhance the tolerance of ischemic tissue in hypoxia (JL and H, 2000, Zhao et al., 1996, Wu et al., 1998, Lo et al., 2003, Sun et al., 2005).

However, recently Wu *et al.* found that there was not significantly positive effect from DanShen agents in patients with acute ischemic stroke (Wu et al., 2007).

In conclusion, traditional Chinese medicines may provide an altenative route to research and find treatments for CHD. However, to effectively reconstitute affected coronary vessels there needs to be formation of coronary vessels to by-pass the narrowed or damaged coronary arteries. One of the challenges for treatment of ischemia is creating new coronary vessels (including capillary vascular networks) naturally to by-pass the narrowed arteries. Up to now, there are still no drugs or therapeutics available that leads to complete correction of the ischemia or formation of sufficient collaterals to an ischemic region.

1.3 A potential alternative treatment for CHD derived from *Geum japonicum* subspecies

1.3.1 General introduction of *Geum japonicum* and its subspecies

There are two subspecies of *G. japonicum*—*G. japonicum* Thunb. and *G. japonicum* Thunb.var.chinense F.Bolle (Figure 1.2). The main differences between these two are as follows: *G. japonicum* Thunb. is densely covered with white pappus throughout the entire plant. It has a flowering period from April to June, fruit period of September to November. It is mainly distributed in Zhejiang, Anhui, Hubei province of China. *G. japonicum* Thunb.var.chinense F.Bolle is covered with yellow pappus, flowering from June to July, with a fruit phase from August to September, and is mainly distributed in Guizhou, Yunnan and Guangdong and Guangxi province of China.

Figure 1.2 Whole plant of *G. japonicum* Thunb.var.chinense F.Bolle. (Adopt from Xue, 2006)

1.3.2 Chemical ingredients

The chemical analysis of *G. japonicum* was processed by Japanese researchers in the 1980's. Generally, *G. japonicum* mainly contains three classes of components: tannins, triterpenoid and essential oil.

I. Tannins

G. japonicum is abundant with tannins and most of the tannins are water-soluble. Yoshida *et al.* identified the structure of a dimeric ellagitannin - Gemin A, which was the first separated from the leaves of *G. japonicum* in 1982 (Yoshida et al., 1982). Gemin A is an α-glucose and β-glucose dimer ellagic acid tannin. Furthermore, 21 different hydrolysable tannins were isolated. They are 6 dimeric tannins (Gemin A-F) and 15 ellagitannins (potentillin, pedunculagin, tellimagrandin I, tellimagrandin II, 1,2,3-tri-O-galloyl-β-D-glucose，1-Ogalloyl-2, 3-O- [(S)-hexahydroxy diphenoyl-α-D-glucose, casuarictin, casuarinin, praecoxin D, 5-desgalloyl stachyurin, geponin, penta-O-galloyl--glucose, 2,3-(S)-hexahydroxydiphenoyl-D-glucose, 2,6-di-O-galloyl-D-glucose and geraniin) (Yoshida et al., 1985, Xu et al., 1994, Yoshida et al., 1982). An updated study by Liu *et al* found a novel compound-gemin G, which is derived from methonal extract of *G. japonicum* Thunb. var. chinense (Liu et al., 2009).

II. Triterpenoid components

Triterpenoids are another major components in *G. japonicum*. 10 triterpene compounds were isolated from *G. japonicum*: 2α, 19α-dihydroxy-3-oxo-12-ursen-28- ursolic acid, ursolic acid, 19α-hydroxy-epi-ursolic acid, maslinic acid, euscaphic acid (Xu et al., 1996, Fa-Quan Zeng, 1998), 1α-3β-19α-23-tetrahydroxy-ursolic acid-

28-O-β-D-glucoside (niga-ichigoside F1), 2α-3β-19α-trihydroxy-23, 28-di-ursolic acid-28-O-β-D-glucoside (suavissimoside F1), 2α-hydroxy-ursolic acid (Shigenaga et al., 1985), glucosyl tormentate and tormentic acid.

III. Essential oil

A total of 103 kinds of chemical constituents were isolated and 40 were identified by gas chromatography-mass spectrometry (GC-MS) from *G. japonicum*. Constituents of the 40 mainly included fatty acids and their methyl esters, triterpenes and their oxy-derivatives. In detail, components of volatile oils are: palmitic acid, Methyl-11, 14,17-eicosatrienoate, linoleic acid, caryophyllene oxide, eugenol and trans-phytosterol (Zhou et al., 2002) .

1.3.3 Biological research of *G. japonicum*

The genetic lineage of *G. japonicum* is *Plantae, spermatophyta ,angiosperma, Rosale, Rosaceae* (Yuan, 1986). *G. japonicum* is also known as Bluebizen, hudong Shuiyangmei, Riben Lubianqing or Dagencao and is widely used to treat aliments of diarrhea, dysentery, bruises, dizziness and headache in the Guizhou area of China (Zhou et al., 2002).

Some reports demonstrated that methanol extracts of *G. japonicum* Thunb. have anti-virus effects on Herpesvirus hominis (Xu et al., 1994, Kurokawa et al., 1998, Kurokawa et al., 1995). *G. japonicum* Thunb.var.chinense F.Bolle. has been reported to have effects of anti-inflammation (Tan et al., 2006). Scholars from Singapore isolated some active compounds from *G. japonicum*: 2α, 19α -dihydroxy-3-oxo-12-ursen-28-oic acid, ursolic acid, maslinic acid and geumonoid which has displayed the potential for inhibition activity against HIV protease (Xu et al., 2000, Xu et al.,

1996). Gallic aldehyde, geponin and other derivatives that have also been isolated from *G. japonicum* have activity of against herpes simplex virus. In particular, gallic aldehyde was able to inhibit the vacuolated activity of 7401H-I-herpes simplex virus, polio virus and leprosy virus. Also, another group showed the inhibition effects on proliferation of herpes simplex virus type I and type II with tellimagrandin II treatment (Kurokawa et al., 1995, Kurokawa et al., 1998). Furthermore, 19-hydroxyasiatic acid, pedunculagin, tellimagrandin II, casuarinin, praecoxin D and penta-O-galloyl--glucose, tannins separated from the methanol extracts of *G. japonicum* have been identified to significantly prolong the time of rabbit blood coagulation (Fa-Quan Zeng, 1998, Dong et al., 1998). The mechanism of pedunculagin anticoagulant activity is that the tannins could inhibit the thrombin activity. Tellimagrandin II, casuarinin, praecoxin D and penta-O-galloyl-glucose acted as a non-competitive inhibitor of thrombin (Dong et al., 1998).

Recent research has explored some new effects of extracts of *G. japonicum.* The crude extract and the three purified tannins (penta-*O*-galloyl-β-glucoside, casuariin and 5-desgalloylstachyurin) showed vasorelaxant and hypotensive effects mediated *via* endogenous NO and subsequent cGMP formation could be used in anti-hypertensive agents (Xie et al., 2007). The methylene chloride fraction from *G. japonicum* had the capacity for inhibition of tumor metastasis and angiogenesis *via* decreasing CD44 and TIMP-2 expression and increasing the activity of the phospho-JNK signal pathway (Heo et al., 2008). In addition, 3,4,5-Trihydroxybenzaldehyde, isolated from *G. japonicum,* could down-regulate the expression of matrix metalloproteinases (MMP-9 and MMP-2), suppress the binding of NF-kB and AP-1 transcription factors and inhibit the phosphorylation of ERK1/2, p38 and c-JNK,

leading to the inhibition effect of migration of smooth muscle cell (SMC) through MAPK pathways (Suh et al., 2008).

1.3.4 Summary of an herbal derived extraction of *G. japonicum* and its therapeutic effects on MI through its dual actions in coronary angiogenesis and cardiogenesis

Our own studies with *G. japonicum* have revealed the new prospect of effective treatment of MI models. From the beginning we found that the fractions extracted from *G. japonicum* could induce the proliferation and maturation of quiescent satellite cells into myocytes and also result in regeneration of myotubes and myotube bundles in a time-dependent manner in damaged muscle tissues (Cheung et al., 2007, Cheng et al., 2006). These findings stimulated further experimentation in which we already demonstrated the therapeutic effect of the crude extract (MRF) of *G. japonicum* in an acute MI animal model, providing evidence of revascularization and myocardial regeneration (Li et al., 2006).

1.3.5 Aims of study

For effective recondition cardiac circulation in CHD patients, there needs to be natural formation of new vessels to by-pass narrowed vasculature that affects coronary circulation. The epidemiological and economic panorama of CHD and its progressive worsening consequently highlights the need for a new effective treatment method that can help minimize repeated invasive revascularization procedures and their complications, and ideally help provide effective treatment for CHD. In our previous studies, the extract of *G. japonicum* already revealed the promising results for treatment of MI. However, the effects of *G. japonicum* for treatment of CHD, clinically classed by having a partially occluded coronary artery, have not been

clearly proven. Therefore, the present aims of this study are:

1. To isolate the active fraction from *G. japonicum* (AFGJ) and investigate its effect on stimulating the growth of new blood vessels in CHD models.

2. To investigate possible mechanisms underlying the effects of AFGJ and its active compounds (Angio-G) for neovascularization.

Chapter 2 Materials and methods

2.1 Experimental design

2.1.1 Bioassay guided isolation of AFGJ

Isolation of AFGJ and Angio-G was modified from previously described (Cheung et al., 2007). Briefly, *G. japonicum* was collected from Guizhou Province of China and dried. Approximately 500g of dried whole plant was subject to extraction with absolute ethanol at room temperature for 3 days and repeated 3 times. The extract was then concentrated *in vacuo*. The concentrated residue (50g) was suspended in H_2O and partitioned successively with *n*-butanol. All soluble fractions of each partition were filtered and evaporated under reduced pressure at 30-50°C, yielding three different fractions. The *n*-butanol soluble fraction (AFGJ) was then subject to a column of Diaion HP20, equilibrated with 10% ethanol and eluted with increasing concentrations of ethanol in H_2O. Seven fractions were collected (GJ-B-1-7). MTT assay was used to identify the active component responsible for angiogenesis. As shown in Figure 2.1, one component in GJ-B-3, named as Angio-G, stimulated proliferation of HUVECs in culture.

The HPLC system used for this chromatography was an Agilent 1200 instrument (Agilent corp. Ltd., USA). For HPLC, 50mg AFGJ and 10mg Angio-G were dissolved in 1 ml methanol and 20ul sample was injected into reversed phase column (Sinochrom ODS-BP 5μm, 4.6mmx150mm [Dalian Elite Analytical instruments Co., Ltd.]) with constant flow rate of 1ml/min at 25°C. The detection wavelength was set to 254nm. 0.1% Acetic acid-H_2O (solvent A) and methanol (solvent B) were used as the mobile phase. Gradient elution program was listed as followed:

Table 2. Elution program of HPLC

Time (min)	B (Methanol)
0	0%
10	20%
40	30%
50	35%
60	40%
70	50%
80	70%
90	100%

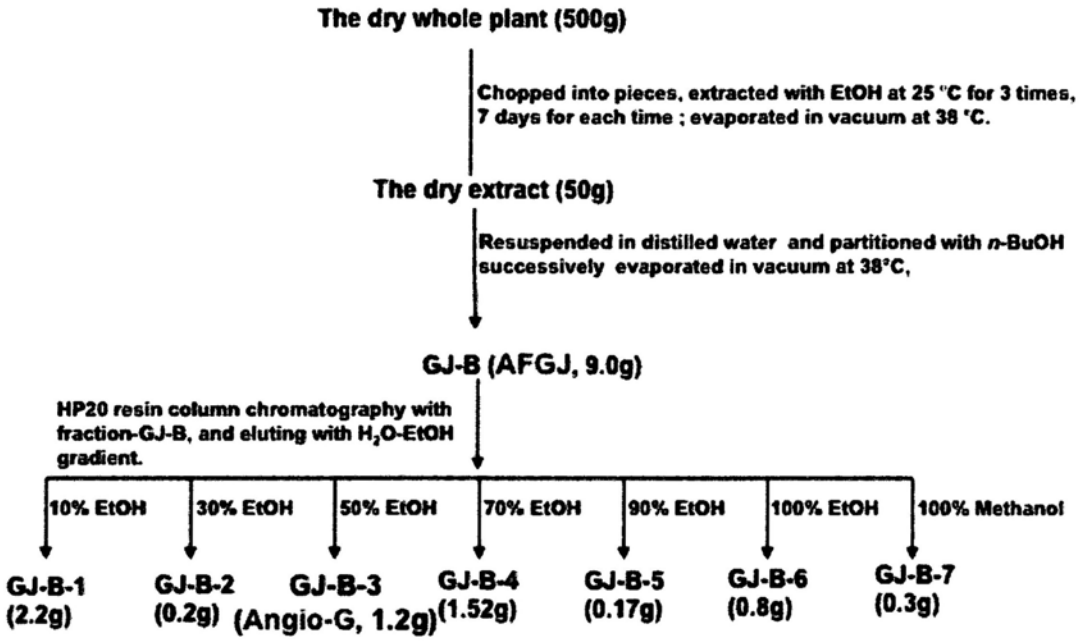

Figure 2.1 Isolation of AFGJ and Angio-G from *G. japonicum.*

2.1.2 Stable CHD animal model production and treatment protocol in rats

Chronic studies were performed to assess the long-term effects of AFGJ on rat ischemic hearts. The experimental procedure is diagrammatically summarized in Figure 2.2. Telemetry transmitters were implanted before CHD surgery and ECG and echocardiography were recorded as baseline (BL) value. Heart ischemia was induced by incompleted ligation of the left anterior descending (LAD) artery. The ECG and echocardiography of rats were recorded one week after CHD surgery to establish a pretreatment record (week 1). The animals were assigned to two groups according to their ECG waveforms to ensure comparable CHD severities. The animals were then treated with AFGJ (at 25mg/ml and 2.5ml) for two weeks by oral feeding. Heart function of the animals were assessed by ECG and echocardiography recorded at different time points (week 3 and week 4). The control rats were received P.O. treatment with H_2O. The hearts were underwent micro-computed tomography (MicroCT) investigation at the 6^{th} week and then the quantitative of the newly formed coronary vessels/collaterals, the morphology, and the ischemia volume were examed.

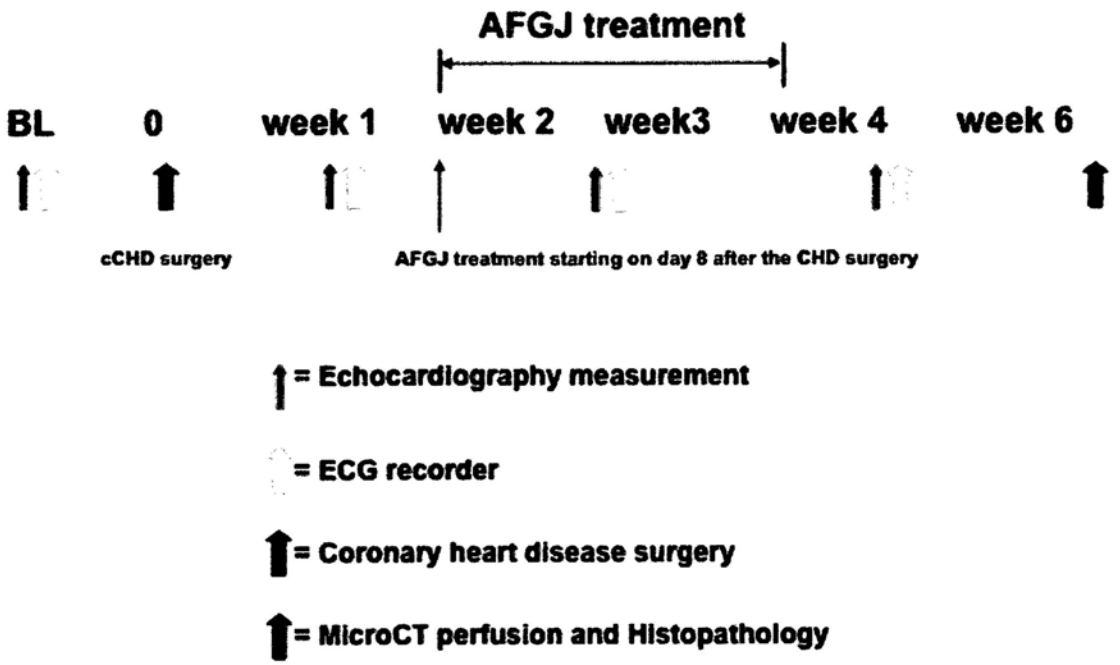

Figure 2.2 Scheme of experimental protocol

2.2 Materials

2.2.1 Animals

Male Sprague-Dawley (SD) rats of 250-300g were used in this study. The rats were housed in plastic cages, four rats in each, which offered proper aeration and free access to drinking water and standard diet. Room temperature in the animal house was maintained at (25±2) °C and a 12-hour light/ dark cycle was programmed automatically. The light period began at 6:00am. All experiments were performed under license from the government of the Hong Kong SAR and endorsed by the animal experimentation ethics committee (AEEC) of the Chinese University of Hong Kong.

2.2.2 Reagents and instruments

All reagent, instruments and solutions was list as following tables:

Table 3. Culture medium, buffers and solutions

Materials	Catalog No.	Suppliers
Fetal bovine serum (FBS)	10270-106	Invitrogen
Penicillin-Streptomycin	15140-122	Invitrogen
Heparin sodium salt	H3149-100KU	Sigma
Endothelial cell growth supplement (ECGs)	356006	BDB FALCON
Kaighn's Modification of Ham's F12K medium	21127-022	Invitrogen

Dulbecco's modified Eagle's medium (DMEM)/F12	11330-057	Invitrogen
Iscove's Modified Dulbecco's Medium (IMDM)	12440-061	Invitrogen
Trypsin-EDTA(0.25% Trypin, 1mM EDTA4Na) Liquid	25200-072	Invitrogen
Recovery-Cell Culture Freezing Medim	12648010	Invitrogen
PBS tablets	E404-200TABS	Amresco
GFR reduced Matrix Matrigel	356230	BDB FALCON
Dimethyl sulfoxide ≥99.5% (DMSO)	D5879-500ML	Sigma
AG490 PTK inhibitor	PHZ 1204	Invitrogen
FicollPaque PLUS	17-1440-02	GE Bio-Sciences
MTT	19265	USB
ThinCert ™ tissue culture Insert for multiwell plates, Pore size: 4 μm transparent)	662641	Greiner bio-one
Poly-L-lysine	P6282-5MG	Sigma

Table 4. Reagents and instruments for animal model

Reagents or instruments Name	Supplier
Ketamine	Alfasan, Holland
Xylazine	Alfasan, Holland
MICROFIL MV-130	Flow Tech, Inc., Carver, Massachusetts
Polyethylene tube for heart perfusion	Smiths Medical LTD.
Syringe	BDB FALCON
37% formaldehyde solution	Advanced Technology & Industrial Co., Ltd

Table 5. Solutions and materials for 2-D gel

Solutions and materials	Suppliers or Recipes
ReadyStrip IPG strips PH 3-10 nonlinear, 7cm, 12	Bio-RAD (163-2002)
Sample lysis buffer	7M Urea, 2M Thiourea, 4% CHAPS in Total 25 ml MilliQ water)
Rehydration buffer	12.5 µl Bio-Lyte 3/10 Ampholyte, 40%, 2 µl 1% Bromophenol Blue, 0.01g DTT in 1 ml Sample lysis buffer
Base Equilibration buffer	6M Urea, 2% SDS, 0.375 M PH 8.8 Tris-

	HCl, 20% Glycerol in total 100ml MilliQ water
Equilibration buffer 1	0.2g DTT dissolved in 10ml base equilibration buffer
Equilibration buffer 2	0.25g Iodoacetamide dissolved in 10ml base equilibration buffer
10X SDS running buffer (161-0732)	Bio-Rad
Mineral oil (163-2129)	Bio-Rad
ReadyPrep Overlay Agarose (163-2111)	Bio-Rad
Seeblue protein Marker (LC5625)	Invitrogen
TEMED (161-0801)	Bio-Rad
Ammonium Persulfate (AP) [161-0700]	Bio-Rad
37.5% Acrylamide/bis (1610125)	Bio-Rad
Protein concentration detection kit (500-0006)	Bio-Rad
Tris-5KG (US75825-5KG)	GE Bio-Sciences
Glycine Ultrapure (US16407-5KG)	GE Bio-Sciences

Bio-Lyte 3/10 Ampholyte, 40% (163-1112)	Bio-Rad
CHAPS (161-0460)	Bio-Rad
Dithiothreitol (DTT) (161-0611)	Bio-Rad
Urea (US75826-5KG)	GE Bio-Sciences
Iodoacetamide (163-2109)	Bio-Rad
Brilliant Blue R, 25g (US32826-25g)	GE Bio-Sciences

Table 6. Ingredients of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Solution	Volume added / 1 gel (ml)	Volume added / 2 gel (ml)
Bis-acrylamide 37.5:1 (A)	11.655	23.31
Tris-HCl 1.5M (B)	8.75	17.5
10% SDS (C)	0.35	0.7
Milli Q water	14.07	28.14
10% APS (D)	0.175	0.35
TEMED (E)	0.01155	0.0231

Table 7. Solutions used for immunostaining

Solutions or antibodies	**Recipes or supplier**
Antigen Retrivol solution	10mM Sodium Citrate, 0.05% Tween 20, pH 6.0
Primary antibody dilution buffer	1%BSA, 0.1% cold fish skin gelatin, 0.5% Triton X-100, 0.05% sodium azide dissolved in 0.01M PBS, pH 7.2-7.4
10% Non-Immune Goat Serum	Invitrogen
PBST	1×PBS + 0.5% Triton-X 100
En Vision+system/HRP, Rabbit DAB+ (K401111)	DAKO
4,6-Damldino-2-phenylindole,dihydrochloride (DAPI) (D1306)	Invitrogen
ProLong Gold Antifade Reagent (P36934)	Invitrogen

Table 8. Solutions used for H&E staining

Materials	Catalog No.	Suppliers or recipes
Xylene	296325	Sigma
100% ethanol	100983	Merck
Hematoxylin Solution (Harris Modified)	HHS32	Sigma
Eosin Y solution	E6003	Sigma (add 1g Eosin Y to 100ml ddH_2O
Acid ETOH	_	Add 1ml HCl to 100ml 70-85% ethanol

Table 9. Antibodies used for immunostaining

Antibodies	Catalog No.	Suppliers
Rabbit polyclonal to Von Willebrand Factor (vWF)	Ab6994	Abcam
Mouse anti-PCNA Monoclonal, Unconjugated, Clone Pc10	Sc-56	Santa cruz
Alexa Fluro 488 goat anti- mouse IgG	A11001	Invitrogen
Alexa Fluro 568 goat anti- Rabbit IgG	A11011	Invitrogen

2.2.3 ECG telemetry recoding system

Traditionally, ECG recording is taken from small animals, in which the wires directly connect to the electrodes, then placed on the limb of anesthetized animals. Both the whole process and results are linked to the performance of machinery and the effects of anesthesia, which has always a direct association with data accuracy. Telemetric recording of physiological parameters in free moving animals is becoming even more prevalent due to the occurrence of commercial products that are more affordable to the majority, which brings vital improvements to the final data. Accuracy of data is achievable due to reactions of conscious animals. Meanwhile, the stress that results from the recording to the animals approaches zero, out of mental impacts in some way. Additionally, the operations could be much easier to be handled by the researchers than before.

The recording system used in this study consisted of three modules, which runs through operations of the implantable transmitters, signal collecting (**Receiver:** RPC-1 model for mice and small animals) and data processers (**Computer control:** DSI data exchange matrix and Dataquest A.R.T system). Implantable transmitters were driven by an internal battery. Researchers may easily control the switching on and off of the machine with a magnet. The transmitter collected locomotor activity, body temperature and biopotential signal (EEG, ECG or EMG). All of these signals were emitted from the radio through FM frequency waves, and then received by a nearby receiver.

Signal collection was achieved through the translation between analog and digital data, which were sent from nearby transmitters to an A/D card installed in the computer. However, the problem was that the transmitter emits its signals by FM

frequency waves while the receiver was non-selective to the signals it receives. As a result, sometimes the receiver not only got the signals from the corresponding transmitter, but also from other transmitters nearby. To avoid the interference from non-desirable nearby transmitters except the implantable transmitters, dense copper meshes were inserted between cages. Before recording, signals from each receiver were carefully examined to make sure that no cross talking occurred among the transmitters nearby.

Data processing was conducted through computers so that the digitized data could be stored, displayed and analyzed by software. These activities enabled data to be saved onto hard disk and reviewed or analyzed off-line, later.

All facilities were purchased from Corporation of Data Sciences International, USA. The specifications of transmitter were list below:

Table 10. Transmitter specifications

Model	Weight (g)	Minimum Animal weight (g)	Biopotential channels	Temperature Operating Range (°C)	Battery Life (Months)
EA-F20	3.9	20	-	34~41	4
ETA-F20	3.9	20	1	34~41	4
CA-F40	7.0	175	-	34~41	6
CTA-F40	7.0	175	1	34~41	6

2.3 Methods

2.3.1 CHD animal model and recording system

2.3.1.1 Transmitter implantation

ECG telemetry implantation was modified from what was previously described (Day et al., 2006, Zimmermann et al., 2006). Male SD rats (n=18) were anesthetized with a mixture of ketamine (60mg/kg) and xylazine (7.5mg/kg) through intraperitoneal (I.P.) injection. A 3cm incision was made on the back skin of the rats. For each rat a sterilized telemetry transmitter (PhysioTel CTA-F40) was inserted into a subcutaneous pocket, which was extended beneath the abdomen by inserting a blunt scissor from the incision and the transmitter placed in the pocket. The negative lead of the telemetry device was placed in the area of the right chest and the positive lead was placed to the left of the xiphoid space and caudal to the rib cage, approximating the standard subcutaneous lead II positions (Figure 2.3). The animals were treated with temgesic (0.3mg/Kg) for 15-30 min prior to surgery to provide pre-emptive analgesia. Post-operation analgesia was applied with temgesic (0.3mg/Kg) for every12 hours to each subject for 2-3 days after surgery, and allowed to recover for 1 week before recording.

