

# **Functional Characterisation of Cardiac Progenitors from Patients with Ischaemic Heart Disease**



**Huajun Zhang**

Wolfson College

**Supervisors: Dr Enca Martin-Rendon  
Professor David Taggart**

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Nuffield Department of Surgical Sciences  
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# Abstract

Ischaemic heart disease (IHD) is the leading cause of death worldwide. Currently, even optimal medical therapies do not attenuate deterioration of the left ventricular (LV) function completely. Stem cell therapies, and recently cardiac stem cell therapies, have emerged as potential novel treatments for IHD. However, clinical evidence from randomised controlled studies has shown mixed results. Thus understanding what patient-related factors may affect the therapeutic performance of the cells may help improving treatment outcomes. The studies described in this thesis aim to understand how cardiac progenitor cells (CPCs) can re-vascularise ischaemic myocardium and promote functional repair of the heart. Resident CPCs were isolated and expanded from the right atrial appendage of 68 patients following the ‘cardiosphere’ method (cardiosphere-derived cells or CDCs). They resemble mesenchymal progenitors as they lack the expression of endothelial and haematopoietic cell surface markers but express mesenchymal progenitor cell markers (e.g. CD105, CD90). Cell function was evaluated by support of angiogenesis, mesenchymal lineage differentiation potential *in vitro*, and improvement in heart function *in vivo*. Notably *in vitro*, CDC from different patients differed in their angiogenic supportive and differentiation potentials. In a rodent model of myocardial infarction (MI), transplantation of CDC reduced infarct size significantly ( $p<0.05$ ). However, only those CDCs with a robust pro-angiogenic ability *in vitro* improved vessel density and heart systolic function ( $p<0.05$ ) *in vivo*. A multiple regression model, which accounted for 51% of the variability observed, identified New York Heart Association (NYHA) class, smoking, hypertension, type of ischaemic disease and diseased vessel as independent predictors of angiogenesis. In addition, gene expression analyses revealed that differential gene expression of several extracellular matrix components (e.g. *CUX1*, *COL1A2*, *BMP1* genes and microRNA-29b) could explain the differences

observed in CDC's vascular supportive function. In summary, this is the first description of variability in the pro-angiogenic and differentiation potential of CDCs and its correlation with their therapeutic potential. This study indicates that patient stratification may need to be included in the design of future trials to improve the efficacy of cell-based therapies.

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## Abbreviations

|               |                                            |
|---------------|--------------------------------------------|
| <b>3'-UTR</b> | 3'-untranslated region                     |
| <b>2/3D</b>   | two/three dimensional                      |
| <b>A</b>      | atrium/atrial                              |
| <b>ACE</b>    | angiotensin-converting enzyme              |
| <b>Ad (5)</b> | adenovirus (type 5 )                       |
| <b>AIC</b>    | Akaike information criterion               |
| <b>ALDH</b>   | alcohol dehydrogenase                      |
| <b>ALP</b>    | alkaline phosphatase                       |
| <b>AMSC</b>   | adipo tissue-derived mesenchymal stem cell |
| <b>ANOVA</b>  | analysis of variance                       |
| <b>ANP</b>    | atrial natriuretic peptide                 |
| <b>APC</b>    | allophycocyanin                            |
| <b>ARB</b>    | angiotensin II receptor blocker            |
| <b>AS</b>     | aortic stenosis                            |
| <b>AVR</b>    | aortic valve replacement                   |
| <b>AWT</b>    | anterior wall thickening                   |
| <b>B2M</b>    | beta-2-microglobulin                       |
| <b>BM</b>     | bone marrow                                |
| <b>BMNC</b>   | bone marrow mononuclear cell               |
| <b>BMP1</b>   | bone morphogenetic protein 1               |
| <b>BMSC</b>   | bone marrow-derived mesenchymal stem cell  |
| <b>BSA</b>    | albumin from bovine serum                  |

|                 |                                                                 |
|-----------------|-----------------------------------------------------------------|
| <b>[C]</b>      | concentration                                                   |
| <b>CABG</b>     | coronary artery bypass grafting                                 |
| <b>CAD</b>      | coronary artery disease                                         |
| <b>CCS</b>      | Canadian Cardiovascular Society angina score                    |
| <b>CDC</b>      | cardiosphere-derived cell                                       |
| <b>cDNA</b>     | complementary DNA                                               |
| <b>CEM</b>      | complete explants medium                                        |
| <b>CGM</b>      | cardiosphere growth medium                                      |
| <b>CHD</b>      | congenital heart disease                                        |
| <b>CHF</b>      | congestive heart failure                                        |
| <b>CHRD</b>     | chordin                                                         |
| <b>COL1A2</b>   | type I collagen chain A2                                        |
| <b>CPC</b>      | cardiac progenitor cell                                         |
| <b>cRNA</b>     | complementary RNA                                               |
| <b>CS</b>       | cardiosphere                                                    |
| <b>CSC</b>      | cardiac stem cell                                               |
| <b>Ct</b>       | threshold cycle                                                 |
| <b>CUX1</b>     | cut-like homeobox 1                                             |
| <b>DAPI</b>     | 4',6-diamidino-2-phenylindole                                   |
| <b>DAVID</b>    | Database for Annotation, Visualization and Integrated Discovery |
| <b>DE genes</b> | differentially expressed genes                                  |
| <b>DEPC</b>     | diethylpyrocarbonate                                            |
| <b>DLR</b>      | dual luciferase reporter                                        |
| <b>DM</b>       | diabetes mellitus                                               |

|                                    |                                            |
|------------------------------------|--------------------------------------------|
| <b>DMEM</b>                        | Dulbecco's modified eagle medium           |
| <b>DMF</b>                         | dimethylformamide                          |
| <b>DMSO</b>                        | dimethylsulphoxide                         |
| <b>dNTP</b>                        | deoxyribonucleotide triphosphates          |
| <b>dT</b>                          | deoxythymidine                             |
| <b>DTT</b>                         | DL-Dithiothreitol                          |
| <b>ECM</b>                         | extracellular matrix                       |
| <b>EF</b>                          | ejection fraction                          |
| <b>EGM-2</b>                       | endothelial growth medium-2                |
| <b>ES</b>                          | enrichment score                           |
| <b>ESV</b>                         | end systolic volume                        |
| <b>FAC</b>                         | fractional area change                     |
| <b>FCS</b>                         | foetal calf serum                          |
| <b>FDR</b>                         | false discovery rate                       |
| <b>FGF</b>                         | fibroblast growth factor                   |
| <b>FHx</b>                         | family history                             |
| <b>FITC</b>                        | fluorescein isothiocyanate                 |
| <b>FS</b>                          | fractional shortening                      |
| <b>GAPDH</b>                       | glyceraldehyde 3-phosphate dehydrogenase   |
| <b>GCP4</b>                        | glypican 4                                 |
| <b>GFP</b>                         | green fluorescence protein                 |
| <b>H<sub>2</sub>SO<sub>4</sub></b> | sulphuric acid                             |
| <b>HAPLN1</b>                      | hyaluronan and proteoglycan link protein 1 |
| <b>has</b>                         | Homo sapiens                               |

|                                |                                                    |
|--------------------------------|----------------------------------------------------|
| <b>HBChol</b>                  | hypercholesterolaemia                              |
| <b>HBp</b>                     | hypertension, hypertensive                         |
| <b>HCl</b>                     | hydrogen chloride                                  |
| <b>HCL</b>                     | hierarchical clustering                            |
| <b>HEK293FT</b>                | human embryonic kidney 293 cell for transfection   |
| <b>HEPES</b>                   | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| <b>HF</b>                      | heart failure                                      |
| <b>HGF</b>                     | hepatocyte growth factor                           |
| <b>HIV-1</b>                   | human immunodeficiency virus-1                     |
| <b>HPC</b>                     | haematopoietic progenitor cell                     |
| <b>HSC</b>                     | haematopoietic stem cell                           |
| <b>HSPG</b>                    | heparan sulfate proteoglycan                       |
| <b>HUVEC</b>                   | human umbilical vein endothelial cell              |
| <b>IC</b>                      | intracoronary infusion                             |
| <b>IGF</b>                     | insulin growth factor                              |
| <b>IHD</b>                     | ischaemic heart disease                            |
| <b>IL-1</b>                    | interleukin-1                                      |
| <b>Imc</b>                     | intramyocardial injection                          |
| <b>INF-<math>\gamma</math></b> | interferon gamma                                   |
| <b>IV</b>                      | intravenous infusion                               |
| <b>IVSd</b>                    | interventricular septum diastolic thickness        |
| <b>IVSs</b>                    | interventricular septum systolic thickness         |
| <b>LAD</b>                     | left anterior descending artery                    |
| <b>LCx</b>                     | left circumflex artery                             |

|                 |                                                      |
|-----------------|------------------------------------------------------|
| <b>LV</b>       | left ventricular; lentiviral vector                  |
| <b>LVArea d</b> | left ventricle end-diastolic area                    |
| <b>LVArea s</b> | left ventricle end-systolic area                     |
| <b>LVAWd</b>    | left ventricle anterior wall end-diastolic thickness |
| <b>LVAWs</b>    | left ventricle anterior wall end-systolic thickness  |
| <b>LVd</b>      | left ventricle end-diastolic diameter                |
| <b>LVs</b>      | left ventricle end-systolic diameter                 |
| <b>M</b>        | million                                              |
| <b>MACS</b>     | magnetic activated cell sorting                      |
| <b>MCS</b>      | multiple cloning site                                |
| <b>MFI</b>      | median fluorescence intensity                        |
| <b>MHC</b>      | myosin heavy chain                                   |
| <b>MI</b>       | myocardial infarction                                |
| <b>minithx</b>  | mini-invasive thoracotomy                            |
| <b>miR</b>      | microRNA                                             |
| <b>MMP</b>      | matrix metalloproteinase                             |
| <b>MNC</b>      | mononuclear cell                                     |
| <b>MOI</b>      | multiplicity of infection                            |
| <b>MRI</b>      | magnetic resonance imaging                           |
| <b>MSC</b>      | mesenchymal stem cell                                |
| <b>MSCM</b>     | mesenchymal stem cell medium                         |
| <b>mTLD</b>     | mammalian tolloid                                    |
| <b>N</b>        | equivalence per litre (Eq/L)                         |
| <b>NO</b>       | nitric oxide                                         |

|                 |                                                        |
|-----------------|--------------------------------------------------------|
| <b>NOC4L</b>    | nucleolar complex associated 4 homolog                 |
| <b>NRE</b>      | negative regulatory element                            |
| <b>NTC</b>      | non-targeting control mimic                            |
| <b>NYHA</b>     | New York Heart Association                             |
| <b>OC</b>       | outgrowth cell                                         |
| <b>OD</b>       | optical density                                        |
| <b>OFT</b>      | outflow tract                                          |
| <b>P(1)</b>     | passage (1)                                            |
| <b>p(hVEGF)</b> | plasmid expressing gene of (human VEGF)                |
| <b>PBS</b>      | phosphate buffered saline                              |
| <b>PC</b>       | percutaneous                                           |
| <b>PCA</b>      | principal component analysis                           |
| <b>PCI</b>      | percutaneous intervention                              |
| <b>PCP</b>      | procollagen C endo-peptidase                           |
| <b>PDL</b>      | poly-D-lysine                                          |
| <b>PE</b>       | phycoerythrin                                          |
| <b>PerCP</b>    | peridinin chlorophyll protein                          |
| <b>PIGF</b>     | placenta derived growth factor                         |
| <b>QoF</b>      | quality of life                                        |
| <b>qRT-PCR</b>  | quantitative real-time polymerase chain reactions      |
| <b>r(hVEGF)</b> | recombinant (human vascular endothelial growth factor) |
| <b>RA</b>       | right atrium                                           |
| <b>RCA</b>      | right coronary artery                                  |
| <b>RCT</b>      | randomised controlled trial                            |



|                                |                                                    |
|--------------------------------|----------------------------------------------------|
| <b>RR</b>                      | response ratio                                     |
| <b>RRR</b>                     | relative responsive ratio                          |
| <b>RT</b>                      | reverse transcription                              |
| <b>RTL</b>                     | relative tubule length                             |
| <b>SD</b>                      | standard deviation                                 |
| <b>SE</b>                      | standard error                                     |
| <b>sFFv</b>                    | spleen focus-forming virus                         |
| <b>SM</b>                      | skeletal muscle/myoblast                           |
| <b>STAT1</b>                   | signal transducer and activator of transcription 1 |
| <b>T150</b>                    | 150cm <sup>2</sup> cell culture flask              |
| <b>TGF-<math>\beta</math></b>  | transforming growth factor beta                    |
| <b>TLR</b>                     | toll-like receptor                                 |
| <b>TNF-<math>\alpha</math></b> | tumour necrosis factor alpha                       |
| <b>TnI</b>                     | troponin I                                         |
| <b>TTE</b>                     | transthoracic echocardiography                     |
| <b>TTL</b>                     | total tubule length                                |
| <b>UNG</b>                     | uracil N-glycosylase                               |
| <b>UTP</b>                     | uridine triphosphate                               |
| <b>V</b>                       | ventricular (septum); volume                       |
| <b>VEGF</b>                    | vascular endothelial growth factor                 |
| <b>VEGFR</b>                   | vascular endothelial growth factor receptor        |
| <b>Vs.</b>                     | versus                                             |

## **Chapter 1   Introduction**

## **1.1 Ischaemic heart disease**

Ischaemic heart disease (IHD) is characterised by an inadequate supply of blood to the myocardium resulting in an imbalance between myocardial oxygen supply and demand. This causes clinical manifestations such as sudden death, myocardial infarction, angina, heart failure, arrhythmia or asymptomatic survival. The major cause of IHD is atherosclerosis, leading to low perfusion of the region of the myocardium supplied by the culprit artery [1].

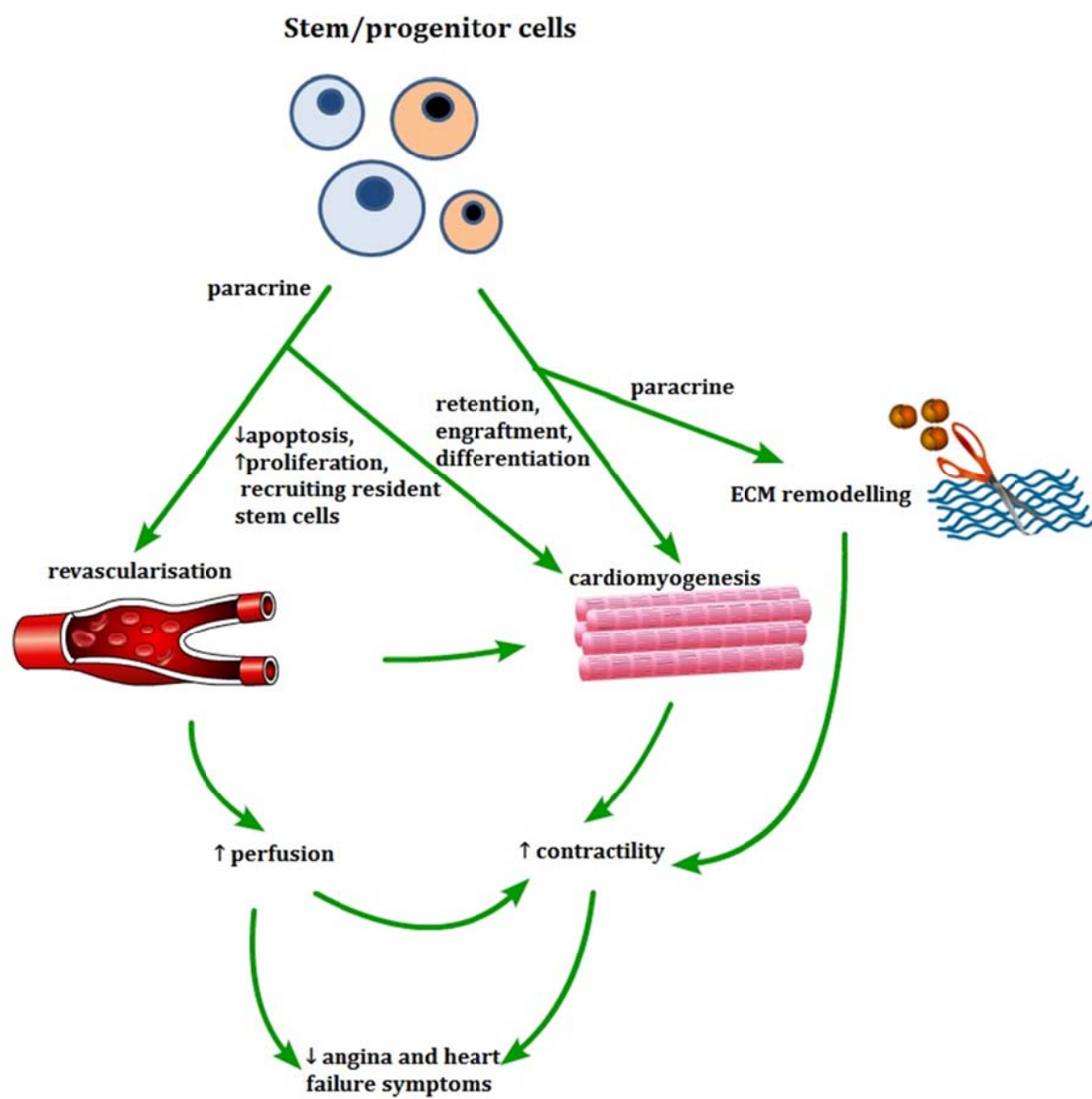
Coronary artery disease (CAD) remains the top ‘medical’ killer in the world. In the United Kingdom alone, a total of around 80,000 deaths were caused by CAD in 2010, with newly diagnosed cases of myocardial infarction (MI) and angina being estimated to be over 123,000 cases annually. The total prevalence of CAD in the UK was estimated at nearly 3.5 million by 2010. [2], [3] In addition to CAD, pathologies such as aortic stenosis and hypertrophic cardiomyopathy can also result in ischaemic damage to the myocardium in due course [4 – 8].

During the past decade, treatment for IHD has generated improved regimes of medical therapy, which combines anti-platelets agents,  $\beta$ -blockers, statins, ACE-inhibitors etc., offering the optimal prevention of coronary artery disease and anti-remodelling effects. In addition, percutaneous coronary intervention (PCI) and highly advanced surgical technology provide substantial gains for patients surviving ischaemic episodes by focusing on revascularising the ischaemic territory, whilst decreasing the subsequent effect of both acute haemodynamic instability and chronic unfavourable LV re-modelling [9 – 12]. However, even treatments meeting today’s highest standard of medical care are unable to halt the progression of IHD in many patients to chronic heart failure [13].

## **1.2 Stem cell therapy: mechanisms, cell types and limitations**

Stem cell therapy may give an opportunity to improve revascularization, to restore impaired left ventricular (LV) function and delay the progression of disease [14]. The mechanism of how stem cells work is not fully understood and various theories have been proposed. Stem cells transplanted into the heart may proliferate and engraft into the damaged myocardium. The engrafted cells may fuse with local cardiomyocytes or enhance their survival, release paracrine factors which recruit resident cardiac stem cells to differentiate into new vasculature and cardiomyocytes or may modify the extracellular matrix and reduce proliferation of the granulation tissue [15–23]. Therefore, stem cell therapy aims to attenuate or reverse adverse LV remodelling following MI and to improve LV function (Figure1.1).

In rodents, stem cells can induce blood vessel formation through secretion of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), interleukins and metalloproteinases [24], resulting in an increased capillary density in the infarct border zone [25]. Such a pro-angiogenic effect has also been confirmed in large animal models of MI [16]. Although in the last decade a plethora of studies has been reported in this field, only stem cell types tested in clinical trials for IHD are reviewed in this Thesis. These stem cells derived from specific tissue sources include skeletal myoblasts, bone marrow (BM)- and blood-derived stem cells, and resident cardiac stem cells.



**Figure 1.1** Goals and mechanisms of stem cell therapy for myocardial regeneration.

### **1.2.1 Skeletal myoblasts (SM)**

Early preclinical evidence showed that SM could repair the damaged myocardium [26], but their clinical efficacy is debatable [27]. Initially, several non-randomised controlled safety studies generated promising results when SM were administered in combination with coronary artery bypass grafting (CABG) in patients suffering from congestive heart failure (CHF). The results showed an improved global and regional contractility and/or myocardial viability in the infarct zone. Long-term follow-up (up to 4 years) also showed improvement in left ventricular ejection fraction (LVEF) associated with an improvement in myocardial viability, and no adverse effects of the treatment [28]. The only safety concern has been an increased risk of arrhythmias as some of the trials reported episodes of sustained ventricular tachycardia following cell transplantation [29], [30]. However, the multicentre, randomised-controlled, double-blind Phase II MAGIC (Myoblasts Autologous Grafting Ischaemic Cardiomyopathy) trial (Table 1.1), using two doses of autologous *ex vivo* expanded SM, found that global and regional LV functions were not significantly changed after 6 months and significant reduction in LV volumes were only observed in the high-dose treated group [31]. Although no significant increase in arrhythmic events was reported in the MAGIC trial, arrhythmias seemed to be clustered in the early post-operative period in the SM-treated groups [31]. Recently, another Phase II trial, the SEISMIC trial (Table 1), reported that injection of SM in patients with CHF is safe but no significant improvement in exercise tolerance or LVEF was observed in the SM-treated group compared with the control group [32]. There was no evidence for SM-induced sudden death in the above mentioned randomised controlled trials (RCTs), however SM are still considered pro-arrhythmogenic and thus the development of SM-based cell therapy for heart disease has been halted.

**Table 1.1 Major completed RCTs of the cell therapies**

| Origin | Cell type | Trial                          | Phase | Administration | Patient cohort | Patient numbers | Main effect                                                                    |
|--------|-----------|--------------------------------|-------|----------------|----------------|-----------------|--------------------------------------------------------------------------------|
| SM     | myoblast  | MAGIC [31]                     | 2     | Imc            | ICM for CABG   | 97              | significant reduction in LV volumes in 800M cell group but prone to arrhythmia |
|        |           | SEISMIC [32]                   | 2     | Imc            | ICM no option  | 40              | Safe. No significant improvement in exercise tolerance or LVEF                 |
| BM     | MNC       | Repair-AMI [33], [34]          | 2     | IC             | AMI            | 204             | improved LVEF, decreased mortality                                             |
|        |           | ASTAMI [35]                    | 2     | IC             | AMI            | 97              | negative                                                                       |
|        |           | Janssens <i>et al.</i> [36]    | 2     | IC             | AMI            | 67              | reduced scar size                                                              |
|        |           | BOOST [37–39]                  | 2     | IC             | AMI            | 60              | improved EF in short term with a single dose                                   |
|        |           | TIME/Late TIME [40], [41]      | 2     | IC             | AMI            | 120             | negative                                                                       |
|        |           | Cao <i>et al.</i> [42]         | 1/2   | IC             | AMI            | 86              | safe, improved EF                                                              |
|        |           | FINCELL [43]                   | 1/2   | IC             | AMI            | 80              | safe, improved EF                                                              |
|        |           | HEBE [44]                      | 2     | IC             | AMI            | 200             | negative                                                                       |
|        |           | BONAMI [45]                    | 2     | IC             | AMI            | 101             | improved LV viability                                                          |
|        |           | Hu <i>et al.</i> [46]          | 2     | Imc            | ICM CABG       | 60              | improved LV function and exercise tolerance                                    |
|        |           | Perin <i>et al.</i> [47]       | 2     | Imc pc         | ICM no option  | 92              | negative                                                                       |
|        |           | Pokushalov <i>et al.</i> [48]  | 2     | Imc pc         | ICM no option  | 109             | improved mortality, LV function, HF symptoms at 12-month,                      |
|        | CD34+     | Patel <i>et al.</i> [49]       | 1/2   | Imc            | ICM CABG       | 20              | further improved LV function                                                   |
|        |           | Regent [50]                    | 2     | IC             | AMI            | 200             | negative                                                                       |
|        |           | Losordo <i>et al.</i> [51]     | 2     | Imc pc         | angina         | 167             | improved exercise time, reduced frequency of chest pain                        |
|        | ALDH+     | Perin <i>et al.</i> [52]       | 1/2   | Imc pc         | ICM no option  | 20              | improved LVESV, potentially improved perfusion and oxygen consumption          |
|        |           |                                |       |                |                |                 |                                                                                |
|        | MSC       | Chen <i>et al.</i> , 2004 [53] | 1/2   | IC             | AMI            | 69              | improved LV function                                                           |
|        |           | Chen <i>et al.</i> , 2006 [54] | 1/2   | IC             | ICM for PCI    | 45              | improved LV function, exercise time, symptoms and myocardial perfusion         |
|        |           | Prochymal allogeneic [55]      | 1/2   | IV             | AMI            | 53              | improved EF                                                                    |
|        |           | POSEIDON [56]                  | 1/2   | Imc pc         | ICM no option  | 30              | autologous cells improved exercise time, allogeneic cells reduced LVESV        |
| Fat    | MSC       | APOLLO [57]                    | 1     | IC             | AMI            | 13              | improved perfusion and reduced scar size                                       |
|        |           | PRECISE [58]                   | 1     | IC             | ICM no option  | 27              | Safe. Improved oxygen consumption and reduced scar size.                       |
| Heart  | CDC       | CADUSEUS [59]                  | 1     | IC             | Recent MI      | 25              | reduced scar size                                                              |
|        | C-kit+    | SCIPIO [60]                    | 1     | IC             | ICM no option  | 16              | improved LV function and reduced scar size                                     |

SM= skeletal muscle, BM= bone marrow, MNC= mononuclear cells, ALDH=alcohol dehydrogenase, MSC= mesenchymal stem cells, Imc= intramyocardial, pc= percutaneous, IC=intracoronary, IV= intravenous.