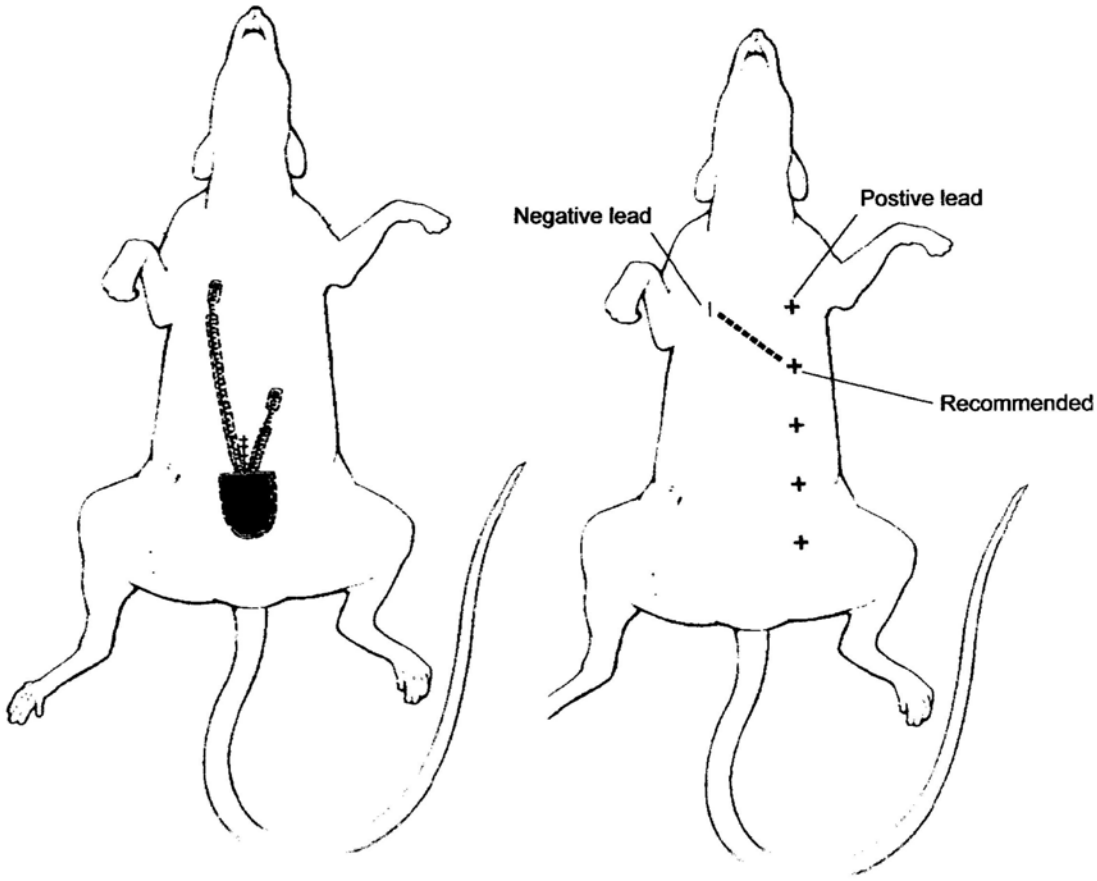

Figure 2.3 Standard lead II positions of electrode of transplanted transmitters for ECG recording

2.3.1.2 ECG assessment of heart function

ECG data was acquired at a sampling rate of 1000 kHz using Dataquest A.R.T (v 3.01, DataSciences). Parameters and waveform of each animal were stored in a PC for later off-line analysis. The transmitters were calibrated based on the data sheet shipped with them before recording. ECG recordings were processed for 1-3 hours at each time point. In theory and clinical diagnosis, evaluation or depression of ST segment represents myocardial injury, ischemia or some medications before infarction develops (Springhouse, 2006, Lewis and Handal, 2000). Furthermore, the depression of ST segment indicates myocardial ischemia if the ST segment is considered depressed above 0.1mV (Lu, 2007, Springhouse, 2006). For the measurement of ST segment depression, a representative ECG signal was shown in Figure 2.4. The depression of ST segments was measured with Clampfit (v9.0) and further statistical analysis was done by GraphPad Prism 5.0 (GraphPad Software Inc., USA).

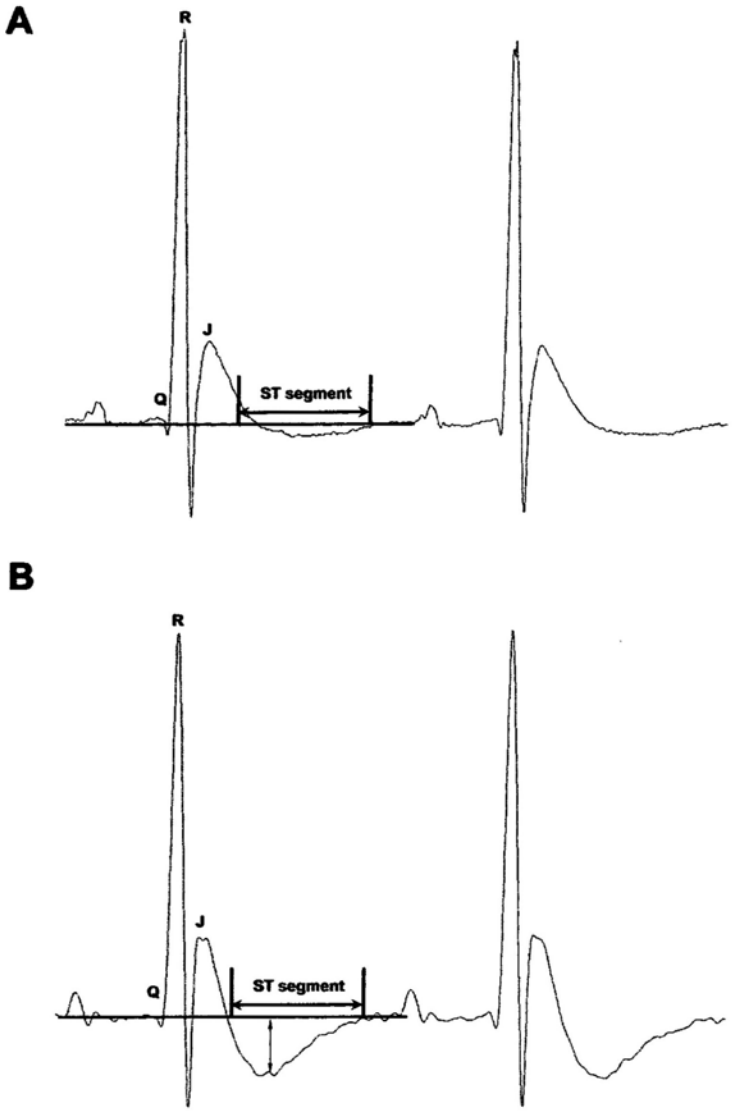

Figure 2.4 A typical example of ST segment evaluation. A: Sample ECG waveforms obtained from a single rat 1 wk after transmitter implantation. B: Sample ECG waveforms obtained from a single rat after surgery (red arrow: the lowest point of ST segment which was recorded.)

2.3.1.3 CHD animal model

The CHD animal model was modified and anesthetized as previously described (Yoshinari et al., 2005). A left thoracotomy was performed and the pericardium was opened which lead to exposure of the LAD. An 8-0 silk suture was placed under the first branch of diagonal artery and a short segment of 11-0 silk suture was laid on the myocardium over the LAD, then the sutures were tied up. The 11-0 silk suture was then drawn out gently to create an only partially blocked LAD. The rats were monitored for 1 hour with continuous ECG recording 1-week post surgery. Prior to treatment, the rats were left to recover for 1 week then treated with AFGJ at 25mg/ml and 2.5ml through P.O. For the control group (n=7), the same operation was conducted but using H_2O instead of AFGJ. All specimens harvested were sectioned for histological and immunohistochemistry analyses.

2.3.1.4 Procedure of heart perfusion for MicroCT reconstruction

To investigate the vascular bypass situation, MicroCT detection was used with MICROFIL injection compounds (MICROFIL MV-130, Flow Tech, Inc.). Briefly, the animal was anesthetized with the mixture of Ketamine (60mg/kg) and Xylazine (7.5mg/kg) intraperitoneally. The thoracic cage was opened rapidly, the thoracic aorta isolated, and a polyethylene cannula inserted distally. The arterial cannula was connected to a 50ml syringe containing PBS and the right atrium was opened to serve as a drain vent. The rat was perfused with PBS until all of the visceral blood volume was flushed out and the perfusate drained through the arterial vent was essentially free from blood. Then the solution was changed into 250ml of 4% formadythine to fix the tissues. During perfusion, the curing agent was added to the MICROFIL injection mass. When perfusion was complete, the mixture was infused

through the aortic cannula by syringe. MICROFIL compound infusion was continued until the injection mass flowed freely from the atrial vent. The atrium and arterial cannula were then clamped and the rat was placed in Room temperture for 30min to allow polymerization. Following these steps, the heart was taken by careful dissection and placed in 4% formadythine at 4 °C overnight and stored in 70% ethanol before MicroCT examination.

2.3.1.5 MicroCT reconstruction analysis of ischemic heart remodeling

Perfused hearts were scanned with high-resolution micro-computed tomography (micro-CT) (VivaCT40, Scanco Medical AG, Bruttisellen, Switzerland). The scan was performed along the long axis of the heart with a voxel size of 21 micron. One thousand projects were made for each stack of images. Each heart was encompassed with minimum of 800 slices.

The image data were analyzed with software provided by the system to quantify the blood vessel volume of hearts. The procedure of reconstitution of hearts was carried out as followed:

1. Acquiring a 3D heart image for gross assessment of the alignment and ligation site.

The 3D architectures of whole hearts calculated in 3D evaluation were preformed with the application of Gaussian filter (Sigma: 0.8, Support: 2). In 3D view program, the rotation and elevation parameters of ligation site were recorded in order to designate following directions of region of interest (ROI).

2. Contour of the heart and the non-vessels microfil blocks.

Due to the perfusion from the thoracic aorta, blocks of the MICROFIL contrast

agent were present in the atriums and ventricles. It is necessary to dissect the peripheral blood vessels of the heart on the acquired images with semi-manual tracing to ensure more specific evaluation of the blood vessels. The dissection was done with identification of the Microfil in the atriums and ventricles and used manual tracing to contour the blocks. Then the whole blocks were contoured with computer-aided edge finding algorithm with set of inner and outer parameter (99 and 45, respectively). Afterward, the heart contour was identified with similar manner. Using the Image Processing Language in the micro-CT system, the non-specific microfil blocks in the atriums and ventricles were subtracted from the heart blood vessels images (Figure 2.5).

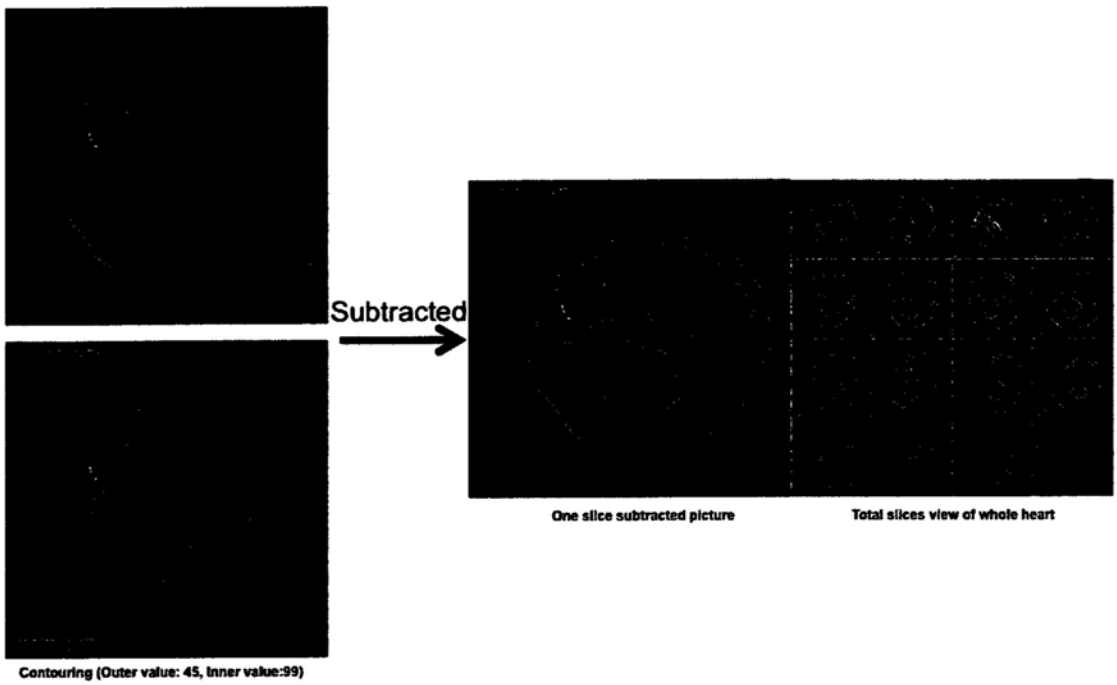

Figure 2.5 Diagram of defining the boundary of analysis area of heart in 2 dimension slices view.

3. Re-alignment of the heart image with a proper anatomical axis for image analysis between different groups.

This step was performed the Z-axis alignment of hearts because position of each heart was not the same, even totally in reverse direction. In detail, use the 3D viewing program to position the VOI with the help of "cutplan %" function and define the z- and x- axis by selecting 3 coordinates (origin_point, z-axis_point, xz-plane_point). The original aim file was read in under IPL command and run ipl> align_z which the coordinates of selected points were input. And then the aligned file was written to the corresponding measurement folder.

4. Defination of the region of interest (ROI) and volume of the interest (VOI).

A 3D image of the heart blood vessels was generated after the evaluation (Figure 2.6). Since the perfusion protocol and the imaging protocol were standardized, the image analysis of the heart blood vessels was done with same segmentation parameters for comparable results between groups. The VOI was generated from the subtraction and a three-dimensional (3D) evaluation of the heart blood vessels was preformed with the application of Gaussian filter (Sigma: 0.8, Support: 2).

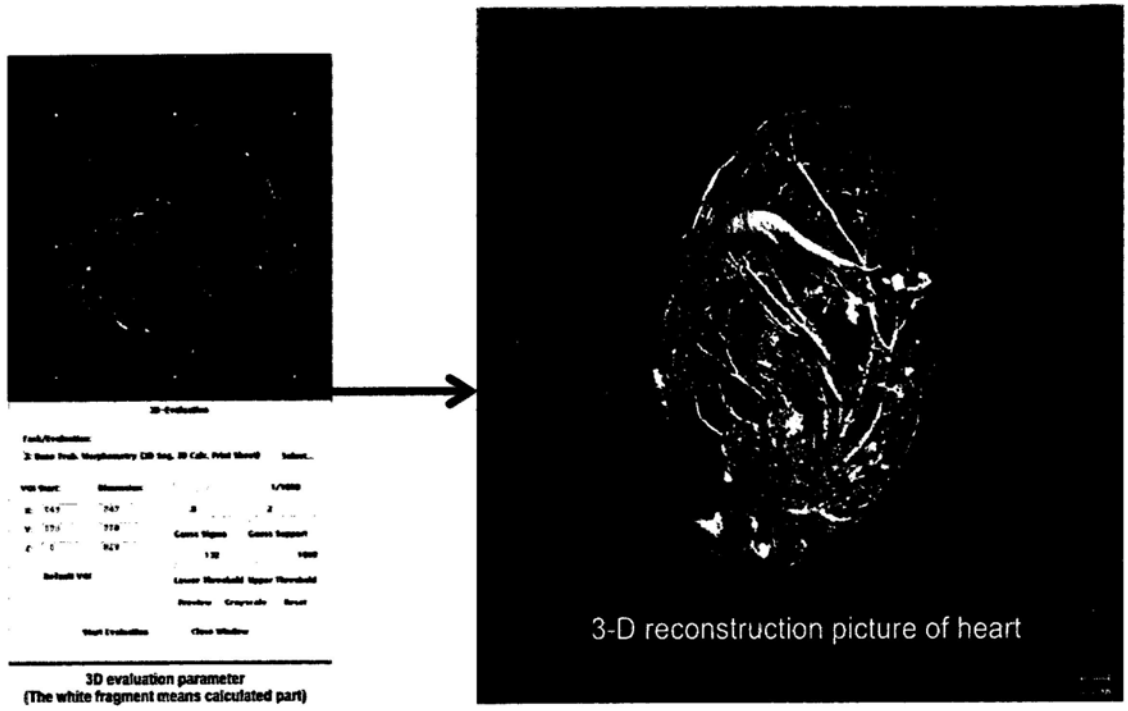

Figure 2.6 The construction parameter and 3-D picture of reconstructed heart.

5. Evaluation of the 3D image with standardized Gaussian filter and threshold parameter.

In this step, 3D evaluation of the aligned and subtracted hearts was recalculated with the application of Gaussian filter (Sigma: 0.8, Support: 2) to produce the new 3D images of hearts.

6. Parameters used for comparison and description of each parameters.

The heart ischemic area was defined as ROI and VOI, which began from ligation position down to 500 slices. 2-D evaluation was counted in the area of vessel within contoured total area to evaluate the blood vessel density (%) every 4 slices. 3-D evaluation was performed the following index: blood vessels volume in ischemic area (VV, mm^3), counted the volume of vessels within contoured total volume (VV/TV) and vessel branching Number (vb.N, 1/mm) which meaned the number of intersections between vessel and nonvessel components per total length of test lines applied to a specimen. The images were represented by three-dimensional. With this procedure, it provided clear visualization of the newly formed nature bypass blood vessels.

2.3.1.6 Echocardiography assessment of heart function

Each of 7 experimental rats received baseline echocardiograms before experimentation. Echocardiograms were recorded under controlled anesthesia (ketamine (60mg/kg) and xylazine [7.5mg/kg]) using a PST-AT6.5MHz phased-array transducer and a TOSHIBA Xario® system. M-mode tracing and 2-D echocardiography images were recorded from parasternal long- and short-axis views.

Systolic and diastolic wall thickness of hearts, defined as left ventricular end-systolic and end-diastolic dimensions, was measured following the leading edge convention of the American Society of Echocardiography in M-mode tracing. For each measurement, at least three consecutive cardiac cycles were recorded. Left ventricular fractional shortening (FS) and ejection fraction (EF) were calculated by a program on the TOSHIBA Xario® system.

2.3.1.7 Histopathological analysis and quantification of capillary density

All hearts were fixed and vibratome-sectioned to 5 mm in thickness, and the resultant uniform transverse sections were routinely processed and paraffin-embedded for histology. Vascular density was determined from H&E stained sections by counting the number of vessels within the ischemic zone using a light microscope under high power field (×40). All vessels in 10 random and non-overlapping view fields within the ischemic field were counted in each section of all AFGJ treated and control hearts. The number of vessels in each view field was averaged and expressed as the number of vessels per view field. Vasculature was counted by two investigators in a blinded fashion.

2.3.1.8 Haematoxylin and Eosin (H&E) staining

Slides were placed in a slide holder and sections were deparaffinized through a sequence of immersions in the followed solutions:

Table 11. Deparaffinization processes

Xylene I	10min
Xylene II	10min
100% Alcohol I	10-30sec
100% Alcohol II	10-30sec

95% Alcohol I	10-30sec
95% Alcohol II	10-30sec
85% Alcohol	10-30sec
70% Alcohol	10-30sec

After rinse with tap water (3 times for 3 minutes), the slides were incubated in hematoxylin solution for 10 minutes and rinsed with tap water. Then sections were differenced in 1% acid ethanol before going into eosin Y solution for 2 minutes. The dehydration process were also conducted through a sequence of immersions, using the solutions as follows:

Table 12. Dehydration processes

95% Alcohol I	10-30sec
95% Alcohol II	10-30 sec
100% Alcohol I	10-30sec
100% Alcohol II	10-30 sec
Xylene I for clear	10 min
Xylene II for clear	10 min

Following dehydration, place a drop of per-mount on the slide using a glass rod. Angle the coverslip and let the coverslip fall gently onto the slide. Allow the per-mount to spread beneath the coverslip, covering all the tissue. Dry overnight in the hood.

2.3.1.9 Immunohistochemistry of paraffin section of CHD heart tissues

Rat hearts were processed and embedded in paraffin. Thin sections (5μm thick) were cut from each heart and processed for immunohistochemistry with DAKO En

Vision DAB kit. Sections were deparaffinized and rehydrated following the procedure below:

Table 13. Deparaffinization and rehydration processes

Xylene I	10min
Xylene II	10minu
100% Alcohol I	30sec with shaking
100% Alcohol II	30sec with shaking
95% Alcohol I	30sec with shaking
95% Alcohol II	30sec with shaking
85% Alcohol I	30sec with shaking
85% Alcohol II	30sec with shaking
Rinse in distilled water	1 min

After rehydration, the slides were put in a plastic jat containing pre-boiled 100ml of 0.01M citrate buffer (+0.05% Tween), at a PH of 6.0, then applied to 350W microwave irradiation for 15 minutes, then left to cool in room temperature for 40 minutes. After cooling, tap off excess buffer and apply enough peroxidase block from Bottle 1 of DAKO kit to cover specimens. Incubate for 1 hour, and then rinse gently three times (5min per wash) with PBS and tap off excess buffer and wipe slides as before. Apply enough optimally diluted primary antibody or negative control reagent to cover specimens (mouse anti-PCNA 1:200 or rabbit anti-vWF 1:200). Sections were incubated overnight at 4 °C, and then rinsed gently with PBST three times (5min each time) and apply enough labeled Polymer from bottle 2. After the final rinse, sections were incubated for 30 minutes and again rinsed three times with PBST. Prepared liquid DAB+substrate-chromogen solution was then poured on specimens followed by incubation for 30sec-5mins to allow DAB reactions to take

place. The reaction was stopped with distilled water and specimens were restained with eosin for 45sec or counterstained with hematoxylin for 30 sec. Dehydration and clearing procedures were done by the following steps:

Table 14. Dehydration and clearing processes

95% Alcohol I	30sec with shaking
95% Alcohol II	30sec with shaking
100% Alcohol I	30sec with shaking
100% Alcohol II	30sec with shaking
Xylene I for clear	10min
Xylene II for clear	10min

Slides were mounted as previously described for H&E staining.

2.3.2 Assessing the effect of AFGJ and Angio-G *in vitro*

2.3.2.1 Cell culture

Human umbilical vein endothelial cells (HUVEC) (American Type Culture Collection [ATCC], Maryland, USA) were cultured on gelatin-coated flasks in Ham's F12K medium with 2mM L-glutamine supplemented with 15%FBS, 6U/ml heparin, 30ug/ml endothelial cell growth supplement, 100U/ml penicillin and 100ug/ml streptomycin. Cells were incubated at 37°C with 95% air and 5% carbon dioxide (CO_2) until confluent. When cells have grown greater than 90% confluency (75 cm^2 Flask), cells were washed once with 1X PBS and detached with 2ml 0.25% trypsin- EDTA for 2 minutes. Dislodged cells were resuspended with culture medium to inactivate the trypsin and were then centrifuged at 1000rpm for 5 minutes. Centrifuged cells were resuspended in 9ml culture medium and re-seeded into three new 75cm^2 culture flask and returned to the incubator. The culture medium

was refreshed every three days and frequency of making subcultures did not exceeded 20 passages for experimentation.

2.3.2.2 Primary isolated GFP-MSCs from mice

The tibias/femurs of transgenic mice were removed and the BM was flushed out of the bones with DMEM/F12 culture medium. The BM was mixed well and centrifuged at 1500 rpm for 5 minutes. The cell pellet was suspended with 5ml culture medium. While the cell suspension was carefully put on 5ml Ficoll solution to minimize disturbance and centrifuged at 2000 rpm for 30 minutes. The second layer was transferred into a tube and washed twice with PBS to remove Ficoll (1200 rpm for 5 minutes). The cell pellet was resuspended in DMEM/F12 culture medium containing 20% heat inactivated FBS and 1% penicillin/streptomycin antibiotic mixture and used for *in vitro* experimentation. Non-adherent cells were discarded after 48 hours of culturing. The adherent cells were cultured by changing medium every 3 days and the cells became nearly confluent after 14 days of culturing.

2.3.2.3 Flow cytometry (FCM) to identify GFP-MSC cells

GFP-MSCs were trypsinized with 0.25% trypsin-EDTA for 2 minutes and centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded and cells resuspended with 0.01M PBS containing 1% BSA. 10^6 GFP-MSCs were incubated with anti-mouse Sca-1 antibody-PE conjugated at 4°C for 45 minutes. These cells were then washed for three times with 0.01M PBS in order to wash out any unbounded antibodies. Fluorescence was detected on a FACS machine (Becton–Dickinson) using Cell Quest Software.

2.3.2.4 MTT assay and analysis of tube-like structure formation on HUVECs

For cell proliferation analysis, MTT assay was used following the modified ATCC protocol (Vignais et al., 1996). HUVECs (2×10^3/well) were seeded into a 96-well

culture plate with growth medium (Ham's F12K medium with 15% FBS, 6U/ml heparin and 30μg/ml endothelial growth factor supplements). After cell attachment, the medium was changed to Ham's F12K medium with 2% FBS for 12 hours. Cells were then treated with Angio-T of gradient concentrations 0, 5, 10, 25, 50, 100 and 200μg/ml, respectively for 36 hours. The OD value was measured by Tecan Sunrise plate reader (GmbH, Australia). Cell proliferation rates were statistically analyzed with Prism 5.0.

For the evaluation of capillary tube formation, BD BioCoat™ Angiogenesis System: Endothelial Cell Tube Formation was used. HUVECs were seeded onto growth factor reduced (GFR) BD Matrigel™ Matrix coated on 96-well plates, then incubated at 37°C with 5% CO_2 for 18 hours with different treatments. AG490, as a JAK/STAT signal pathway inhibitor, was used in a capillary tube formation assay. HUVECs were seeded onto GFR BD Matrigel™ Matrix as described above, and treated with different doses of AG490 (5, 10, 20μg/ml) with Angio-G (5μg/ml) for 18 hours. Tubules were visualized with a Zeiss LSM510 META confocal microscope. Endothelial cell tube areas were quantitated using Adobe Photoshop 7.0 and expressed as units in pixels.

2.3.2.5 MSCs and HUVECs co-culture system

GFP-MSCs and HUVEC were trypsinized and the cell number was counted. GFP-MSCs were seeded onto a poly-L-lysine coated coverslip which was placed in a 24-well plate. HUVECs were seeded in culture inserts for 24-well plate (Figure 2.7). Cells density of both cell types was 1×10^4/well. After cells attached, the medium was changed into co-culture medium (DMEM/F12+3%FBS+1% penicillin/streptomycin antibiotic mixture) with different treatments as listed below:

Table 15. Groups and treatments of co-culture system

Groups	GFP-MSCs	HUVECs	Medium and Drugs	Primary antibody
I	+	-	Co-culture medium only	PBS
II	+	-	Co-culture medium + AFGJ 125μg/ml	Rabbit anti-vWF
III	+	+	Co-culture medium only	Rabbit anti-vWF
IV	+	-	Co-culture medium +Angio-G 10μg/ml	Rabbit anti-vWF
V	+	+	Co-culture medium + AFGJ 125μg/ml	Rabbit anti-vWF
VI	+	+	Co-culture medium +Angio-G 10μg/ml	Rabbit anti-vWF

All groups of cells were incubated in 37°C and 5% CO_2 for 7 days. Medium was changed every 3 days. Then cells were fixed by 4% paraformaldehyde (PFA) and processed immunofluorescence staining.

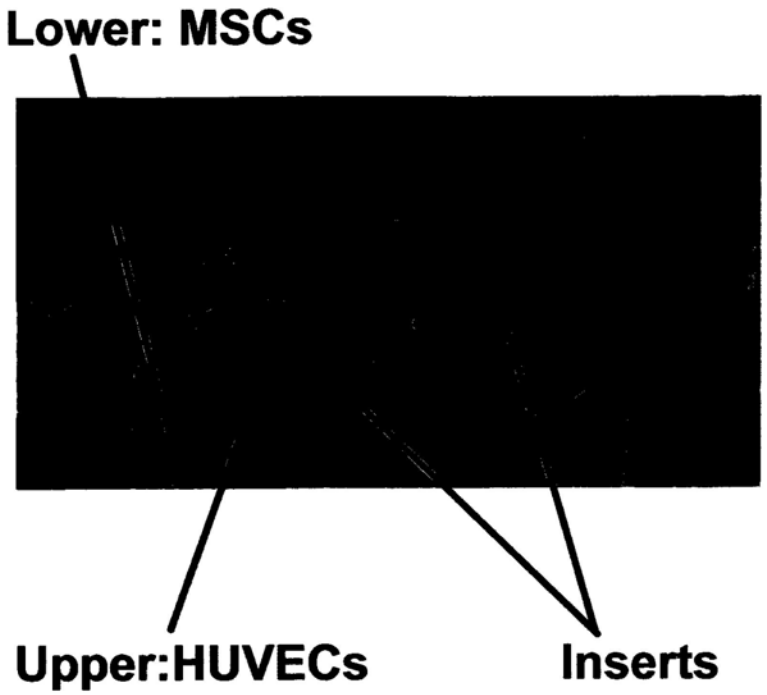

Figure 2.7 Diagram of GFP-MSCs and HUVECs co-culture system

2.3.2.6 Immunofluorescence of fixed cells

Cultured cells were fixed with 4% PFA in PBS for 30 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. Cells were washed 3 times (5 minutes per wash) with PBST and incubated in 10% non-immune goat serum for 1 hour to avoid non-specific sites of antibody adsorption. Diluted rabbit anti-vWF (1:200) was covered on specimen overnight at 4 °C. The primary antibody solutions were removed from the wells and the cells were washed 3 times (10 minutes each) with 0.05% Tween-20 in 1×PBS. Then another wash was conducted with PBS 3 times (5 minutes each). Secondary antibodies (goat anti-rabbit IgG antibodies conjugated with peroxides or fluorophore [Alexa fluro 568, Invitrogen]) were added for 1 hour at room temperature. The nuclei were stained with 300nM DAPI. In all cases, slides were mounted with ProLong Gold Antifade Reagent (Invitrogen) and visualized on Zeiss LSM510 META confocal microscope using 20× and 40× objectives.

2.3.2.7 Capillary tube like formation assay.

GFP-MSCs and HUVECs were co-cultured as described in 2.3.2.4 in 6-well plate (using inserts for 6-well plate) for 5 days. The co-cultured GFP-MSCs were trypsinized and the cell number was counted. The counted cells were mixed with equivalent HUVECs (1:1, 1×10^4/well) and seeded onto GFR BD Matrigel™ Matrix coated on 96-well plates, then incubated at 37°C with 5% CO_2 for 18 hours with presence of Angio-G (5μg/ml). Tubules were visualized and pictured with a Zeiss LSM510 META fluorescence microscope.

2.3.3 Proteomic analysis of repaired hearts in CHD rat model and HUVECs treated with AFGJ and Angio-G

2.3.3.1 Preparation of proteins from CHD hearts

A small slice of each heart from the above-prepared ischemic rats with or without AFGJ treatment were collected and put into liquid nitrogen immediately after incision. The tissues were stored in liquid nitrogen prior to processing. About 60mg of the frozen specimens were ground to a fine powder in liquid nitrogen and transferred into a sample grinding tube which contained grinding resin (Amersham Biosciences Corp.). The tissue powder was further homogenized using a handheld homogenizer for 1 minute in 200μl of 2-D lysis buffer containing a mixture of protease inhibitors (PMSF, 20mM; pepstain, 0.34mg/ml; benzamidine HCl, 5.6mg/ml; leupeptin, 1mg/ml; E-64, 0.36mg/ml) at 4 °C. After grind 3 times, samples were centrifuged at 16,000rpm for 10 minutes. The clear supernatant was carefully transferred into another tube and the concentration of the extract was measured using the Bradford protein assay kit (see 2.3.3.3 for details).

2.3.3.2 Preparation of proteins of HUVECs treated with AFGJ and Angio-G

HUVECs were cultured in a 150cm^2 culture flask coated with gelatin and starved in 5% FBS F12K medium for 12 hours before treatment. The starved cells were treated with AFGJ (125μg/ml) or Angio-G (100μg/ml) for different time points (12 hours and 24 hours) in F12K containing 15% FBS with 6U/ml heparin and 1% P/S. The cells were washed with 1×PBS and detached with 2ml 0.25% trypsin-EDTA and centrifuged at 1000rpm for 5 minutes. The pellet of cells was then wash 2 times with 220mM Tris-HCl (PH 7.4) in order to reduce the level of ions. The cell

pellet was then transferred into a grinding tube handled according to the procedure described in 2.3.3.1.

2.3.3.3 Protein concentration

Total protein concentration of the samples was measured with a protein assay kit (Bio-RAD, USA). Here bovine serum albumin (BSA) was used as the standard protein solutions. The linear range of the BSA at a microtiter plate assay is 0.5 mg/ml to approximately 8 mg/ml. All the protein solutions were assayed in triplicate. Dye reagent was diluted five times with MilliQ water and added 200μl into 96-well plate, then transferred the standard protein solution or tested samples into well which containing the dye reagent. The plate was shook 2 minutes for color reaction and measured the OD value at 570nm. The protein concentrations were calculated with Excel software (Microsoft corp.)

2.3.3.4 Two-dimensional electrophoresis (2-D PAGE)

It has been recognized that the two-dimensional electrophoresis (2-D PAGE) can effectively separate thousands of proteins at the same time. Therefore it is possible to be in the vanguard of the technology to high resolution for protein separation.

The first dimensional isoelectric focusing electrophoresis (IEF) was performed with a PROTEAN IEF cell to focusing proteins on the basis of their pI which is defined as the pH at a charged proteins not to migrate in electric field. 300μg total protein (for tissue samples) or 160μg total protein (for HUVECs lysis) were separated on 7cm ReadyStrip IPG strips PH 3-10 NL (Bio-RAD, USA). Each strip was covered with 2-3 ml of mineral oil to prevent evaporation during the focusing process. IEF was operated at 17 °C using the following program:

Table 16. IEF program of 2D gel

Steps	Voltage	Voltage Ramping Modes	Time
Rehydration	50V	-	12 hours
S1	250V	Liner	30 minutes
S2	500V	Fast	30 minutes
S3	8000V	Liner	4 hours
S4	8000V	Fast	25,000 Volt hours
S5	500V	Fast	5 hours

After IEF, the IPG was prepared to transfer to the second dimension. Place the focused strip in each channel and filled the channels successively with the equilibration buffers I for 15 minutes, then decant. Recover the strip with equilibration buffer II and incubated again for 15 minutes. After equilibration, remove the IPG strip and embed it onto the prepared second-dimension gel.

The second-dimension separation was performed with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a vertical tank. The separating gel was prepared for suitable gel concentration (10%) with solution A-E (see 2.2.5 for details). The gel was placed 20 to 30 minutes at room temperature to be polymerized.

The strips were then transferred into the cathode side of the prepared SDS-PAGE gel and sealed with 0.5% Ready Prepared Overlay agarose. The electrophoresis started to run under 60V constant voltage for 10 minutes and increased to 160V running for 1 hour in 1×SDS running buffer. In all experiments, the second dimensional SDS-PAGE was performed immediately after IEF.

The resolved proteins were stained by 0.25% commassive blue solution. The stained gels were scanned using a Bio-Rad GS-800 calibrated densitometer. The

digital gel images were analyzed in a TIFF format with PDQuest^{TM} software (version 7.3).

2.4 Biostatistics

All morphometric data were collected blindly. Results are presented as mean±SEM computed from mean measurements obtained from each rat heart. Statistical significance for comparison between two measurements was determined using the unpaired 2-tailed Student *t*-test. One way or two way ANOVA was used for determing the 3 measurements. Values of $p<0.05$ were considered significant.

Chapter 3 Effect of active fraction of *G. japonicum* (AFGJ) on the coronary heart disease (CHD) rat model

3.1 Introduction

Coronary heart disease (CHD) is a primary disease that can lead to premature death. Major risk factors have been extensively identified, in which effective therapeutic control strategies had been executed in order to decrease the death rate from CHD. However, according to clinical figures, the strategies developed so far have failed to overturn the fact that CHD is still the most prevalent cause of death, especially in the aged population. Hence, current strategies are lacking significantly desired effect in practical treatment. In our previous study (Cheng et al., 2009) we have demonstrated the dual actions of angiogenesis and cardiogenesis of a methanol extract from *G. japonicum* (EGJ) and an active molecule (cardiogenin). Both EGJ and cardiogenin greatly enhanced myocardial regeneration of endogenous MSCs in the infarcted region of experimental rat hearts. Another study of ours demonstrated that another extraction of EGJ (Angio-T) could stimulate the growth of new vessels in muscle ischemia animal model with activation of various angiogenic factors, such as VEGF, EGF and FGF, all of which are related to angiogenesis (Cheung et al., 2007, Gu et al., 2006). Based on our previous studies, we hypothesize that active fractions of *G. japonicum* (AFGJ) may effectively stimulate the growth of new coronary vessels and the formation of sufficient collaterals to the chronic ischemic region of CHD hearts, and therefore provide a possible effective treament of CHD by presenting improved blood perfusion to ischemic regions.

One of the objectives of this study was to screen for the active fraction of *G. japonicum* (AFGJ) for the development of a therapeutic drug for CHD. According to the guidelines of the FDA and the ICH, we reduced the number of potential toxicity residual solvents during the isolation process. The guideline from ICH-IMPURITIES: GUIDELINE FOR RESIDUAL SOLVENTS Q3C (R3) offers a pharmaceutical interpretation for residual solvents. It is a type of organic volatile

chemicals used or produced in the manufacture of drug substances or excipients, or in the preparation of drug products. If appropriately selected to make a synthetic drug, the solvents tend to increase the yield, determine characteristics about crystal form, purity and solubility. On the other hand, there is a warning that some solvents are linked to cause unacceptable toxicities (*Class 1*). In order to protect patients from potential adverse effects, academically speaking, all residual solvents should be removed, for non-therapeutic effects could be achieved. Despite the advanced manufacturing techniques, such solvents could not be thoroughly eliminated. Nevertheless, this clearance should be strictly controlled within safety standards to protect any patients from being harmed by residual solvents when using drug products, as well as to meet fundamental quality requirements that cover aspects including product specifications and good manufacturing practices. Any solvents used that serve no good purpose should be strongly justified. We aimed to limit the solvents associated with severe toxicity, in the production of drug substances, excipients, or drug products, as well as in a risk-benefit assessment. Methanol, mainly used in previous studies for extraction of *G. japonicum*, belongs to the *Class 2* solvents in the ICH Q3C guideline and has a maximum concentration limit of 3000ppm. Chloroform and hexane are also listed as *Class 2* solvents in the guidebook. Therefore, we modified the extraction procedures and tested the prolifertation effect of AFGJ and Angio-G (an active fraction derived from AFGJ) on HUVECs.

3.2 Results

3.2.1 Isolation and characterization of AFGJ and Angio-G from *G. japonicum* Thunb. Var. Chinense F. Bolle

As mentioned before, to avoid the potential toxicity of methanol and other residual solvents during the extraction of *G. japonicum*, the extraction method previously used was modified. Approximately 50g of dried powder was produced from ethanol extraction of 500g-dried whole plant. Successive partitions of the dried powder with *n*-butanol yielded AFGJ. AFGJ was evaporated under reduced pressure (48°C) and was further separated using reverse phase column chromatography with a gradient eluting program. Seven fractions were resolved from this chromatography. Activity test demonstrated that fraction 3 (GJ-B-3, named as Angio-G) from current isolation displayed a potent effect on promoting the proliferation of HUVECs *in vitro*. The fingerprint of AFGJ and Angio-G was analyzed by reversed phase HPLC. It was shown that approximately 21 absorption peaks were resolved from the elution peaks of AFGJ (Figure 3.1A), and 12 peaks were resolved from the chromatography of Angio-G, which was further classified into three parts according to elution time. The peaks of 7, 8, 9, 10 were the major compounds representing more than 50% of the composition in Angio-G (Figure 3.1B). Furthermore, the comparison between the footprints of AFGJ and Angio-G displayed that 5 peaks derived from Angio-G chromatography could match with the peaks derived from AFGJ chromatography at the same retention time (Figure 3.1C), which indicated Angio-G was derived from AFGJ.

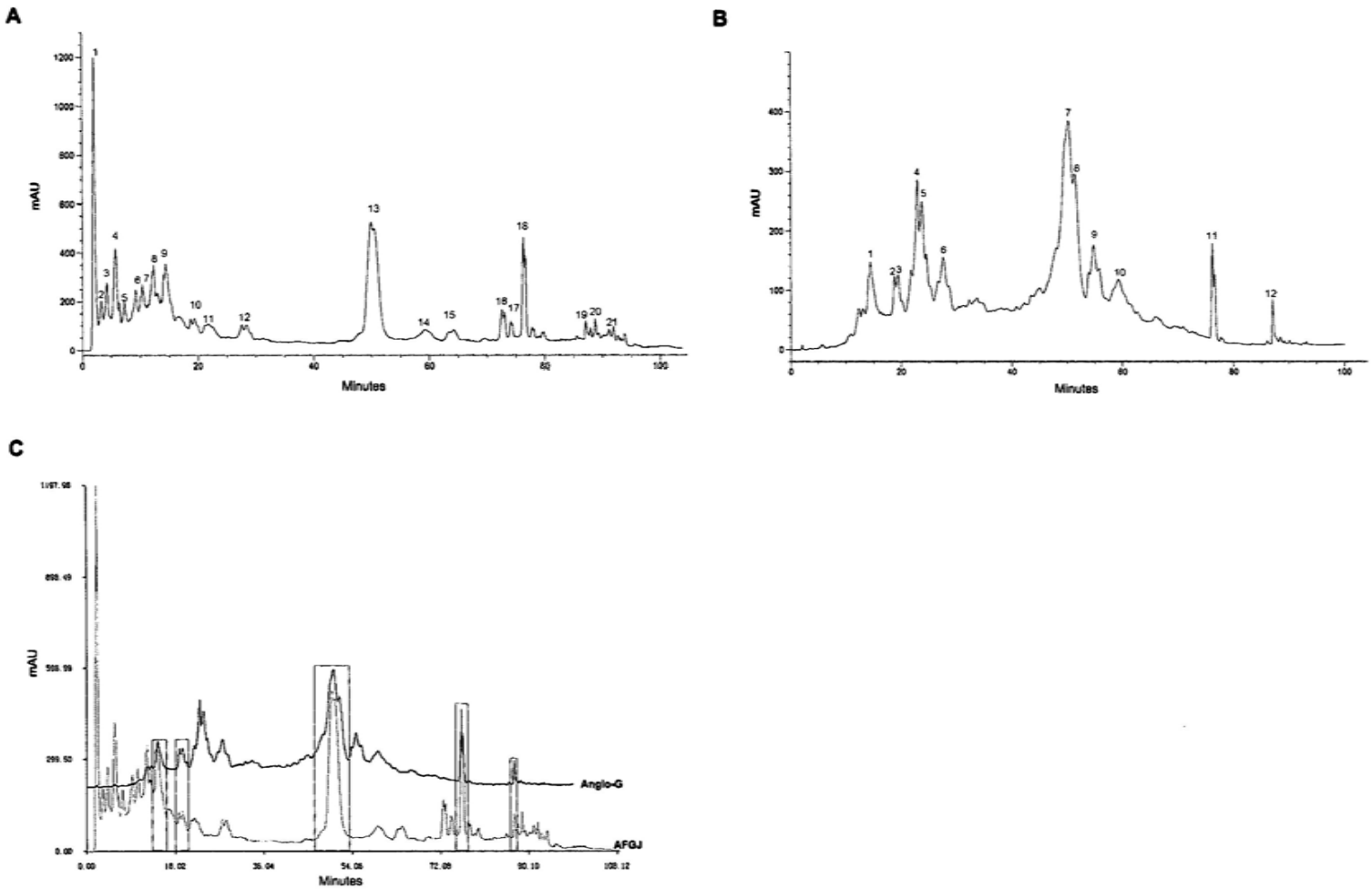

Figure 3.1 HPLC chromatograms of ethanolic isolated AFGJ and Angio-G. (A) Representative of AFGJ chromatogram. (B) Chromatogram of Angio-G. (C) Comparison of AFGJ and Angio-G showed 5 peaks displayed similar waveform at the same retention time (green rectangle).

3.2.2 Cell culture confirmation of the effects of AFGJ and Angio-G

The dynamic process of angiogenesis includes endothelial cell proliferation, migration, lumen formation and maturation (Adams and Alitalo, 2007). Therefore, we first tested the effect of AFGJ and Angio-G on endothelial cell proliferation. MTT assay was used to assess the effect of AFGJ and Angio-G on proliferation in vessel endothelial cells (HUVECs). HUVECs were treated with various concentrations of AFGJ or Angio-G. Our results revealed that while AFGJ slightly stimulated proliferation of HUVECs at a high concentration, Angio-G significantly and drastically stimulated the proliferation of HUVECs in a dose-dependent manner (Figure.3.2).

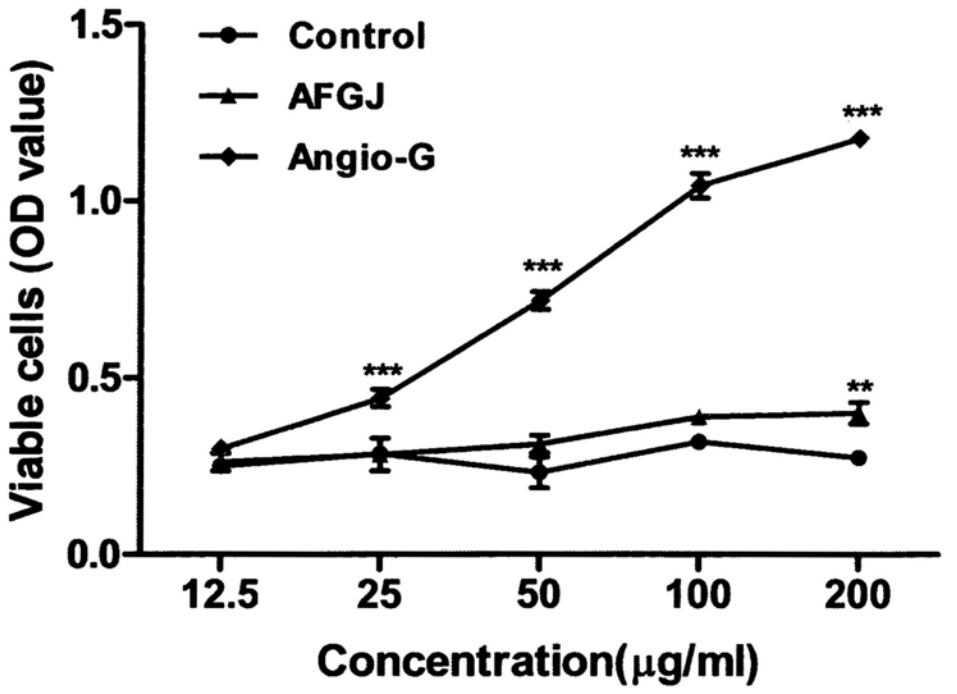

Figure 3.2 AFGJ and Angio-G induced proliferation of HUVECs. There was no obvious increase of proliferation effects in lower concentrations of AFGJ and Angio-G, but the proliferation of the cells began to sharply increase when the concentration of Angio-G was beyond 50μg/ml (***, $p<0.0001$). AFGJ treatment was shown the proliferating difference at 200μg/ml (**, $p<0.01$).

3.2.3 Establishment of CHD rat animal model

A total of 18 rats were enrolled for the CHD rat model and 15 successfully completed the procedure (including 1 sham rat for reference data). One rat had died by the 5th day of treatment, and 1 rat was excluded because of insufficient effect of the occluder under ECG monitor. Lastly, 1 rat had failed to be perfused with MicroFil dye.

3.2.4 AFGJ improves heart function in CHD rat model

To demonstrate whether AFGJ-enhanced neovascularization in ischemic heart tissue would lead to improved heart functional performance, ECG and echocardiography were used to measure the ischemia and functional performance in both treated and non-treated control hearts at different time points before and after AFGJ treatments in CHD rats. It was found that similar waveform expressions of ECG in both vehicle and AFGJ treated groups at baseline level before and after incomplete LAD ligation were recorded. Interestingly, the significantly lowered ST segments of ECG were progressively restored to the normal level 2 weeks post AFGJ treatment, indicating the restoration of blood perfusion to the ischemic hearts. By contrast, the ST segments of ECG in the vehicle hearts remained significantly depressed, suggesting remained ischemia of the vehicle-treated hearts (Figure 3.3A). Quantitative analysis of the obtained ECG data demonstrated that the absolute value of ST segment depression was significantly restored in AFGJ-treated group (means±SD: 0.036±0.025 mV) compared to the values in vehicle group (means±SD: 0.128±0.048mV), in which $p<0.01$ (Figure 3.3B).