### ***1.2.2 Bone marrow mononuclear cells (BMNC)***

Unselected BMNC are clearly the most investigated cell-based therapy for heart disease in clinical studies, with the longest follow-up lasting up to 5 years [61–65]. As an attractive novel treatment for acute and chronic MI, BMNC are relatively easy to harvest, easy to process in a short timeframe from whole bone marrow aspirates using standardised techniques (e.g. density gradient centrifugation and cell sorting) and usually yield large quantities of cells. This makes them extremely amenable to treat acute MI patients.

In this patient cohort, BMNC have a beneficial but moderate effect on heart function, measured by LVEF [61–65]. Not surprisingly, following the expectations raised by the early-phase clinical studies, several RCTs have generated mixed result (Table 1.1). The largest trial, the REPAIR-AMI trial, showed an improvement of global LVEF in the treatment group without significant changes of left ventricular (LV) end-diastolic volumes 4 to 6 months following cell transplantation [33]. In addition, decreased mortality was observed in the treatment group compared with the control group at 2 years follow-up [34]. In contrast, in other landmark studies, BMNC have not shown the same beneficial effect in this patient cohort. Negative to mixed to transient effects are seen in the ASTAMI [35], the Belgium [36] and the BOOST trials [37], [38] respectively.

Although the definite reasons for these mixed results remain elusive, differences in cell- and patient-related factors are most likely to be the cause. Differences in study protocol and design, including time from reperfusion to cell injection, route of delivery, cell type, cell dose, and cell isolation techniques are all factors that could influence treatment outcome [66]. Importantly, cell function is known to be affected by cardiovascular risk factors [67]. In addition, the methods used for assessing outcomes (e.g. echocardiography, magnetic



resonance imaging (MRI), radionuclide ventriculography, etc.) and the relevant primary outcomes should be carefully selected as each of them have their own limitations [68]. Whereas BMNCs were infused within the first 7 days post-MI in most trials, in the Belgium trial, cells were injected within 24h of MI [36]. In the ASTAMI trial, MRI was not performed until 2-3 weeks after cell transfer, whereas echocardiographic imaging was performed at baseline. Further, cells were prepared differently from the REPAIR-AMI trial [35]. Subsequently follow-up data of the REPAIR-AMI and BOOST trials have become available. In the REPAIR-AMI, the improvement of LV function was sustained after 12 months and was associated with a significant reduction in major adverse cardiovascular events post-AMI in 2 years follow-up [34]. However, in the BOOST trial, the initial difference in LVEF between trial arms was no longer significant after 18 months [38]. Recently, 5-year follow-up data from the BOOST trial did not show a sustained benefit on systolic and diastolic LV function after a single BMNC infusion [39]. Interestingly, subgroup analyses suggested that patients with a more severely impaired LV function may have a benefit from BMNC administration, whereas patients with preserved LV function post-MI may not [39].

During the course of this thesis more clinical data have become available. RCTs such as the HEBE [69], BONAMI [45], FINCELL [43], and TIME [40], [41] studies (Table 1.1) show no significant effect on heart function or contractility between treated and non-treated patients. Recent systematic reviews and meta-analyses, which also included smaller trials, have suggested that BMNC improves LVEF by 3-5% [61], [63], [70], [71]; however, there is no significant reduction on the risk of mortality in AMI patients treated with BMNC compared with control.

Fewer data from RCTs are available in patients with chronic MI and heart failure (Table 1). Intracoronary delivery of BMNC during CABG in patients with chronic MI and HF resulted in significant changes in LVEF and exercise tolerance in favour of the treatment [46]. HF patients, receiving optimal medical treatment and with no option of revascularization have been treated in two other trials. Following the promising results of the Phase I trial, Perin *et al.*, designed a Phase II trial. Surprisingly, no significant improvement in LVESV or maximal oxygen consumption was observed in treated patients compared to controls [47], [48]. In another trial, intramyocardial delivery of BMNC to patients with ischaemic HF improved HF symptoms, and LV function and even improved survival of these patients significantly [47], [48]. In Pokushalov's study, patients suffered from a more severe stage of ischaemic heart failure (NYHA class III-IV) with lower rates of survival compared with those in Perin's study (NYHA class II-IV), and this may be a determinant of the decreased mortality observed in this patient cohort.

Notably, BMNC are a heterogeneous cell population that contains haematopoietic stem and progenitor cells (HSC/HPC) and endothelial progenitor cells (around 2-4%), mesenchymal progenitor cells (MSC) (0.001 to 0.01% of the nucleated cells) and committed progenitor cells and their differentiated progeny [72]. As a consequence, it is difficult to ascertain which cell types in the whole population are more potent or responsible for the therapeutic effect. This has led to the development of clinical trials which use selected cell populations such as HSC or MSC as treatment for IHD.

### ***1.2.3 Haematopoietic stem cells (HSC)***

Orlic *et al.* showed that Lin-/c-kit<sup>+</sup> HSC mobilised from the bone marrow and injected into infarcted myocardium were able to improve heart function in mice and generate new

cardiomyocytes [18]. Human HSC, identified by CD133, CD34 or aldehyde dehydrogenase (ALDH) markers have been tested in clinical trial of IHD. CD133+ or CD34+ HSC can be enriched from mononuclear cells, either from the bone marrow or mobilised peripheral blood, or by immunomagnetic cell separation procedures [49], [73]. ALDH+ cells are isolated from the bone marrow by cell sorting [52].

The first clinical trial with HSC injected CD133+ cells into the infarct border zone of patients undergoing CABG [74]. Promising results from this trial, with no adverse events and improved LV function led to the development of larger RCTs (Table 1.1). More recently, the REGENT trial compared CD34+ CXCR4+ HSC cells to unfractionated BMNC or control in patients who suffered from acute MI and received PCI as a primary intervention (Table 1). However, no significant improvement in LVEF was observed between trial arms [50].

Patients undergoing off-pump CABG with LV dysfunction (LVEF <35%) were treated with bone marrow CD34+ HSC in a small RCT and showed a significant improvement in LVEF compared with control patients [49], [73](Table 1.1). Recently, a newly identified HSC population expressing the alcohol dehydrogenase (ALDH) enzyme has been tested in patients with chronic ischaemia and unsuitable for conventional revascularisation. Perin *et al.* provided preliminary evidence that treatment with ALDH+ cells is safe and may improve perfusion and offer functional benefits to those patients [52].

CD34+ HSC have been tested in a Phase I trial in patient with refractory angina and no option of revascularisation. In this study, patients received G-CSF to mobilise bone marrow cells into the circulation and CD34+ cells were enriched from peripheral blood mononuclear cells prior to injecting then into the myocardium using electromechanical mapping (Table

1.1). Unlike in AMI trials, the primary outcome measured by this trial was a change in angina frequency [51]. Administration of CD34+ cells resulted in a significant reduction of angina frequency (number of angina episodes per week). The results were confirmed in a dose escalation Phase II trial [51]. The first Phase III clinical trial with CD34+ cells as treatment for patients with refractory angina is currently on-going [75].

#### ***1.2.4 Mesenchymal stem/stromal cells (MSC)***

MSC constitute another potential option for stem/progenitor cell-based therapy. MSC, identified by the surface marker expression of CD90, CD105, CD44 and CD73 and the lack of haematopoietic markers such as CD45, CD34, CD14, CD19, and HLA-DR, are rare in the bone marrow (0.002% to 0.02% of the BMNC [76]), but due to their immunomodulatory properties [77], they have attracted the pharmaceutical industry as they can be generated as an ‘off-the-shelf’ product for allogeneic cell transplantation. They have also been identified in other tissues such as adipose tissue.

Autologous and allogeneic MSC transplantation is currently under investigation for IHD among other conditions; however, clinical data are scarce. The first RCT using autologous BM-derived MSC treated patients who had suffered AMI and underwent PCI. The MSC treatment proved to be safe and significantly improved LV function [53]. Autologous BM MSC also improved exercise tolerance, NYHA class and LVEF significantly in patients with severe ischaemic cardiomyopathy [54]. A safety double blinded Phase I trial with intravenous administration of allogeneic BM MSC to treat patients with acute MI showed a significant improvement in LVEF compared with controls [55]. Following this the authors compared autologous and allogeneic MSC, but no placebo, in patients with ischaemic cardiomyopathy [56]. In the Phase I/II POSEIDON trial, three doses of MSC were injected into patients.

Autologous, but not allogeneic MSC, improved exercise capacity and quality of life (QoL) significantly. None of the injected cells improved maximal oxygen consumption or LVEF. In contrast, allogeneic MSC significantly reduced LV end systolic volume (ESV). In this early study, no immunological reaction to MSC therapy was observed. [56]

In addition, the APOLLO and PRECISE trials showed that freshly isolated adipose tissue-derived MSCs is a safe treatment in patients suffering from AMI and chronic MI. MSC infusion resulted in a significant improvement of the perfusion defect and a 50% reduction of myocardial scar formation in AMI patients but there was no improvement in LV function after 6 months [57]. Similarly in patients with no option of revascularization cell transplantation showed a moderate decreased in scar size at 6-month and a significant increase in peak oxygen consumption at 18-months, but no improvement of LV function was reported [58].

#### ***1.2.5 Cardiac stem/progenitor cells (CSC)***

Until recently, the heart has been considered a terminally differentiated organ with no capability for self-repair. However, an elegant study by Bergmann *et al.* showed the first evidence of human cardiomyocyte renewal over an adult life time. According to this study, the rate of cardiomyocyte turn-over was estimated between 1% per year in young adults to 0.5% in the elderly [78]. More recently, evidence showed that experimental injury could stimulate cardiomyogenesis in mice [79], [80]. Additionally, the human heart has a cardiomyocyte renewal rate of approximately 2% in 20 year-old individuals and this declines with age to 0.04% in the elderly [79]. These data suggest that the heart contains a population of stem/progenitor cells which may be involved in steady-state tissue repair and regeneration that occurs under normal and pathological conditions.

In post-natal hearts, tissue-resident cardiac stem and progenitor cells (CSC) were first found in mice [81–83]. Endogenous cardiac progenitor cells have also been identified in humans and seem to be activated in patients with ischaemic cardiomyopathy [84]. These tissue-specific progenitors are characterised by the expression of c-kit<sup>+</sup> [85], Sca-1<sup>+</sup> [86] or Islet-1<sup>+</sup> [87] cell surface markers, the cardiac side population (SP) cells [81] or the formation of clusters called ‘cardiospheres’ [88–90]. Cardiosphere-derived cells (CDCs) have been isolated from human tissue biopsies independently of biopsy location [91], age [92], sex, type and duration of the disease [93]. In animal models, they have been reported to engraft in the ischaemic heart and improve left ventricular function [89], [94]. Although early studies emphasised the cardiogenic potential of CDCs and the presence of c-kit<sup>+</sup> cells in the mixed population [95], during the course of this thesis a recent study has shown that cardiac-derived progenitors also have a paracrine/autocrine effect on cardiac function by increasing re-vascularisation [96].

The first clinical trials using CSC as treatment for MI, the CADUCEUS and SCPIO trials, have now been completed and have demonstrated that autologous transplantation of CSC is safe [59], [60]. In the CADUCEUS trial, patients who had suffered from a recent MI received CDCs or placebo. The results showed a significant improvement in myocardial viability and reduced scar size 4-6 months following cell transplantation. Interestingly, the reduction in infarct size was not accompanied by an improvement in heart function [59], [60]. However, the reduction in infarct size correlated with an increase in viable myocardium in the peri-infarct areas. No cardiomyocyte hypertrophy was observed in treated patients in the infarct border zone, suggesting that the myocardium had been replaced in this area by new cardiomyocytes. In animal models of MI, CDC transplantation increases the number and decreases the size of cardiomyocytes in the infarct border zone, demonstrating evidence for

myocardial regeneration [60], [97]. In the SCIPIO trial, end-stage HF patients with LVEF < 40% who did not respond to bypass surgery received c-kit<sup>+</sup> CSCs. The results confirmed a significant reduction in infarct size in the treated group compared to the control group. In addition, there was a significant increase in LVEF [60]. Although not evident from the first clinical trial, preclinical data obtained with c-kit<sup>+</sup> CSCs demonstrate functional cardiac regeneration and repair following c-kit<sup>+</sup> CSCs transplantation [80].

### ***1.2.6 Limitations of cell therapies***

The divergent results of these trials are not surprising, as evidenced by their clinical heterogeneity: different cohort of patients, cell type, cell dose, method of sampling and administration. Experimentally, in a head to head comparison with the c-kit<sup>+</sup> cell fraction from CDC, bone marrow mesenchymal/stromal cells (BMSC), adipose tissue MSC and BMMNC, unsorted CDC have shown a greater therapeutic ability in a rodent model of MI, where it was reported that CDC improved cardiac function, possessing greater myogenic and angiogenic differentiation potentials than other progenitor cells [98]. Their mode of action has been attributed to paracrine mechanisms. Whilst promising, current cell therapies are facing challenges such as understanding the clinical conditions, the function of the relevant cell type(s) in health and disease, and what influences their therapeutic performance. Risk factors associated with ischaemic heart disease are known to affect not only the numbers, but the mobilisation, homing and engraftment of cells, both resident in the bone marrow and mobilised into the peripheral circulation [67], [99].

## **1.3 The role of revascularisation and angiogenesis in heart repair**

Advanced revascularization technologies such as CABG and PCI have contributed to a remarkable decrease in mortality in IHD. Following primary revascularisation, up to 90% of

patients suffering from an occluded coronary artery will achieve almost immediate patency. Hence, the five-year cardiac event-free survival expectancy of CAD patients receiving primary revascularisation has increased considerably from ~30% to >85% [2].

However, it is estimated that a quarter to a third of patients with CAD undergoing primary revascularisation would experience inadequate or slow myocardial reperfusion even after treatment. This phenomenon which was first studied in a canine model of ischemia-reperfusion injury by Kloner *et al.* in 1974, was named the ‘no-reflow’ phenomenon, and is caused by dysfunction of the microcirculation in the myocardium. Patients with no-reflow are compromised, even with the optimal effect of the primary revascularisation, and face higher risks of developing ventricular remodelling and early death [100]. Currently, no alternative or superior treatment option is available for this patient cohort. The reasons why these patients fail to meet a satisfactory outcome from revascularisation include (i) the diffuse nature of multiple diseased coronary arteries which are ungraftable and (ii) the difficulty in determining the latent culprit lesions to bypass [101 – 105].

In addition, even in patients who successfully undergo primary standard CABG, re-stenosis of the vein grafts is commonly seen after several years, followed by a recurrence of symptoms and decreased survival. They generally require repeated revascularisation, regardless of the normal patency of the grafted conduit [106], [107]. Therefore, these patients continue to experience residual myocardial ischemia despite optimal therapy. As it is important to achieve appropriate myocardial collateral blood flow and perfusion of the myocardium, therapeutic angiogenesis, aiming at restoring the microcirculation in the ischaemic myocardium has emerged as a therapeutic approach for ischaemic heart disease.



Blood vessel formation is induced by ischaemia and is characterised by (i) the sprouting of new blood vessels from existing ones (angiogenesis), (ii) the migration, differentiation and incorporation of endothelial progenitors into the damaged vessels (vasculogenesis) or (iii) the increase in the size of the lumen of pre-existing arterioles by remodelling and growth (arteriogenesis) [108]. The concept of therapeutic angiogenesis was coined over fifteen years ago with the aim of enhancing blood vessel formation by using pro-angiogenic factors. The first promising results of therapeutic angiogenesis in humans were reported in severely symptomatic patients with critical limb ischemia where VEGF was administered directly into the skeletal muscle and expressed constitutively [109]. This followed experimental proof of concept in rabbits with induced unilateral limb ischaemia which received intra-arterial infusion of an acidic FGF encoding vector [110]. Soon after, clinical trials were also extended to patients with advanced symptomatic CAD not amenable to standard revascularization strategies who received either basic FGF during CABG [111] or a VEGF-expressing plasmid via a mini left anterior thoracotomy [112].

By contrast, anti-angiogenic cancer therapies have resulted in cardiac toxicity. Anti-angiogenic drugs such as bevacizumab (a specific VEGF blocking antibody), sorafenib and sunitinib (both tyrosine kinase inhibitors) have been used against tumour angiogenesis [113 – 115]. Although with different specificity and molecular targets, all the above approved drugs have been reported to be associated with cardiovascular side effects such as LVEF reduction, increased incidence of heart failure, hypertension and myocardial ischaemia [116].

## **1.4 Angiogenic growth factors**

Numerous growth factors with the potential to promote angiogenesis (e.g. hepatocyte growth factor (HGF) [117], or insulin-like growth factor (IGF) [118] ) are expressed in the ischemic

myocardium. However, the VEGF and FGF families are the best studied growth factors in angiogenesis following MI.

#### ***1.4.1 VEGF family***

VEGF family members include 5 variants of VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF in human. They have high affinity with their receptors, VEGFR1 and VEGFR2, present on endothelial cells upon stimulation of angiogenesis. Receptor-ligand interactions mediate the production of nitric oxide (NO), which is tightly regulated through signalling cascades in the endothelial cells [119], [120]. The role of VEGF-B and PlGF in angiogenesis remains controversial and their therapeutic potential has not been assessed in clinical trials [121]. Therefore, the following discussion is focused on VEGF-A, the member of the family that has been investigated in large clinical trials. VEGF-A gene expression is induced by hypoxia [122]. *In vivo*, VEGF-A production is significantly up-regulated by ischemia in pigs and rats [123], [124], suggesting that VEGF-A is a likely mediator in the natural process of ischaemia-induced myocardial neo-vascularisation. Animal experiments showed that VEGF treatment was effective in treating acute and chronic MI. The administration of adenoviral vectors (Ad)-encoded VEGF gene (AdVEGF) could improve capillary density and LV function, while reducing collagen deposition in AMI rat model [125]. Pig models of chronic IHD showed that the injection of an adenovirus coded with the VEGF<sub>121</sub> gene into stunned myocardium increased collateral vessel flow and improved cardiac function [126]. Two different isoforms of VEGF-A have been tested in clinical trials VEGF-A<sub>165</sub> (containing 165 amino acids) and VEGF-A<sub>121</sub> (a shorter form of VEGF-A consisting of 121 amino acids). Early trials and promising results led to the development of RCTs (Table 1.2) where recombinant protein, plasmid DNA or adenoviral vectors expressing the angiogenic factors were used as treatment.

In the VIVA trial, patients with stable IHD were randomised to receive placebo or recombinant human VEGF (rhVEGF-A<sub>165</sub>) protein followed by an intravenous infusion [127]. There were no significant differences in exercise tolerance, QoL and myocardial perfusion at 60 days between the treated and placebo groups. At 120 days, high dose of rhVEGF-A<sub>165</sub> improved angina class only and, hence, the trial was terminated prematurely. No other large trial with recombinant protein has been performed. In the EUROINJECT-ONE [128] and NORTHERN [129] RCTs, patients with severe stable IHD and no other treatment option received plasmid DNA expressing VEGF<sub>165</sub> (phVEGF-A<sub>165</sub>). None of the trials found significant improvement in myocardial perfusion in the treated groups compared with the placebo groups despite some improvement in wall motion and LV function in the EUROINJECT-ONE trial [128].

The REVASC study enrolled patients with intractable angina and no option of revascularization to receive VEGF-A<sub>121</sub> expressed in an adenoviral vector (AdVEGF-A<sub>121</sub>). The treatment was delivered by direct intramyocardial injections via a minithoracotomy and the control group received maximal medical therapy and no placebo. There were significant improvements in exercise tolerance and QoL in the AdVEGF-A<sub>121</sub> treated patients compared to the controls [130].

The NOVA trial was a double-blinded, placebo-controlled, multicentre study investigating the safety and efficacy of BIOBYPASS (AdGVVEGF<sub>121</sub>.10NH) gene therapy in patients with refractory advanced CAD. An injection of AdGVVEGF<sub>121</sub> did not improve exercise capacity or myocardial perfusion in a 52-week follow-up and the study was terminated prematurely [131].

**Table 1.2 RCTs of the therapies with pro-angiogenic factors**

| Family    | Factor                                | Trial                     | Phase | Administration                              | ICM Patient cohort  | Patient numbers               | Main effect                                                                                            |
|-----------|---------------------------------------|---------------------------|-------|---------------------------------------------|---------------------|-------------------------------|--------------------------------------------------------------------------------------------------------|
| VEGF      | VEGF-A165                             | VIVA [127]                | 2     | IC and IV                                   | CCS II–III          | 178                           | Safe. High-dose improved CCS and trend in exercise time and angina frequency.                          |
|           | AdVEGF165 or plasmid/liposome VEGF165 | KAT [132]                 | 2     | IC                                          | CCS II–III for PCI  | 103                           | Safe. Improved myocardial perfusion at 6 months.                                                       |
|           | AdVEGF121                             | REVASC [130]              | 2     | Imc minithx                                 | CCS II–IV           | 67                            | improved time to 1 mm ST-segment depression on ECG at 26 weeks                                         |
|           | AdVEGF121                             | NOVA [131]                | 1/2   | Imc pc                                      | CCS II–IV           | 17/129(premature termination) | Safe. Negative effect.                                                                                 |
|           | VEGF165 plasmid                       | EUROINJECT-ONE [128]      | 2/3   | Imc pc                                      | CCS III–IV          | 74                            | negative                                                                                               |
|           | VEGF165 plasmid                       | NORTHERN[129]             | 2/3   | Imc pc                                      | CCS III–IV          | 120                           | negative                                                                                               |
| FGF       | rFGF-2                                | Laham <i>et al.</i> [133] | 1/2   | Epicardial implantation in ungraftable area | CCS III–IV for CABG | 24                            | Safe. Beneficial for angina symptoms and improved myocardial perfusion at 3 years with a high dose.    |
|           | rFGF-2                                | FIRST [134]               | 2     | IC                                          | CCS III–IV          | 337                           | Safe. Trend towards 3-month improvement in angina. No effect on exercise time or myocardial perfusion. |
|           | Ad5-FGF4                              | AGENT [135]               | 1/2   | IC                                          | CCS II–III          | 79                            | Safe. Improved exercise time.                                                                          |
|           | Ad5-FGF4                              | AGENT-2 [136]             | 2     | IC                                          | CCS II–IV           | 52                            | improved myocardial perfusion.                                                                         |
|           | Ad5-FGF4                              | AGENT-3 [137]             | 3     | IC                                          | CCS II–IV           | 416                           | negative with a low dose                                                                               |
|           | Ad5-FGF4                              | AGENT-4 [137]             | 3     | IC                                          | CCS II–IV           | 116                           | improved exercise tolerance and time in female with a high dose                                        |
| VEGF+ FGF | Naked VEGF A165/ FGF-2 plasmid        | VIF-CAD [138]             | 2     | Imc percutaneous                            | CCS III–IV          | 52                            | negative                                                                                               |

R= recombinant, Ad(5)= adenovirus (type 5), IC= intracoronary, IV= intravenous, Imc= intramyocardial, minithx= mini-invasive thoracotomy, CCS= Canadian Cardiovascular Society angina score.