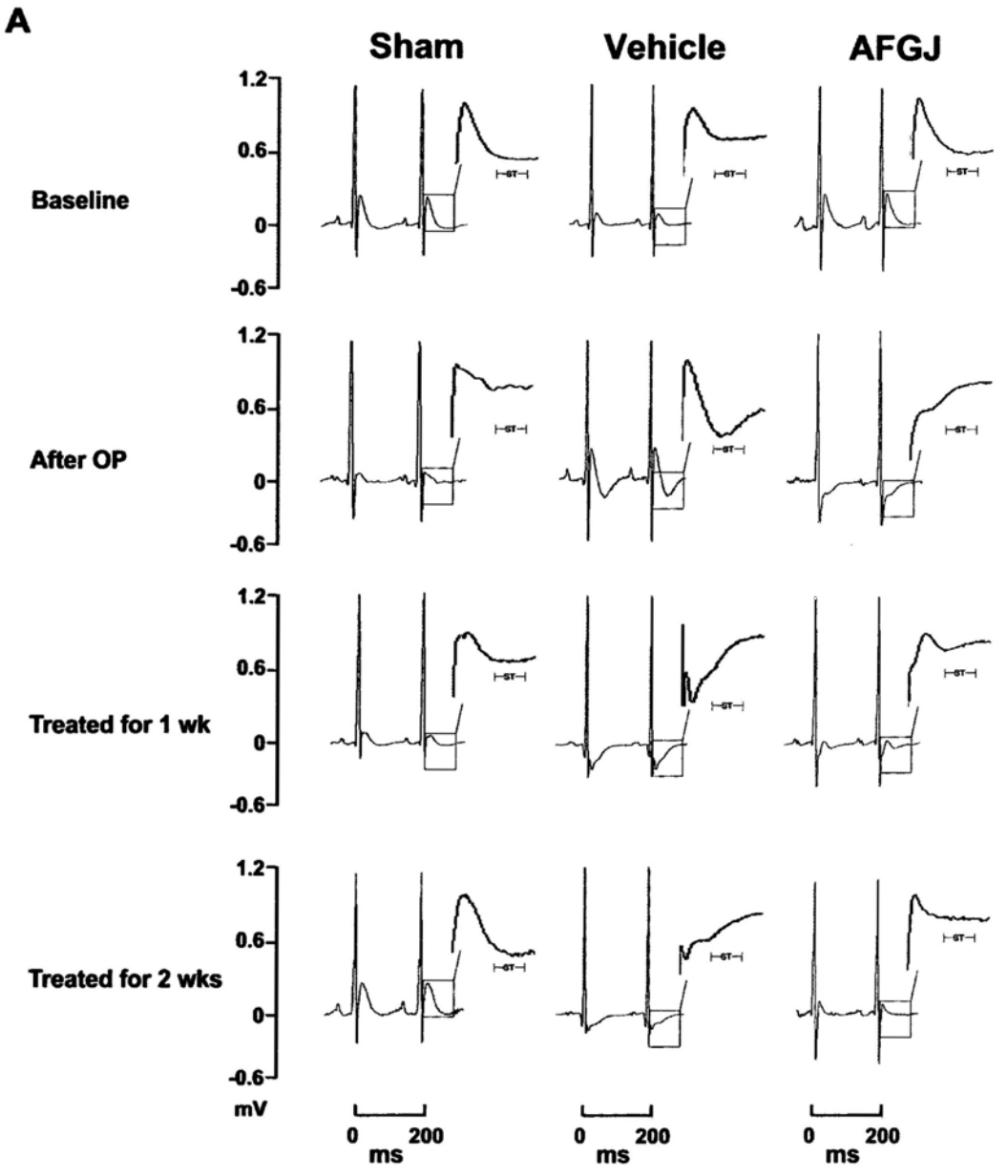

Figure 3.3 Electrocardiograms of CHD rats. (A) Two cycles of ECG recordings were taken in one of the animals from sham, vehicle and treated rats in our partially occluded LAD model. The waveforms showed typical ECG waves in sham group. ECG showed similar waveforms before and after operation between AFGJ treated and vehicle rats. However, note the dipping of the ST segment is more pronounced after surgery in both non-treated and AFGJ treated ECGs, confirming successful ischemia induction. After AFGJ treatment, ECG waveforms between the non-treated and treated group were clearly different. In week 1 and week 2 the dip is still present in the vehicle, whereas in the treated the dip is leveled.

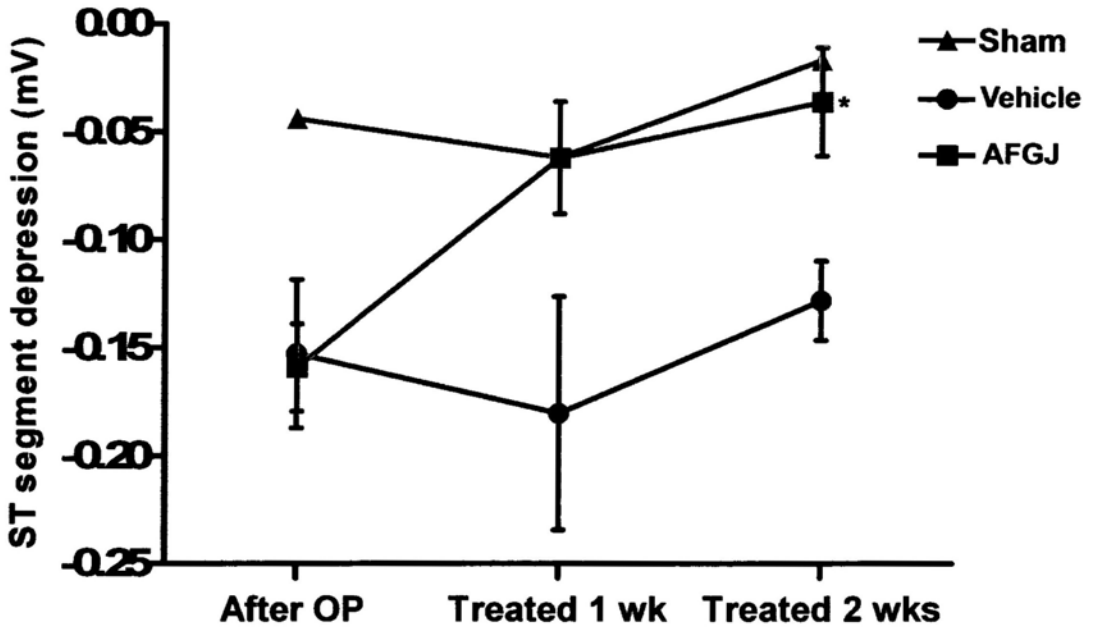

Figure 3.3 Electrocardiograms of CHD rats. (B) Mean data and statistics for ST segment depression. The baseline number was normalized as zero. Normally, the ST segment should be <0.1mV. Ischemia was significant (means of ST decrease at >0.1mV) in the vehicle group compared with the AFGJ treated group (AFGJ *vs* vehicle: *, $p<0.05$).

Nowadays, Echocardiography as an effective diagnosis method is widely applied in heart function investigation. The most important index of echocardiography-the left ventricular ejection fraction (LVEF) and fractional shortening (LVFS) were measured by using TOSHIBA Xario® system. Significantly enhanced heart function, as measured by echocardiography, was observed in the AFGJ treated group. LVFS in the AFGJ treated group was higher than in the vehicle group after treatment for 2 weeks with mean±SEM of 49.7±3.62 *versus* 36.94±3.27 (Figure 3.4A). While LVEF in the AFGJ treated group showed the improvement effect after treatment of 2 weeks with mean±SEM 73.63±5 *versus* 60.16±4.87 (Figure 3.4B).

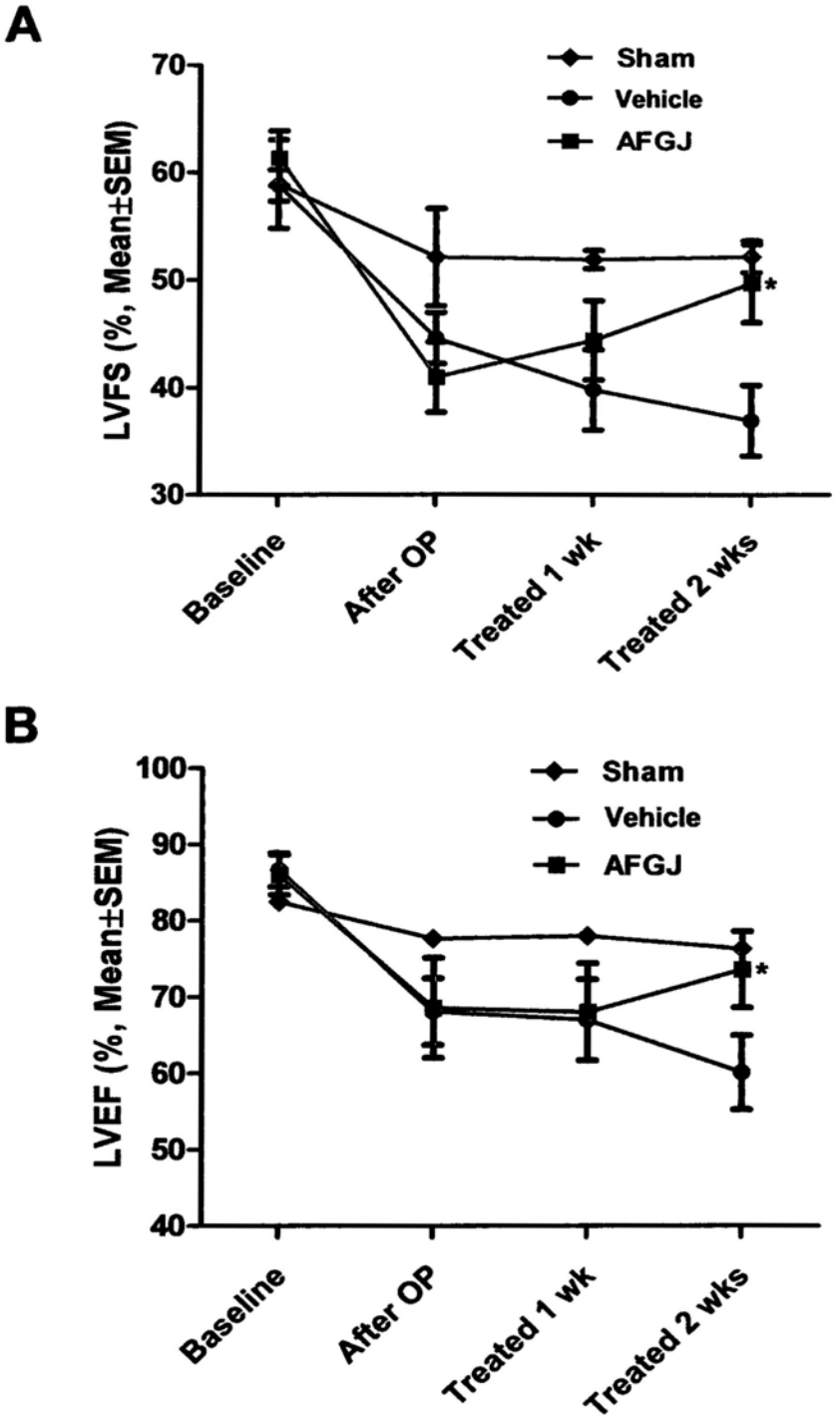

Figure 3.4 Serial echocardiography measurements. LVFS was significantly improved in the AFGJ treated group compared with that in the vehicle group (AFGJ *vs* vehicle: *, $p<0.05$). After surgery, FS continued to lower in the vehicle group while AFGJ treated was significantly increased, and consecutively so from week 1 to 2. Meanwhile, LVEF was similar before 1-week treatment and significantly ameliorated when treated for 2 weeks with AFGJ (AFGJ *vs* vehicle: *, $p<0.05$).

3.2.5 AFGJ stimulates formation of new bypass vessels

3.2.5.1 Coronary microvasculature: MicroCT based analysis

To investigate the functional vascular formation in ischemic hearts directly, MicroCT analysis was applied to reconstruct the vascular network of ischemic hearts. Hearts were perfused with MicrofiL® dye (Fig.3.5) and were scanned by Vivi CT and reconstituted by VICT program.

The 3D architecture of the heart vessels is shown in Figure 3.6. The AFGJ-treated hearts had more microvessels and bypass vessels compared to vehicle hearts. Interestingly, the distribution of average diameters of heart vessels (Figure. 3.7) demonstrated that the ratio of arterioles or venules (21-63μm) and microvesseles (63-210μm) in the AFGJ-treated group was significantly higher than that of the vehicle group, which were similar to that of the sham heart. However, there was no significant difference among different groups in the number of vessels when the diameters of vessels exceeded 210μm. It is suggested that more microvessels were formed as newly bypass vessels in treated hearts compared to the vehicle hearts.

Figure 3.5 CHD rat heart perfused with MICROFIL® mixture.

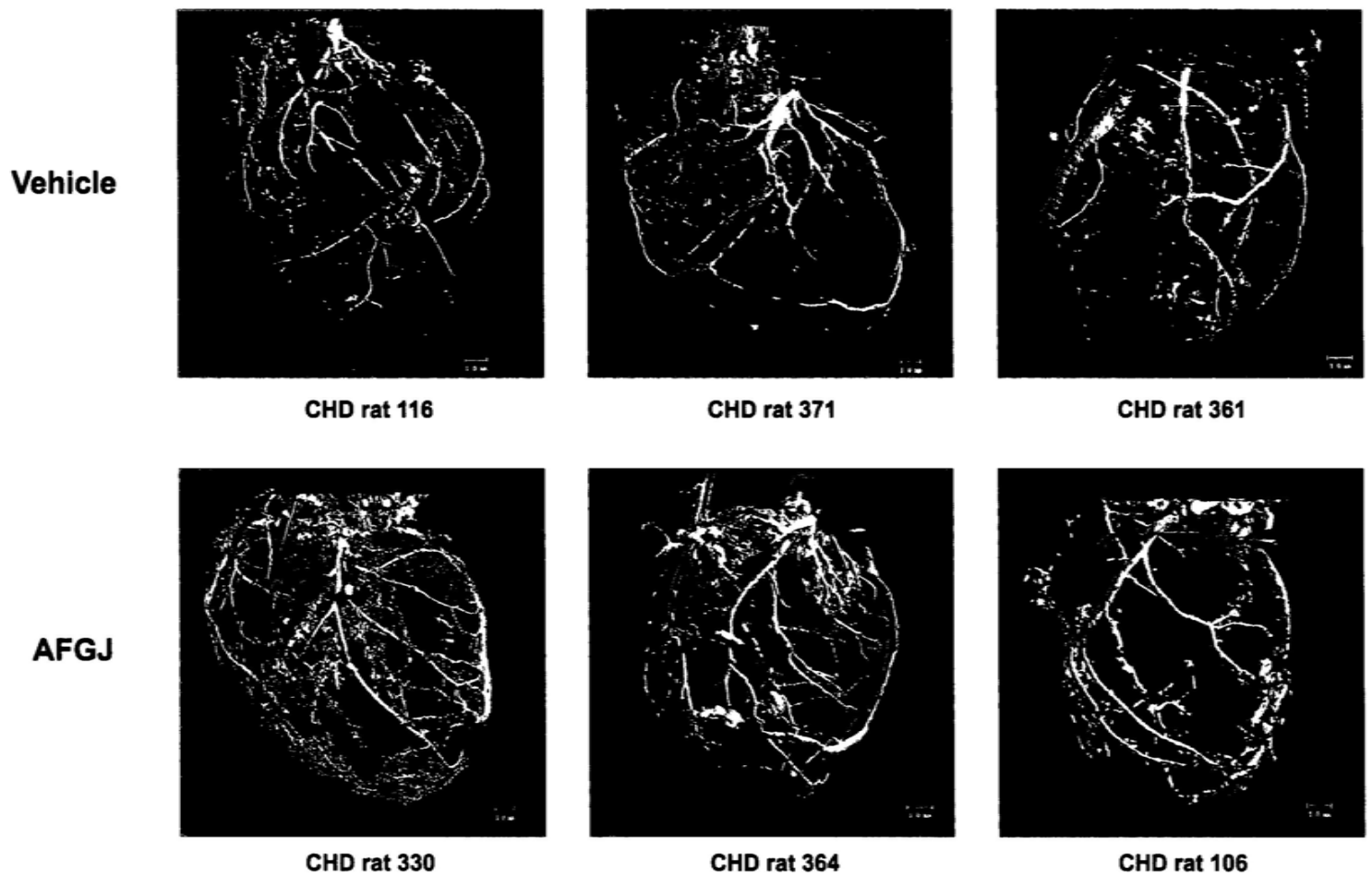

Figure 3.6 Representative 3D images of perfused hearts in CHD rat model. (A) Images of ischemic hearts. Red rectangle highlights the uncompleted ligation position.

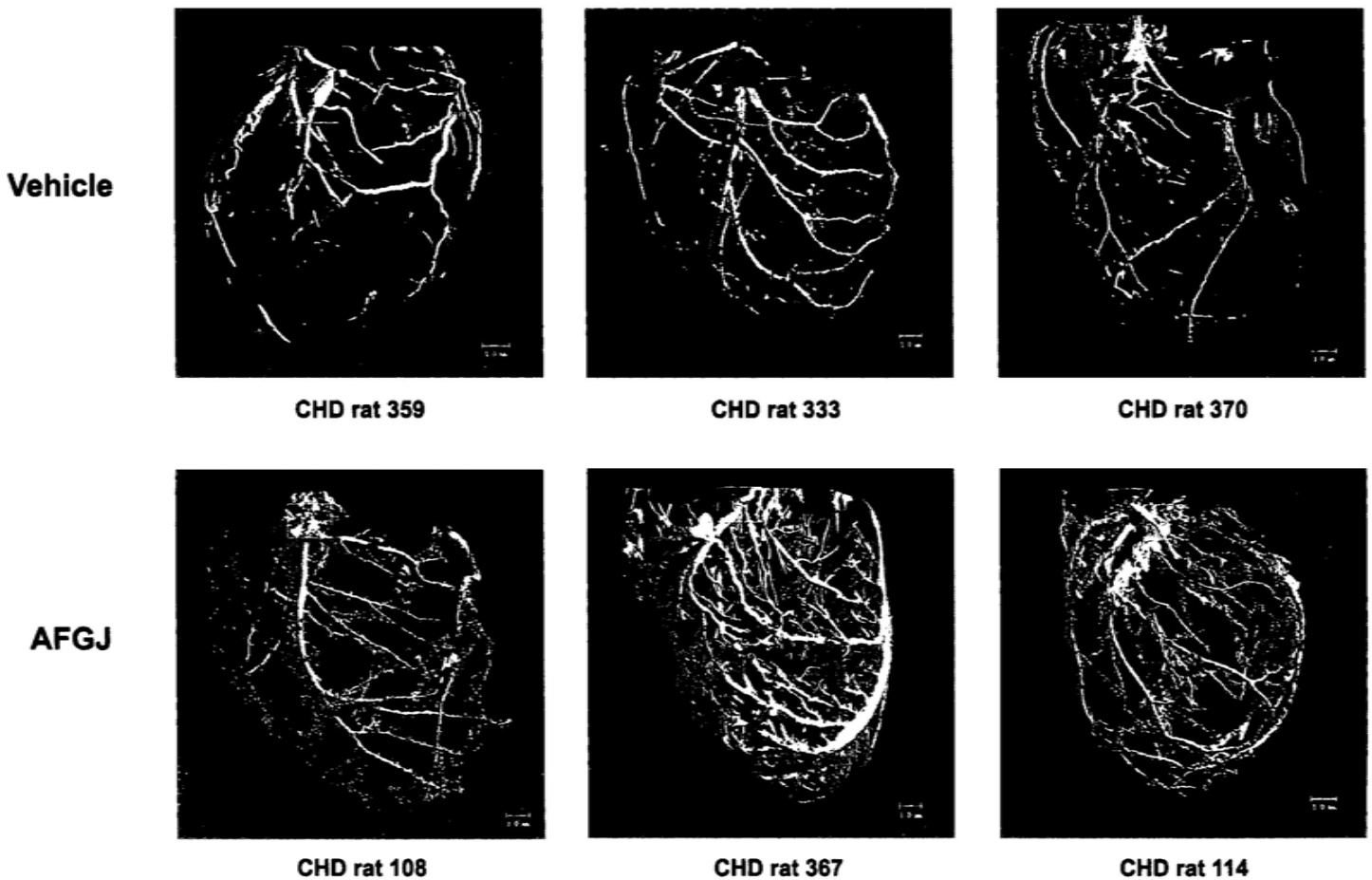

Figure 3.6 Representative 3D images of perfused hearts in CHD rat model. (B) Images of ischemic hearts. Red rectangle highlights the uncompleted ligation position.

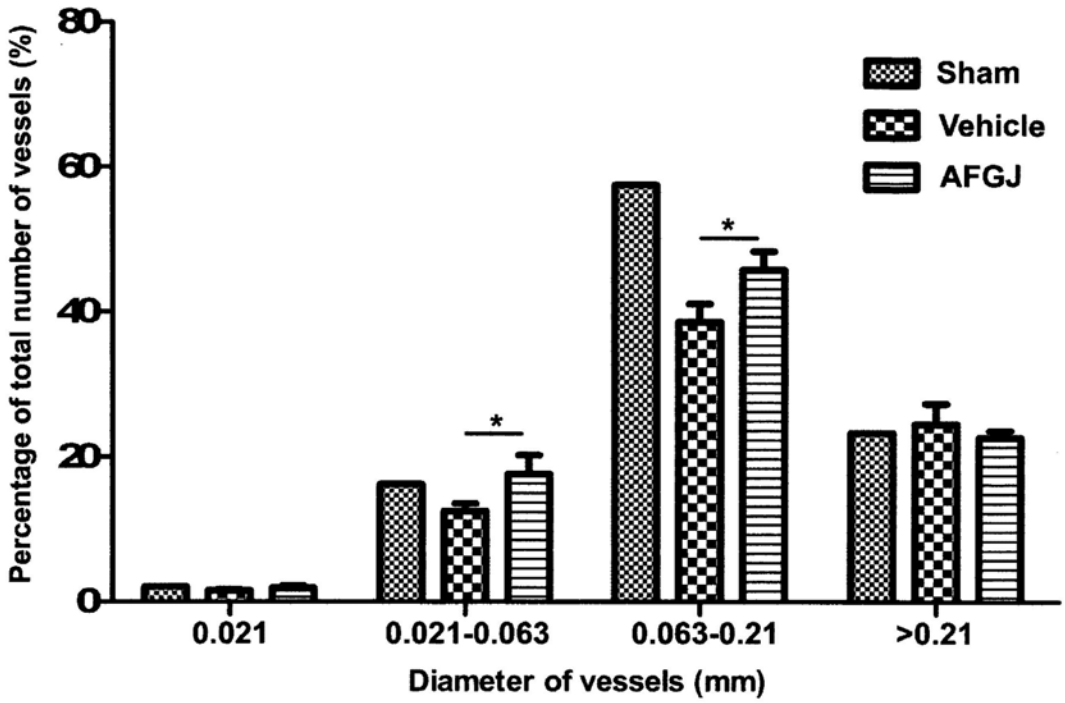

Figure 3.7 Distribution of different diameters of vessels. The ratio of arterieos or venules (at the diameter 0.021-0.06mm) and microvessels (at the diameter 0.063-0.21mm) was drastically higher in AFGJ-treated group compared to the vehicles (*, $P<0.05$). In contrast, the ratio in vehicle group was no significant difference compared with that of AFGJ-treated group when the diameter below 0.021mm and exceeded 0.21 mm.

After analyzing whole heart datasets, the ischemic area (ROI/VOI) was selected and neovascularization in the ischemic area was analyzed to provide more confident data to reflect the effects of AFGJ. The 3D reconstructive images of ischemic area are shown in Figure 3.8. Figure 3.8 also displays the similar morphologies and more details to the images of whole hearts showed in Figure 3.6. Note that the vessels formed more connections with each other in the AFGJ-treated group compared to the vehicle group.

The quantitative analysis of heart neovascularization demonstrated a significant increase of microvessel formation in the AFGJ-treated group compared to the vehicle group. The vessels in unit area in ROI displayed significantly higher vascular densities in different parts of ligated hearts of the AFGJ-treated group compared to the vehicle group (Figure 3.9). Specifically, the density of vessels was remarkably higher in the AFGJ-treated group than that of the vehicle group within the first 100 slices in ischemic areas (mean± SEM: 4.5±0.5 versus 2±0.35).

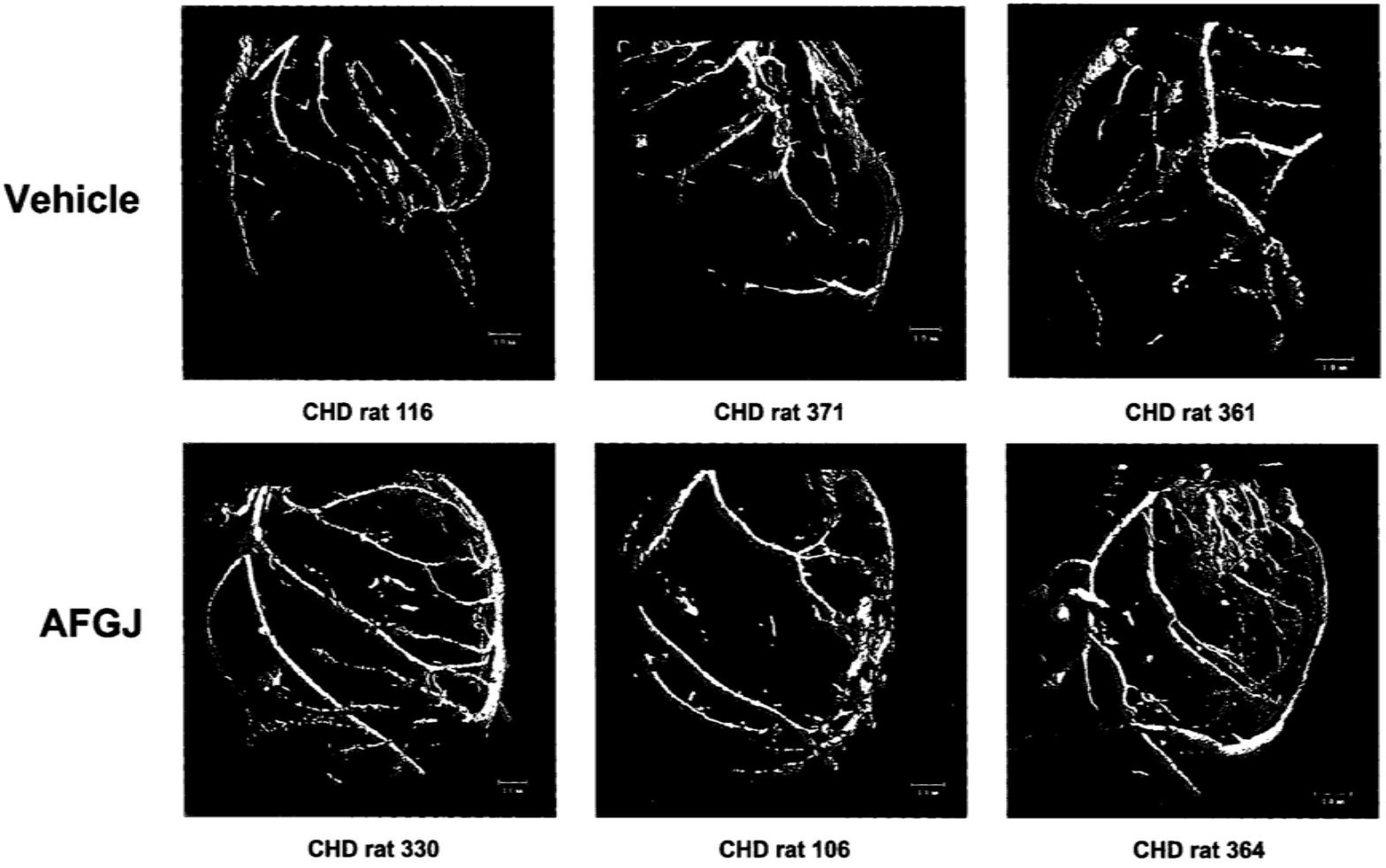

Figure 3.8 Representative 3D images of ischemic area of CHD hearts. (A) Images of the vehicle and AFGJ treated group.