Finally, the 8-year follow up of the Kuopio angiogenesis trial (KAT) showed that VEGF-A<sub>165</sub> expressed transiently in either an adenoviral vector or a plasmid was safe, did not increase the risk of mortality and it was well tolerated.[132], [139] However, no significant improvements were observed compared to controls. In the AdVEGF-A<sub>165</sub> group, myocardial perfusion was improved compared to the plasmid VEGF-A<sub>165</sub> group at six months [124].

#### ***1.4.2 FGF family***

The FGF family comprises several members with known ability to promote angiogenesis in pre-clinical studies [119]. The existence of 22 ligands and 4 receptors make this system highly complex. The best studied members of the family are FGF-1 and FGF-2 and together with them, FGF-4 has been tested in clinical trials to improve angiogenesis post-MI.

The safety and feasibility of recombinant FGF for therapeutic angiogenesis has been evaluated in patients with chronic myocardial ischemia during CABG [133] and in subjects with stable angina pectoris secondary to CAD [134]. Whilst the Phase I trial [133] delivering a single injection of rhFGF-2 showed signs of therapeutic efficacy, the larger Phase II trial [134] did not revealed any significant improvement in exercise tolerance or ischaemic areas in the treated patients. A significant improvement in angina class and angina frequency was only observed in the sicker patients [134].

Like VEGF, FGF gene transfer was based on adenoviral vectors. In the Angiogenic GENE Therapy (AGENT) double-blind RCT, the administration of 5 increasing doses of Ad5-FGF4 in patients with stable angina pectoris showed a trend towards clinical

improvement in exercise tolerance. Administration of Ad5-FGF4 was safe and well tolerated with no immediate adverse events [135]. The efficacy of Ad5-FGF4 therapy was further examined in the AGENT-2, AGENT-3 and AGENT-4 studies. The AGENT 2 study showed an encouraging trend for improved myocardial perfusion in patients treated with Ad5FGF-4 compared to the placebo group [136], whereas the results of the AGENT-3 and AGENT-4 trials showed no significant differences between treatment and placebo in angina symptoms, angina class or exercise tolerance. However, when stratified by gender, the data indicated that in women the placebo effect was small and the treatment had a significant effect [137].

#### ***1.4.3 Limitations of angiogenic therapies***

The results of these trials seem disappointing and fail to fulfil the early promise of therapeutic angiogenesis shown in preclinical studies. However, the lack of treatment effect might be due to inadequacies in the current approaches. For instance, the half-life of recombinant proteins delivered to the heart might be short and a major limitation of the present gene transfer approach is the transient expression of the desired gene and the low transduction efficiency of the target tissues. Future clinical trials may have to take into consideration that monotherapies do not seem to work and hence stimulating collateral arteriogenesis and vessel maturation as opposed to capillary formation only, might be more suitable.

### **1.5 Aims of this thesis**

At the outset of this study, mixed results from experimental and clinical studies using either angiogenic factors or bone marrow- and blood-derived stem cells were

available and cardiac progenitor cells seemed promising alternative cell therapies. The cardiogenic potential of human CDCs isolated from transplanted hearts had been well established and their therapeutic ability tested in preclinical animal models of MI. However, their vascular supportive function had not been fully explored and nothing was known about their variability in supporting vessel formation and maturation or how this was affected by disease state or cardiovascular risk factors.

Based on the preclinical data obtained from animal models and from the first studies with human CDCs obtained from transplanted hearts, we hypothesised that the heart of IHD patients also contained resident cardiac progenitor cells which could potentially be isolated from cardiac atrial biopsy and developed for clinical use. Like BMSC, the autologous cardiac progenitor cells would potentially improve heart function, mainly through a paracrine fashion to enhance local revascularisation around the ischaemic area. We also hypothesised that the therapeutic potency of the cardiac progenitor cells from different individuals might vary due to the diverse disease status and/or cardiovascular risk factors. In order to prove our hypotheses, the aim of this thesis was to isolate and characterise CDCs from patients suffering from IHD for their potential use in cell therapy applications, with focus on promoting revascularisation and functional heart tissue repair. Firstly, the phenotype and differentiation potential of CDC isolated from patients undergoing cardiac surgery were determined and found to resemble mesenchymal progenitors (Chapter 3). Secondly, a robust *in vitro* angiogenesis assay was established to assess their vascular supportive function. Variability in their pro-angiogenic phenotype was confirmed in a rodent model of MI and could be predicted by some cardiovascular risk factors (Chapter 4). Finally, in an attempt to gain a greater understanding of how their

vascular supportive function is regulated, transcriptional and post-transcriptional analysis of gene expression assessed the differences in pro-angiogenic phenotype observed (Chapter 5). The novel findings of this thesis are summarised and discussed in Chapter 6. Nevertheless, although quite interesting and of paramount clinical significance, the head-to-head comparison of autologous BMSC and resident cardiac progenitors was not the main objective of this thesis.



## **Chapter 2   Materials and methods**

## **2.1 Ethics, patient selection and consent**

Ethical approval (Ref 07/H0607/95) was obtained from the Mid/South Bucks Research Ethics Committee. Adult patients undergoing cardiac surgery conducted by Professor David P. Taggart were eligible to participate in this project, regardless of age, gender, heart function and operation type. Written consent (detailed in Appendix 4) was obtained on the day before surgery. Samples were anonymised for the duration of the research project. The risk factors of coronary artery disease including co-morbidities (diabetes mellitus, hypertension and hypercholesterolaemia), history of cigarette smoking and family history of cardiovascular disease were recorded by Prof Taggart's clinical team and retrieved from the patients' medical notes to conduct further statistical analyses. Ethical approval for the animal experiments was obtained accordingly from the Hospital La Fe, University of Valencia, Spain by Dr Sepulveda.

## **2.2 Cells, cell culture and plasmids**

### ***2.2.1 Human umbilical vein endothelial cells (HUVECs)***

HUVECs were kindly provided by Emma Pepperell, Stem Cell Research Laboratory, Oxford. Approximately  $1 \times 10^6$  cryopreserved HUVECs were cultured in 25mL of complete Endothelial Growth Medium-2 (EGM-2) containing 2% (v/v) Foetal Calf Serum (FCS) and 1% (v/v) Penicillin/Streptomycin (all from Lonza Biologics) and plated in a 75cm<sup>2</sup> cell culture flask with vent cap (Corning) (T75). Cells were maintained in a Nuaire DH autoflow incubator at 37°C with 21% O<sub>2</sub> and 5% CO<sub>2</sub>. Fresh medium was replaced twice a week until the cells reached approximately 90% confluency.

### ***2.2.2 Human bone marrow mesenchymal stem cells (BMSCs)***

BMSCs were purchased from Lonza Biologics and cultured in 25mL of complete Mesenchymal Stem Cell Medium (MSCM, Lonza Biologics) in a 150cm<sup>2</sup> cell culture flask with vent cap (Corning) (T150). Cells were maintained in culture at 37°C with 21% O<sub>2</sub> and 5% CO<sub>2</sub>. Fresh media was replaced twice a week until the cells reached approximately >90% confluency.

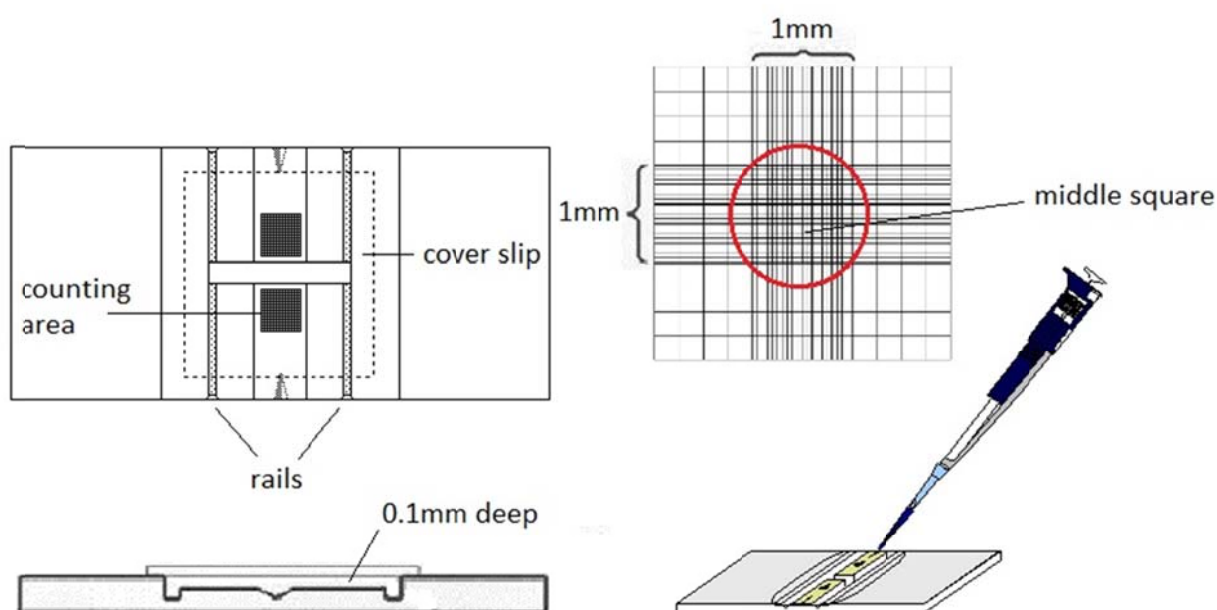
### ***2.2.3 Human embryonic kidney 293 cells for transfection (HEK293FTs)***

HEK293FTs were obtained from Invitrogen (Invitrogen Life Technologies) and cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose (PAA Laboratories GmbH) containing 10% (v/v) FCS, 6mM glutamine (PAA Laboratories GmbH), 0.1mM MEM non-essential amino acids (Life Technologies), and 500µg/mL Geneticin (Gibco) in a T150 flask. Cells were maintained in a Nuaire DH autoflow incubator at 37°C with 21% O<sub>2</sub> and 5% CO<sub>2</sub>. Fresh media was replaced every two days until the cells reached approximately 80% confluency.

### ***2.2.4 Passaging, maintenance and storage***

Cells were kept in the incubator with fresh media replaced regularly and cell morphology checked under microscope. Removed from the incubator, the cell culturing medium was decanted out of the flask, which was followed by rinsing three times with the same volume of 1× phosphate buffered saline (PBS, Sigma-Aldrich). Then, cells were incubated at 37°C for 5 min in 0.05% Trypsin-EDTA (PAA laboratories GmbH) to enable adherent cells to detach (10ml Trypsin-EDTA for T150 flasks or 5ml for T75 flasks). Trypsin activity was neutralised by adding to the flasks

the same volume of culturing media containing serum and the cell suspension was transferred to conical tubes. Cells were collected by centrifugation in a Hettich Rotina 46R centrifuge (DJB Labcare) at 180G, room temperature for 5 min and the cell pellet was re-suspended in 1mL of their respective culturing media. Cells were counted in 10 $\mu$ L of the cell suspension using a Neubauer haemocytometer (Figure 2.1).



**Figure 2.1 A Neubauer haemocytometer and cell counting technique.** The depth between the cover slip and the haemocytometer was approximately 0.1mm, and the area of the middle square of each counting area was 1 $\times$ 1 mm<sup>2</sup>, thus the volume of the cell suspension to be quantified was approximately 0.1 $\mu$ L (=1 $\times$ 10<sup>-4</sup>mL). Cell counting of both the upper and lower middle squares were averaged.

The total cell number in the suspension was calculated by the formula below:

$$\text{Total cell number} = \frac{C_{\text{upper}} + C_{\text{lower}}}{2} \times 10^4 \text{ cell/mL} \times 1\text{mL}$$

( $C_{\text{upper}}$  = cell count from upper middle square,  $C_{\text{lower}}$  = cell count from lower middle square).

Cells were diluted to the desired density and plated into tissue culture flasks accordingly. For routine culture, cells were maintained in their own culturing media in a Nuair DH autoflow incubator at 37°C with 21% O<sub>2</sub> and 5% CO<sub>2</sub>, unless otherwise mentioned.

For long-term storage, cells were harvested by centrifugation again at 180G, room temperature for 5 min. The supernatant was removed completely and the cell pellet re-suspended in freezing mix (90% (v/v) Foetal Bovine Serum (FBS, PAA Laboratories GmbH), 10% dimethylsulphoxide (DMSO) (Sigma-Aldrich Ltd.) at a concentration of 1×10<sup>6</sup>/mL. Aliquots containing 1mL of cell suspension per cryovial were frozen gradually to -80°C for 24 hours before transferring then to liquid nitrogen (-186°C).

### ***2.2.5 Plasmids***

Plasmid DNA including pLV-GFP (lentiviral vector genome), pΔ8.91 (lentiviral packaging vector) and pVSV-G (lentiviral envelope vector) for genetic tracking of HUVECs, pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) and pmirGLO containing the 3'-untranslated region (3'-UTR) of genes such as COL1A2, BMP1, mTLD, and CHRD were kindly provided by Dr Enca Martin-Rendon, Stem Cell Research Laboratory, Oxford. Schematic maps of these plasmids are provided in Appendix 1.

## **2.3 Isolation and expansion of cardiac progenitors from heart biopsies**

### ***2.3.1 Biopsy collection, handling and process***

Cardiac-derived cells were isolated and expanded in culture as previously described [90] with minor modifications.

A 6-well plate was coated by human fibronectin at  $0.8\mu\text{g}/\text{cm}^2$  surface area. Approximately  $50\mu\text{L}$  of 0.1% fibronectin from human plasma (Sigma-Aldrich Ltd.) was diluted in 12mL of  $1\times$  PBS at a working concentration of  $4\mu\text{g}/\text{mL}$ . Then 2mL of the working solution was added to each well of the 6-well plate and the plate incubated at  $37^\circ\text{C}$ , 21%  $\text{O}_2$ , 5%  $\text{CO}_2$  for 30 min. Before seeding the explants, the wells were thoroughly washed with  $1\times$  PBS three times to remove unbound fibronectin.

Full-thickness biopsies were taken from the right atrial appendage of patients undergoing surgery. Following sternotomy, a purse string was placed in the right atrial appendage and the whole layer of myocardium in the middle of the purse string was removed with or without clamping with Duval forceps.

The biopsy was immediately transferred to a sterile 50ml test tube with 10mL of cold Complete Explant Medium (CEM) (See Appendix 2), delivered in an icebox and processed within two hours. Blood and connective tissue were removed by two rinses with 2mL of  $1\times$  PBS in two separate wells of a new 6-well plate and cut into small slices in 2mL of  $1\times$  PBS in a third well to enable deep wash and facilitate trypsinisation. Then, the biopsy slices were transferred to 2 mL 0.05% trypsin-EDTA

in a fourth well and incubated at 37°C for 5min. Neutralised with 2mL of CEM, the biopsy slices were minced into  $\sim 1 \text{ mm}^3$  explants and maintained in the medium for seeding.

After removing the free fibronectin from the wells by PBS wash as described above, 9-13 explants were placed onto the fibronectin-coated 6-well plates. Then, 1mL of CEM was slowly added into a well in order to keep the explants attached. Explants were maintained in a Nuair DH autoflow incubator, at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub>, with 1mL fresh CEM per well changed every 3 to 4 days until outgrowth cells in most wells reached >90% confluency.

### ***2.3.2 Harvest of outgrowth cells for cardiosphere formation***

After 1-2 weeks in culture, explants were surrounded by stromal-like cells that serve as supportive monolayer for small, round, phase-bright cells to migrate over. These cells were called outgrowth cells (OCs).

A Costar<sup>®</sup> 24-well flat bottom cell culture plate (Corning Life Sciences) was coated with Poly-D-Lysine (PDL) (Sigma-Aldrich Ltd.) at  $4\mu\text{g}/\text{cm}^2$  surface area. In practice, 5mg of lyophilised PDL was re-suspended in 2.5mL 1× PBS to make up the working stock at the concentration of 2mg/mL. Approximately 0.1mL of PDL working stock was further diluted into 12mL of 1× PBS. Then, 0.5mL of the diluted PDL was added to each well of the 24-well plate and the plate was incubated at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> for 30-60 min. The wells were rinsed with 1mL of 1× PBS to remove the free PDL before seeding the OCs.

Wells containing OCs were rinsed three times with 1mL of 1× PBS prior to detaching the OCs with 1mL of 0.05% trypsin-EDTA at 37°C for 5 min. After neutralisation with an equal volume of CEM containing 10% (v/v) FBS, the cell suspension was harvested by centrifugation at 150G, room temperature for 8 min and the cells were re-suspended in 1mL Cardiosphere Growth Medium (CGM) (See Appendix 2) for quantification according to the method described in section 2.2.4.

After count, the cell density was adjusted to  $1 \times 10^5$  cells /mL of CGM for seeding. 300µL of cell suspension (approximately 30,000 cells) was placed onto each well of the PDL coated 24-well plate. The OCs cells were incubated at 37 °C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> and OCs began to aggregate and form floating or semi-floating multicellular clusters, called ‘cardiospheres’, after 1-2 weeks in culture. Every 3 to 4 days, the culturing medium was removed gently by fine aspiration, leaving cardiospheres intact, and replaced by 300µL of fresh CGM per well.

### ***2.3.3 Harvest of cardiospheres for expansion of cardiosphere-derived cells (CDCs)***

Cardiospheres were harvested two weeks later and transferred to a fibronectin-coated T150 culture flask for expansion of CDCs.

On the previous day, a T150 flask was coated with human fibronectin at 0.33µg/cm<sup>2</sup> surface area and incubated at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> for 30- 60 min. Free fibronectin was removed by washing the wells with 15mL of 1× PBS three times.

Cardiospheres were detached using a 1mL pipette and gently aspirating the medium up and down several times. The cardiosphere suspension was then harvested by



centrifugation at 100G, room temperature for 10min and re-suspended in 15mL CEM. Cardiospheres were seeded into the fibronectin-coated T150 flasks and incubated at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> with 25mL of fresh medium replaced twice a week to enable optimal cell growth. CDCs grew as a monolayer on fibronectin-coated flasks.

CDCs were passaged as the cells reached >90% confluency. Cells were harvested, counted and seeded into new fibronectin-coated T150 flasks at a cell density of approximately  $1 \times 10^6$  cells/T150 flask in 15mL of CEM. CDCs were maintained at 37 °C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> with 25mL of fresh medium replaced twice a week.

Long-term storage of CDCs was achieved by placing  $1 \times 10^6$  cells per mL of freezing mix according to the procedures described in Section 2.4.

For treatment with TGF- $\beta$ 1, cells were maintained as above in T75 flasks. The original culturing media was replaced by 8mL of fresh EGM-2 (0ng/mL TGF- $\beta$ 1), containing 5ng/mL or 10ng/mL TGF- $\beta$ 1 (R&D Systems). Cells were incubated at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> for 48h before total RNA was isolated from the cells.

## **2.4 Flow cytometric analysis**

Flow cytometric analysis was used in this study to quantitate the expression of cell surface markers. A general protocol is detailed below. Test and isotope control antibodies, along with relevant information about fluoro-chromes, suppliers and dilution factors are summarised in Table 2.1. The principle of flow cytometric analysis is shown in Figure 2.2.

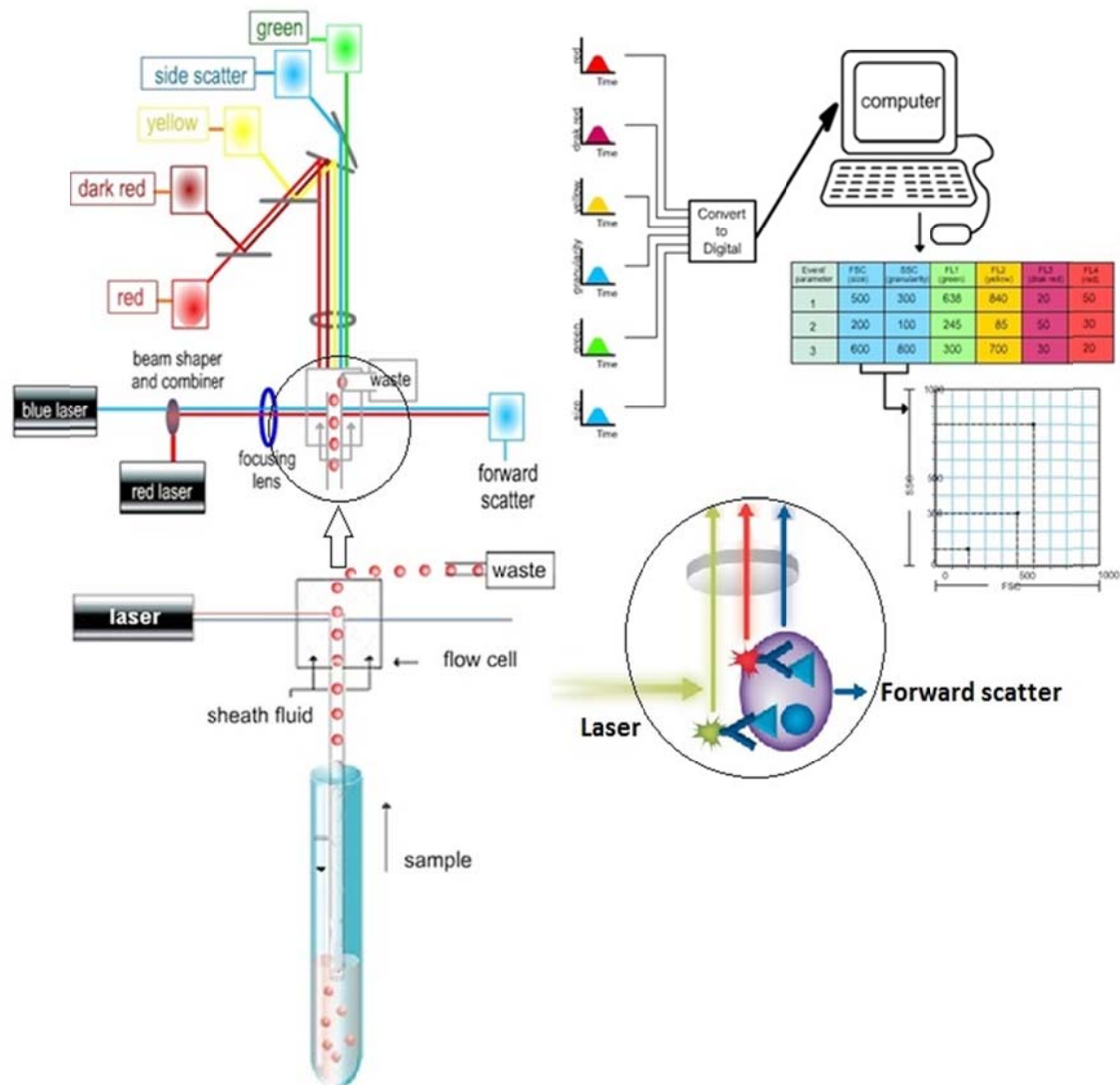
**Table 2.1 Antibodies used for flow cytometric analysis.**

| <b>Antibody</b> | <b>Isotype</b> | <b>Chanel</b> | <b>Company</b>      | <b>Stock concentration<br/>(µg/µL)</b> | <b>Final antibody concentration<br/>(µg/µL)</b> |
|-----------------|----------------|---------------|---------------------|----------------------------------------|-------------------------------------------------|
| Isotype         | mIgG1          | PE            | BD Biosciences      | 0.05000                                | 0.005                                           |
| CD31            | mIgG1          | PE            | BD Biosciences      | 0.00625                                | 0.005                                           |
| Isotype         | mIgG1          | FITC          | BD Biosciences      | 0.05000                                | 0.020                                           |
| CD90            | mIgG1          | FITC          | BD Biosciences      | 0.50000                                | 0.020                                           |
| Isotype         | mIgG1          | PE            | R&D System          | 0.02500                                | 0.005                                           |
| CD105           | mIgG1          | PE            | R&D System          | 0.02500                                | 0.005                                           |
| Isotype         | mIgG1          | PerCP         | BD Biosciences      | 0.20000                                | 0.005                                           |
| CD45            | mIgG1          | PerCP         | BD Biosciences      | 0.02500                                | 0.005                                           |
| Isotype         | mIgG1          | APC           | BD Biosciences      | 0.00625                                | 0.005                                           |
| CD117           | mIgG1          | APC           | BD Biosciences      | 0.20000                                | 0.005                                           |
| Isotype         | mIgG1          | FITC          | DAKO                | -                                      | -                                               |
| CD146           | mIgG1          | FITC          | Millipore(Chemicon) | 1.00000                                | 0.005                                           |

#### ***2.4.1 Cell preparation***

Cells were harvested as described in section 2 and counted in 1mL of MACS buffer (2mM EDTA and 0.5% BSA (bovine serum albumin) in 1× PBS) (all from Sigma-Aldrich Ltd.). After counting, the cells were collected by centrifugation at 180G, room temperature for 5min.