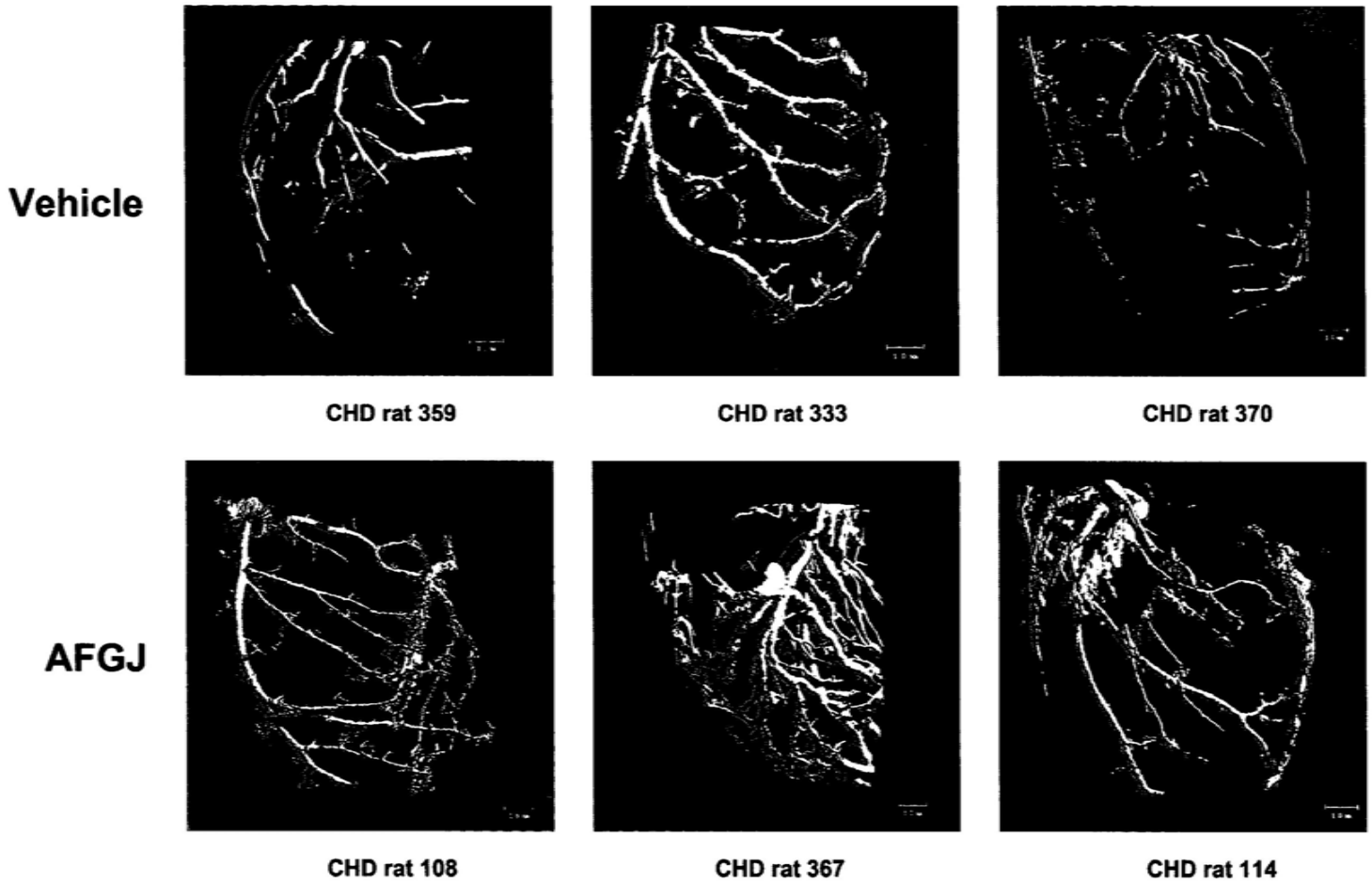

Figure 3.8 Representative 3D images of ischemic area of CHD hearts. (B) Images of the vehicle and AFGJ treated group.

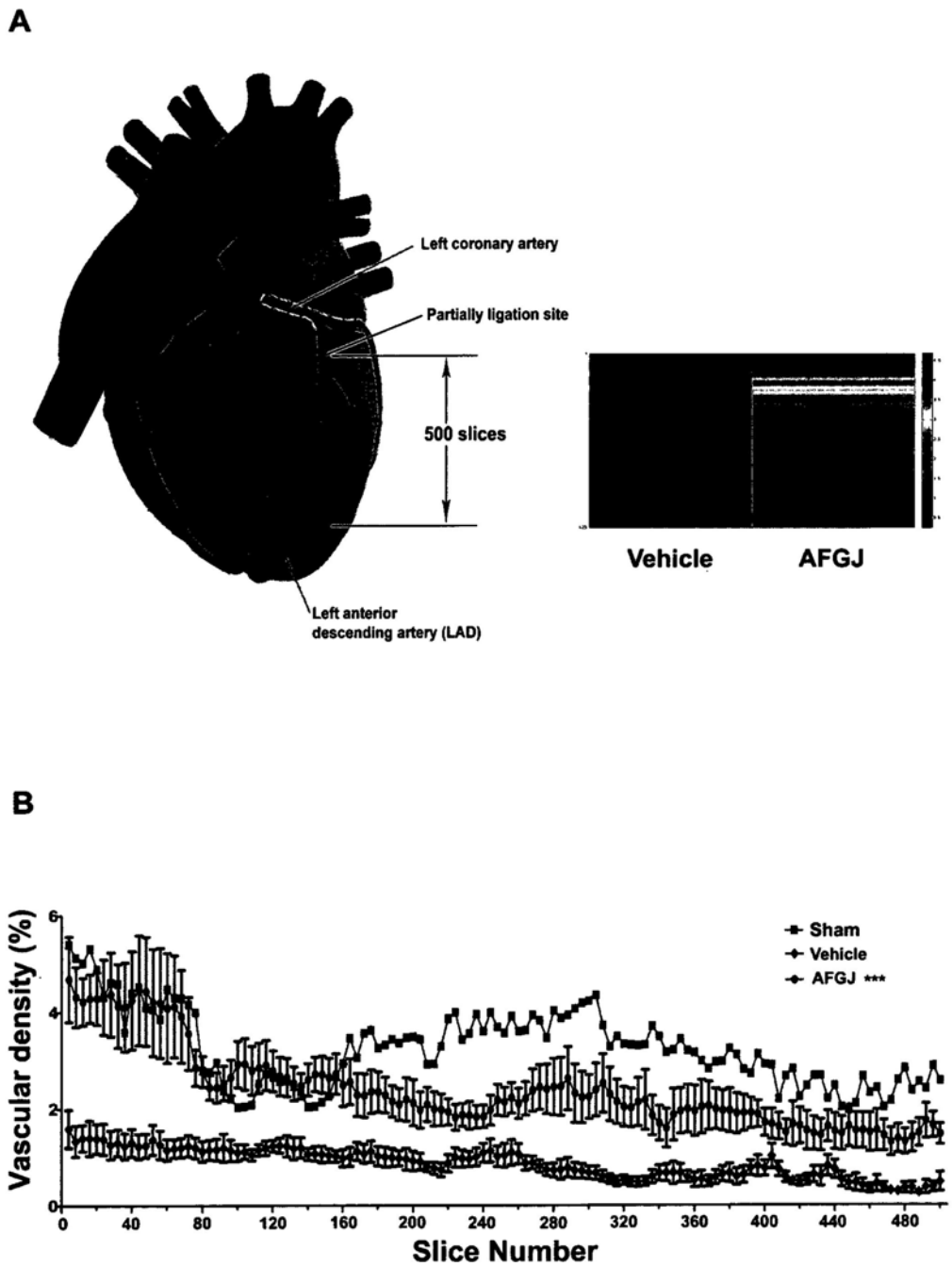

Figure 3.9 Heat map and quantification of ischemic area. (A) Representative 2D evaluation of unit area of vessels in ROI which counted the 125 data sets interval every 4 slices in total 500 slices and heatmap displaying the ischemic degrees (More blue, more ischemia). (B) The average density of vessels of AFGJ-treated hearts was significant more than that of vehicle hearts (***, $p<0.001$).

The quantification of microvessels of VOI was measured by using a 3D evaluative method. The vascular volume (VV) of the AFGJ-treated group was 10.75±1.39mm^3 compared with that of the vehicle group of 5.22±1.03mm^3. Furthermore, the VV/TV in VOI showed a higher ratio of microvessel volume within total vessel volume in AFGJ-treated hearts (mean±SEM: 0.024±0.005) than vehicle hearts (mean±SEM: 0.01±0.002), indicating an increase in microvessel growth in the AFGJ-treated hearts compared to vehicle hearts (Figure 3.10). In addition, both the values of VV and VV/TV in the AFGJ-treated hearts were nearly closed to sham group (mean: 10.7 to 11.02 and 0.024 to 0.027), indicating that AFGJ treatment was effective to induce the newly growth of blood vessels.

Importantly, the number of intersections between vessel and non-vessel components per total length (vb. N) of vessels in VOI provided information on vessel branching points or segment cross-sectional vessel number (Figure 3.11). There were substantial branching points in AFGJ-treated group compared to sham and vehicle group (mean±SEM: 0.94±0.08 *versus* 0.87±0.0001 and 0.65±0.10), suggesting that there is significant new formation of by-pass vessels in the AFGJ-treated group in CHD hearts.

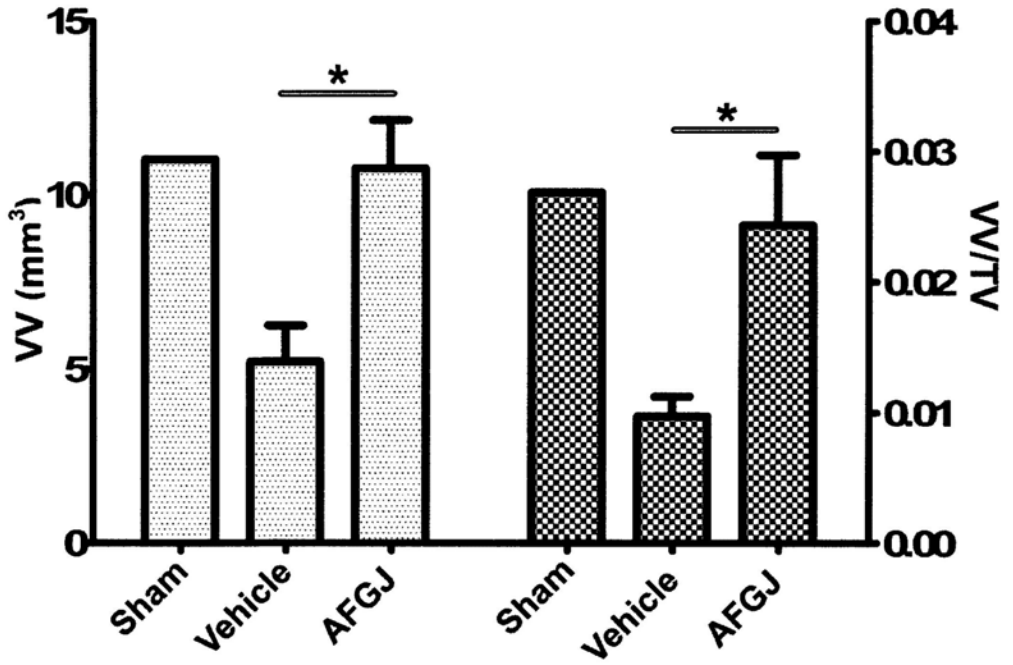

Figure 3.10 Quantitative volumetric measurements of heart vascular angiogenesis. The left Y axis represents the vascular volume (VV) of ischemic area. VV was significantly increased in AFGJ-treated hearts compared with vehicle hearts (*, $p<0.05$). The right Y axis illustrates the ratio of VV within total volume (TV) which also displays the noticeably increase in the AFGJ treated group compared to the vehicle group (*, $p<0.05$).

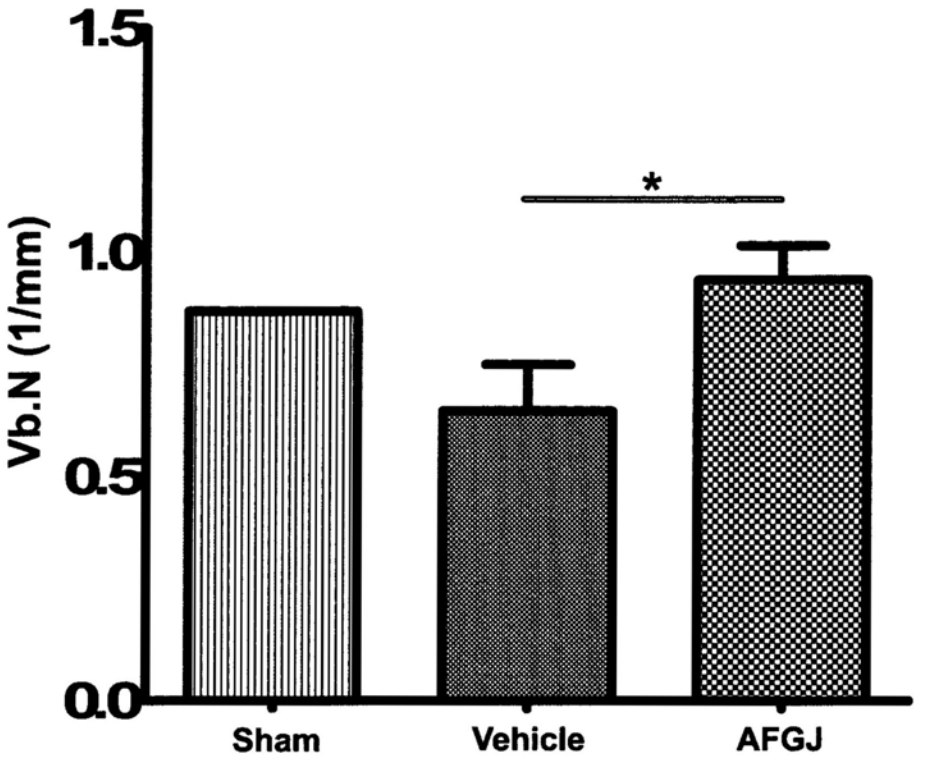

Figure 3.11 Quantitative measurement of heart angiogenesis represented by vessel branching points. Bar graph showing the detailed information that the average of segment cross-sectional vessel number in AFGJ-treated hearts was increased compared with vehicle hearts (*, $p<0.05$).

3.2.5.2 Histological analysis on angiogenesis of CHD hearts

Histological studies were performed after MicroCT analysis. H&E images of CHD hearts showed no obvious heart infarction both in the vehicle and AFGJ-treated group. However, many minute sac-like structures were found in the cytoplasm of cardiomyocytes, and the cell integrity was also decreased as indicated by the broken cell membrane in the cells of vehicle hearts (Figure 3.12). In addition, analysis of vascularization of ischemic hearts of CHD revealed that the capillary density in AFGJ-treated ischemic hearts is approximately 68.1±6.4 per field averaged from 5 randomly selected fields of each slide, with 6 slides each from the 7 treated hearts. This density has no significant difference with the capillary density in sham hearts (74.5±6.7 per field). In contrast, significantly fewer blood vessels (27.4±2.6 view field) were observed in the ischemic area of the untreated control hearts (Figure 3.13). Together, the data of MicroCT and quantitative analysis of vascular density demonstrated that the proliferation-promoting effect of AFGJ in vessel endothelial cells could be remarkably translated into enhanced neovascularization in ischemic hearts of the CHD model.

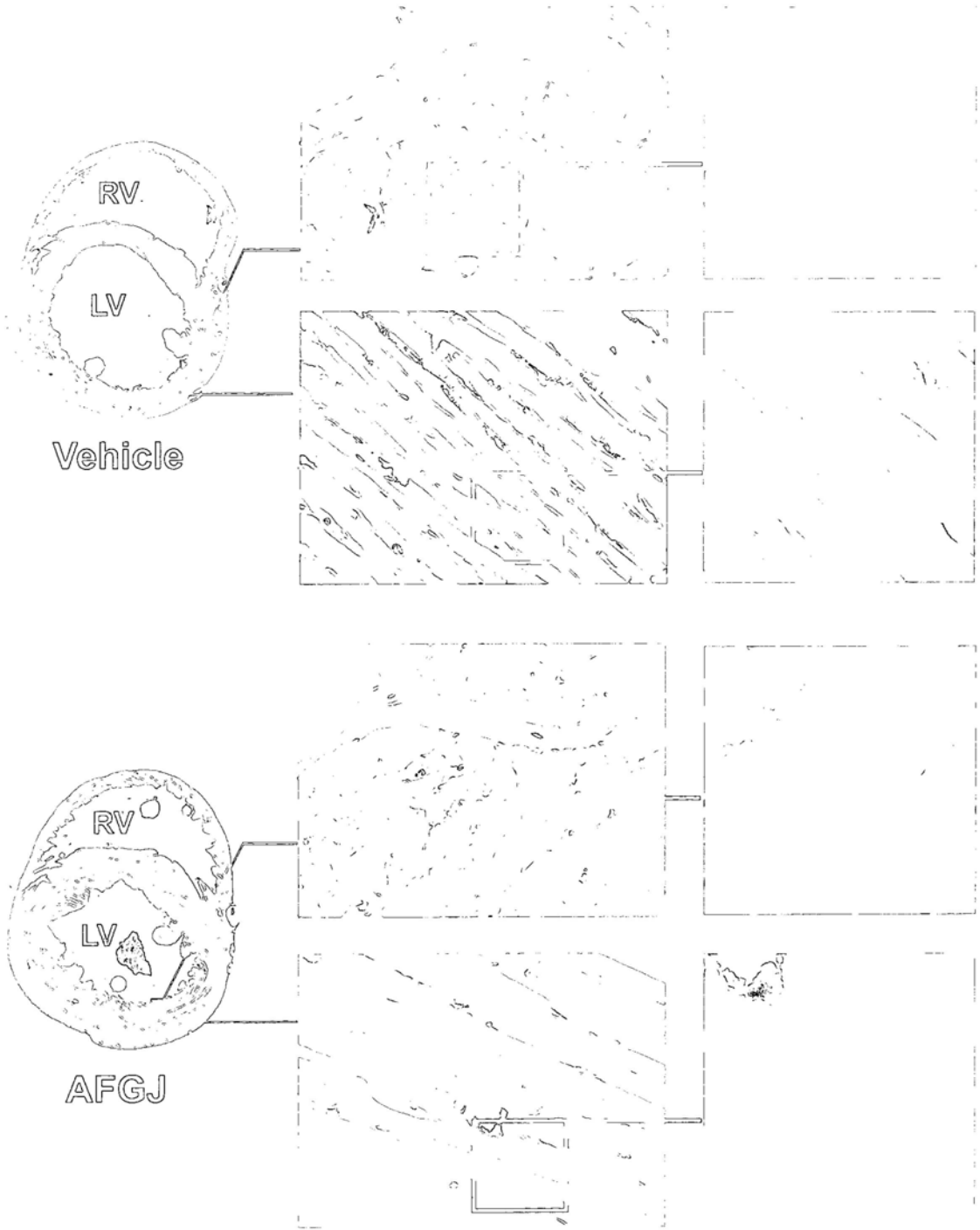

Figure 3.12 Histopathology of CHD hearts that received AFGJ therapy. Cytoplasm of myocytes in ischemic area of the vehicle hearts showed porous morphology and less integrity compared to the treated hearts (Blue rectangles).

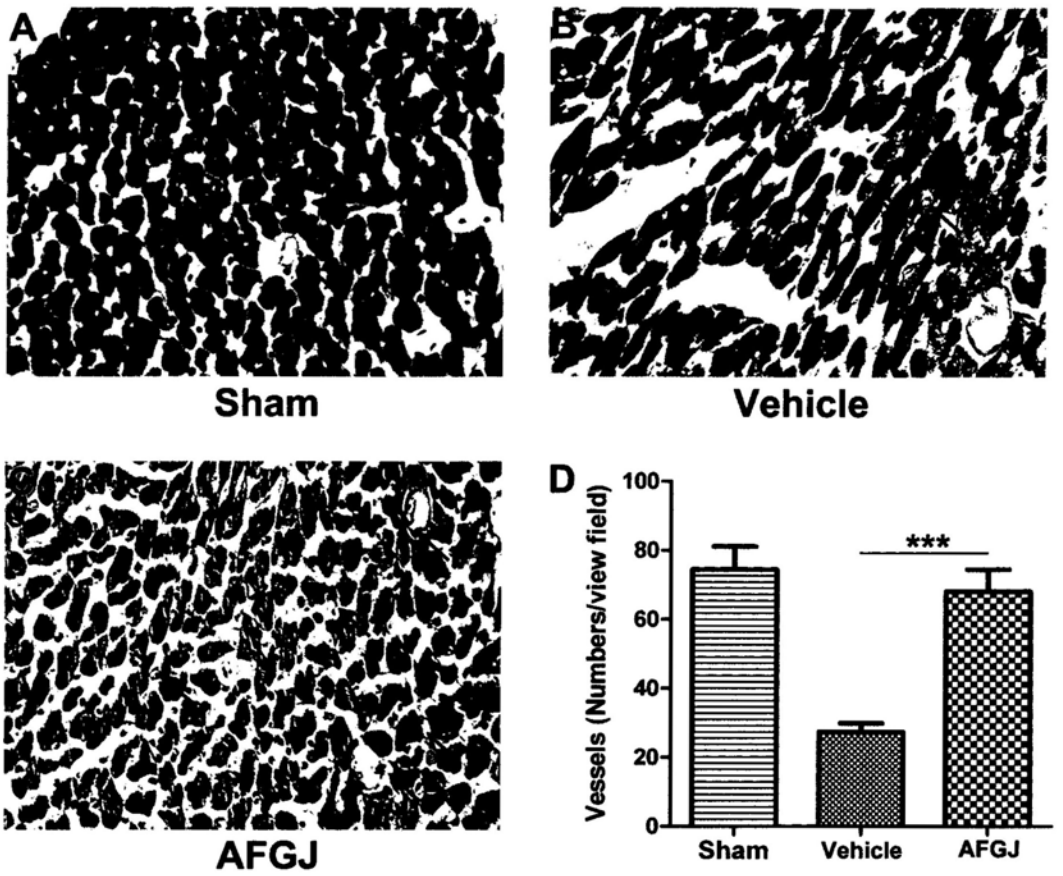

Figure 3.13 Revascularization in ischemic area of CHD models. Total blood vessels in the ischemic area of the vehicle (B) were less than sham (A) and AFGJ-treated hearts (C), and the vascular density (D) in the AFGJ treated ischemic hearts averaged higher than that of vehicle hearts and simlar with in that of sham hearts (***, $p<0.0001$).

3.2.5.3 AFGJ-induced self-repair or renewal of endothelial cells

It has already been revealed that numerous microvessels were neovascularized in the ischemic area in AFGJ-treated hearts. In addition, analysis of the proliferation or self-repair of endothelial cells derived from AFGJ-treated and vehicle hearts was conducted by proliferating cell nuclear antigen (PCNA) staining, which assessed cell proliferation (Figure 3.14). There were significantly more PCNA positive endothelial cells in AFGJ-treated hearts compared with vehicle hearts (Figure 3.14B&C), while there was few PCNA expression in endothelial cells of sham hearts (A). Interestingly, the cardiomyocytes were also labeled with PCNA, suggesting that myocytes might have been benefited from blood flow improvement. Quantitative analysis indicated that a higher number of proliferation/self-repair endothelial cells occurred in AFGJ-treated hearts with the PCNA positive ratio 15.65±0.87 *versus* 8.13±0.58 in vehicle hearts, while the sham hearts kept the lowest expression of PCNA at 3.15±0.42 (Figure 3.14D). This result indicated that AFGJ induced endothelial cells to undergo proliferation, contributing to the neovascularization.