Cell pellets were re-suspended in 10% human FcR block in MACS buffer (Miltenyi Biotech) at a cell density of  $1 \times 10^6$  cells/mL. The cell suspension was placed on ice for 30 min and the cells harvested by centrifugation as above.



**Figure 2.2 The principle of flow cytometric analysis.** Cells labelled with antibodies conjugated with different fluoro-chromes are dragged into a narrow chamber to produce a single file of cells by hydrodynamic focusing. The cells passed through the fluidics system are stimulated by a beam of lasers at different wave lengths. Light scattering, including forward scattering and side scattering, or fluorescence emissions can be detected after passing a set of specific optical filters, which would provide information about the cell properties. A current is generated as the light is sensed by a photo detector and the voltage is associated with the photons detected. The electric signals are amplified and converted to digital signals which could be plotted graphically in a computer.

### ***2.4.2 Antibody incubation***

Test and isotype control antibodies (see Table 2.1) were titrated with MACS buffer into wells of a Costar<sup>®</sup> V-shaped-bottom 96-well plate (Corning Life Sciences) to obtain a final volume of 50µL and placed at 4°C protected from light.

Cell pellets were washed with the same volume of ice-cold MACS buffer and re-suspended in MACS buffer at a final cell suspension of  $2 \times 10^6$  cells/mL. 50µL of the cell suspension, containing  $1 \times 10^5$  cells, was added into each well of the 96-well plate and incubated in the presence of specific antibodies or their isotype controls at 4°C, protected from light, for 30 min. Then the cell-antibody mix was centrifuged at 210G, 4°C for 5 min and the supernatant removed. Cells were subsequently washed in 200µL  $1 \times$  PBS to give lower background, re-suspended in 250µL  $1 \times$  PBS and transferred to a FACS tube (BD Biosciences) for flow cytometric analysis.

### ***2.4.3 Data acquisition and data analysis***

The viability of the cells was assessed by adding 2.5 µL of 1mM To-Pro<sup>®</sup>-3 Iodide stain (Life Sciences) into each FACS tube to label the dead cells so that the viable cells could be distinguished for accurate analysis. Cells incubated with isotype-matched control antibodies were used to set up negative thresholds in the analysis.

The expression of cell surface antigens was analysed in a minimum of 100,000 events using a BD LSRII flow cytometer (BD Biosciences) with 670/14 filter and parameters listed in Table 2.2. The threshold of positive population was set above the 99th

percentile of a negative control cell population. The data were analysed using BD FACS Diva 6 software (BD Biosciences).

**Table 2.2 Typical human CDC flow cytometric parameters**

| <b>Detector</b> | <b>Voltage</b> |
|-----------------|----------------|
| FSC             | 280            |
| SSC             | 255            |
| FITC            | 270            |
| PE              | 250            |
| PerCP           | 400            |
| APC             | 400            |

## **2.5 Mesenchymal lineage differentiation assays**

### ***2.5.1 Adipogenic assay***

#### ***2.5.1.1 Adipogenic stimulation***

Cells that were maintained in their respective media were harvested and counted according to section 2.4. The cell suspension was adjusted to  $1 \times 10^5$  per mL by adding its routine culturing medium and seeded in a 6-well plate at  $2 \times 10^5$  cells in 2 mL of medium per well (in triplicates). The cells were then maintained at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> with 2mL of the fresh media changed three times a week until the cultures reached 100% confluency before induction of adipogenic differentiation.

A total of three cycles of induction/maintenance were required to achieve optimal stimulation of adipogenic differentiation. In brief, the media was completely removed from the confluent cultures. Cells were then rinsed twice with 2mL of  $1 \times$  PBS followed by the addition of 2mL of freshly supplemented Adipogenesis Induction Medium (Lonza Biologics) per well. For each sample, cells in the other three wells in the same 6-well plates were used as controls and incubated under non-inducing

conditions in their standard respective media. The cells were maintained under inducing conditions at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> for three days.

Thereafter, the induction medium in each well was carefully replaced by equal volume of supplemented Adipogenic Maintenance Medium (Lonza Biologics), without disruptions to the potential lipid vacuole formation within the cells. Fresh media was also changed in the non-induced controls for maintenance. Cells were kept at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> for 3 days to complete the first cycle of adipogenic stimulation. This first cycle was followed by the next two induction/maintenance cycles.

#### *2.5.1.2 Lipid staining*

The successful differentiation into adipocytes could be assessed by observing, under bright field of a Nikon Eclipse TS100 light microscope (Nikon UK Ltd.), numerous lipid vacuoles within the cells whilst no such change was observed in the non-induced controls.

Lipid anabolism was confirmed by staining cells with Oil Red O after fixation with 100% methanol. Briefly, the culture media was removed and cells were rinsed three times with 2mL of 1× PBS before adding 1mL of ice cold 100% methanol per well (Sigma Aldrich Ltd.) and maintaining the cells at -20°C for 5 min. Then the methanol was removed from the wells followed by two washes with 1mL of distilled water per well.

A 0.3% (w/v) oil red O isopropanol (both from Sigma-Aldrich Ltd.) stock solution was diluted in water at a ratio of 3:2 and filtered using a 0.2µm filter, to make up the working solution. Approximately 0.5mL of the freshly made oil Red O working solution was added into each well of 6-well plates and incubated at room temperature on a shaking platform for 20 min. The staining solution was then removed and the cells were gently washed with 0.5mL of distilled water per well. Finally, 0.2mL of water was added into each well to keep the wells from drying. Images of the cultures were taken under bright field of a Nikon Eclipse TS100 light microscope. Hamamatsu ORCA-ER camera controlled by SimplePCI automated image capture camera device software (Hamamatsu Photonics UK Ltd.) was used to record the images from all wells.

### ***2.5.2 Osteogenic assay***

#### *2.5.2.1 Osteogenic stimulation*

As above (section 5.1 Adipogenic assay), approximately  $3 \times 10^4$  cells in 2 ml of medium were seeded in each well of a 6-well plate and maintained at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> for 24 to 48 hours to enable cell adhesion before the induction of osteogenesis. Following adhesion, cells were rinsed twice with 2mL of 1× PBS and 2mL of freshly supplemented Osteogenic Induction Medium (Lonza Biologics) was added to each well whilst standard culture medium was added to the non-inducing controls. The cells were incubated at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> with fresh osteogenic induction or their respective culture media replaced twice a week. The culture was monitored for osteogenic differentiation under the light microscope every time before media changes.

Usually, gaps forms within the monolayer of cells and cells delaminate as the morphology starts to change from spindle shaped to cuboidal shaped during the period of osteogenesis and mineralization between 2 to 3 weeks from the start of the induction. As soon as morphological changes were observed, and prior to delamination occurring, cells were stained for alkaline phosphatase expression.

#### *2.5.2.2 Staining for alkaline phosphatase*

Naphthol AS-MX is a substrate of alkaline phosphatase (ALP) and the coupled diazo product can be stained pink by fast red TR salt. ALP expression is detected by staining with fast red/ naphthol AS-MX in alkaline staining solution.

Every 20mL of osteogenic staining solution was made up of 19mL of 0.1M Tris-HCl pH=9.2, 1mL dimethylformamide (DMF), 4mg naphthol AS-MX, and 20mg fast red (all from Sigma-Aldrich Ltd.). After the media was removed from the cultures and gently washed with 2mL of 1× PBS, 1mL of osteogenic staining solution was added into each well of the 6-well-plates and the plates were incubated at room temperature for 30 min. Then, the staining solution was removed, and the wells were washed with 1mL of 1× PBS per well. Cells were fixed with 1mL of 4% paraformaldehyde (Sigma-Aldrich Ltd.) per well at room temperature for 10min. Images were taken under the light microscope thereafter as described in section 2.5.1.2.

#### *2.5.3 Chondrogenic assay*

##### *2.5.3.1 Reagent preparation*

Preparation of 20µg/mL TGF-β3 working stock was made no longer than 6 months prior to assay. Approximately 2µg of lyophilised TGF-β3 (Lonza) was re-suspended



in 100 $\mu$ L of 4mM HCl (Sigma-Aldrich Ltd.) supplemented with 1 mg/mL BSA. TGF- $\beta$ 3 solutions were kept in -80°C for future use.

#### *2.5.3.2 Chondrogenic stimulation*

As above (section 2.5.1 Adipogenic assay), cells were maintained in their culture media until they reached confluency. Cells were then harvested, counted and re-suspended in supplemented Incomplete Chondrogenic Induction Medium (Lonza Biologics) at a density of  $7.5 \times 10^5$  cells/mL. Approximately 1mL of the cell suspension was transferred to two 15ml conical tubes separately, one for chondrogenic stimulation and the other as control. A cell pellet was formed in each tube by centrifugation at 150G, room temperature for 5min.

One pellet was re-suspended in 1.5mL of Complete Chondrogenic Induction Medium (Lonza Biologics) to achieve a density of approximately  $5 \times 10^5$  cells/mL and equally split into three 15mL conical tubes with  $2.5 \times 10^5$  cells in each tube. Cell pellets were formed as described above. Another 0.5mL of Complete Chondrogenic Induction Medium was gently added to each tube to make the total volume of 1mL per tube.

The other pellet was re-suspended in 3mL of its culturing medium and split into three 15mL conical tubes with 1mL in each containing  $2.5 \times 10^5$  cells and spun down at 150G, room temperature for 5min.

Cell pellets were incubated at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> without disturbance for 48h. Media changes were performed three times per week for the following 4 weeks.

#### *2.5.3.3 Immunohistochemical staining for type 2 collagen*

The pellets were processed for immunohistochemistry staining to detect type II collagen synthesis 28 days after induction of chondrogenesis.

Briefly, pellets were harvested from the culturing tubes into small aluminium foil cups, fixed and embedded with Raymond A Lamb Waxes and Embedding Media OCT (Thermo Scientific) and snap frozen at approximately -150°C in liquid nitrogen. Sections of 10µm thickness were taken from each tissue block using a Leica CM3050 S cryostat (Leica Biosystems) and mounted onto Superfrost Plus microscope slides (Thermo Scientific Gerhard Menzel).

Approximately 1mL of permeabilisation buffer (1× PBS containing 2% BSA and 0.2% Triton ×100 (Sigma-Aldrich Ltd.)) was applied onto the tissue sections surrounded by a water-repellent barrier made with an ImmEdge™ Hydrophobic Barrier Pen (Vector Laboratories) and incubated at room temperature for 1 hour.

After the permeabilisation buffer was removed, 100µL of the test antibody (goat anti-collagen II IgG, from Abcam) or the isotype control (goat IgG isotype control, from R&D systems) was applied onto the tissue sections and incubated at room temperature for 1h. Sections were then washed three times with 100µL of 1× PBS.

Immediately after, 100µL of rabbit anti-goat AlexaFluor® 488-conjugated antibody (Molecular Probes) , diluted 1:100 (v/v) in permeabilisation buffer, was added to the sections and these were incubated at room temperature for 30 min. Three washes with 100µL of 1× PBS were required to remove the excess of secondary antibody. A

drop of Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) was added onto each section to stain the nuclei before covering it with a glass slip and sealing it.

Fluorescence from immunohistochemistry was detected under UV light using a Nikon Eclipse TS100 light microscope (Nikon UK Ltd.). A Hamamatsu ORCA-ER camera controlled by SimplePCI automated image capture camera device software (Hamamatsu Photonics UK Ltd.) was used to record the images from all sections.

## **2.6 Clonogenic assay**

In order to assess the clonogenicity of CDCs, four Corning® Costar® 96-well plates were coated with human fibronectin at  $0.33\mu\text{g}/\text{cm}^2$  surface area as previously described (section 2.3.3). Plates were then incubated at  $37^\circ\text{C}$ , 21%  $\text{O}_2$ , 5%  $\text{CO}_2$  for 30- 60 min.

CDCs were harvested and counted as described in section 2.3.3 and a cell suspension of 10cells/mL was prepared. After the wells were washed to remove the free fibronectin, 0.1mL of the cell suspension was placed in each well of the four 96-well plates to achieve seeding only one cell per well. The plates were maintained at  $37^\circ\text{C}$ , 21%  $\text{O}_2$ , 5%  $\text{CO}_2$ , with full media changes every 3 days, for a minimum of 4 weeks or until any cell proliferation was observed under the light microscope. The total number of wells that had cell division or formed a colony was counted. The clonogenic potential was calculated by the formula below.

$$\text{Clonogenicity} \approx \frac{\text{number of wells with colonies formed}}{400} \times 100\%$$

## 2.7 Vector particle production and titration

The self-inactivating lentiviral viral vector system used in this study is based on the human immunodeficiency virus-1 (HIV-1) and was kindly provided by Professor Adrian Thrasher, Institute of Child Health, London (Appendix 1). The lentiviral vector genome expresses green fluorescent protein (GFP) under the control of the spleen focus-forming virus (sFFv) promoter. Lentiviral vector particles for gene transfer and labelling of cells were produced as previously described [140].

Briefly, plasmid DNA was obtained using a PureLink<sup>R</sup> HiPure Plasmid Maxiprep kit (Life Technologies) following the manufacturer's instructions. For transfection,  $1 \times 10^7$  low passage (P2-P5) HEK293FT cells were seeded into a T150 flask in 30mL of the standard culturing medium and incubated at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> overnight.

As illustrated in Figure 2.3, cells that reached 90% confluency were transfected with 50µg of lentiviral vector genome (LV-GFP; A), 32.5µg of lentiviral packaging vector (pΔ8.91; B) and 17.5µg of lentiviral envelope construct (pVSV-G; C) using 200µL of Lipofectamine<sup>®</sup> 2000 reagent (Invitrogen, Life technologies) in 30mL of Gibco<sup>®</sup> Opti-MEM<sup>®</sup> Medium (Life Technologies). Cells were returned to the incubator and were cultured at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> to produce lentiviral vector particles. Approximately 48h post-transfection, the supernatant of the transfected cells was cleared of cellular debris by centrifugation at 500G, room temperature for 5 min and stored in 1mL aliquots at -80°C for future use (D).

### A. Vector genome

pLV-GFP



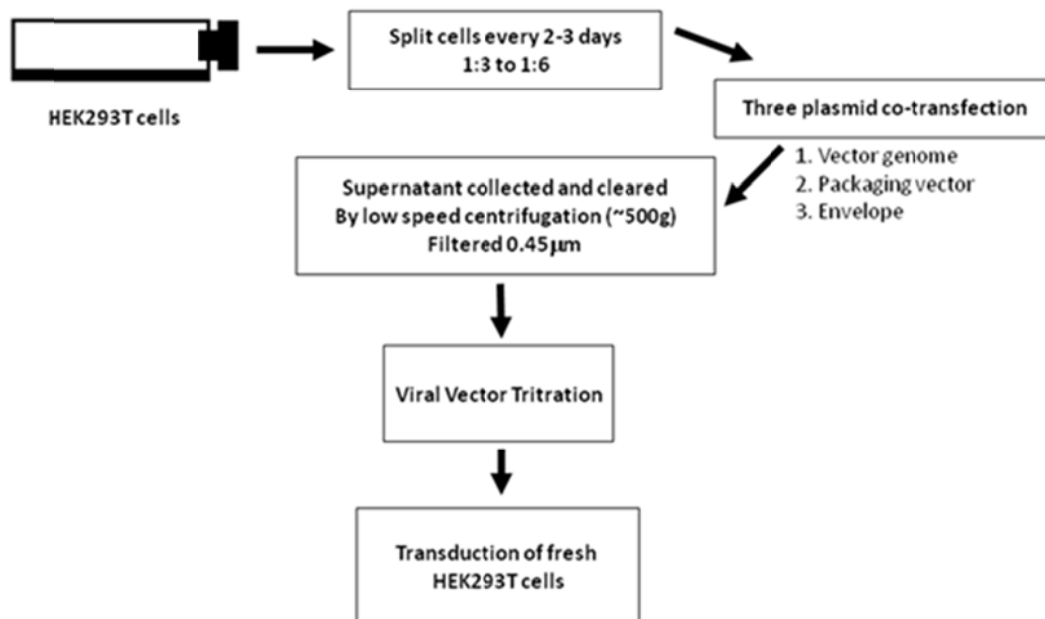
### B. Packaging vector



### C. Envelope expression vector



### D. Production of lentiviral vector particles and viral titration



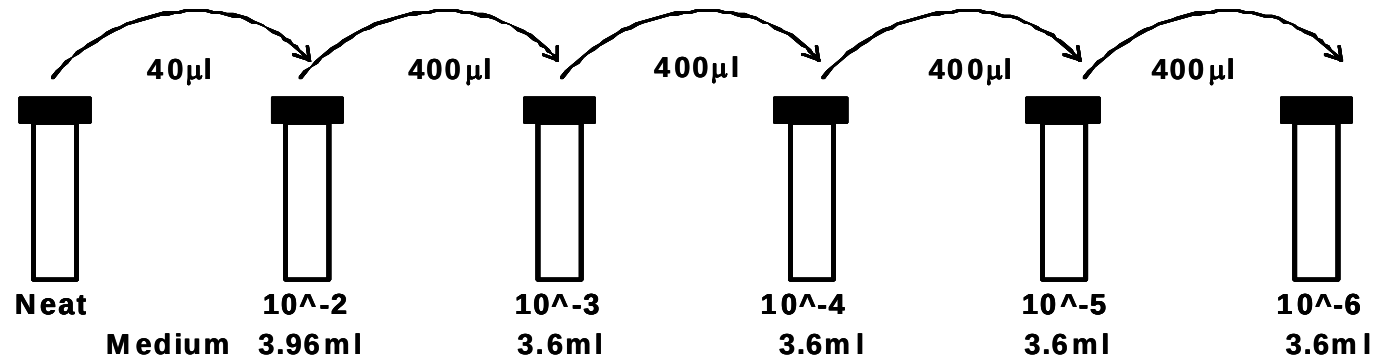
**Figure 2.3 A schematic diagram of lentiviral vector particle production and titration.** (A) The self-inactivating (SIN) vector genome expresses green fluorescent protein (GFP) under the control of the spleen focus-forming virus (sFFV) promoter and contains the Woodchuck Post-transcriptional Regulatory Element (WPRE) for optimal expression. (B) The packaging vector contains the human immunodeficiency virus type I (HIV-1) *gag* and *pol* genes under the control of the cytomegalovirus (CMV) promoter. (C) The envelope plasmid expresses the Vesicular Stomatitis Virus envelope Glycoprotein (VSV-G) under the control of the CMV promoter. (D) Lentiviral vector particle production is achieved by transient co-transfection of HEK293T cells with the three plasmids. The particles are harvested from the cell supernatant and titrated by serial dilution on the target cells.

Lentiviral vector stocks were titrated by transducing target cells. Briefly, HEK293FT cells were seeded in 12-well plates at  $5 \times 10^4$  cells/well the day before. Lentiviral vector stocks were thawed at 37°C and diluted in the standard culturing medium as illustrated below (Figure 2.4). Serial dilutions at 1:1000, 1:10000, 1:100000 and 1:1000000 of the lentiviral vector stocks were added to the cells in 1mL medium per well in triplicates. Cells were incubated at 37°C, 21%O<sub>2</sub>, 5% CO<sub>2</sub> for 48-72 hours. The number of GFP-expressing cells or colonies (green) was counted under UV fluorescence using a Nikon Eclipse TS100 light microscope. The viral titre was estimated as transducing units (TU) per mL of lentiviral particle stock by the formula below.

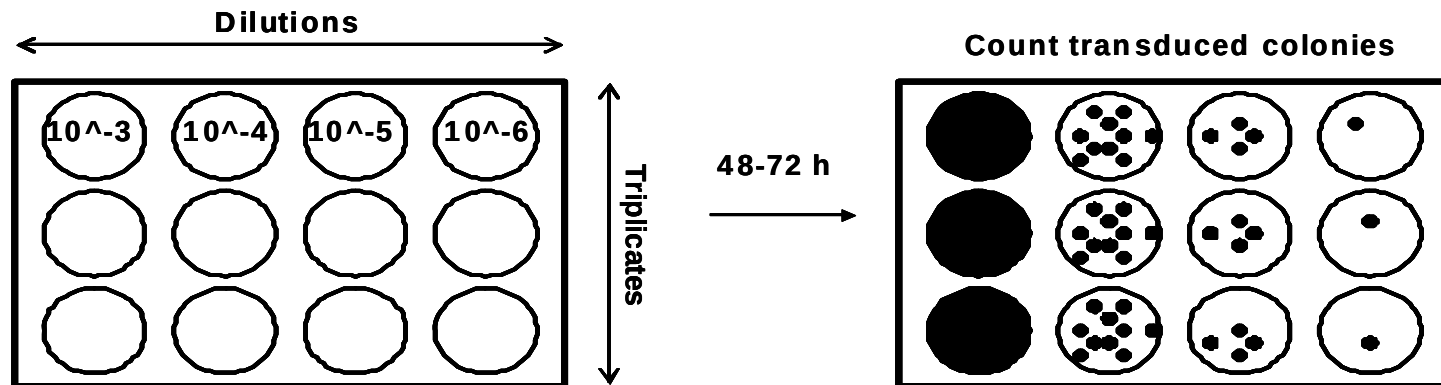
$$\text{Viral particle titre (TU/mL)} = \frac{\text{number of transfected cells or colonies}}{\text{dilution factor}}$$

For angiogenesis assays, HUVECs were transduced with LV-GFP vector stocks at a multiplicity of infection (MOI) of 3.

**1. Vector stock serial dilution**



**2. Take 1ml of your diluted stocks and place it in each well of the 12xwell plate**



**3. Titer= Number of transduced cells or colonies / dilution factor= Transducing Units (T.U.)/ml**

**Figure 2.4 Titration of lentiviral vector particles on target cells using serial dilutions.**

## **2.8 Angiogenesis assay**

In this study angiogenesis has been assessed using an *in vitro* assay consisting of a co-culture of GFP-labelled HUVECs and stromal supportive cells such as CDCs or BMSCs. The expression of GFP allows the monitoring of the live cultures over time.

### **2.8.1 Transduction of HUVECs**

Approximately  $1 \times 10^6$  HUVECs were seeded in a T150 flask and maintained at  $37^\circ\text{C}$  with 21%  $\text{O}_2$  and 5%  $\text{CO}_2$  for 24h. LV-GFP stocks with an average titre of  $3 \times 10^6$  TU/mL were thawed at  $37^\circ\text{C}$  in water bath and diluted in 25mL of EGM-2 to achieve an MOI of 3. The transduced cells were incubated at  $37^\circ\text{C}$  with 21%  $\text{O}_2$  and 5%  $\text{CO}_2$  until they reached 90% confluency. The cells were then harvested, counted, stored in liquid nitrogen and passaged as described in Section 2.2.4.