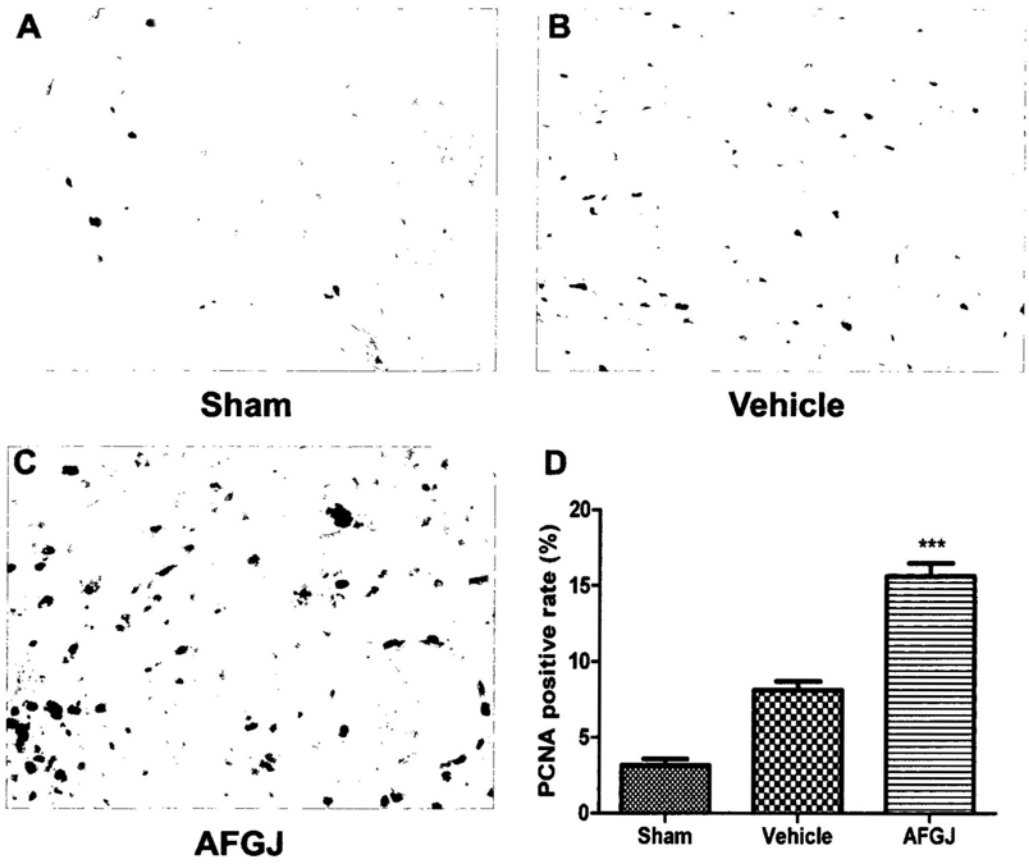

Figure 3.14 Self-renewal or repairing actions induced by AFGJ treatment. Immunohistochemistry images (A, B&C) of sections from sham, AFGJ treated and vehicle hearts were stained with anti-PCNA (brown color) and eosin. PCNA positive endothelial cells in AFGJ treated hearts were more than that of vehicle hearts (red arrows). Some myocytes were also PCNA positive. (D) Quantification of PCNA positive endothelial cells by counting positively stained cells in 3 randomly selected fields in 7 rats is given (***, $p< 0.0001$ *vs* other groups).

3.3 Summary

In this study, the promoting effects of AFGJ and Angio-G on proliferation of vessel endothelial cell culture, suggest its potential in neovascularization *in vivo*. The therapeutic effect of AFGJ isolated from *G. japonicum* on a CHD rat model was predominantly examined instead of Angio-G. The choice of AFGJ was due to inadequate amount of Angio-G to offer a practical dosage for oral administration.

The present findings have provided the evidence for AFGJ-mediated neovascularization in CHD and suggested its potential application for clinical treatment of ischemic heart diseases. The demonstrated effective therapeutic effect in the present CHD animal model by oral administration of AFGJ, represented by progressively restoration of ST segments of ECGs, time-dependent improvement of heart functional performance and increased vascular density in the ischemic region of AFGJ-treated hearts, suggests its protential clinical use. In addition, newly formed microvessels may be beneficial to stimulation of endothelial cells and cardiomyocyte self-repair. More importantly, many neovascularized vessels act as natural by-pass and compensate for the function of the ischemic LAD, as detected through MicroCT investigations. It has been found that MicroCT provides a detailed visualization of vascular networks of CHD hearts. Interestingly, this technique has allowed us to measure vessel area and analyze vascular formation, suggesting that AFGJ treatment markedly increases microvessels number (0.063-0.2mm) that contributes to the reduction in heart ischemia.

Taken together, our results show that the neovascularization induced by AFGJ may have been translated into effective growth of new coronary vessels/collaterals in

ischemic hearts of a chronic CHD rat model, with progressively restored heart functional performance to overcome CHD.

Chapter 4 Molecular mechanisms underlying the effect of AFGJ on microvessel formation

4.1 Introduction

New blood vessel growth, known as neovascularization, could be traced to two different cellular mechanisms: angiogenesis and vasculogenesis. Angiogenesis is where vascular cells derived from pre-existing vessel proliferation migrate to form new blood vessels (Ball et al., 2007). Vasculogenesis is where undifferentiated stem cells are recruited, egress into the growing vessel wall to form cell clusters, then proliferate to form the new vessels network (Tepper et al., 2005).

Previous results from my study have demonstrated that AFGJ and Angio-G were able to significantly stimulate the proliferation of HUVECs. Furthermore, the newly formed by-pass vessels remodeling, triggered by AFGJ, was confirmed by MicroCT based analysis.

Although results showed proliferation induced by AFGJ and Angio-G, the mechanisms underlying the AFGJ effects remain to be elucidated. There are two major problematic complexities. Firstly, it needs to be determined whether AFGJ or Angio-G could induce capillary-like tube formation or just induce proliferation. The formation of neovascularized vessels most likely occurs through angiogenesis. The molecules involved in this process therefore need to be identified. Secondly, it is already known that there are two different ways of new blood vessel formation-angiogenesis and vasculogenesis. Therefore one may ask the question whether the differentiation of MSCs could lead to vasculogenesis to exhibit such rapid new blood by-pass vessels formation by AFGJ, apart from angiogenesis effects. The major objective of the study in this chapter is to investigate the potential mechanisms of microvessel formation induced by AFGJ or Angio-G.

4.2 Results

4.2.1 Angio-G-induced capillary tube formation *in vitro* is inhibited by JAK inhibitor AG490

The process of capillary tube-like vessel formation on matrigel recapitulates the angiogenic process, in which endothelial cells also undergo attachment, migration and differentiation events to form interconnected networks of endothelial cell-lined tubes. To determine whether Angio-G could also enhance capillary tube formation of vessel endothelial cells in addition to its proliferation-promoting effect, HUVECs were starved for 6 hours in 2% FBS F12K medium and cultured on GFR matrigel-coated 96-well plates with different concentrations of Angio-G in a growth medium without endotherial cell growth supplement (ECGS). Tube-like structures formed after 18 hours. Cells treated with Angio-G formed more tube networks than control cells (Figure 4.1A). To quantify the effects of Angio-G on capillary tube formation, the tube length was measured. The length of formed tubes significantly amplified with increasing concentrations of Angio-G compared to control ($p<0.05$). Moreover, at an Angio-G concentration of 5μg/ml the greatest tube length was detected with increased tube formation by 51% compared to control (Figure 4.1B).

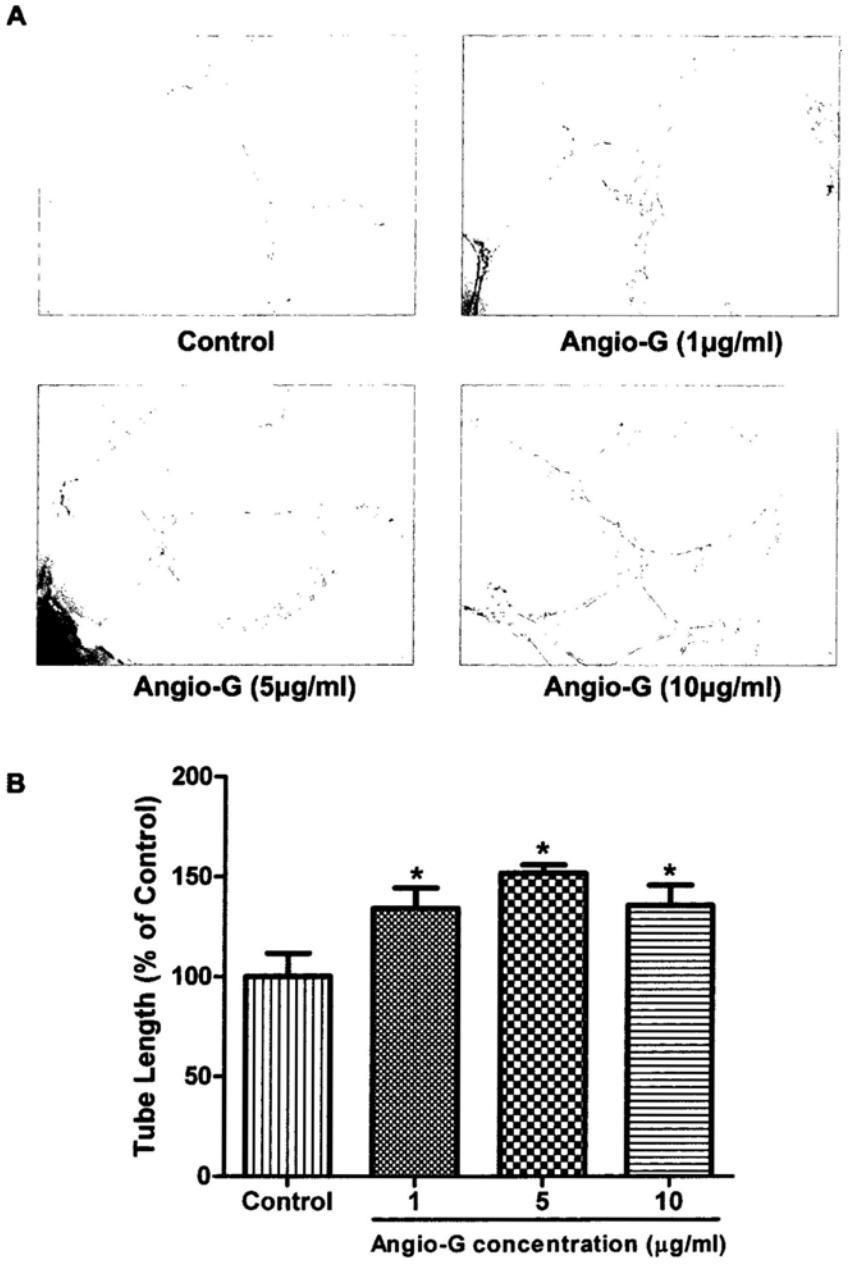

Figure 4.1 Angio-G induces endothelial cell tube formation in growth factor reduced matrigel. (A) Representative micrographs of tube formation in HUVECs incubated with Angio-G (1-10μg/ml) for 18 hours, whereas (B) shows quantitative measurements of tube formation in HUVECs derived from data obtained in (A). The tube length of capillary tube-like structure was increased with raising concentrations of Angio-G (n=3 per group, each experiment repeated twice).

In our previous study, fifteen genes had been identified to be significantly up-regulated upon Angio-T treatment in infarcted heart tissue (data not shown). Five of which were JAK/STAT related (including genes involved in the NFκB pathway). The JAK/STAT pathway consists of four non-receptor tyrosine kinases, JAK1, JAK2, JAK3 and TYK2 (Kisseleva et al., 2002). Therefore AG490, a specific inhibitor of JAK2, JAK3/STAT and JAK3/MAPK pathways (Gazit et al., 1991, Cetkovic-Cvrlje et al., 2003) was used to investigate the involvement of this particular pathway in tube-like formation induced by Angio-G. As shown in Figure 4.2, the total length of tubes induced by 5μg/ml of Angio-G was reduced with increasing AG490 concentrations. This suggests that AG490 inhibits Angio-G-induced tube-like structure formation through inhibition of JAK/STAT pathway and that activation of JAK/STAT pathway by Angio-G plays a crucial role in capillary tube formation.

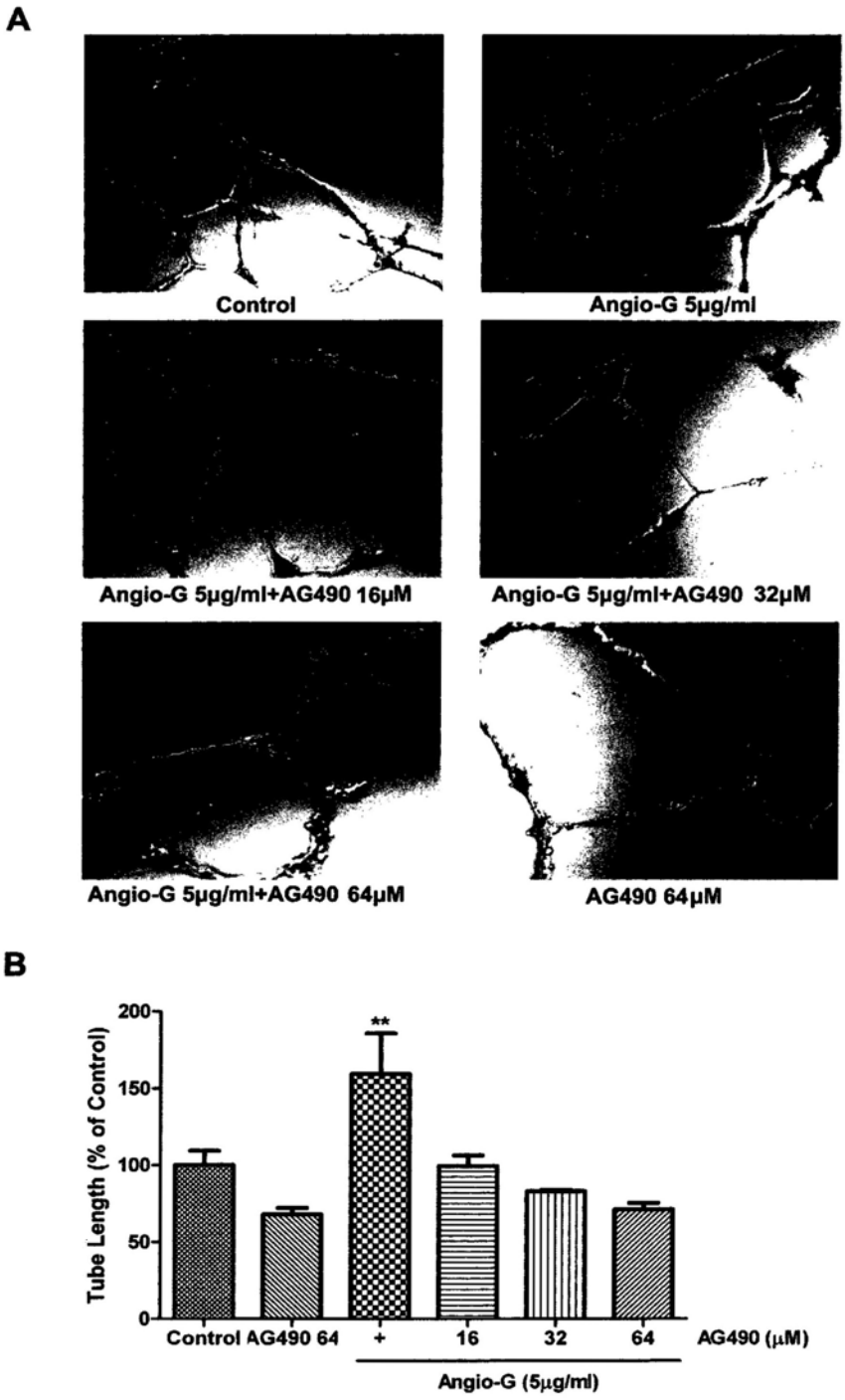

Figure 4.2. Inhibition effect of AG490 on Angio-G enhanced tube formation in vessel endothelial cells. (A) Representative micrographs of tube formation in HUVECs incubated with AG490 and Angio-G for 18 hours. (B) is the quantitative analysis of tube formation assay. The total length of tubes was significantly reduced during increasing concentrations of AG490 with 5μg/ml of Angio-G. (**, $p<0.01$ vs other groups)

4.2.2 Induction of MSCs differentiation into vascular endothelial cells and involved in tube-like formation by AFGJ and Angio-G

4.2.2.1 GFP-MSCs characteristics *in vitro*

Following seeding in MSC-culture medium, typical colonies could be detected after 1 week and 2 weeks later forming a monolayer of adherent cells. The homogenous population of spindle-shaped cells was observed under microscope. GFP-MSCs were subcultured to 4 passages to be used in the experiments. Flow cytometric (FCM) analysis revealed that GFP-MSCs were positive for the mesenchymal markers Sca-1 (Figure 4.3).

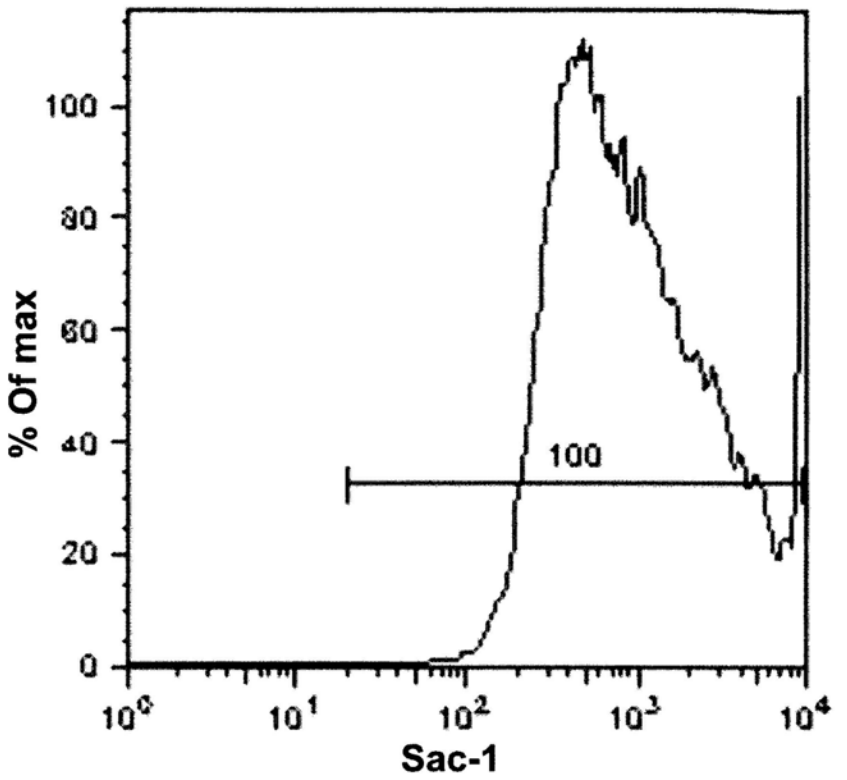

Figure 4.3 FCM analyses of MSCs using antibodies against Sac-1.

4.2.2.2 AFGJ and Angio-G-induced MSC differentiation into vascular endothelial cells in co-culture system

It has been reported that MSCs can differentiate into endothelial cells both *in vitro* and *in vivo* (Oswald et al., 2004, Nagaya et al., 2004, Silva et al., 2005). In current study, a co-culture system was established to induce that GFP-MSCs differentiation into endothelial-like cells. GFP-MSCs were seeded on the poly-L-lysine coated coverslip and HUVECs were seeded into the upper culture insert chamber. The GFP-MSCs and HUVECs were not contacted directly, while the culture medium and proteins could be exchanged *via* a transparent membrane (Pore size: 0.4um) in this co-culture system. After 7 days treatment of AFGJ (125μg/ml) or Angio-G (10μg/ml), GFP-MSCs were stained by anti-Von Willebrand factor (vWF) antibody. Undifferentiated GFP-MSCs, AFGJ-treated GFP-MSCs, as well as co-cultured GFP-MSCs all exhibited vWF negative staining (Figure 4.4: I, II, IV). But after 7 days of co-culture in the presented of AFGJ or Angio-G, vWF positive signal was detected both in AFGJ and Angio-G treated group (Figure 4.4: V, VI). The numbers of differentiated MSCs (elongated and vWF-positive staining) in AFGJ-treated group were more abundant than that of Angio-G treated group. Interestedly, it was also a detected few vWF positive cells in the group of MSCs cultured alone but treated with Angio-G but the cells morphology was still kept in the shape of MSCs, representing the cells might underwent the progress of differentiation (Figure 4.4: III). It was showed much more irregularly GFP-MSCs and rarely vWF-positive in control group (I, II, III, IV) compared to groups of AFGJ (V) and Angio-G (VI) treated co-cultured cells (Figure 4.5).

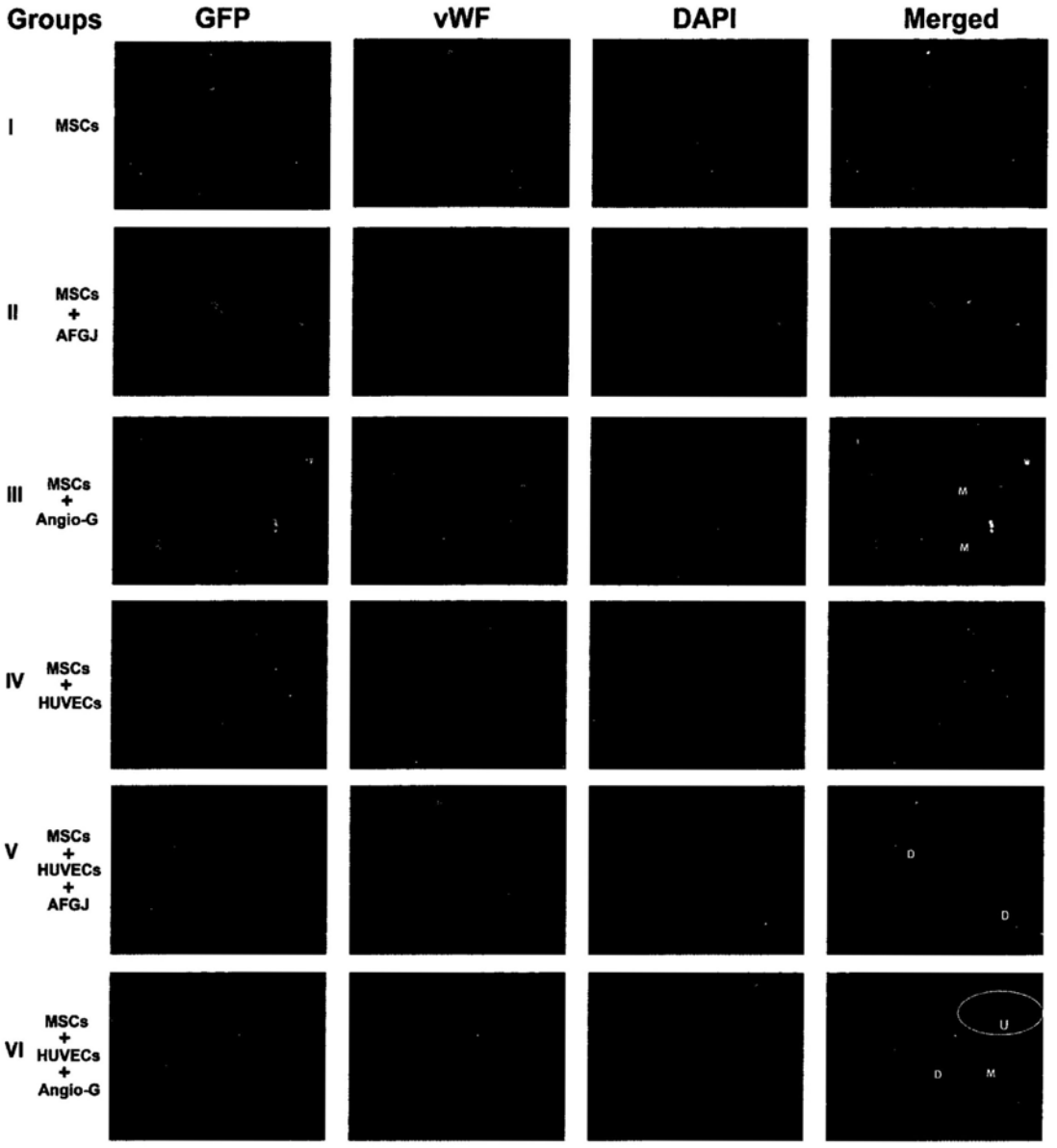

Figure 4.4 vWF immunoflurorescence of co-cultured GFP-MSCs (20X). Cultured GFP-MSCs only (I), GFP-MSCs treated with AFGJ only (II) and GFP-MSCs co-cultured with HUVECs for 7 days (IV) were negative staining of vWF. However, 7 days post treatment of co-cultured cells, GFP-MSCs were immunocytochemically stained against vWF (red), and the nuclei were stained blue with DAPI. Note the cells of presenting GFP and DAPI-positive but vWF-negative cytoplasma among other vWF-positive cytoplasma in Angio-G-treated co-cultured MSCs (VI, white circle U) was possibly due to a proportion of MSCs had not committed to endothelial cell differentiation, in which may also serve as a negative control. The cells of the shape still displayed typical morphology of MSCs but presented the GFP, DAPI and vWF-positive cytoplasma in GFP-MSCs only treated with Angio-G and co-cultured MSCs treated with AFGJ (III, V: M), illustrating the underwent differentiation of MSCs. Both AFGJ and Angio-G-treated co-cultured MSCs were elongated appearance and GFP, vWF-positive (V, VI: D).