### **2.8.2 Co-culture of HUVECs and stromal cells**

Cells were cultured as described in section 2.2 and 2.3. Passage 1-2 (P1-P2) CDCs or P4-P5 BMSCs and P1-P2 GFP-labelled HUVECs were maintained at  $37^\circ\text{C}$  5%  $\text{CO}_2$  in their respective culture media replaced every three to four days until the cells reached >90 % confluency.

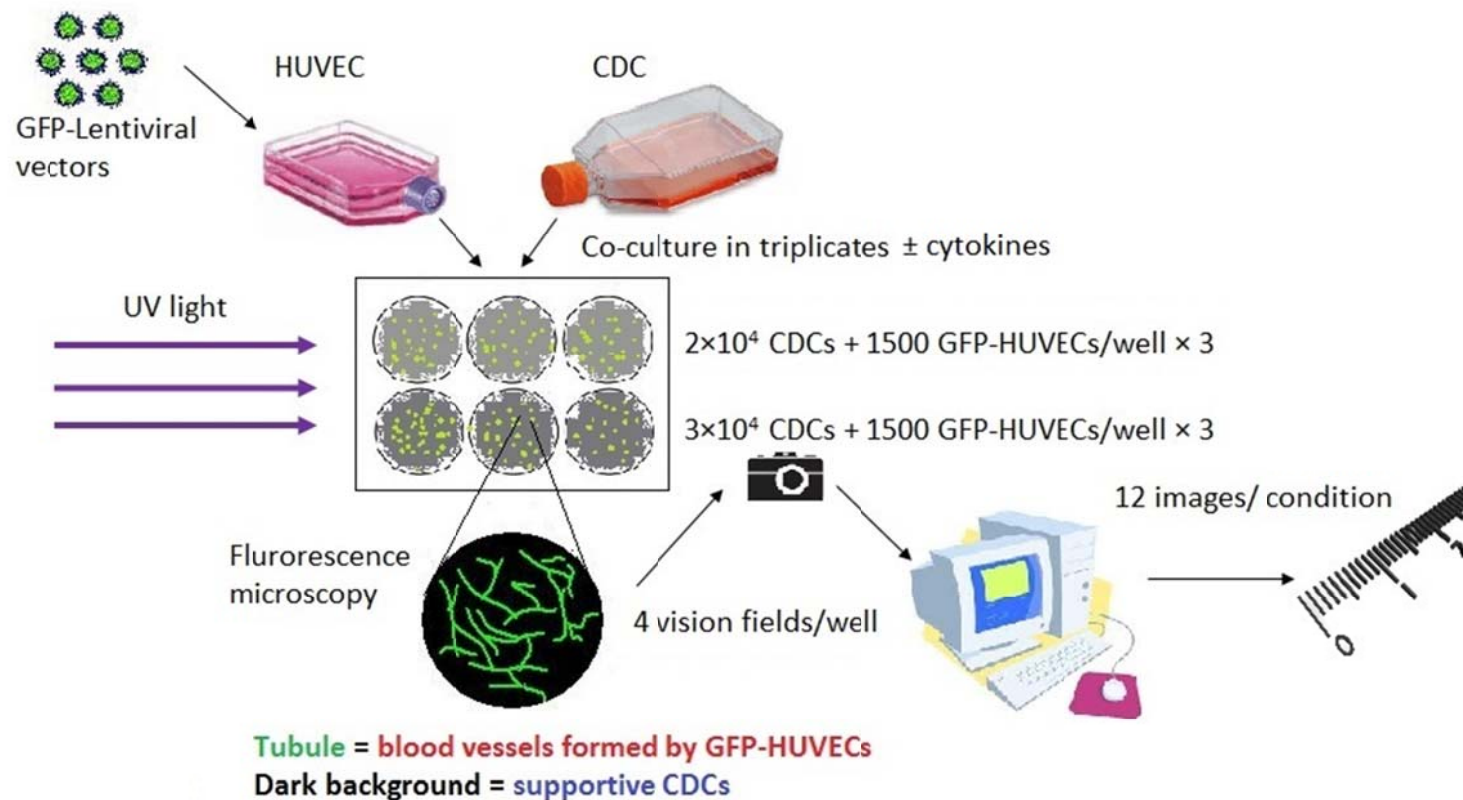
On the day before setting up the co-culture, a 48-well plate was coated with human fibronectin at  $0.8\mu\text{g}/\text{cm}^2$  surface area for 30-60 min. Before seeding the cells, the wells were washed three times with 0.5mL per well  $1 \times$  PBS to remove the free



fibronectin. Cells were harvested and cell numbers were adjusted to  $1 \times 10^5$  CDCs,  $1 \times 10^5$  BMSCs and  $1.5 \times 10^4$  HUVECs/mL respectively in complete EGM-2.

Approximately 0.1, 0.2 or 0.3 mL of cell suspension containing 1, 2 or  $3 \times 10^4$  of CDCs or BMSCs were seeded onto fibronectin-coated 48-well plates in triplicates as illustrated in Figure 2.5. Subsequently, 0.1mL of GFP-HUVEC cell suspension, containing approximately  $1.5 \times 10^3$  cells were added to each well. EGM-2 medium was added to the wells to make a final volume of 500 $\mu$ L/well. The co-cultures were incubated at 37°C, 5% CO<sub>2</sub>, for 14 days with medium changed 3 times a week. Cytokines were supplemented at certain concentrations with the fresh medium as required in the relevant experiments as listed in Appendix 3.

Tubule formation was monitored using a Nikon Eclipse TE2000U fluorescence microscope (Nikon) and Hamamatsu ORCA-ER camera controlled by SimplePCI software was used to take 4 images from each well (12 images in total). All images were saved in TIFF format for processing.



**Figure 2.5 Illustration of the angiogenesis assay.** HUVECs were transduced with GFP-lentiviral vector particles and passaged in culture. In the meantime, CDCs were isolated from each patient's right atrial biopsy and cultured to passage 2. After harvesting the cells, co-cultures of CDCs and GFP-HUVEC were mixed at different ratios in triplicate. Cells were cultured for 14 days in endothelial growth media and green tubules formed by HUVECs could be visualised under the fluorescence microscope. Four images, from four fields of view, were taken from each co-culture totalling 12 images from each culture condition. The images were analysed and the tubules formed quantitated using the AngioSys software.

### **2.8.3 Image processing and data analysis**

Images were processed with Photoshop® CS6 software package (Adobe). The angiogenesis images were transformed into greyscale mode with signal/background colour converted, so that the tubules signal became black whilst the background was shown in white. The levels of the images were adjusted manually to facilitate recognition by the angiogenesis analytical software.

The number of tubules, number of junctions, mean tubule length, and total tubule length in each image were automatically quantitated using the AngioSys software package (TCS Biologicals). The data was collected and saved as MS Excel 2007 documents for statistical analysis.

## **2.9 Human CDC transplantation into the immunocompromised nude rat model of ischaemic heart disease**

A myocardial ischaemia (MI) rat model was established in collaboration with Dr Pilar Sepulveda, University of Valencia, Spain to investigate the therapeutic potential of transplanted CDCs from IHD patients. A diagram summarising the experimental design is shown in Figure 2.6. Human CDCs were isolated, passaged and *in vitro* assayed by myself in the Oxford laboratory before being sent to Dr P. Sepulveda's laboratory in Spain for transplantation. Ethical approval to conduct the animal experiments was obtained accordingly from the relevant committee at Hospital La Fe, University of Valencia, Spain. All animal handling, surgery, husbandry, euthanasia and parameter measurements were performed by Dr Sepulveda's group according to regulations for animal experimentation in Spain upon the provision of the human

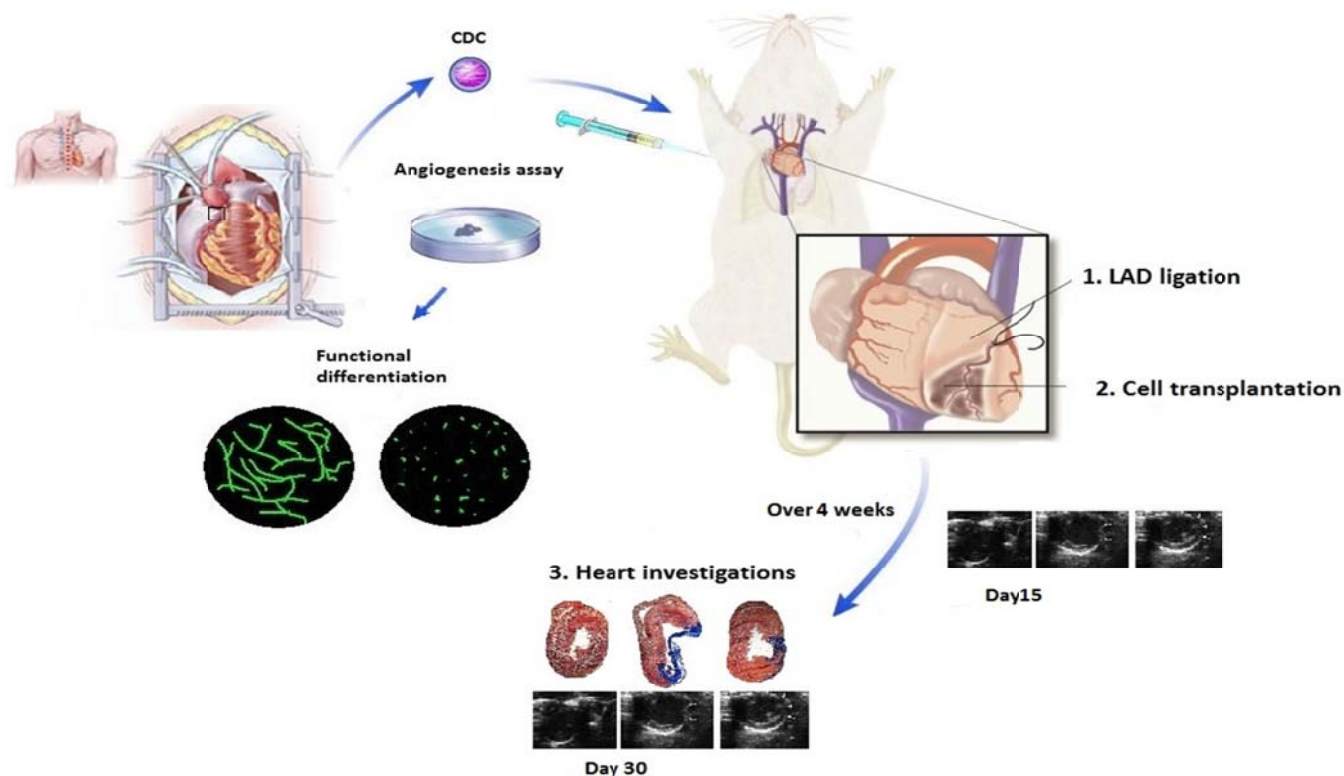
CDC samples in a blind manner. Raw measurement data were returned to Oxford for statistical analysis and interpretation.

### ***2.9.1 Rat myocardial ischaemia (MI) model***

The model was established as previously described [141], [142]. In brief, six to eight week old adult athymic nude rats (*HHH-Foxn1<sup>rnu</sup>*) were purchased from Charles River Laboratories, Inc., (Wilmington, Massachusetts). Systolic and diastolic parameters of LV chamber and anterior wall were measured by transthoracic echocardiography before surgery. The rats were then anaesthetised with sevoflurane inhalation (2.5% v/v) followed by an intraperitoneal injection of fentanyl (0.05 mg/kg). The midline sternotomy was performed with aseptic techniques and MI was induced by LAD- artery ligation [143]. Animals with fractional shortening (FS) above 35% following MI were excluded from the study.

### ***2.9.2 CDC transplantation***

Seven days following LAD- artery ligation, animals were divided into three groups; two groups were treated with  $1 \times 10^6$  cells in 5 $\mu$ L 1 $\times$  PBS, namely CDC good and CDC poor, the third group was injected with saline (PBS) and used as control. Cells and saline were injected directly into the myocardium with a Hamilton syringe at five locations around the infarct border zone. The rats were monitored for up to 4 weeks. Transthoracic echocardiography (TTE) was performed at baseline, on Day15 and Day30 (final) post-transplantation to assess the changes in cardiac function. Animals were sacrificed with an overdose of ketamine (125mg/kg), valium (10mg/kg), and atropine (50 mg/kg), and hearts removed for immunohistological examination.



**Figure 2.6 Experimental design of CDCs transplantation in ischaemic rat hearts.** CDCs from patients with IHD were isolated and tested in the *in vitro* angiogenesis assay. Myocardial ischaemia (MI) was induced by ligation of the Left Anterior Descending (LAD) artery ligation in adult nude rats. Passage 2 CDCs were selected according to their support of angiogenesis *in vitro*. Approximately  $1 \times 10^6$  CDCs from ‘good’ and ‘poor’ supporters of angiogenesis were transplanted per rat 7 days following LAD-artery ligation. The rats were monitored for 4 weeks following cell transplantation. Left ventricular function was measured by echocardiography at baseline and at the end of the study. The animals were sacrificed and the hearts underwent immunohistological examination.

### ***2.9.3 Transthoracic echocardiography (TTE) measurements and evaluations of LV function***

LV end-systolic and end-diastolic parameters including diameters, areas, anterior wall and septum thickness were measured by TTE and used to derive the values for percentage of LV Fractional Area Change (FAC%), Fractional Shortening (FS%), Anterior Wall Thickening (AWT%) and Ejection Fraction (EF%) according to the formulas below.

$$FAC\% = \frac{LVArea\ d - LVArea\ s}{LVArea\ d} \times 100\%$$

$$FS\% = \frac{LVd - LVs}{LVd} \times 100\%$$

$$AWT\% = \frac{LVAWs - LVAWd}{LVAWd} \times 100\%$$

$$LVEDV = \frac{7.0}{(2.4 + LVd)} \times LVd^3$$

$$LVESV = \frac{7.0}{(2.4 + LVs)} \times LVs^3$$

$$EF\% = \frac{LVEDV - LVESV}{LVEDV} \times 100\%$$

*FAC% = left ventricle per cent fractional area change, LVArea d = left ventricle end-diastolic area, LVArea s = left ventricle end-systolic area, FS% = left ventricle per cent fractional shortening, LVd = left ventricle end-diastolic diameter, LVs = left ventricle end-systolic diameter, AWT% = left ventricle anterior wall per cent thickening, LVAWs = left ventricle anterior wall end-systolic thickness, LVAWd = left ventricle anterior wall end-diastolic thickness, EF% = left ventricle per cent ejection fraction, LVESV = left ventricle end-systolic volume, LVEDV = left ventricle end-diastolic volume.*

#### **2.9.4 Immunohistochemistry**

Rat hearts were excised from the animals for immunohistochemical examinations in order to assess the effect of CDC transplantation on neovascularisation *in vivo* and the therapeutic potential to reduce the infarct size on Day30 and processed following the methods described elsewhere [141], [143]. In brief, hearts were washed with 1× PBS and fixed in 2% paraformaldehyde. Then the hearts were perfused with 20% sucrose and embedded in Tissue-Tek OCT Compound (Sakura Finetek) prior to being cryopreserved. Transverse sections of 14µm thickness (1 slice each 200µm of tissue) were obtained from apex to base.

Capillaries were stained with anti-rat CD31 antibody (Chemicon International) and an Alexa 488 conjugated secondary antibody. Images of 10 fields of approximately 0.15 mm<sup>2</sup> in the infarct border zone of each animal heart were taken at 200× magnification using a fluorescence microscope. The number of capillaries was counted with the Image Pro-Plus 5.1 software (Media Cybernetics, Inc.) and the capillary density was referred as the number of capillaries per mm<sup>2</sup>.

The LV infarct size was measured in 8 to 12 transverse sections of 14µm thickness slices stained with Masson's trichrome stain. The fibrotic area and the total LV cross section area were determined using Image Pro-Plus 5.1 software (Media Cybernetics, Inc.). The infarct size of a heart was calculated as the mean of the percentages of the fibrotic area against the total LV cross section area in all the slices from this heart.

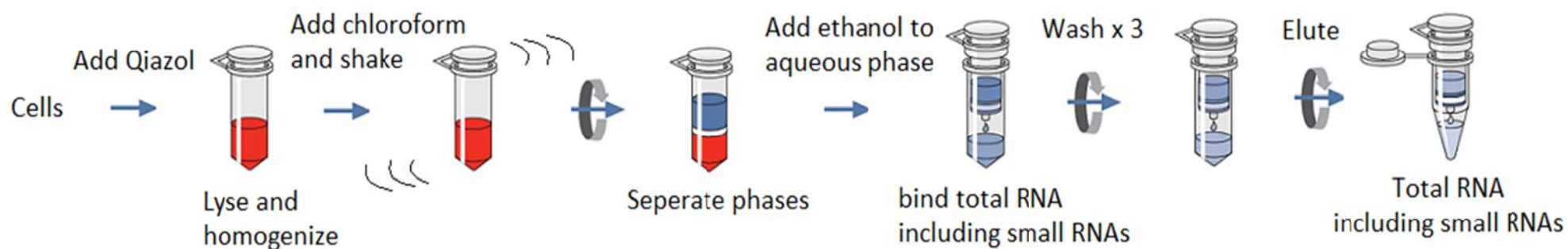
## **2.10 Total RNA isolation**

Total RNA, including mRNA and microRNA was isolated from human cells using miRNeasy Mini Kit (QIAGEN Ltd.) (see Figure 2.7). Briefly, cells were cultured, and harvested as described in section 2.2 and 2.3. Approximately 700µL of QIAzol Lysis Reagent (QIAGEN Ltd.) was added to the cell pellet after the supernatant was completely removed and the lysate was mixed by pipetting several times and vortexing the microtube vigorously for 1 min to enable optimal homogenisation. The homogenate was transferred to a 1.5mL tube and placed on the bench for 5 minutes to promote dissociation of nucleoprotein complexes before continuing with the isolation of RNA or the storage at -80°C for later use.

Approximately 140µL of chloroform (Sigma-Aldrich Ltd) was added to the homogenate and thoroughly mixed by vigorous shakes for 15 seconds. The mixed emulsion was separated into phases by centrifugation at 12,000 G, 4°C for 15 min. The sample then separated into 3 phases: an upper, colourless, aqueous phase, a white interphase, and a lower, pink, organic phase.

The upper aqueous phase (approximately 350µL), which contains RNA, was carefully removed and transferred to a 1.5mL collection tube without contamination of the white interphase. Approximately 1.5 times of the aqueous phase volume (usually 525µL) of 100% ethanol (Sigma-Aldrich Ltd.) was added to the collection tube mixed thoroughly by pipetting.





**Figure 2.7 Isolation of total RNA.** Cells were re-suspended in QIAzol (QIAGEN) in order to lyse them and prepare a cell homogenate. Nucleic acids were extracted from the cell homogenate by the addition of chloroform followed by vigorous shaking and centrifugation. As a result, the homogenate separated into two phases: the upper aqueous phase containing the nucleic acid suspension and the lower solvent-rich phase with a thin interphase containing cell debris. The aqueous phase was carefully removed and ethanol (100%) was added to the suspension in order to precipitate the RNA. Total RNA in the suspension was bound to an ion exchange column (QIAGEN), washed to remove all traces of ethanol remaining in the column and eluted from the column.

Approximately 700µL of the sample, including any precipitate formed was transferred into a RNeasy Mini spin column in a 2 mL collection tube and spun down at >8000 G for 15 seconds at room temperature to enable RNA to bind to the column membrane. The procedure was repeated until all the suspension has been used and the flow-through discarded. The column was washed with 700µL RWT buffer, followed by two washes with 500µL RPE buffer following the manufacturer's instructions.

The RNeasy Mini spin column with RNA bound to it was carefully removed from the collection tube so that the column did not contact the flow-through to avoid carryover of ethanol and placed into a new 1.5mL collection tube. Approximately 30µL RNase-free water was added directly onto the column for RNA elution. The eluted RNA solution was placed on ice before quantification.

To assess the RNA yield, 2µL of RNA sample was placed into the reading platform of a NanoDrop<sup>®</sup> Spectrophotometer (Thermo Fisher Scientific) calibrated by applying 2µL of Ambion<sup>®</sup> Nuclease-free Water (not DEPC-treated) (Life technologies) before measurement. The RNA quality was determined by the ratio of absorbance at 260nm to 280nm.

## **2.11 Enzyme-linked immunoabsorbent assay (ELISA) of cell culture supernatant**

### ***2.11.1 Cell culture and conditioned media preparation***

Approximately  $5 \times 10^5$  cells were cultured in 8mL of standard culturing media in a T75 flask (coated with fibronectin for CDC) until they adhered to the flask bottom.

Standard culturing medium was then replaced with 8mL of freshly made EGM-2 and cells incubated at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> for 48-72 hours. Conditioned media (cell culture supernatant) was collected and cell debris removed by centrifugation at 150G, room temperature, for 5 min. Aliquots of the conditioned media were stored at -80°C for future experiments.

### ***2.11.2 Human TGF- $\beta$ 1 and TGF- $\beta$ 2 ELISA***

Human TGF- $\beta$ 1 and TGF- $\beta$ 2 levels in conditioned media were quantitated by ELISA using the Human TGF- $\beta$ 1 or TGF- $\beta$ 2 Quantikine ELISA kits (R&D Systems) according to the manufacturer's instructions.

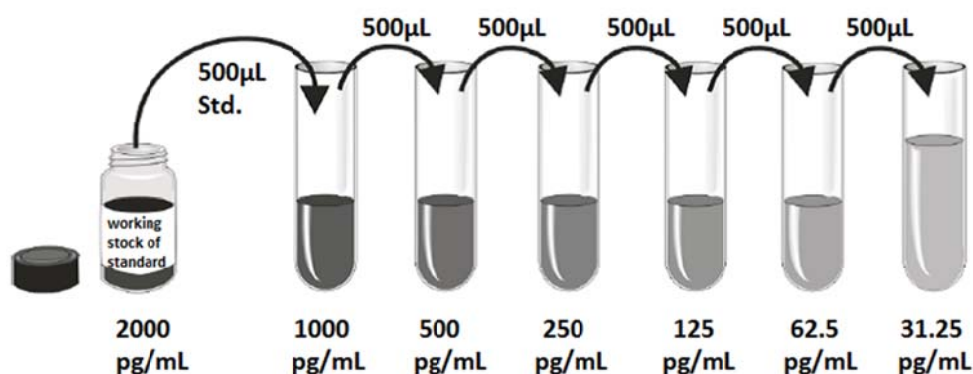
#### ***2.11.2.1 Activation of TGF- $\beta$ 1 and TGF- $\beta$ 2***

The latent forms of TGF- $\beta$ 1 and TGF- $\beta$ 2 in the conditioned media were activated by treating 500 $\mu$ L culture supernatant with 100 $\mu$ L of 1M HCl and incubating them at room temperature for 10 min. The acid was neutralised with 100 $\mu$ L 1.2M NaOH/0.5M HEPES. Therefore, the sample concentration had been diluted to 1: 1.4, with dilution factor 1.4.

#### ***2.11.2.2 Preparation of the protein standards***

Recombinant human TGF- $\beta$ 1 and TGF- $\beta$ 2 standards were reconstituted with of 2mL of 1 $\times$  Calibrator Diluent RD5-53 included in the ELISA kit, to obtain protein working solutions at a concentration of 2000pg/mL.

As illustrated in Figure 2.8, two-fold serial dilutions of TGF- $\beta$ 1 and TGF- $\beta$ 2 protein standards were prepared by adding 500 $\mu$ L of the working stock or last dilution in 500  $\mu$ L of 1 $\times$  Calibrator Diluent RD5-53 at final concentrations of 1000pg/mL, 500pg/mL, 250pg/mL, 125pg/mL, 62.5pg/mL, and 31.25pg/mL, respectively.



**Figure 2.8 Preparation of recombinant human TGF- $\beta$ 1 and TGF- $\beta$ 2 standards.**

The lyophilised protein standards (TGF- $\beta$ 1 and - $\beta$ 2) were reconstituted with 2mL of 1 $\times$  Calibrator Diluent RD5-53 (R&D Systems) to make up the working stock at a concentration of 2000pg/mL. Approximately 500 $\mu$ L of 1 $\times$  Calibrator Diluent RD5-53 was added into 6 empty microtubes. Then, 500 $\mu$ L of the working stock was diluted in the first tube to a concentration of 1000pg/mL, which was subsequently diluted 1:2 in the next tube. The process was repeated to reach the concentration of 31.25pg/mL.

#### *2.11.2.3 Binding and detection of TGF- $\beta$ 1 and TGF- $\beta$ 2*

Approximately 50 $\mu$ L of TGF- $\beta$ 1 or 100 $\mu$ L of TGF- $\beta$ 2 protein standards or ‘activated’ cell culture supernatant was placed in each well of a TGF- $\beta$ 1 or TGF- $\beta$ 2 microplate. Following this, 50 $\mu$ L of 1 $\times$  Assay Diluent RD1-21 or 100 $\mu$ L 1 $\times$  Assay Diluent RD1-17 was added to each well and the solutions mixed by gently tapping of the plate, prior to covering it with adhesive strips and incubating it at room temperature for 2 hours. After incubation, the supernatant was removed and plate was washed three

times with 400µL per well of 1× wash buffer (containing surfactant). The excess of liquid was removed by blotting on tissue paper.

Approximately 100 µL of TGF-β1 or 200 µL of TGF-β2 Conjugate, supplied in the kit, were added to each well. The plate was then covered with adhesive strips and incubated at room temperature for 2 hours. The unbound antibodies were removed from the wells by washing three times with 400µL of 1× wash buffer.

In order to detect the HRP-conjugated antibody bound to complexes of TGF-β1 or TGF-β2 and their capture antibodies, 100 µL or 200 µL of Substrate Solution containing 1:1 mixture of H<sub>2</sub>O<sub>2</sub> and tetramethylbenzidine was added to each well and the plate was incubated at room temperature for 30 min protected from the light. The reactions to generate luminal fluorescence from TGF-β1 or TGF-β2 were completed by adding 100 µL stop solution (1N HCl) or 50 µL of (2N H<sub>2</sub>SO<sub>4</sub>) to each well and mixing thoroughly by tapping the plate.

#### *2.11.2.4 Quantitation of TGF-β1 and TGF-β2*

The optical density (OD) of each well was determined within 30min of the end of the reactions by measuring the luminal absorbance using a Bio-Rad Microreader Model 680 (Bio-Rad Laboratories) set at 450nm, and corrected by readings at 570nm to correct for optical imperfections in the plate.

The duplicate readings for standards, blank controls and samples were averaged with MS Excel 2007 software package. Following this, a regression equation with its

standard curve was generated and the protein concentration estimated using the methods described below.

Means of optical densities (OD) of the standards on the Y-axis were plotted against their corresponding concentrations on the X-axis. The best fit line was drawn through the points of the graph by linear regression analysis performed by the software. The linear equation with its coefficient of determination (R-square) was in the format shown below:

$$y = kx + b$$

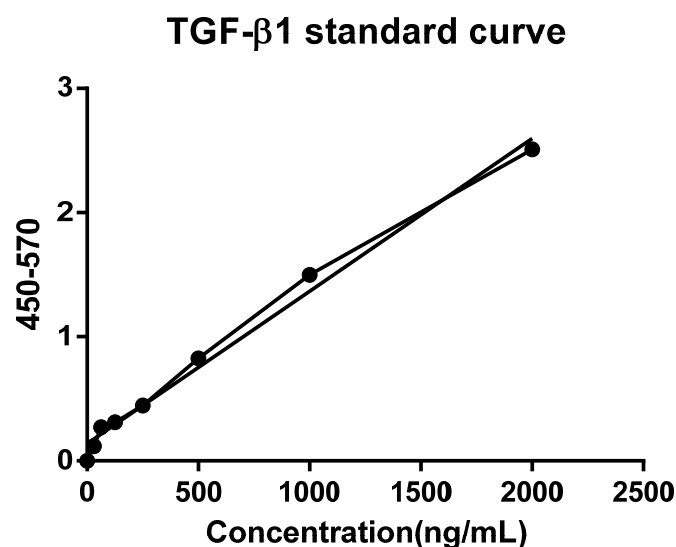
*(y – OD value, x – protein concentration, b – intercept , k – regression coefficient)*

Given the OD value of the protein samples, their concentration was determined by the inverse function of the regression equation above, expressed as below:

$$y = \frac{1}{k} (x - b)$$

*(y – protein concentration, x – OD value, b – intercept , k – regression coefficient)*

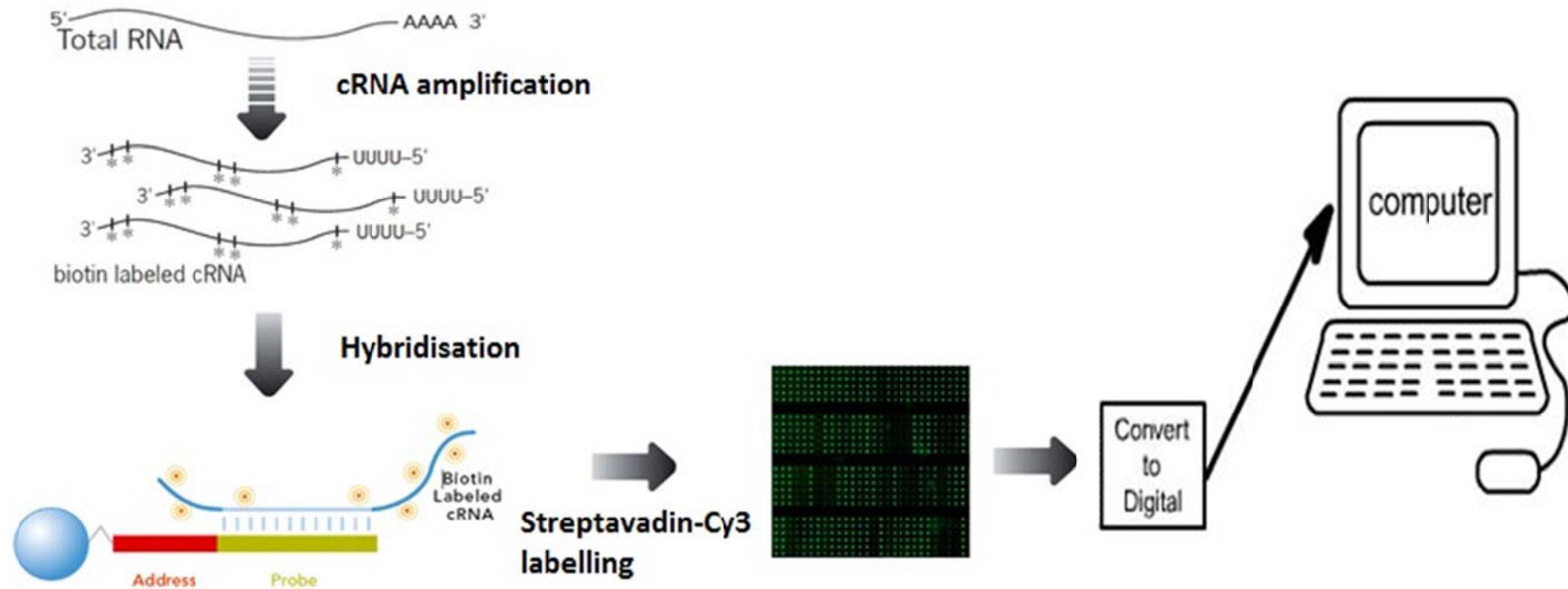
As the sample was diluted during the activation stage, the actual concentration from each sample was determined by multiplying the estimated protein concentration in the assay by the final dilution factor used to prepare the samples.



**Figure 2.9** An example of standard curve for TGF- $\beta$  ELISA. The Y axis represents the optical density or absorbance obtained for each sample (OD 470nm corrected at 570nm) whilst the X axis represents the protein concentration in ng/mL. Here, the linear regression equation is  $y = 0.001229x + 0.1380$ ,  $R^2 = 0.9890$ .

## 2.12 Human whole genome expression and gene ontology analysis

Biotin-labelled total complementary RNA (cRNA) was hybridised to human whole genome Illumina beadchips to quantitate differences in gene expression profiles of CDC samples with different pro-angiogenic ability. Total RNA from CDC samples tested in the *in vitro* angiogenesis assay and categorised as ‘good supporter’(n=6) and ‘poor supporter’(n=6) were isolated and stored in  $-80^{\circ}\text{C}$  according to the method described in section 2.9. Human whole genome expression service, including the biotin labelling of cRNA, beadchip hybridisation and data acquisition was performed in the University of Oxford, Genomic Service at the Department of Cardiovascular Medicine. The data were analysed by me on advice from Dr Theodosios Kyriakou, Department of Cardiovascular Medicine, University of Oxford.  $5\mu\text{g}$  of total RNA from each selected CDC sample was provided by, and the generated cDNA array data analysis was performed by the thesis writer (see Figure 2. 10).



**Figure 2.10 Summary of the Illumina beadchip cDNA array protocol.** Biotin-UTP, alongside total RNA template, was used for *in vitro* transcription and amplification of complementary RNA (cRNA) with biotin label. The purified biotin-labelled cRNA was hybridised to the probes on a human genome Illumina beadchip. The probes were located by the conjugated address which was calibrated by the manufacturer. The array was processed using streptavidin-Cy3 labelling and the signal developed was quantified by a bead array reader before data analysis.



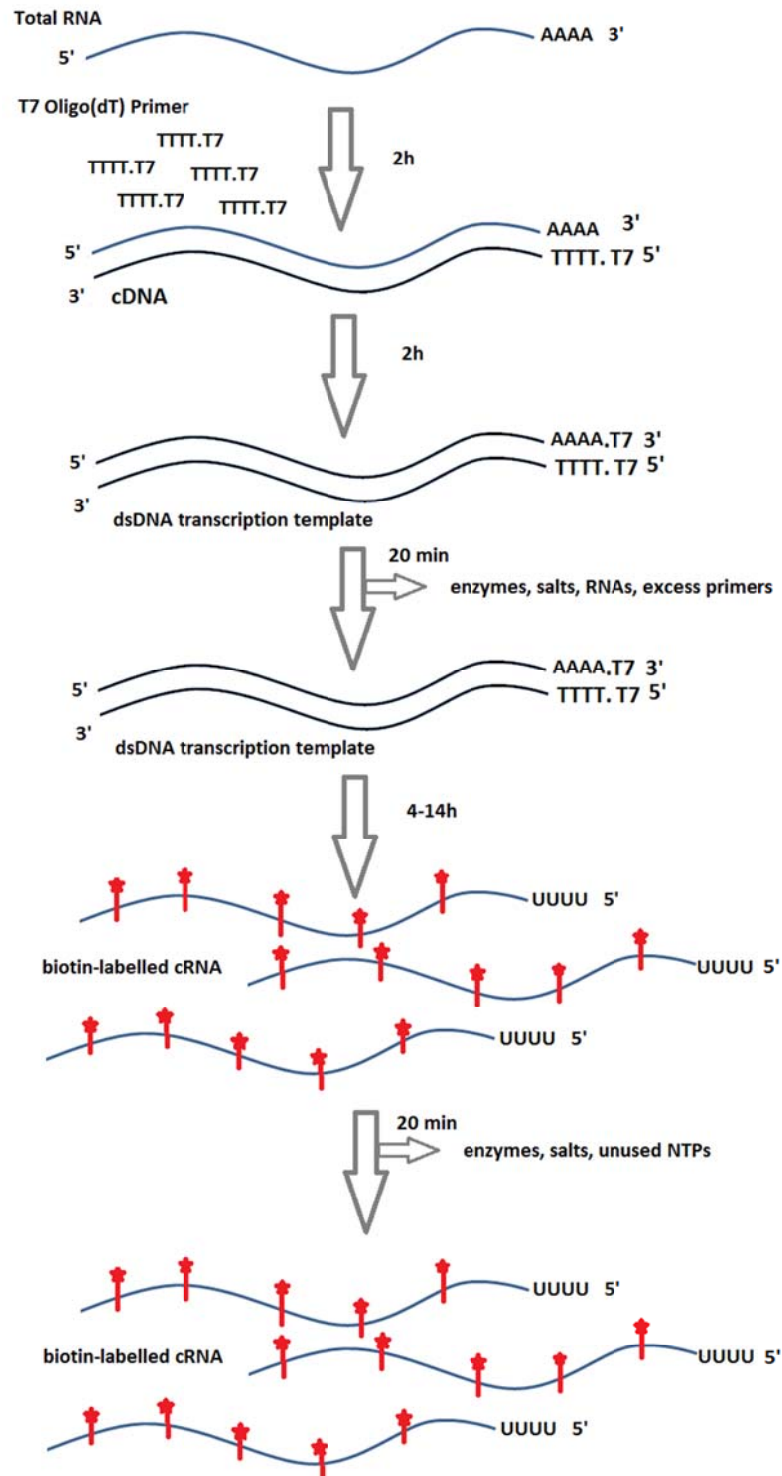
### ***2.12.1 cRNA amplification, labelling and hybridisation***

Biotin-labelled cRNA was generated from high-quality total RNA with the Ambion<sup>®</sup> Illumina<sup>®</sup> TotalPrep RNA amplification kit (Life Technologies Ltd.) according to the manufacturer's instructions. In brief, 50ng of total RNA was reverse transcribed with an oligo(dT) primer bearing a T7 promoter. The first-strand complementary DNA (cDNA) was used to produce the second strand. The double stranded cDNA was purified and, along with biotin-labelled UTPs, was then used as template to generate biotinylated, antisense complementary RNA (cRNA) from each mRNA sample in an *in vitro* transcription reaction with the T7 RNA Polymerase (see Figure 2.11). The size distribution profile and quantitation of the biotin-labelled cRNA samples were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.).

After cRNA labelling, 1.5µg of purified, labelled cRNA from each sample was hybridised at 55°C overnight with a Sentrix Human-6 v3 expression Illumina Beadchip (Illumina, Inc.) containing 48,804 probe sets. The beadchip was washed the following day and probed with Streptavidin-Cy3. The fluorescent emission by Cy3 from each chip was quantitated by scanning the chips using an Illumina BeadArray Reader (Illumina, Inc.).

### ***2.12.2 Data collection and processing***

Expression profiling was performed using Illumina Human WG6 version 3 beadchips (Illumina Inc.) using 48,804 probes as described above. Raw fluorescence intensity values from the scanned chips were generated using the Illumina Inc. Beadstudio



**Figure 2.11 Biotin-labelled cRNA amplification.** First strand complementary DNA (cDNA) was reverse transcribed following T7 Oligo(dT) Primer binding to the 3' poly(A) tail of mRNA template. The first strand cDNA was used to synthesise the second strand cDNA and obtain double stranded cDNA (dsDNA). dsDNA was used as template for *in vitro* transcription of cRNA along with biotin-labeled UTPs. Biotin-labelled cRNA was purified by washing out cDNA, unused NTPs, enzymes and salts.

software package (Illumina Inc.). Custom script written in R (v2.14.1) using the open source vsn Bioconductor package (Bioconductor.org) was used to normalise the fluorescent signal intensities according to the beadchip batch, subtract the background and also to remove any probes which had a detection score of less than 95% in any of the samples.

### ***2.12.3 Data analysis***

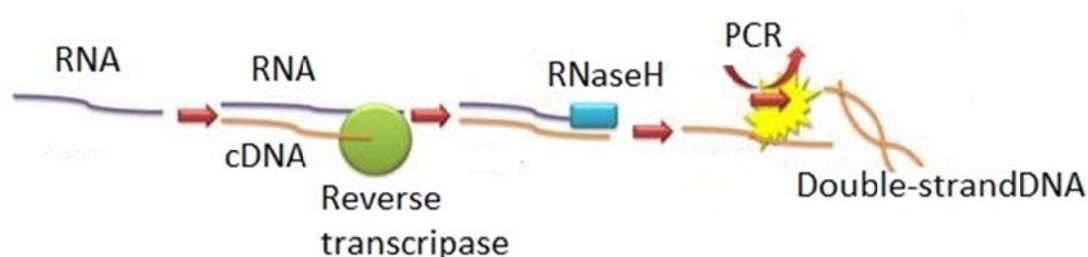
Data analysis was performed using the commercial statistical package Partek R Genomics SuiteT v6.11.0701 (Partek Incorporated) and the open source software MeV v4.8.1. Multiple descriptive approaches, including boxplots of detection count and overall signal density distribution curves, were used to detect any outliers amongst the samples tested. Probe profile data was normalised according to log2 transformation and equality of fluorescence intensity was diagnosed by boxplots of logarithmic average signal intensities. Principal component analysis (PCA) and hierarchical clustering (HCL) analysis were used to perform unbiased analysis of the raw data, with 2D and 3D mapping plots and dendrograms generated using the Partek R Genomics SuiteT v6.11.0701 software.

Differentially expressed (DE) genes were determined using two-independent-sample *t*-test in MeV v4.8.1 and one-way ANOVA in Partek R Genomics SuiteT v6. P values lower than 0.05 were considered significant. Additional conditions such as fold change, Bonferroni's correction or/and false discovery rate (FDR) were applied where appropriate.

Functional annotation of the list of DE genes was conducted using the public online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 [144] by separately uploading the up- and down-regulated genes onto the database.

## 2.13 Quantitative reverse transcription and polymerase chain reaction (qRT-PCR)

qRT-PCR was used to quantify the relative level of gene and microRNA expression from total RNA. Two consecutive steps were included in the experiment, namely reverse transcription and quantitative real-time PCR.



**Figure 2.12 The principles of qRT-PCR.** The RNA sample is used as template for complementary DNA (cDNA) synthesis by the reverse transcriptase (in green). The RNA is then degraded by RNase H (in blue) before quantitation. Chemical fluorescence is generated during the amplification of the cDNA by polymerase chain reaction (PCR) and its intensity accumulated as double-stranded DNA is amplified each PCR cycle. The accumulated fluorescence directly correlates with the relative quantity of DNA produced and the initial concentration of the template.

### 2.13.1 Reverse transcription

Complementary DNA (cDNA) synthesis was performed using ThermoScript™ RT-PCR system (Life Technologies). For each RT reaction, up to 9µL of RNA solution containing up to 5µg total RNA was mixed with 1µL of Random Hexamers primers, 2µL of 10mM High quality deoxyribonucleotide triphosphates (dNTP) Mix and adjusted to a final volume of 12µL with Ambion® Nuclease-free Water (not DEPC-

treated) into a 0.2mL thin-wall PCR tube (Thermo Fisher Scientific). The mix was heated at 65°C for 5 min to denature any secondary structures in the RNA and then placed on ice for another 5 min.

RT master mix was prepared on ice. Briefly for each RT reaction, 8µL of the RT master mix comprising of 4µL of 5× cDNA Synthesis Buffer (250mM Tris acetate (pH=8.4), 375mM potassium acetate, 40mM magnesium acetate, stabiliser), 1µL of 0.1M DL-Dithiothreitol (DTT), 1µL of DEPC-treated water, 1µL of RNase Out™, and 1µL of ThermoScript™ Reverse Transcriptase (all from Life Technologies) was required.

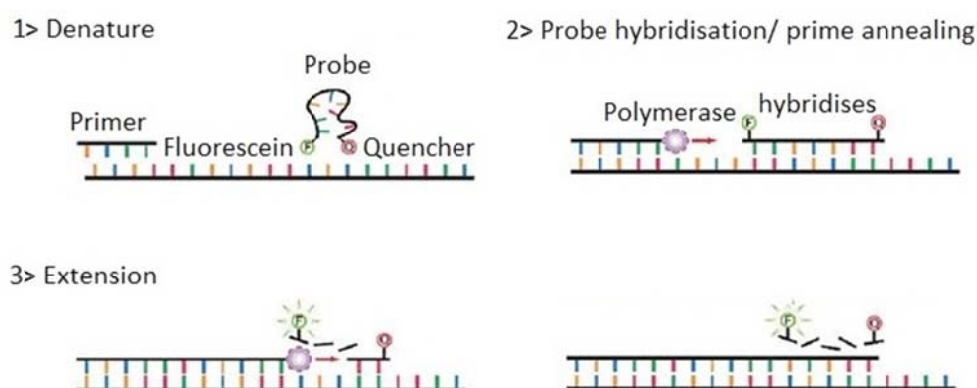
The RT reaction was performed in a Biometra T3000 Thermocycler System (Biometra GmbH) with the operating programme of incubation at 25°C for 10 minutes, 55°C for 50 minutes, and then 85°C for 5 minutes to terminate the RT reaction before cooling down to 4°C.

Following RT, the remaining RNA template in the reaction was removed by treating the samples with *E. coli* RNase H and incubating them at 37°C for 20 min. There was no accurate way to determine the concentration of the cDNA, therefore it was estimated on assumption that the reverse transcription reaction was 100% efficient. This being the case, the maximal concentration of cDNA was estimated to be 238.1ng/µL following the equation below

$$[c]_{cDNA\ max} = [c]_{RNA\ max} = 5000ng / 21\ \mu L = 238.1ng/\mu L$$

### 2.13.2 Quantitative real time PCR (qRT-PCR)

qRT-PCR was performed in a MicroAmp® Optical 96-Well Reaction Plate (Life Technologies) using the ViiA™ 7 Real Time PCR System (Life Technologies).



**Figure 2.13 Illustration of the qRT-PCR (TaqMan) method.** During double-stranded DNA denaturing, the fluorescein reporter in the probe set is inhibited by its quencher and no proton was emitted. As the temperature drops and Taq polymerase is activated, the primer anneals to the template and the probe set hybridises with the template DNA. The Taq polymerase extends the DNA by adding nucleotides whilst removing the probe from the template, which causes the separation of fluorescein from the quencher, resulting in fluorescence signals. The more the template, the faster the fluorescence accumulates.

Each gene of interest was tested in triplicate in single reactions. PCR reactions were set up in a final volume of 25µL by adding 0.105µL (25ng) cDNA diluted in 11.25µL of Ambion® Nuclease-free Water (not DEPC-treated) (Life technologies), 12.50µL of Taqman PCR Supermix (Life Technologies), and 1.25 µL 18µM TaqMan® Gene Expression Assay (20× primers and probe mix) (Life Technologies). The total amount of reagents to be used was estimated by multiplying by the number of reactions. Human β2-microglobulin and GAPDH gene expression assays (Life Technologies) were used as endogenous controls. Reactions were cycled in the ViiA™7 using the programme settings with 9600 emulation, 0.1mL plates, using the comparative Ct

method, 20µL final volume, with the FAM™ reporter dye and the standard thermal cycling parameters listed below.

**Table 2.3 Thermal cycling parameters for TaqMan® q-RT PCR**

| Step             | AmpliTaq Gold®<br>Enzyme Activation | PCR               |               |
|------------------|-------------------------------------|-------------------|---------------|
|                  | Hold                                | Cycle (40 cycles) |               |
|                  |                                     | Denature          | Anneal/Extend |
| Time             | 10 min                              | 15 sec            | 60 sec        |
| Temperature (°C) | 95                                  | 95                | 60            |

### **2.13.3 Data analysis of qRT-PCR**

The amount of PCR product was measured after each cycle and reported in fluorescence levels generated after probe-specific amplification. The threshold cycle (Ct) value denoted the number of PCR cycles required for the fluorescence to reach the threshold level. Thus, the more target cDNA present in a sample, the lower the Ct value was, as the threshold was reached sooner.

Ct values of the reactions were saved to a MS Excel document and comparative Ct methods including  $\Delta Ct$ , relative level against endogenous controls (% of control) and/or  $\Delta\Delta Ct$ , fold change were calculated for data analysis according to the following formulas.

$$\Delta Ct = Ct_{test} - Ct_{loading\ control} \quad (1)$$

$$relative\ level = \frac{RNA\ level_{test}}{RNA\ level_{loading\ control}} = \frac{2^{-Ct_{test}}}{2^{-Ct_{loading\ control}}} = 2^{-\Delta Ct} \times 100\% \quad (2)$$

$$\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{placebo} \quad (3)$$

$$fold\ change = \frac{relative\ level_{treatment}}{relative\ level_{placebo}} = \frac{2^{-\Delta Ct_{treatment}}}{2^{-\Delta Ct_{placebo}}} = 2^{-\Delta\Delta Ct} \quad (4)$$

#### 2.13.4 qRT-PCR for microRNA

Total RNA isolated as described in section 2.10 also contained microRNAs (miRNAs) from the cells, thus it was appropriate to use it as template for RT. However, unlike mRNA, each miRNA required an individual RT with a specific primer. Therefore, the cDNA product was only useful for quantification of a specific miRNA. TaqMan<sup>®</sup> MicroRNA Reverse Transcription Kit and TaqMan<sup>®</sup> MicroRNA Assays (both by Applied Biosystems) were used for RT of miRNA and qRT-PCR, which followed similar principles and steps as of mRNA.