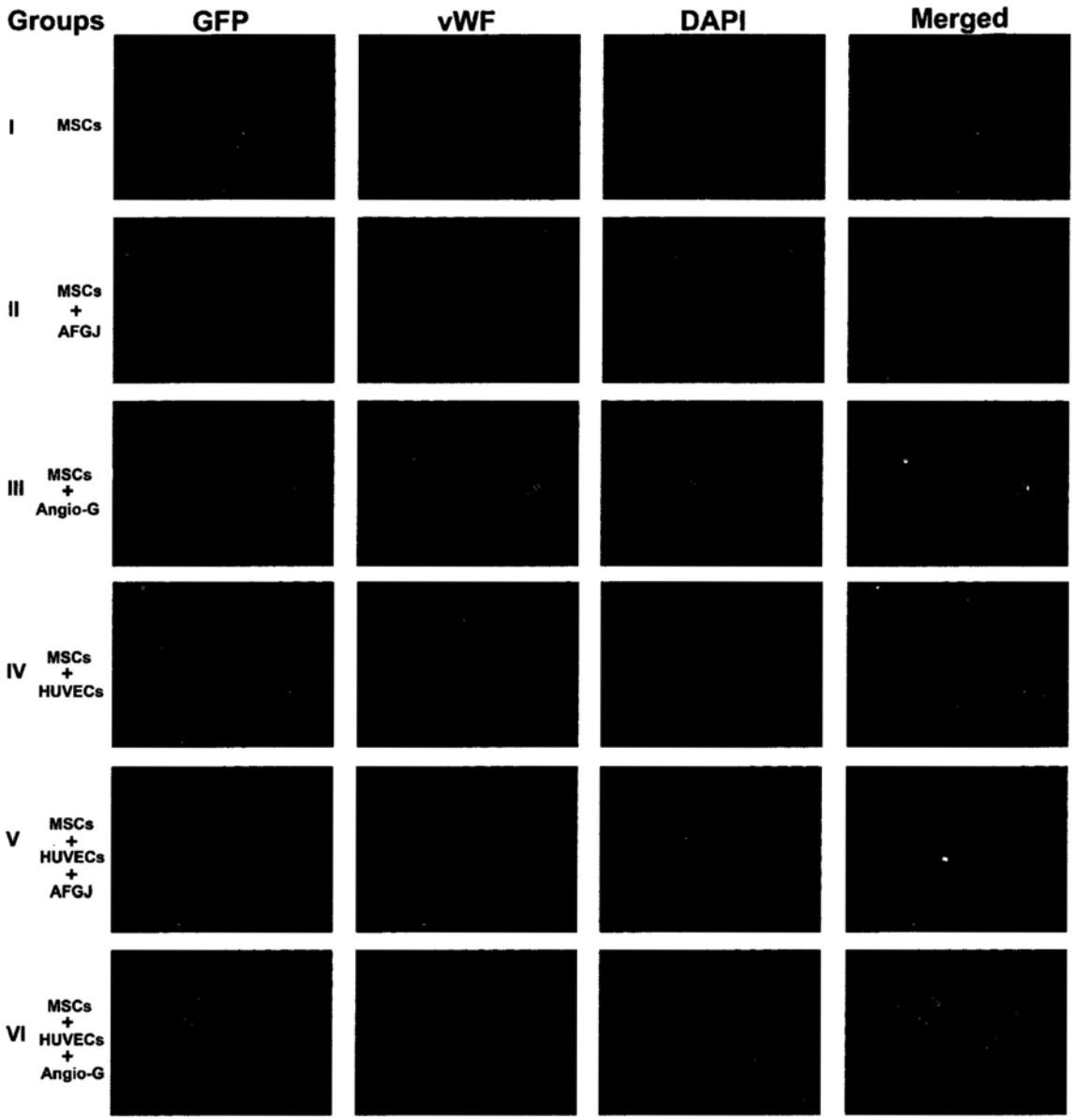

Figure 4.5 vWF immunofluorescence of co-cultured GFP-MSCs (40X). 40X magnified photos displayed more clear morphology and staining of co-cultured MSCs. AFGJ and Angio-G treated co-cultured MSCs were elongated and GFP, vWF-positive (V, VI) in contrast to the irregularly shaped MSCs and absence of red fluorescence in sham-treated cells (I, II, IV). GFP-MSCs treated with Angio-G for 7 days showed few vWF-positive differentiating cells (III).

4.2.2.3 Involvment of differentiated MSCs in capillary tube formations

Previous results had shown that Angio-G could induce tube formation of HUVECs and several studies have reported that MSCs had the potential to differentiate into ECs in favored condition (Wu et al., 2005). Thus, there are questions to be raised: What is the relationship between existing endothelial cells and MSCs in vasculature? Are MSCs involved in tube formation through connection with exsiting ECs to form new tube-like structure? To answer these questions, GFP-MSCs were trypsinized and mixed with equivalent number of HUVECs, and the mixed populations of cells were seeded on the GFR-reduced Matrix gel for 18 hours with the presence or absence of AFGJ (125μg/ml) or Angio-G (5μg/ml).

As shown in figure 4.6, there was no significant fluorescence detected in MSCs alone with or without Angio-G or with AFGJ treatment, indicating that undifferentiated MSCs do not participate in the process of tube-like formation *in vitro*. However, when co-cultured MSCs described before were trypsinized and mixed with equivalent number of HUVECs, and seeded on the GFR-reduced Matrix gel for 18 hours in presence of AFGJ, they exhibited significantly higher potency in tube-like formation (Figure 4.7: II) compared with the control group (Figure 4.7: I). Surprisingly, co-cultured MSCs treated with Angio-G failed to exhibit tube-like formation, even though these cells expressed specific endothelial cells marker as demonstrated in section 4.2.2.2 (Figure 4.7: III).

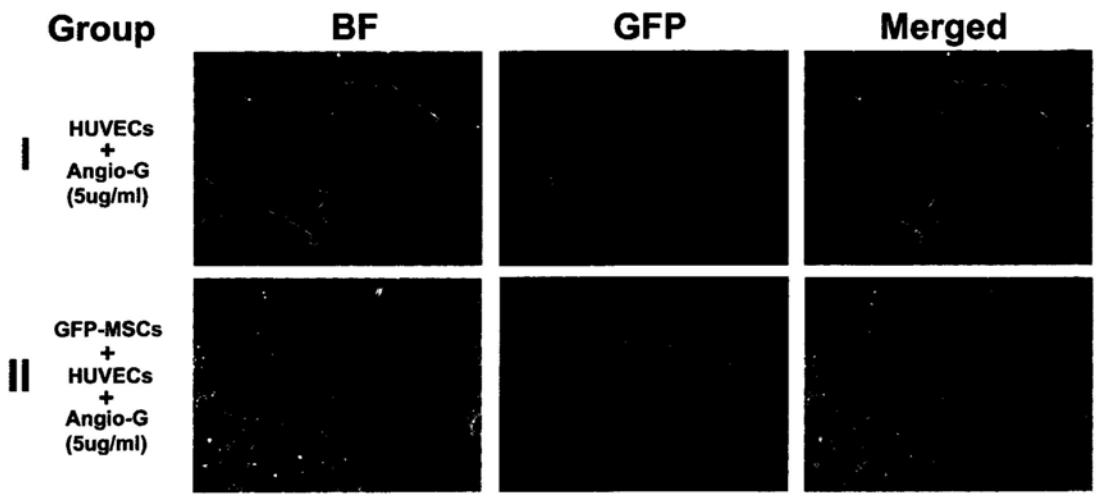

Figure 4.6 GFP-MSCs could not be involved in capillary tube formation. Representative micrographs of tube formation in co-cultured GFP-MSCs and HUVECs incubated with Angio-G (5μg/ml) for 18 hours.

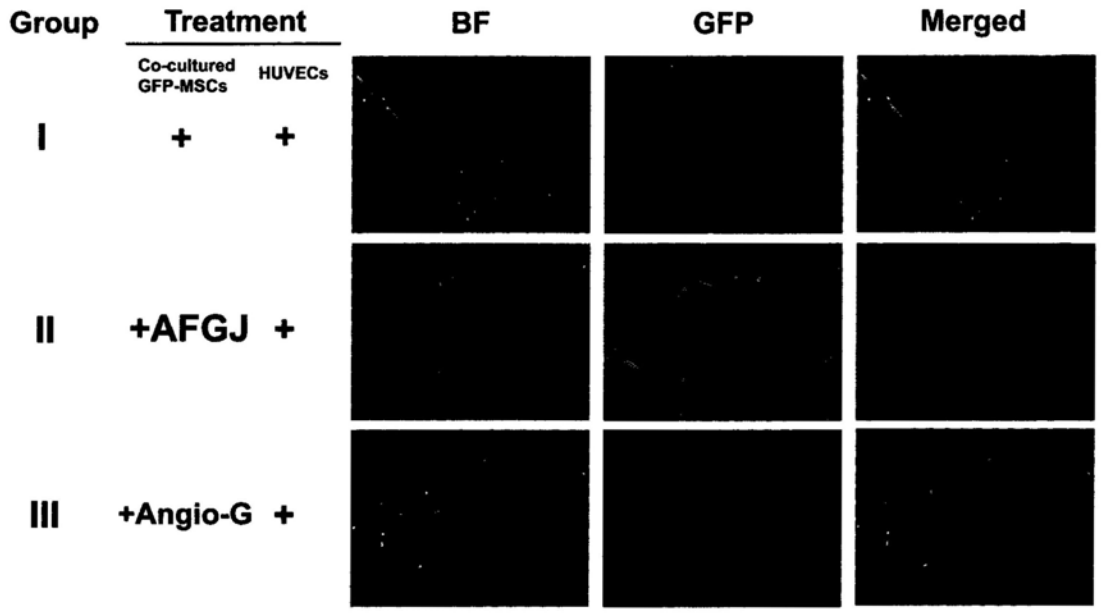

Figure 4.7 Differentiated GFP-MSCs contributed in capillary tube formation. Representative micrographs of tube formation in co-cultured GFP-MSCs and HUVECs incubated with Angio-G (5μg/ml) for 18 hours. The co-cultured MSCs without treatment (I) and co-cultured MSCs treated with Angio-G (10μg/ml) for 5 days (III) did not shown the obviously capacity of formation of tube-like structure. Only MSCs co-cultured with HUVECs in the presence of AFGJ (125μg/ml) have remarkably ability to contribute capillary tube like structure formation, which showed as green GFP fluorescence combined with bright field tubules (II).

4.2.3 Proteomics approach to screen the potential factors involved in mediating the effect of AFGJ

4.2.3.1 Alteration in rat cardiac proteome in CHD rat model

Proteomics refers large-scale characterization of proteins, including quantitative expression patterns, where proteins are localized, their physical modifications and interactions, their activities, regulations, and the physiological context of their activities (Qi, 2008). It is a powerful tool in studying the alteration of proteins in disease process. Combination of 2-D PAGE and MS technology is suitable for a direct comparision of the difference in the levels of protein expression between abnormal and normal samples. It has been reported that 2-D electrophoresis can be applied to identify the changes in protein expression in myocardial infarct tissue (De Celle et al., 2005). Therefore, in the present study I focused on the changes in the CHD heart proteome, and the relationship of these changes with recovery of the CHD heart treated with AFGJ.

For 2-D gel of CHD heart extracts I used normal range strips (PH 3-10) for the first dimension and 10% SDS-PAGE in the second dimension. Figure 4.8 shows the protein spots on the 2-D gel spot patterns that were only expressed in AFGJ-treated hearts compared with their respective vehicle and sham hearts. 16 spots were cut out for identification by MALDI-TOF/TOF MS with MS/MS in combination with database searching. As a result, 8 spots expressed in AFGJ-treated hearts were identified (Table 17). The majority of the protein spots identified are metabolism-related proteins and antioxidant proteins. Intriguingly, 3 identified proteins in this study were also found changed in some researches on hearts proteomics. These included decrease of fumarate hydratase (spot 2) in chronic heart failure (CHF)

(Cieniewski-Bernard et al., 2008), Glutathione S-transferase omega-1 (spot 9) expressed in spontaneously hypertensive rats (Jin et al., 2006), α-Enolase 1 (spot 5) down-regulated in ischemia/reperfusion injury animal model (Fert-Bober et al., 2008).

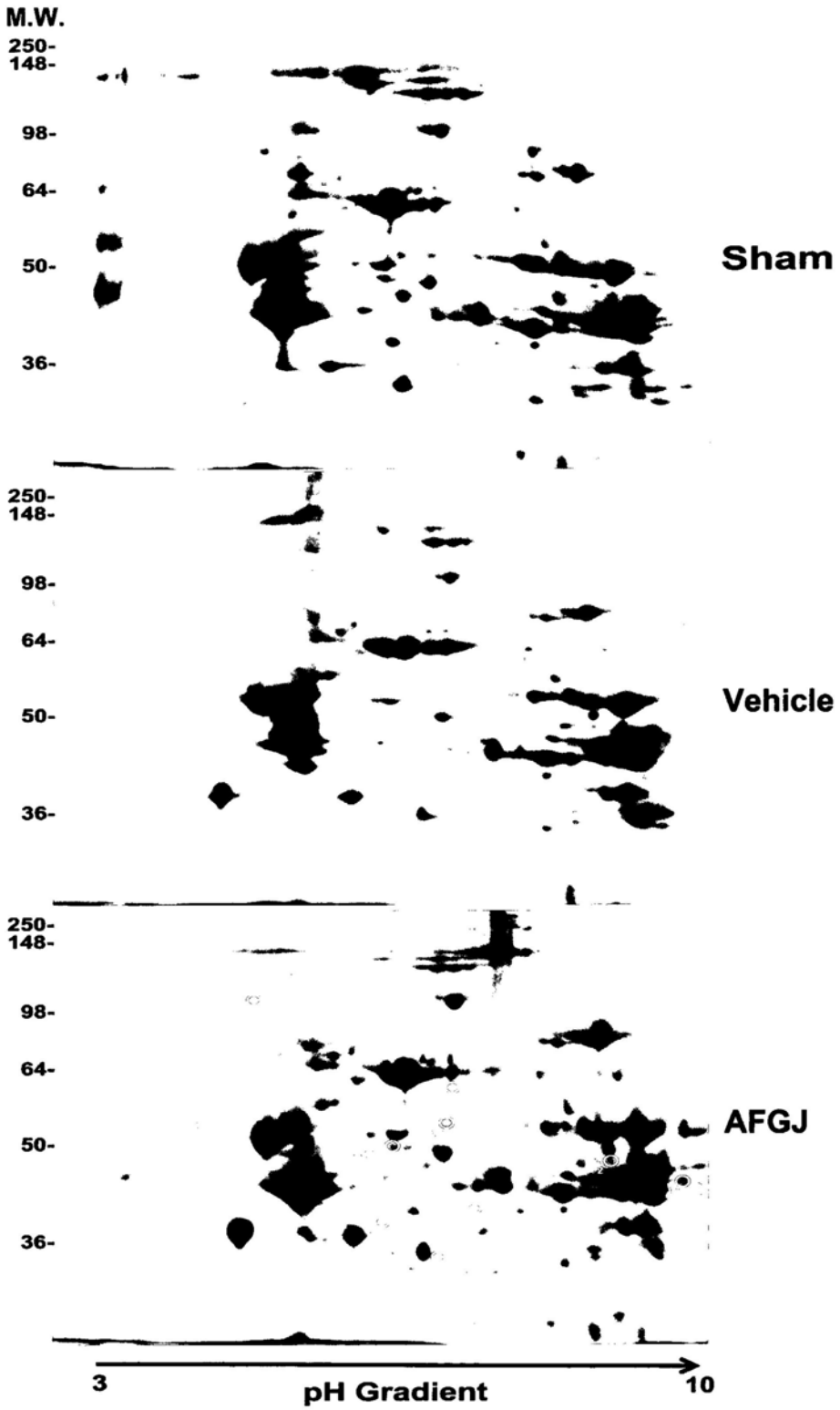

Figure 4.8 Representative 2-D PAGE of proteins from different treated hearts. Circles show the protein spots only expressed on treated heart (white circles and Number) indicated the protein spots from qualitiative analysis using PDQuest measurement software. N=3 *per* group.

Table 17. Identification of protein spots from CHD rats

Spot	Protein identification	Accession no.	MW (kDa)	Function
1	Peptide chain release factor 1 （RF-1)	B0JYC5.1	41.22	Termination of translation
2	Fumarate hydratase	P14408	54.7	Carbohydrate metabolism; tricarboxylic acid cycle
3	Not identified			
4	UDP-N-acetylenolpyruvoylglucosamine reductase	Q133X3.1	33.07	Cell wall formation
5	Enolase 1, alpha	P04764	47.8	Glycolysis, Carbohydrate degradation
6	Not identified			
7	Not identified			
8	Not identified			
9	Glutathione S-transferase omega-1 （GSTO 1-1）	Q9Z339.2	27.9	Antioxidant reactions
10	Not identified			
11	Not identified			
12	Not identified			
13	Sarcalumenin	Q90577.1	54.56	Regulation of calcium transport
14	Not identified			
15	Dihydropyrimidinase-related protein 2 （DRP-2)	O02675.1	62.63	Plays a role in axon guidance, neuronal growth cone collapse and cell migration
16	Ktelc1 protein	BC091408.1	41.8	-

4.2.3.2 Alteration in HUVECs proteome during angiogenesis *in vitro*

Previous results of this study have shown that Angio-G induced the proliferation of HUVECs in a dose-dependent manner. After Angio-G treatment for 24 hours, the proliferation rate was significantly increased compared with non-treat group. In addition, results of the CHD rat model indicated that AFGJ (Angio-G as the main compounds) had the capacity of promoting neovascularization in ischemic hearts. Therefore, both AFGJ and Angio-G were applied to treat the HUVECs for 12 hours and 24 hours. Figure 4.9 shows representative gels treated with AFGJ and Angio-G at different time point. A total of 17 spots were sent for identification and 11 spots were identified, and the results are listed in table 18. The greatest changes in protein levels *versus* the control group were detected in Angio-G treatment for 12 hours. Interestingly, 5 proteins, Glutamate dehydrogenase 1 (spot 3), TCP-1-beta (spot 5), Lamin A protein (spot 12), Pyruvate kinase (spot 13) and EH domain-containing protein 1 (spot 17) in AFGJ treatment for 24 hours, were observed at the same position in Angio-G 12 hour's treatment, indicating that the effects of AFGJ on inducing angiogenesis was less potent than that of Angio-G treatment *in vitro*. This delayed effect of AFGJ may be due to the less concentration of Angio-G in AFGJ. Furthermore, spot 1 (Elongation factor 2), 4 (Guanine nucleotide-binding protein subunit beta-2-like 1), 10 and 11 (Not identified) were only detected on Angio-G-treated gels. In addition, all identified proteins were searched in DAVID bioinformatics database in order to get the information of protein function and potential pathways involved (Huang da et al., 2009, Dennis et al., 2003). Glutamate dehydrogenase 1 (spot 3), TCP-1-beta (spot 5), Lamin A protein (spot 12), Pyruvate kinase (spot 13) and EH domain-containing protein 1 (spot 17) were involved in the process of nucleic acid binding, Glutamate dehydrogenase 1 (spot 3), TCP-1-beta

(spot 5) and EH domain-containing protein 1 (spot 17) were also considered having ATP-binding activity. Pyruvate kinase (spot 13) and Protein DJ-1 (spot 15) were involved in the process of glycolysis.

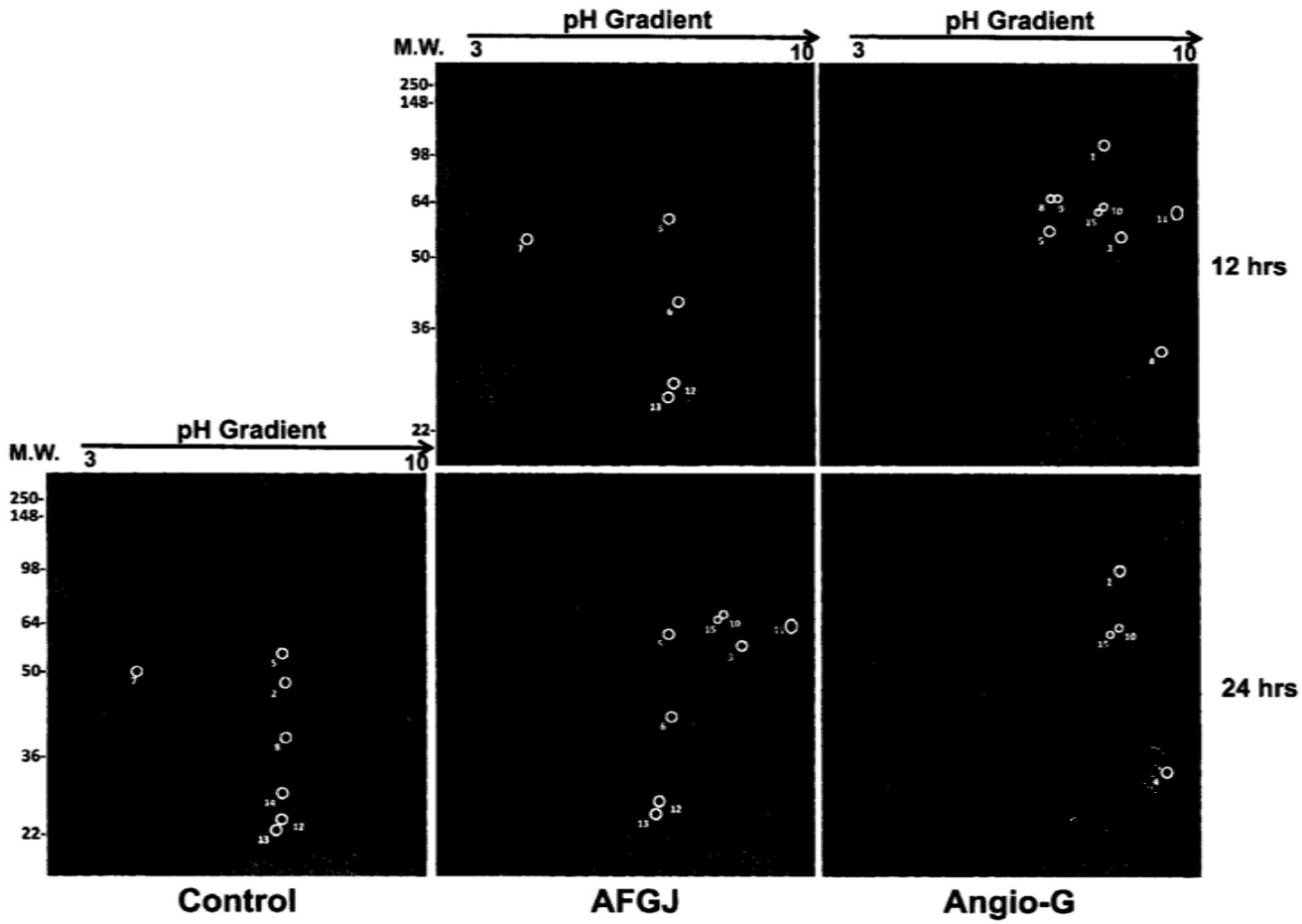

Figure 4.9 Representative 2-D PAGE of proteins from different treated HUVECs. Circles show changed protein spots (white circles and Number) indicate the protein spots from quantitative analysis using PDQuest measurement software. N=3 *per* group.

Table 18. Identification of protein spots from HUVECs treated with AFGJ and Angio-G

Spot	Protein identification	Accession no.	MW (kDa)	Function
1	Elongation factor 2 （EF-2)	Q3SYU2.3	96.27	Promotes the GTP-dependent translocation
2	Not identified			
3	Glutamate dehydrogenase 1 （GDH)	P00367.2	61.7	Allosteric regulation
4	Guanine nucleotide-binding protein subunit beta-2-like 1	P63245.3	35.5	Up-regulation of the activity of kinases such as PKC via binding to KRT1
5	T-complex protein 1 subunit beta （TCP-1-beta）	P78371.4	57.8	Molecular chaperone
6	Not identified			
7	Not identified			
8	Annexin A1	P04083.2	38.9	Calcium/phospholipid-binding protein
9	Not identified			
10	Not identified			
11	Not identified			
12	Lamin A protein	M13452.1	57.7	-
13	Pyruvate kinase	P14618.4	58.47	Glycolytic enzyme
14	Proteasome subunit beta type-3	P49720.2	23.21	Cellular metabolism
15	Protein DJ-1	Q99497.2	20	Positive regulator of androgen receptor-dependent transcription; Protects neurons against oxidative stress and

				cell death; cell-growth promoting activity and transforming activity
16	Endoplasmic reticulum protein ERp29	P81623.2	28.84	Processing of secretory proteins within the ER
17	EH domain-containing protein 1 (EHD1)	Q9H4M9.2	60.64	Acts in early endocytic membrane fusion and membrane trafficking of recycling endosomes

4.3 Discussion

Previous results of this study indicated that Angio-G derived from AFGJ has the capacity of stimulating tube-like structure formation in HUVECs. Our previous study has implicated the involvement of the JAK/STAT signaling pathway in Angio-G induced capillary tube formation (data not shown). AG490, which is an inhibitor of JAK/STAT, was used in a tube formation test and the results showed that it reduced the tube length induced by Angio-G, suggesting that activation of the JAK/STAT pathway by Angio-G plays a crucial role in capillary tube formation.