##### 2.13.4.1 Reverse transcription

Total RNA template for RT was diluted in Ambion<sup>®</sup> Nuclease-free Water (not DEPC-treated) (Life technologies) at 4°C. Each qRT-PCR reaction required 0.9ng of cDNA and every 15µL RT reaction allowed a maximum of 5µL RNA containing up to 10ng total RNA. Therefore, 5µL of 2ng/µL total RNA would provide abundant RNA template for tests of a specific miRNA in triplicates in 96-well plate.

The RT Master Mix was made up according to the total number of RT reactions. Each 15µL reaction contained the components listed in Table 2.4. 7µL of the master mix,



5µL of total RNA and 3µL of RT primer were mixed into a 0.2mL thin-wall PCR tube on ice and mixed by centrifugation at 150G, room temperature for 1min.

**Table 2.4 Components of a 15µL reverse transcription reaction**

| <b>Component</b><br>(all from Life Technologies) | <b>Amount (µL)<br/>for 5µL RNA</b> |
|--------------------------------------------------|------------------------------------|
| 100mM dNTPs (with dTTP)                          | 0.15                               |
| MultiScribe™ Reverse Transcriptase (50 U/µL)     | 1.00                               |
| 10× Reverse Transcription Buffer                 | 1.50                               |
| RNase Inhibitor, 20U/µL                          | 0.19                               |
| Nuclease-free water                              | 4.16                               |
| <b>Total</b>                                     | <b>7.00</b>                        |

The RT was performed in a Biometra T3000 Thermocycler System (Biometra GmbH) with the operating programme activating enzyme at 15°C for 30 minutes, followed by incubation at 42°C for 30 minutes and then termination of the reactions at 85°C for 5 minutes. There was no accurate way to determine the concentration of the cDNA but it was estimated on assumption that the reverse transcription reaction was 100% efficient. Hence, the maximal concentration for cDNA was 0.67ng/µL base on the formula below.

$$[c]cDNA = [c]RNA = 10ng/15 uL = 0.67ng/uL.$$

#### 2.13.4.2 Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR was performed in a MicroAmp® optical 96-well reaction plate using the ViiA™7.. Each miRNA of interest was tested in triplicate by adding 1.33uL containing 0.9ng cDNA, mixed with 10.00uL of TaqMan™ 2× Universal PCR Master Mix and 1.00uL of TaqMan® Gene Expression Assay (18µM, 20× primers and probe mix) in a single reaction, which was adjusted to a final volume of 20µL by adding 7.67µL of Ambion® Nuclease-free Water (not DEPC-treated). miR-16 was used as

endogenous control. Data acquisition and analysis was carried out as described in section 2.13.3.

## **2.14 Luciferase assays**

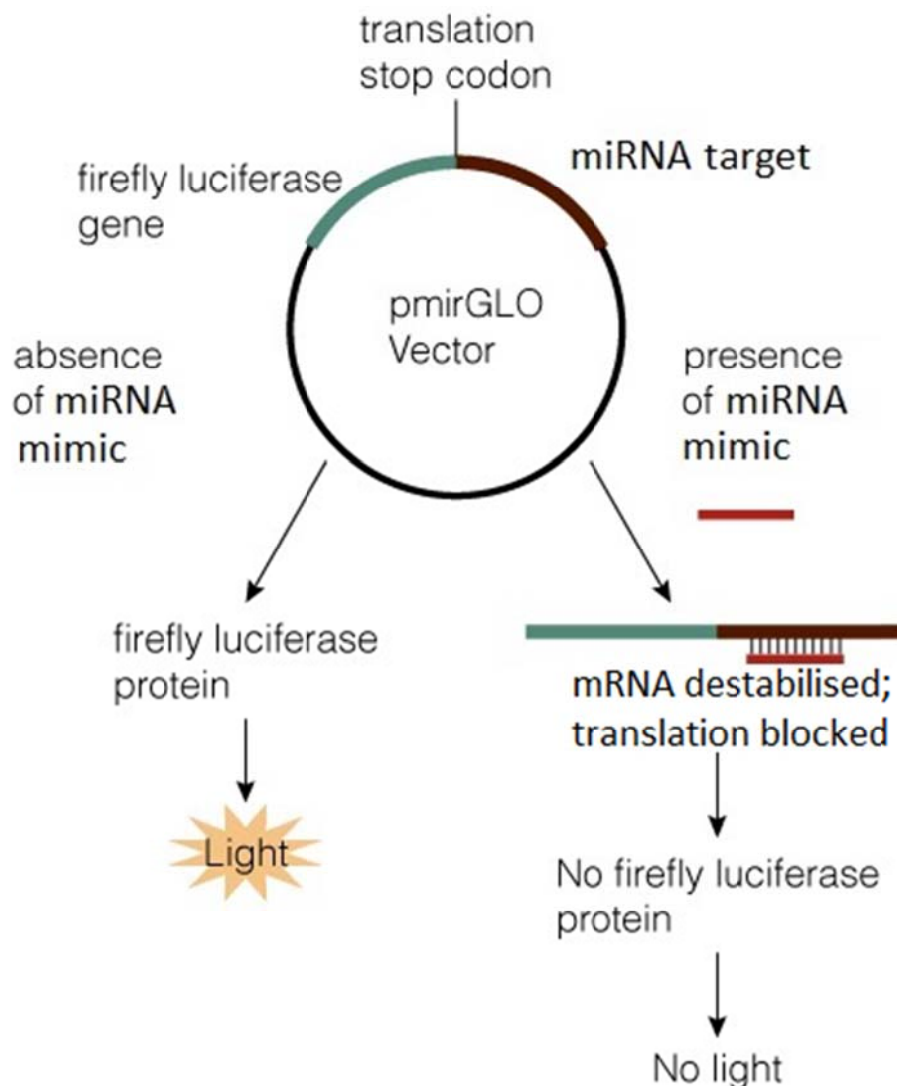
### ***2.14.1 Overview***

The luciferase assay used in this study is an *in vitro* assay designed to investigate the antagonising effect of miRNA on the genes of interest (see Figure 17.1). By coupling the transcriptional regulation to the expression of the reporter luciferase gene, the physical responses to the miRNA binding can be analysed through quantification of the luciferase activity from the reporter which reflects its expression level. A second reporter gene whose expression level will not be affected by miRNA binding is used as endogenous control in order to normalise the expression of the experimental reporter gene.

### ***2.14.2 miRNA target expression vectors***

The 3'-UTR regions of the human *COL1A2*, *BMP1/mTLD*, and *CHRD* genes were cloned downstream the firefly luciferase gene in the pmiRGLO Dual-Luciferase miRNA Target Expression Vector (Promega Ltd.). In brief, the 3'-UTR regions of the above genes were amplified by PCR using human genomic DNA from BMSC as template. The PCR fragments containing the restriction sites for *SacI* and *XhoI* endonucleases were cloned into the pmiRGLO expression vector using the same sites which are present in the multiple cloning site (MCS) (see Appendix1). The recombinant plasmids were verified by DNA sequencing and were kindly supplied by

Dr E. Martin-Rendon, Stem Cell Research Laboratory, Oxford. The plasmid DNA was diluted with Ambion® Nuclease-free Water (not DEPC-treated) to 20ng/μL before use.



**Figure 2.14 Illustration of the luciferase binding assay system.** The putative miRNA binding sequence is cloned downstream the stop codon of the firefly luciferase gene in the pmirGLO plasmid. In the absence of miRNA mimic, the luciferase gene is expressed and the enzymatic activity of the firefly luciferase can be measured by the emission of light. In the presence of miRNA mimic that binds to the target sequence, the translation of the luciferase gene will be blocked resulting in the absence of enzymatic activity and therefore no light will be emitted.

#### ***2.14.3 Transient transfection of HEK293FT cells with plasmid DNA***

HEK293FT cells were cultured, harvested and counted as described before (section 2.3). Cells were seeded in triplicate at  $1 \times 10^4$  cells per well (0.1mL) in a Corning® Costar® clear flat-bottom 96-well plate (Corning Life Sciences) and incubated at 37°C, 21% O<sub>2</sub>, 5% CO<sub>2</sub> overnight (approximately 16-20h) in a Nuaire DH autoflow incubator before transfection took place.

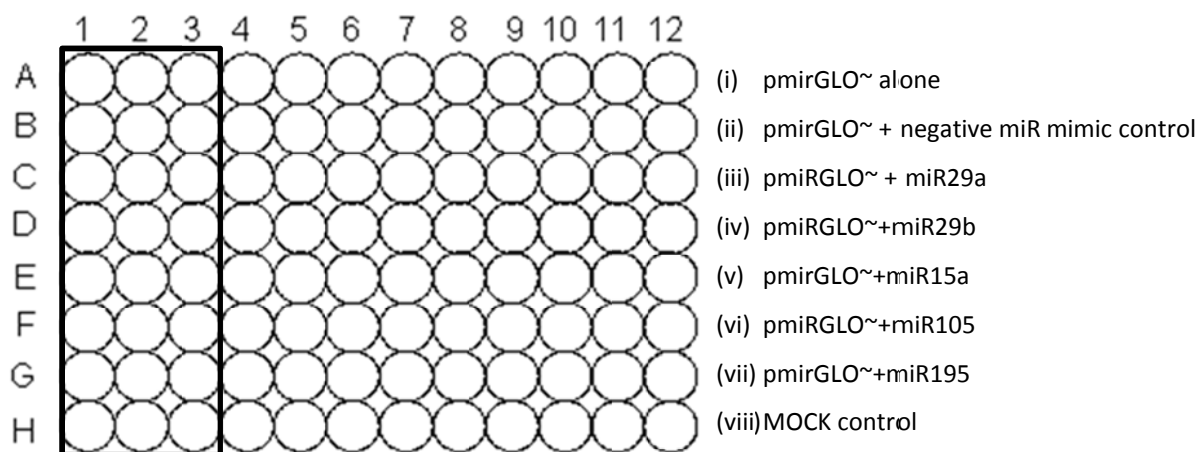
On the following day, the medium was removed from the wells by gentle suction and replaced by 100µL of DMEM serum free transfection mix in each well. The transfection mix was made up of two key components, namely a specific plasmid and DharmaFECT® DUO transfection reagent (Thermo Scientific Dharmacon®), diluted in serum free DMEM medium (See Appendix2). DharmaFECT® DUO transfection reagent, supplied as a 50× concentrated solution was diluted with serum free DMEM to obtain a 1× working solution. Each transfection mix contained the following: 17.5µL plasmid DNA (20ng/µL), 35µL of 1× DharmaFECT® DUO transfection reagent and DMEM serum free medium up to 350µL. Transfection with the reagents alone was used as mock control (see Figure 2.15). Transfected cells were incubated at 37°C, 5% CO<sub>2</sub> for 48 hours before measuring luciferase activity.

#### ***2.14.4 Co-transfection of plasmid vector and miRNA***

HEK293FT were prepared as described above (section 2.2.3) the day before. On the day of the transfection, the media was removed by gentle suction and replaced by 100µL of DMEM serum free media. miRNA mimics and non-targeting control (NTC) miRNA mimics (5nmol) were reconstitute in 250µL nuclease-free water to a stock

solution of 20 $\mu$ M. 1 $\mu$ L of the stock solution was further diluted in 400 $\mu$ L water to a working stock of 50nM for transfection using 1 $\times$  DharmaFECT<sup>®</sup> DUO transfection reagent.

Co-transfections of plasmid DNA and miRNAs were performed in triplicate in a 96-well plate with 350 $\mu$ L of the transfection mix per well: 17.5 $\mu$ L plasmid DNA (20ng/ $\mu$ L), 8.5 $\mu$ L miRNA mimic or NTC mimic (50nM), 35 $\mu$ L DharmaFECT<sup>®</sup> DUO transfection reagent (1 $\times$ ) and DMEM serum free medium up to 350 $\mu$ L. Transfection with the plasmids and reagents alone were used as positive and mock controls, respectively (Figure 2.15). Cells were incubated at 37°C, 5% CO<sub>2</sub> for 48 hours prior to assessing luciferase activity.



**Figure 2.15 Typical layout of co-transfection conditions in a 96-well plate.**

#### ***2.14.5 Luciferase assay***

In this study, the Dual-Luciferase<sup>®</sup> Reporter Assay and Dual-Glo<sup>®</sup> Luciferase Assay Systems (both from Promega) were used. Both systems measure the expression of two independent luciferase genes from two distinct species – the firefly luciferase as experimental reporter gene and the Renilla luciferase as control reporter. The Dual-Luciferase<sup>®</sup> Reporter Assay was used to determine luciferase activity following the cloning of 3'-UTRs downstream the firefly luciferase gene. The Dual-Glo<sup>®</sup> Luciferase Assay was used to assess the effect of miRNA species on the expression of firefly luciferase containing different 3'-UTRs. The latter system is more appropriate for multiple assays due to its stabilised and prolonged luminescence when compared with the Dual-Luciferase<sup>®</sup> Reporter assay system. The stabilised luminescence allows measuring of luciferase activity between 10-30 min after the reagents are added to the cells with no reduction of luciferase activity.

#### ***2.14.6 Dual Luciferase Reporter assay***

The Dual Luciferase Reporter (DLR) assay was performed according to the manufacturer's protocols 48h after transfection. The plasmids used in this study are detailed in Appendix 1.

##### ***2.14.6.1 Preparation of cell lysates***

This procedure allows the passive lysis of cells in multiwell plates. In order to make up 1× Passive Lysis Buffer, 1 volume of 5× Passive Lysis Buffer was added to 4× volumes of distilled water. Transfected HEK293FT cells were washed with 100µL of 1× PBS and 20µL of 1× Passive Lysis Buffer was added to each well of the 96-well

plates. The plates were incubated at room temperature on a gently shaking platform, for 15 min.

#### *2.14.6.2 Preparation of reagents*

Luciferase Assay Reagent II was prepared by mixing the Luciferase Assay Substrate provided with 10mL of Luciferase Assay Buffer II. Stop & Glo<sup>®</sup> Reagent was prepared by diluting 200 $\mu$ L of 50 $\times$  Stop & Glo<sup>®</sup> Substrate into 10ml of Stop & Glo<sup>®</sup> Buffer.

#### *2.14.6.3 Luciferase assay procedure*

The assays for firefly and Renilla luciferase activities are performed sequentially in a single well of a 96-well plate. Approximately 100 $\mu$ L of Luciferase Assay Reagent II were added to 20 $\mu$ L cell lysate and mixed by pipetting 2 or 3 times. The luminescent signal of the firefly luciferase was measured immediately using a Victor<sup>™</sup> X5 Multilabel Plate Reader (Perkin Elmer). The reading lasted for 10 seconds without repetition and the result was recorded before continuing with the measurement of the Renilla luciferase activity in the same sample. The luminescence signal of Renilla luciferase was measured by adding 100 $\mu$ L of Stop & Glo<sup>®</sup> Reagent into the same well and reading it immediately. The procedure was repeated for all the samples and samples were prepared at least in triplicate.

#### **2.14.7 Dual-Glo<sup>®</sup> luciferase assay**

The Dual-Glo<sup>®</sup> Luciferase assay was performed 48h after co-transfection of plasmid DNA and miRNA into the HEK293FT cells in 96-well plates.

#### *2.17.7.1 Preparation of reagents*

Assay reagents supplied with the kit were prepared following the manufacturer's instructions. In brief, the lyophilised Dual-Glo<sup>®</sup> Luciferase Substrate was re-suspended in 10mL of Dual-Glo<sup>®</sup> Luciferase Assay Buffer II (Promega) to make up the Dual-Glo<sup>®</sup> Luciferase Reagent and 100µL of Dual-Glo<sup>®</sup> Stop & Glo<sup>®</sup> Substrate was added to 10ml of Dual-Glo<sup>®</sup> Stop & Glo<sup>®</sup> Buffer to make up the Dual-Glo<sup>®</sup> Stop & Glo<sup>®</sup> Reagent at room temperature.

#### *2.17.7.2 Luciferase assay procedure*

To achieve maximum reproducibility, transfected HEK293FT cells were equilibrated in media at room temperature by adding 75µL of media to each well of the 96-well plate. Following equilibration, 75µL of the Dual-Glo<sup>®</sup> Luciferase Reagent were added to each well. The plates were incubated at room temperature for 10 min prior to measuring the firefly luciferase activity using a Victor<sup>™</sup> X5 Multilabel Plate Reader.

Light was measured in each well for 0.5 second and the whole plate was read three times. Acquired readings were recorded and saved as a MS Excel document. To measure the Renilla luciferase activity, 75µL of Dual-Glo<sup>®</sup> Stop & Glo<sup>®</sup> Reagent was added to each well and the plates were incubated at room temperature for another 10 min to achieve complete quenching effect. The plate was read in the same order as above for three times. The acquired data was also saved as a second MS Excel document.



### 2.14.8 Data analysis

Typical datasets included readings for firefly (reporter) and Renilla (control) luciferase activities. The ratio of luminescence from the reporter to luminescence from the control luciferase was calculated for each well. This is the Response Ratio (RR), and was calculated as follows.

$$RR = \frac{\text{Firefly luminescence intensity}}{\text{Renilla luminescence intensity}}$$

Then the RRs of each experimental sample were standardised to the ratio of control as Relative Response Ration (RRR) using the formula below.

$$RRR = \frac{RR_{test} - RR_{control}}{RR_{positive\ control} - RR_{mock\ control}}$$

The impact of an inhibitor or miRNA on the putative target sequence downstream the firefly luciferase gene was estimated by RRR.

## 2.15 Statistical analysis and multiple regression model

The continuous data were described in the fashion of mean±standard deviation (SD) while the categorical data were expressed in the format of percentage.

For normally distributed data sets with equality of variance, the significance of the difference between two groups was tested against a *t*-distribution, and the difference among multiple groups was tested using one-way analysis of variance (ANOVA), whilst non-parametric tests, including Mann-Witney test and Kruskal-Wallis test, were used for data sets that did not display normal distributions as per data settings. P

value < 0.05 was considered statistically significant, and p value less than 0.01 was considered highly significant. Bonferroni's correction for multiple testing was performed when appropriate.

A multiple regression model was developed to test whether any clinical parameter may be an independent predictor of angiogenesis in a blinded manner. Total tubule length (TTL) obtained in the angiogenesis assay was used as a quantitative dependent variable. However, TTL did not display a normal distribution. In order to satisfy the requirement of approximate normality for fitting a linear model, a Box-Cox transformation of the TTL variable was identified from the profile likelihood of the Box-Cox  $\lambda$  parameter. A multiple regression model was then fitted using the open source programme R 2.12.2 software package with response variable,

$$y = \frac{TTL^\lambda - 1}{\lambda}$$

after calculation by MASS package with TTL against age, sex, NYHA Class, type of disease (including number of diseased coronary arteries), co-morbidity of hypertension, diabetes, and hypercholesterolaemia as independent variables.

The significance of these explanatory variables was tested against a *t*-distribution (Student's *t*-test). The primary selection of significant model candidates was based on the Akaike information criterion (AIC). The adjusted coefficient of determination (R-squared) provided a measure of the variability accounted for by the model, and the analysis was validated by carrying out the usual diagnostics for confirming normality, stability of variance and outlier detection on the model residuals.

### **Chapter 3     The isolation and expansion *ex vivo* of human cardiosphere-derived cells from patients with chronic ischaemic heart disease**

### 3.1 Background

In 2004, the isolation of human cardiac progenitor cells using the ‘cardiosphere’ method was first described by Messina *et al.* [88]. They reported that, using this method, cardiac progenitors could be isolated from the atrium or the ventricular myocardium of patients aged 1-month to 80- years. These cardiac progenitors were expanded *in vitro*, and were able to form 3D cellular aggregates called ‘cardiospheres’ that resemble heart tissue. In 2007, Smith RR *et al.* were able to expand cardiac progenitors even further by culturing cardiosphere-derived cells (CDCs) as monolayers. This added step in the protocol permits the expansion of CDCs from small biopsies to large enough number of cells that can be used for clinical applications. Smith RR *et al.* isolated and expanded CDCs from the endocardium of patients who had a heart transplant. CDCs were described as a mixed population of multi-lineage precursors that expressed the haematopoietic stem cell marker c-kit (CD117), mesenchymal markers such as CD90 and CD105 and cardiomyocyte-specific markers such as TnI, MHC, ANP and Nkx2.5. In addition, CDCs were negative, for CD34, CD45 and Lin. Differentiation of human CDCs into cardiomyocytes that beat spontaneously was achieved by co-culturing them with neonatal rat cardiomyocytes *in vitro*, and their beneficial effect on heart function demonstrated *in vivo* in a rodent model of MI. Nonetheless, the differentiation potential of CDCs for endothelial and smooth muscle cells was also demonstrated *in vivo* [89].

Between 2008 and 2009, two studies reported results that contradicted the previous findings. In the first study, mice explant migrating cells, which would equate to outgrowth cells in the ‘cardiosphere’ method, were found to lack cardiogenic potential

[145]. The second study described that murine CDCs did not contain stem cells with cardiogenic potential [146].

Following the publication of Smith RR *et al.* [89], our laboratory set out to establish the culturing of human CDCs from patients with chronic IHD for clinical applications. Preliminary results obtained by Thomas GN (2009) demonstrated that: (i) CDCs from this patient cohort did not express high levels of c-kit as previously described, (ii) cardiospheres were not 'clonogenic', as they were not able to be generated from single cells, and (iii) specific markers of endothelial and cardiac cells, with the exception of GATA-4, were not expressed in human CDCs or OCs [147]. These strongly contradictory findings raised questions about the origin, phenotype, differentiation and therapeutic potential of human CDCs from patients with IHD.

### **3.2 Aims and objectives**

In order to confirm our primary hypothesis that resident cardiac progenitor cells could be successfully isolated from every IHD patient by the 'cardiosphere' method and they were principally the mesenchymal cells from myocardium that resembled BMSC, the aims of this chapter were to isolate and expand *ex vivo* endogenous cardiac progenitor cells from a population of patients with IHD that could be used in clinical applications. The specific objectives were as follows:

- 1) To recruit a typical cohort of patients to be a sample of the patient population with IHD in the UK and Europe,
- 2) To establish and optimise a reliable method to isolate and culture cardiac progenitors from patients with IHD,

- 3) To identify the cell surface markers of human cardiac progenitors by using flow cytometry,
- 4) To characterise their differentiation potential by using *in vitro* differentiation assays.

### **3.3 Results**

#### ***3.3.1 Patients demographic characteristics***

Participants recruited to this study were adult patients with IHD who were scheduled to undergo cardiac surgery at the John Radcliffe Hospital, Oxford, UK, with Professor David P. Taggart. We continuously approached and recruited appropriate patients on a weekly basis during the course of this study without restrictions. This recruiting method enabled us to 1) include a cohort of patients that was representative of the entire population of severe cardiovascular disease in the UK; 2) minimise the errors in the following analysis caused by the selection bias during recruitment; and 3) compare the biological behaviours of cardiac progenitor cells from IHD patients with that from other non-IHD. Written informed consent was obtained from each patient with ethical approval granted by the Mid/South Bucks Research Ethics Committee (REC number 07/H0607/95) (see Appendix 4). In order to blind the study, each patient sample was anonymised and assigned a study ID number with the matching clinical data being archived to enable the generation of unbiased data in the following stages of the study.