In this chapter, it is demonstrated that MSCs differentiated into endothelial cells in the presence of active fractions from *G. japonicum* in a HUVECs co-cultured system. Moreover, the differentiated MSCs, induced by AFGJ, were involved in capillary tube-like formation. Surprisingly, the Angio-G treatment of co-cultured MSCs failed to incorporate this process. This result indicates that AFGJ is more effective for vessel formation compared to Angio-G, suggesting that MSCs may have an important role in new vessels growth in the adult.

2-D gel and MS have been widely used in biological and medical research areas, such as to distinguish the critical changes involved in post-translational modification in different conditions, protein differential expression, disease subtyping and monitoring, protein-protein interaction and biomarker identification (Qi, 2008, Burbaum and Tobal, 2002). To investigate the alterations of protein expression in CHD rat hearts and proliferation of HUVECs, 2-D PAGE was applied to distinguish the protein differential expression both *in vivo* and *in vitro*. A study by Caroline *et al* analyzed the proteomics of chronic heart failure model and 49 different spots were identified which were classified in 7 different functional groups. One of these

proteins- fumarate hydratase, enzyme of tricarboxylic acid cycle, was down regulated in CHF model (Cieniewski-Bernard et al., 2008). In the present study, this protein was found expressed in AFGJ-treated hearts. Furthermore, another identified protein- α-Enolase was detected reduced in myocardial proteome after ischemia/reperfusion injury in rats model (Fert-Bober et al., 2008). The decreasing of proteins involved in energy metabolism may affect the heart resoponse to ischemia. In the present study, 2 identified proteins, related to metabolism, both were increased in expression in AFGJ-treated group, indicating that AFGJ treatment is effective the protection against ischemia injury of the heart.

Previous results in present study suggested that AFGJ and Angio-G could stimulate the proliferation of HUVECs. To further investigate the molecular mechanism of proliferation induced by AFGJ/Angio-G, we tried to detect the alteration of proteins involved in HUVECs proliferation by using the proteomics approach. As shown in table 18, 11 identified proteins were confirmed with important celluar functions such as protein sequencing and modifications, metabolism and ATP binding activities. In the literature, the regulations of some identified proteins involved in cell proliferation were reported. A study by Vanja *et al* demonstrated that expression of lamina-associated polypeptide 2α – lamin A complexes were involved in controlling cell cycle in proliferation of human diploid fibroblasts (Pekovic et al., 2007). Another study by Marko *et al* reported that EHD1 regulated ß1 integrin recycling which has effects on cell adhesions and migration (Jovic et al., 2007). In our results, the expression of 7 identified proteins was increased after AFGJ/Angio-G treatment, which also included Lamin A (spot 12) and EHD1 (spot 17). Similarly, the increased expression of these proteins which are involved in protein sequencing and metabolisms, might provide the energy and

synthesis of proteins for proliferation of HUVECs. The important proteins identified by MS will be verified in future study, which may provide critical information for the elucidation of the molecular mechanisms underlying AFGJ-induced neovascularization.

CHAPTER 5 General discussions

5.1 Introduction

Coronary heart diseases including chronic CHD and myocardial infarction remain the most prevalent cause of premature death (Isles and Paterson, 2000, Delaney et al., 2008). Heart ischemia results mainly from coronary artery occlusion. Prolong ischemia is the major etiological factor inducing myocardial infarction and cause irreversible death of myocardium (Downey, 1990, Sharma and Das, 1997). During vascular remodeling process, neovascularization to the ischemic heart tissues is insufficient to support the requirement of tissue compensation and results in hypertrophy, even death of the cardiomyocytes (Shah and Shalia, 2009). The most effective way to rescue the cardiac myocytes and heart ischemia is to augment new blood vessel growth that would improve the blood supply, which allow circulating nutrients and growth factors. Restoration of coronary blood flow *via* forming nature-bypassed vessels may offer a direct and effective therapeutic modality to intractable ischemic heart diseases.

In this study, substantial therapeutic effects of AFGJ on heart ischemia in CHD animal models are demonstrated. Evidences, both morphological and functional, are provided indicating that the remarkable therapeutic effect of the AFGJ in repairing ischemic heart lies critically in its ability to enhance coronary vascular neovascularization in ischemic hearts, the root cause of chronic CHD. The cellular and molecular basis of AFGJ and Angio-G actions will be discussed in this chapter.

5.2 Effects of the AFGJ on CHD rat model.

In the present study, we have isolated an active angiogenic fraction (Angio-G) from the extract AFGJ. We firstly demonstrated the promoting effects of AFGJ and Angio-G on proliferation in vessel endothelial cells *in vitro*. More importantly, this

angiogenic potential of AFGJ has shown to promote effective growth of new coronary vessels/collaterals with progressive restoration of heart functional performance in a CHD subclinical rat model. By applying the new technology, MicroCT, in CHD rat model, visible evidences of microvessels formation was achieved instead of the traditional counting vessel density in histochemistry analysis.

In pre-clinical researches, MicroCT has been developed for *in vivo* small animal and specimen scanning, which use specially designed micro-focus X-ray tube to obtain more precision images with the resolution of several micrometers. Some researches have described the capability of MicroCT to detect the alteration of microvasculature in different animal models. Savai *et al* investigated the lung tumor angiogenesis using MicroCT and achieved ideal visualization of the architecture of the mouse lung and lung tumor vasculature. Interestingly, this technique was able to measure vessel area and quantitative measurement of intrapulmonary tumors and their vascular content (Savai et al., 2009). Toyota *et al* successfully established ischemic cardiomyopathy model and the coronary microvascular volume fraction was decreased in this model (Toyota et al., 2007). Another study demonstrated that the reduction of lesion volume in c3Ado treated $ApoE^{-/-}/LDL^{-/-}$ mice was associated with a decreased vasa vasorum growth with characterizing plaque volume and vasa vasorum luminal volume based on the MicroCT analysis (Langheinrich et al., 2009). However, this technology has not yet been used to measure the alteration of coronary vasculature of whole heart in CHD animal model.

In this study, vessel images allowed completely stereoscopic visualization of the 3D architecture of the vascular network through excluding the atrium part that was perfused with Microfil material. MicroCT was suitable to analyze and quantify discrete regions of interest more precisely. More bypass-connected microvessels in

the hearts of AFGJ-treated group were observed compared with that of vehicle group (Figure 3.7). This phenomenon was confirmed with the result of distribution of vascular diameter, suggesting that the more microvessels were neovascularized in AFGJ-treated hearts which may improve the blood supply of the myocardium, while the big vessels were not obviously changed. Furthermore, the measurement of vascular density of ROI, vascular volume in VOI, and analysis of the number of vascular branches revealed that AFGJ treatment substantially promoted the growth of microvessels that contribute to the myocardium nutrition. However, AFGJ treatment had less influence on mature big vessel in ischemic heart, more affecting the small vessels in ischemic area. In addition, the quantities of neovascularization in histology analysis and improved heart function induced by AFGJ in ischemic hearts from this partially occluded LAD model are consisted with the findings in a completely occluded LAD model (data not shown).

Although our studies have demonstrated the benifical effect of AFGJ and its potential in developing into an effective therapeutic curative for CHD, no toxicity studies have been conducted to prove its safety for use in humans. However, it has been reported that a fraction isolated from *G. japonicum* (Angio-T), used as single dosage of 0.5mg/kg, was able to increase the formation of vessels as 2-fold in acute ischemic muscles and relieve the muscle ischemia (Cheung et al., 2007). In vitro study, Angio-G could stimulate the proliferation of HUVECs at a dosage as high as 200μg/ml, without detectable cytotoxity on the cells of the pure Angio-G. Moreover, in the present study, the intramuscularly administration were replaced with oral administration with AFGJ of 75mg/rat/day of CHD rats for 2 weeks. H&E staining from this study revealed that cells treated with AFGJ appeared normal and healthy. Hence, it can be suggested that concentrations of AFGJ used in this study do not seen

to have acutely toxicity to rat cardiomyocytes and other organs with the subchronic treatment of 2 weeks. However, the duration of AFGJ treatment and possible toxicity in human beings requires further investigation.

The molecular mechanism for AFGJ mediated coronary reconstitution remains unclear. Our recent study (Cheung et al., 2007) has demonstrated the up-regulation of some traditional angiogenic factors, such as EGF, FGF7, VEGFb in Angio-T induced angiogenesis in ischemic muscles. In the present study, in vitro test also demonstrated a proliferation-promoting effect induced by Angio-G in HUVECs. In addition, the present study showed that MSCs could differentiate into endothelial cells, indicating that the other model of vessels formation method, vasculogenesis, might be involved in the microvessels neovascularization. Consistent with this notion, it has been reported that MSCs could differentiate into smooth muscle cells and endothelial cells in canine chronic ischemia model (Silva et al., 2005).

5.3 Angiogenesis induced by AFGJ and Angio-G

In normal condition, endothelial cells stay in the static state and less than 0.1% of endothelial cells are labeled as proliferating cells (Zubar, 2005b). When the body is injured or insulted, the cells in wounded area are activated by the released angiogenic growth factors such as VEGF, PDGF and FGF. The activated endothelial cells secret proteases to degrade the basement membrane and migrate to surrounding interstitial tissues from pre-existing vessels where spouting endothelial cells proliferate and form capillary structures (Zubar, 2005a, Patan, 2000, Conway et al., 2001). Nowadays, effects of angiogenic factors on angiogenesis are well documented, which mainly include: VEGF, transforming growth factors (TGF), FGF, urokinase-type plasminogen activator (u-PA), angiogenin, and PDGF. These factors promote

angiogenesis by stimulating endothelial cells proliferation, and/or migration through activating the corresponded tyrosine kinase receptors such as VEGFR, Tie2, u-PA receptor, Eph and PDGFR.

In general, VEGF family plays a central role in both vasculogenesis and angiogenesis during development as well as in the adult. VEGFs are homodimeric, secreted glycoproteins which are responsible for the regulation of the vasculature. VEGF family includes 5 members: VEGF-A to -D and placental growth factor (PlGF) and 3 receptors involved in initiating tyrosine kinases phosphorylation termed VEGFR-1, VEGFR-2 and VEGFR-3. VEGFR-3 also play important roles in the development of embryonic vascular system and postnatal is restricted in endothelial cells of lymphatic vessels and specialized fenestrated capillaries (Dumont et al., 1998).

The JAK/STAT pathway is one of pleiotropic cascades to induce many signal transductions for development and homeostasis in animals. In mammals, the JAK/STAT pathway plays an important role in triggering cytokines and growth factors which regulate various biological processes such as cell proliferation, differentiation, cell migration, and apoptosis (Singh et al., 2005, Vignais et al., 1996, Tarnawski and Jones, 1998). Our previous study has demonstrated that the up-regulation of some angiogenic factors, such as VEGF, EGF and FGF in Angio-T induced angiogenesis in ischemic muscles (Cheung et al., 2007). The importance of angiogenic factors and signaling pathways in cell survival and overcoming ischemia has been highlighted in a few studies. Microvascular endothelial cells were able to become tolerant to hypoxia by up-regulated VEGF expression and activation of the JAK2/STAT5 pathway (Dudley et al., 2005). Another study (Zeng et al., 2002) found that activated JAK2/STAT and PI3-kinase increased the expression of bFGF-2

and resulted in the growth of human microvascular endothelial cells (HMVECs). In addition, it has been reported that VEGF-VEGF receptor ligation selectively induces activation and peri-nuclear translocation of STAT3 through receptor-mediated endocytosis, which accounted for the VEGF induction of anti-apoptotic effects in CLL B-cells (Lee et al., 2005). AG490 had been reported to inhibit EGF receptor phosphorylation and cell growth through inactivation of JAK2, JAK3/STAT and JAK3/MAPK pathways (Gazit et al., 1991). It has also been reported that AG490 is able to inhibit the growth of leukemic cells both *in vitro* and *in vivo* (Meydan et al., 1996). Therefore, we used AG490 to investigate whether the JAK/STAT signaling pathway is involved in this process of revascularization or not, in the presence of Angio-G.

The present study demonstrated that AFGJ could increase the vascular density in ischemic area in CHD model. Furthermore, Angio-G was able to stimulate the proliferation of HUVECs and promote tube-like capillary formation capacity. However, AG490 was able to inhibit capillary network construction capacity *in vitro*. The JAK inhibition experiment from this study suggested that the JAK/STAT pathway may be involved in Angio-G-induced angiogenesis. Hence, in combination with the results of earlier studies, the JAK/STAT pathway plays a critical role in making efficient revascularization in ischemic hearts possible. A possible mechanism of AFGJ in rectifying ischemic hearts can be proposed: AFGJ (Angio-G) may activate the JAK/STAT signal transduction pathway and activation of the JAK/STAT pathway results in high expression of angiogenic genes in cells and tissues. In turn, these angiogenic factors induce endothelial cells proliferation, which eventually yield new vasculature, leading to improve or increase blood transport for efficient heart function.

5.4 Adult vasculogenesis occurs through MSCs differentiation induced by AFGJ and Anigo-G.

Adult neovascularization has traditionally considered being limited to angiogenesis. However, the isolated endothelial progenitor cells (EPCs) from the adult bone marrow, with a capacity of differentiation into endothelial phenotype, broke this convention in 1997 (Asahara et al., 1997). Recent studies have led researchers to come back to this orthodox process of angiogenesis. EPCs express CD34, CD133 and VEGFR-2 as common markers. Studies using ischemic animal models have suggested that EPCs might participate in new vessels formation when ischemia happens. EPCs, expanded *in vitro* and implanted into hind limb ischemia models, were able to promote angiogenesis in ischemic area (Urbich et al., 2003, Kalka et al., 2000, Kawamoto et al., 2001, Murohara et al., 2000). There are more and more evidences showing that EPCs can be incorporated into postnatal neovascularization. Recently, a study of transplantation of autologous EPCs in a swine ischemic model demonstrated the improvement of left ventricular function and reduction of the myocardial ischemia (Kawamoto et al., 2003). In addition, Tepper *et al.* established a new ischemic model to investigate the contribution of EPCs to angiogenesis. It was demonstrated that EPCs were recruited to ischemic tissue and formed the functional vessels derived from labeled EPCs (Tepper et al., 2005).

However, to our knowledge no studies have demonstrated that EPCs are able to differentiate into VSMCs which are necessary for all kinds of artery except capillary vessels. Furthermore, the phenotype of EPCs is overlapped with hematopoietic cells and normal endothelial cells, and thus, controversy remains regarding identification of the origin of EPCs (Urbich and Dimmeler, 2004, Schmeisser and Strasser, 2002). Therefore, MSCs, as pluripotent stem cells, seem to be a better candidate in

restoration of vascular disease. It was reported that MSCs differentiated into endothelial cells *in vitro* (Oswald et al., 2004) and improved the capacity of migration in hypoxia (Annabi et al., 2003). It was also demonstrated that MSCs participated in the vascular repair in animal model. A study by Silva *et al.* provided the evidence that MSCs might induce angiogenesis *via* differentiation into endothelial phenotype cells and resulted in the improvement of heart function in a canine ischemia model (Silva et al., 2005). Recent study has shown evidence for MSCs' involvement in vascular regeneration with MSCs transplantation in pig myocardial infarct model (Dib et al., 2006).

In the present study, we demonstrated that co-cultured MSCs in the presence of AFGJ and Angio-G could differentiate into ECs with positive staining of vWF. Surprisingly, Angio-G treated co-cultured MSCs failed to participate in the process of capillary tube-like formation, indicating that the effect of Angio-G in new blood vessel formation mainly to its stimulation of the proliferation and tube formation of ECs. There may be other compounds in AFGJ to promote the differentiation of MSCs in the process of new vessels formation. However, MSCs treated by AFGJ only was vWF negative and become vWF positive when co-cultured with HUVECs, suggesting that MSCs have the milieu-dependent differentiation potential to endothelial lineage when co-cultured with mature ECs and AFGJ and Angio-G are necessary in this MSCs differentiation.

5.5 Angiogenesis *vs* vasculogenesis

Based on the aforementioned findings by others and the present study, I propose possible mechanisms of neovascularization by which AFGJ is able to promote newly blood vessels formation *via* both adult vasculogenesis and angiogenesis (Figure 5.1). It has been reported that newly blood vessels growth is initiated as a

response to ischemia (Tepper et al., 2005). Ischemia releases cytokines/growth factors which subsequently recruit MSCs from bone marrow. AFGJ treatment may enhance these cytokines secretion and MSCs homing to ischemic area where neovascularization is needed. MSCs then differentiate into ECs and VSMCs *in situ* forming cordlike vascular. Soon after, newly formed vascular cords from differentiated MSCs are extended and connected to existing vasculature in the presence of AFGJ. On the other hand, Angio-G, compounds contained in AFGJ, stimulates the proliferation of ECs. Proliferated ECs are recruited toward angiogenic stimulus which is released by ischemic tissue and recruits MSCs and distant to the leading tip cell of the sprout. In this progress, ECs might be connected with differentiated MSCs to form cell-cell gap-junctions and activate signaling pathways to induce the maturation of newly growth vessels. By MicroCT perfusion after surgery 6 weeks, there is evidence of functional microvessels formed and these newly by-passed vessels significantly improved the heart function. However, the other linages of progenitor cells such as CSCs and EPCs were also reported that these cells could differentiate into ECs or VSMCs (Dib et al., 2006). These progenitor cells might be contributed as complementary in newly blood vessels formation and have the similar effects with MSCs. Further investigation is needed to determine whether AFGJ stimulates differentiation of other progenitors.

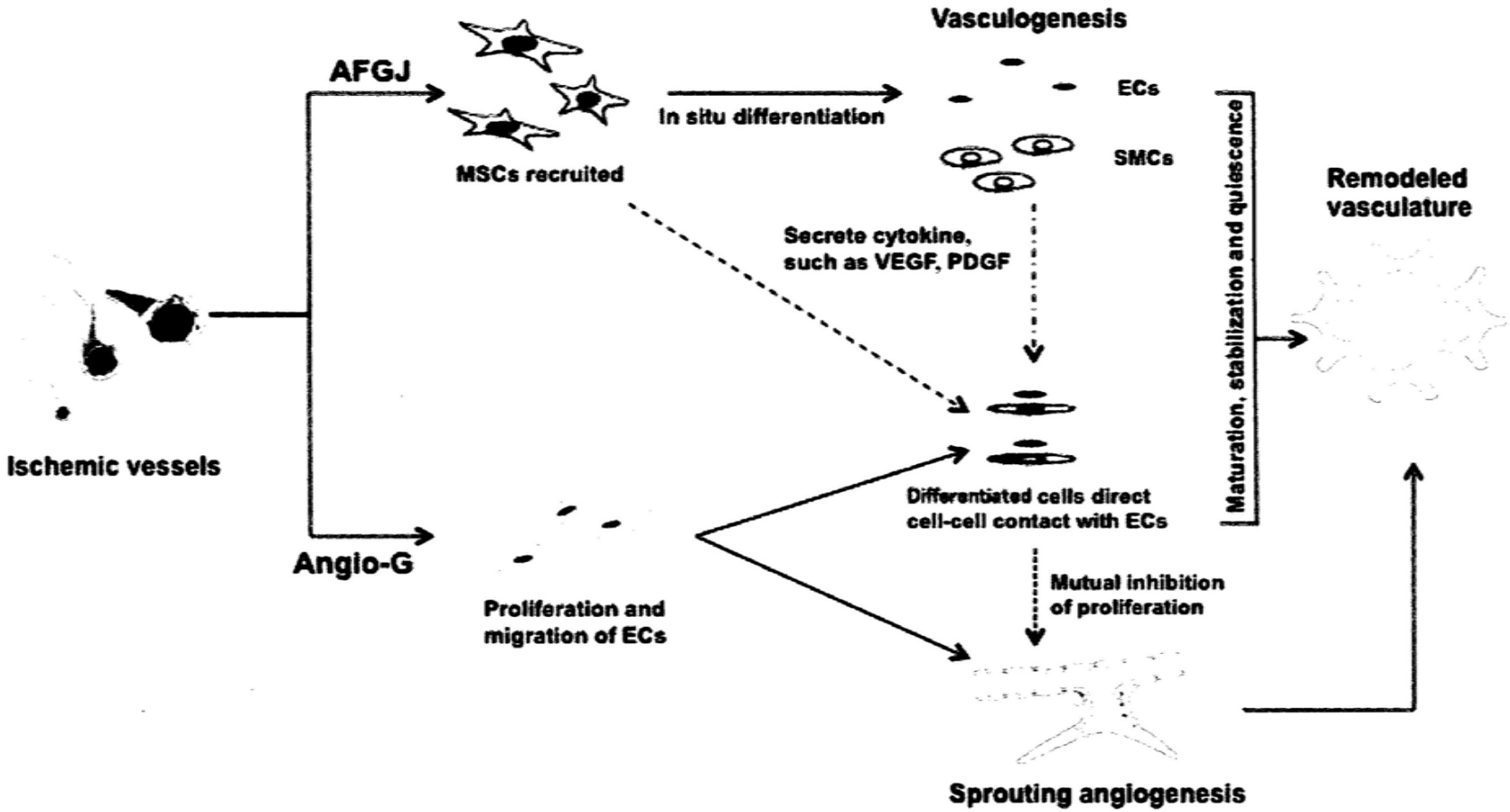

Figure 5.1 Schematic representation of the potential mechanism by which AFGJ induced neovascularization in CHD ischemic heart.

5.6 Conclusions and future prospective

In the present study, I examined the therapeutic effect of AFGJ isolated from *G. japonicum* on a CHD rat model. The main findings are summarized as follows: 1) an active fraction (AFGJ) and further isolated compounds (Angio-G) were obtained and identified from *G. japonicum*; 2) AFGJ does promote effective growth of coronary vessels/collaterals in ischemic hearts of CHD rat model with a novel technology (MicroCT) providing visualized evidence of neovascularization; 3) an Angio-G, compounds derived from AFGJ, significantly enhances the proliferation and the ability of capillary-like tube formation in HUVECs *in vitro* through activation of JAK/STAT signal pathway; 4) AFGJ could induce MSCs differentiation into ECs and these differentiated ECs involved in the process of tube formation; 5) AFGJ exerts its neovascularization-promoting effect in vessel endothelial cells and ischemic hearts probably through not only activation of several angiogenesis pathways, such as the JAK/STAT signaling pathway, but also promoting the MSCs differentiation and eventually yield new vasculature.

Our findings provide ground for the development of a potentially valuable drug for CHD. Over the past decades, there has been a large amount of concerns in developing methods to promote neovascularization in ischemic disease. Most studies have focused on the delivery of angiogenic agents and stem cells transplantation, many of which are ongoing clinical trails (Diaz-Sandoval and Losordo, 2003, Miller-Kasprzak and Jagodziński, 2007). A new modality for increasing blood vessel growth is through traditional Chinese herbs, which provide a more natural treatment instead of invasive methods. This has been examined in both animals and humans (Lau et al., 2009, Hong et al., 2009, Fan et al., 2006). This study demonstrated that

the ideal neovascularization of CHD induced by AFGJ treatment through oral administration whose advantages are easy to be treated and no pain of operation, despite a limited understanding of the molecular mechanism by which AFGJ stimulates new vessels formation. Therefore, further study will investigated the direct interacting proteins with AFGJ and potential angiogenic factors involved or related signaling pathways.

2D-PAGE combined with MS is a useful approach to achieve an overview of proteomic alterations both in AFGJ treated animal and AFGJ/Angio-G treated HUVECs. In the present study, total 19 proteins were identified which included 8 for CHD rat model and 11 for HUVECs. Some of the proteins are related to cell proliferation. However, the expression of 8 proteins in total 19 proteins are involved in celluar energy metabolism and protein modificatons. Protein alterations in different heart injuries have also been reported that proteins involved in energy metabolism are significantly modified (White et al., 2005, Fert-Bober et al., 2008, Jin et al., 2006, Cieniewski-Bernard et al., 2008). Therefore, the increased expression of proteins during AFGJ treatment for ischemia heart is one of possible defense mechanisms. This result has provided a clue for demonstrating the molecular mechanisms in ischemia heart disease treated with AFGJ in further work.

Our elucidation of a mechanism through which AFGJ contributes to neovascularization has important implications. With the supporting evidence that bone marrow-derived cells participate in adult vasculogenesis through *in situ* recruitment, proliferation and differentiation (Tepper et al., 2005). Together with my results, it suggests that AFGJ could promote both angiogenesis and vasculogenesis in the adults. Interestingly, co-cultured MSCs treated with AFGJ were able to participate into tube-like capillary formation but not the Angio-G treated cells,

implying that there might be some other compounds in AFGJ to induce differentiated MSCs being a part of tube-like structure beside Angio-G effects. I propose future experiments focusing on the compound mediators of differentiated MSCs connecting with ECs in ischemic vessels, which will yield functional new blood vessel formation to increase the heart function in CHD model. In addition, the direct effects of molecules in co-culture medium on MSCs differentiation into endothelial cell lineages will be examined, which may help elucidate the exactly signaling pathways involved in mediating the effect of AFGJ.

In conclusion, this study demonstrated therapeutic properties of AFGJ to induce rapid angiogenesis and reconstitution of damaged coronary vessels with newly formed functional coronary collaterals. The new coronary collaterals by-pass the obstructed arteries and correct the ischemia of affected heart. Together with data achieved in chapter 3 and 4, our study suggests that AFGJ treatment could induce the effective angiogenesis in ischemic hearts and *in situ* vasculogenesis in the recover period. This study may provide grounds for the development of a new therapeutic modality for effective treatment of ischemic heart diseases.

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