At the time of writing up this chapter, a total number of 68 IHD patients were included and their clinical conditions are detailed in Table 3.1 and summarised in

Table 3.2. Patients participating in this study were between 40 and 84 ( $68 \pm 10$ ) years of age, with an approximate male to female ratio of 4:1. Among all recruited patients, 50 had coronary artery disease (73.5%) and received simple or combined CABG surgery, 21 had aortic valve stenosis (30.9%) and had valve replacement and 3 had other types of disease (4.4%) causing or resulting from myocardial ischaemia in need of open-heart surgery (aortic root aneurysm repair, left ventricle aneurysm repair and hypertrophic cardiomyopathy septum myomectomy, respectively). All patients shared similar features of pathological condition in terms of chronic ischaemia of the myocardium on the basis of either occlusion of the coronary arteries, due to arterosclerosis, or disease of the left ventricle outflow tract at the aortic root, valve and ventricular septum. The extent of heart failure varied on symptoms of dyspnoea with restriction of physical activity. The patients' heart function was classified according to the NYHA Classification: 13 patients (19.1%) were assorted under Class I, 28 (41.2%) under Class II, 25 (36.8%) under Class III and the remaining 2 patients (2.9%) under Class IV. As for co-morbidities, 47 patients also had hypertension (69.1%), 21 combined with type 2 diabetes mellitus (30.9%) and 54 with hypercholesterolaemia (79.4%). Patients were treated with standard medication such as diuretics,  $\beta$ -blockers, ACE-inhibitors/ARB, oral hypoglycaemic agents, and statins. No long-term steroids user was recruited to this study. History of cigarette smoking was observed in 72.0% of the patients, of whom 81.6% were ex-smokers, abandoning the habit before or after the diagnosis of cardiovascular disease. Family history of cardiovascular disease existed in 52.9% of the cases in this study, a majority of which was described as heart attack, except for one case of heart rhythm problem.

**Table 3.1 Demographic characteristics of each patient whose pro-angiogenic potential was tested in this study**

| ID   | Age | Sex | Procedure | NYHA | AS | CAD | N° Diseased Vessels | LAD | LCX | RCA | DM | HBP | HBChol | Smoker  | FHx |
|------|-----|-----|-----------|------|----|-----|---------------------|-----|-----|-----|----|-----|--------|---------|-----|
| Pt08 | 46  | F   | CABG      | I    | N  | Y   | 3                   | Y   | Y   | Y   | Y  | N   | N      | current | Y   |
| Pt10 | 67  | M   | CABG      | III  | N  | Y   | 3                   | Y   | Y   | Y   | N  | N   | Y      | ex      | Y   |
| Pt13 | 56  | M   | AVR       | III  | Y  | N   | 0                   | N   | N   | N   | N  | Y   | Y      | unknown | Y   |
| Pt14 | 57  | F   | CABG      | I    | N  | Y   | 1                   | Y   | N   | N   | N  | Y   | Y      | ex      | Y   |
| Pt23 | 77  | M   | CABG      | IV   | N  | Y   | 1                   | N   | N   | Y   | Y  | Y   | Y      | ex      | U   |
| Pt25 | 72  | M   | CABG      | II   | N  | Y   | 3                   | Y   | Y   | Y   | Y  | Y   | Y      | non     | U   |
| Pt26 | 70  | F   | AVR       | III  | Y  | N   | 0                   | N   | N   | N   | N  | N   | N      | ex      | N   |
| Pt27 | 52  | M   | CABG      | II   | N  | Y   | 3                   | Y   | Y   | Y   | N  | Y   | Y      | non     | N   |
| Pt28 | 76  | M   | CABG      | I    | N  | Y   | 2                   | Y   | Y   | N   | N  | N   | Y      | ex      | Y   |
| Pt29 | 74  | M   | AVR       | II   | Y  | N   | 0                   | N   | N   | N   | N  | Y   | Y      | ex      | Y   |
| Pt30 | 69  | M   | AVR       | I    | Y  | N   | 0                   | N   | N   | N   | N  | Y   | Y      | ex      | Y   |
| Pt32 | 68  | M   | CABG      | II   | N  | Y   | 3                   | Y   | Y   | Y   | N  | Y   | Y      | ex      | N   |
| Pt33 | 63  | M   | CABG      | II   | N  | Y   | 3                   | Y   | Y   | Y   | Y  | Y   | Y      | non     | Y   |
| Pt34 | 68  | M   | CABG      | II   | N  | Y   | 3                   | Y   | Y   | Y   | N  | Y   | Y      | non     | N   |
| Pt35 | 58  | M   | CABG      | II   | N  | Y   | 3                   | Y   | Y   | Y   | N  | Y   | Y      | ex      | Y   |
| Pt37 | 84  | M   | AVR       | III  | Y  | N   | 0                   | N   | N   | N   | Y  | Y   | Y      | ex      | N   |
| Pt39 | 45  | M   | CABG      | III  | N  | Y   | 1                   | Y   | N   | N   | N  | N   | Y      | current | N   |
| Pt40 | 73  | F   | AVR       | III  | Y  | N   | 0                   | N   | N   | N   | Y  | N   | Y      | non     | Y   |
| Pt41 | 68  | M   | AVR       | I    | Y  | N   | 0                   | N   | N   | N   | N  | N   | Y      | ex      | Y   |
| Pt42 | 80  | F   | Other     | I    | N  | N   | 0                   | N   | N   | N   | N  | N   | N      | non     | N   |
| Pt43 | 65  | M   | CABG      | III  | N  | Y   | 1                   | N   | N   | Y   | N  | Y   | Y      | ex      | N   |
| Pt44 | 69  | M   | Other     | II   | N  | N   | 0                   | N   | N   | N   | N  | N   | Y      | ex      | Y   |
| Pt45 | 84  | F   | CABG      | II   | N  | Y   | 2                   | Y   | N   | Y   | N  | Y   | Y      | non     | Y   |
| Pt46 | 48  | M   | CABG      | IV   | N  | Y   | 3                   | Y   | Y   | Y   | Y  | Y   | Y      | ex      | N   |
| Pt47 | 76  | M   | CABG      | II   | N  | Y   | 2                   | Y   | N   | Y   | Y  | Y   | Y      | current | Y   |
| Pt48 | 65  | M   | CABG      | II   | N  | Y   | 2                   | Y   | Y   | N   | N  | N   | Y      | non     | N   |
| Pt49 | 72  | M   | CABG      | III  | N  | Y   | 3                   | Y   | Y   | Y   | N  | Y   | Y      | ex      | N   |
| Pt50 | 40  | M   | Other     | III  | N  | N   | 0                   | N   | N   | N   | N  | N   | N      | current | Y   |
| Pt51 | 72  | F   | CABG      | III  | N  | Y   | 2                   | Y   | Y   | N   | Y  | Y   | Y      | non     | Y   |
| Pt52 | 82  | F   | AVR       | II   | Y  | N   | 0                   | N   | N   | N   | N  | Y   | Y      | non     | Y   |
| Pt54 | 68  | M   | CABG      | II   | N  | Y   | 3                   | Y   | Y   | Y   | N  | Y   | Y      | ex      | N   |
| Pt55 | 81  | M   | AVR       | II   | Y  | N   | 0                   | N   | N   | N   | N  | Y   | N      | non     | Y   |



(Continued)

| ID   | Age | Sex | Procedure | NYHA | AS | CAD | N° Diseased Vessels | LAD | LCX | RCA | DM | HBP | HBChol | Smoker  | FHx |
|------|-----|-----|-----------|------|----|-----|---------------------|-----|-----|-----|----|-----|--------|---------|-----|
| Pt56 | 77  | M   | CABG      | III  | N  | Y   | 3                   | Y   | Y   | Y   | N  | Y   | Y      | ex      | N   |
| Pt57 | 70  | F   | AVR       | III  | Y  | N   | 0                   | N   | N   | N   | N  | Y   | N      | ex      | Y   |
| Pt58 | 71  | M   | CABG      | II   | N  | Y   | 1                   | Y   | N   | N   | N  | Y   | Y      | ex      | N   |
| Pt60 | 61  | M   | AVR       | II   | Y  | N   | 0                   | N   | N   | N   | N  | Y   | Y      | ex      | Y   |
| Pt62 | 60  | M   | CABG      | II   | N  | Y   | 3                   | Y   | Y   | Y   | Y  | Y   | Y      | ex      | N   |
| Pt63 | 57  | M   | CABG      | III  | N  | Y   | 3                   | Y   | Y   | Y   | N  | N   | N      | ex      | N   |
| Pt64 | 76  | F   | AVR       | III  | Y  | N   | 0                   | N   | N   | N   | N  | Y   | Y      | non     | N   |
| Pt66 | 54  | M   | CABG      | III  | N  | Y   | 3                   | Y   | Y   | Y   | Y  | Y   | Y      | current | Y   |
| Pt67 | 76  | M   | AVR+CABG  | II   | Y  | Y   | 1                   | Y   | N   | N   | N  | N   | Y      | non     | N   |
| Pt68 | 68  | M   | CABG      | II   | N  | Y   | 2                   | Y   | Y   | N   | N  | Y   | Y      | ex      | Y   |
| Pt69 | 71  | M   | CABG+Af   | II   | N  | Y   | 3                   | Y   | Y   | Y   | Y  | Y   | Y      | ex      | Y   |
| Pt70 | 69  | M   | CABG      | I    | N  | Y   | 2                   | Y   | Y   | N   | N  | N   | Y      | ex      | Y   |
| Pt71 | 70  | M   | CABG      | II   | N  | Y   | 3                   | Y   | Y   | Y   | N  | Y   | Y      | ex      | N   |
| Pt72 | 64  | M   | CABG      | II   | N  | Y   | 3                   | Y   | Y   | Y   | Y  | Y   | N      | non     | Y   |
| Pt73 | 77  | M   | CABG      | III  | N  | Y   | 3                   | Y   | Y   | Y   | Y  | Y   | Y      | ex      | Y   |
| Pt74 | 74  | M   | AVR       | I    | Y  | N   | 0                   | N   | N   | N   | N  | Y   | Y      | ex      | Y   |

ID- identification, Pt- patient followed by identification number, AS- aortic stenosis, AVR- aortic valve replacement, CABG- coronary artery bypass graft, CAD- coronary artery disease, F- female, LAD- left coronary artery anterior descending branch, LCX- left coronary artery circumflex branch, M- male, NYHA- New York Heart Association, RCA- right coronary artery, DM- diabetes mellitus, HBP- hypertension, HBChol- hypercholesterolaemia, FHx- family history, TTL- total tubule length, Y- yes, N- no, U- unknown.

**Table 3.2 Summary of the demographic characteristics (n=68).**

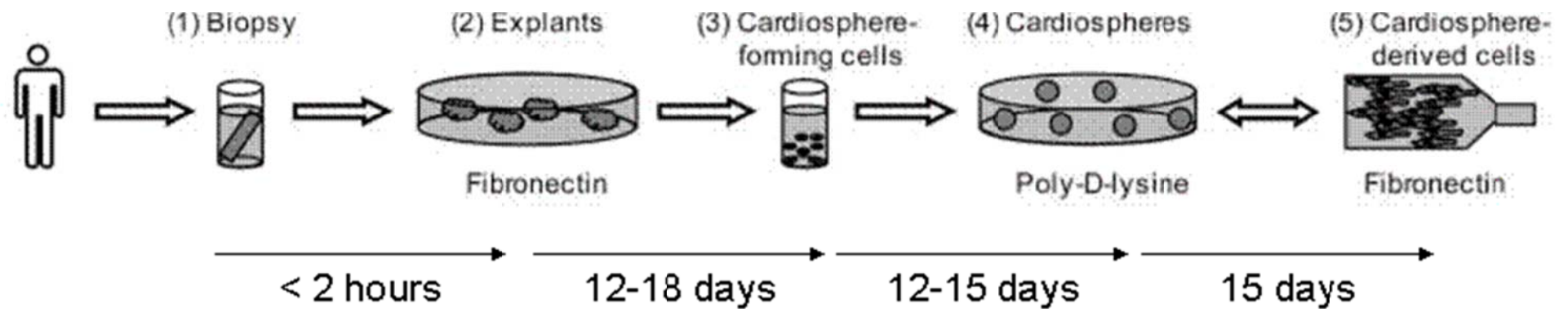
| <b>Factor</b>                     | <b>Subgroup</b> | <b>Mean <math>\pm</math>SD</b> |
|-----------------------------------|-----------------|--------------------------------|
| <b>Age at surgery (yrs)</b>       |                 | 68 $\pm$ 10 (68)               |
| <b>Gender</b>                     | Male            | 79.4% (54)                     |
|                                   | Female          | 20.6% (14)                     |
| <b>NYHA classification</b>        | Class I         | 19.1% (13)                     |
|                                   | Class II        | 41.2% (28)                     |
|                                   | Class III       | 36.8% (25)                     |
|                                   | Class IV        | 2.9% (2)                       |
| <b>Procedure</b>                  | CABG            | 64.7% (44)                     |
|                                   | AVR             | 22.1% (15)                     |
|                                   | CABG + AVR      | 8.8% (6)                       |
|                                   | Other           | 4.4% (3)                       |
| <b>Number of diseased vessels</b> | 0               | 26.5% (18)                     |
|                                   | 1               | 14.7% (10)                     |
|                                   | 2               | 17.6% (12)                     |
|                                   | 3               | 41.2% (28)                     |
| <b>Coronary Artery Disease</b>    | Total ischaemia | 73.5% (50)                     |
|                                   | Ischaemic RCA   | 52.9% (36)                     |
|                                   | Ischaemic LAD   | 69.1% (47)                     |
|                                   | Ischaemic LCX   | 51.5% (35)                     |
| <b>Aortic Stenosis</b>            |                 | 30.9% (21)                     |
| <b>Family History</b>             | Yes             | 52.9% (36)                     |
|                                   | No              | 41.2% (28)                     |
|                                   | Unknown         | 5.9% (4)                       |
| <b>Diabetes mellitus</b>          |                 | 30.9% (21)                     |
| <b>Hypertension</b>               |                 | 69.1% (47)                     |
| <b>Hypercholesterolaemia</b>      |                 | 79.4% (54)                     |
| <b>Smoker</b>                     | No              | 26.5% (18)                     |
|                                   | Yes             | 13.2 % (9)                     |
|                                   | Ex-smoker       | 58.8% (40)                     |
|                                   | Unknown         | 1.5% (1)                       |

AVR, aortic valve replacement; CABG, coronary artery bypass graft; LAD, left anterior descending artery; LCX, left circumflex artery; NYHA, New York Heart Association, Class I= asymptomatic, Class II= mild limitation, Class III= moderate limitation, Class IV= severe limitation; RCA, right coronary artery.

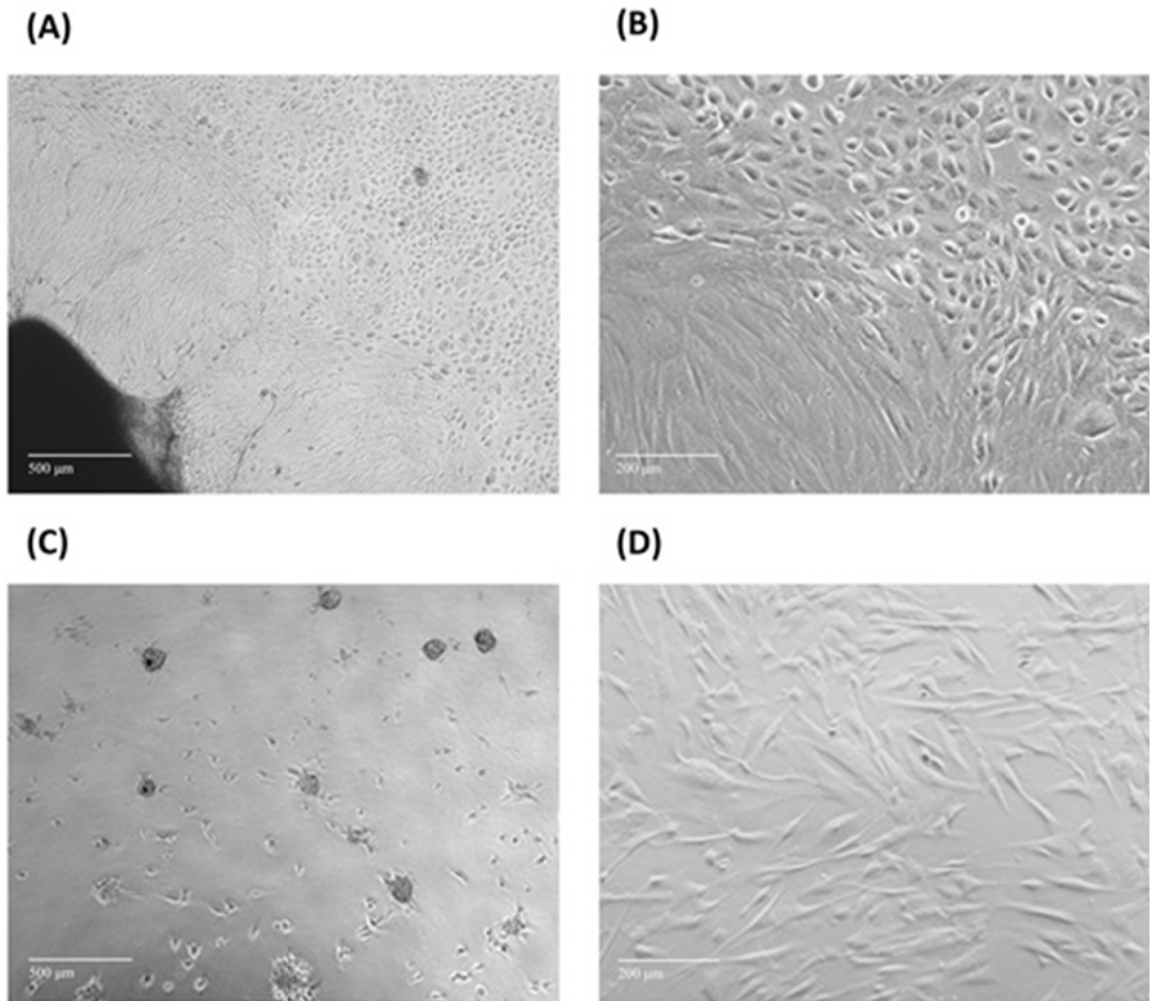
### **3.3.2 Generation of human CDCs**

Following the methods described in chapter 2 (section 2.3.1), heart biopsies were collected from the right atrial appendage during cardiac surgery and processed within 2 hours (Figure 3.1). The standard protocol established previously by us was followed [90] and CDCs were cultured and expanded *in vitro* from all 68 biopsies within 4-6 weeks (Figure 3.1).

Explants up to 1mm<sup>3</sup> of size were seeded onto fibronectin-coated wells (Figure 3.2A). Typically, slim and paving-stone like OCs began to appear from the edge of the explants from Day 6 to 7 after seeding. The OCs proliferated and migrated until the cell monolayer covered the whole surface of the well (Figure 3.2B). The OCs were harvested and the cell suspension was seeded onto poly-D-lysine coated wells in which cells aggregated to form 3D spheres, called 'cardiospheres' (Figure 3.2C). Although the seeding density of OCs onto the poly-D-lysine wells was the same for all samples, the starting time of multicellular clustering ranged between 24h and 1 week. By the end of the second week from seeding OCs, a majority of cardiospheres became semi-detached in the culture medium. No synchronised spontaneous contraction of cardiospheres was observed. The loosely attached cardiospheres were harvested and CDCs (Figure 3.2D) were expanded for two passages prior to further characterisation.



**Figure 3.1 Process of CDC isolation and expansion** (adapted from [31]). The right atrial biopsy was collected from patients with ischaemic heart disease during cardiac surgery and cut into small explants before being seeded onto fibronectin coated plates within 2 hours following removal. After incubation at 37°C for 12-18 days, the outgrowth cells (OCs) were harvested and transferred to wells coated with poly-D-lysine. The cells aggregated into clusters named ‘cardiospheres’ within 12 to 15 days. Cardiospheres were then harvested and seeded into fibronectin-coated flasks for cell expansion as cardiosphere-derived cells (CDCs) which took another 15 days to achieve confluency.

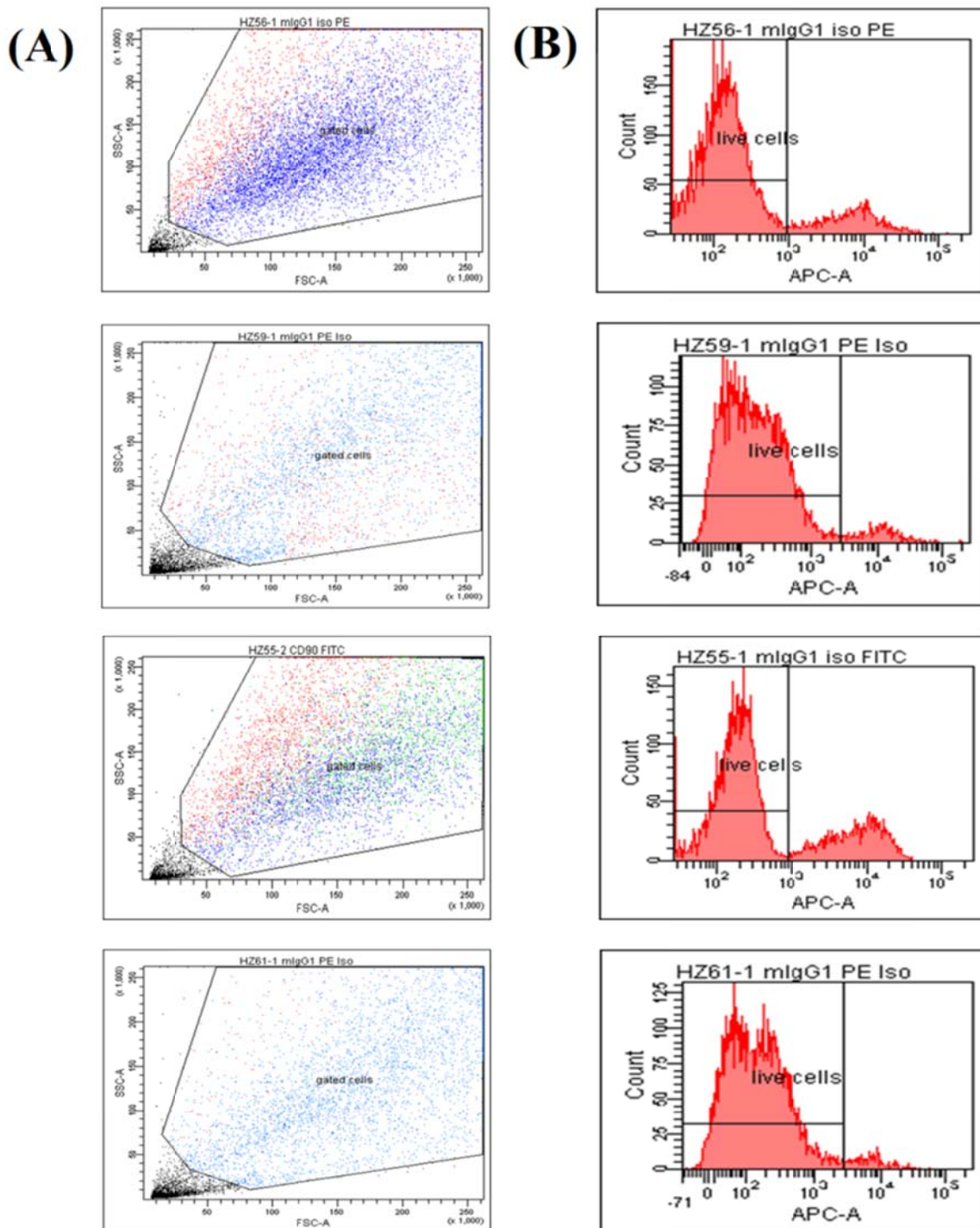


**Figure 3.2 Isolation and culture of CDCs.** Representative images of (A) the border of cardiac explants seeded onto fibronectin-coated wells (magnification 6 $\times$ ), (B) outgrowth cells (OCs) (magnification 15 $\times$ ), (C) cardiospheres formed in Poly-D-lysine coated plates (magnification 6 $\times$ ) and (D) cardiosphere-derived cells (magnification 15 $\times$ ) growing on fibronectin-coated plates (scale bar=500 $\mu\text{m}$  for 6 $\times$  magnification; scale bar=200 $\mu\text{m}$  for 15 $\times$  magnification ).

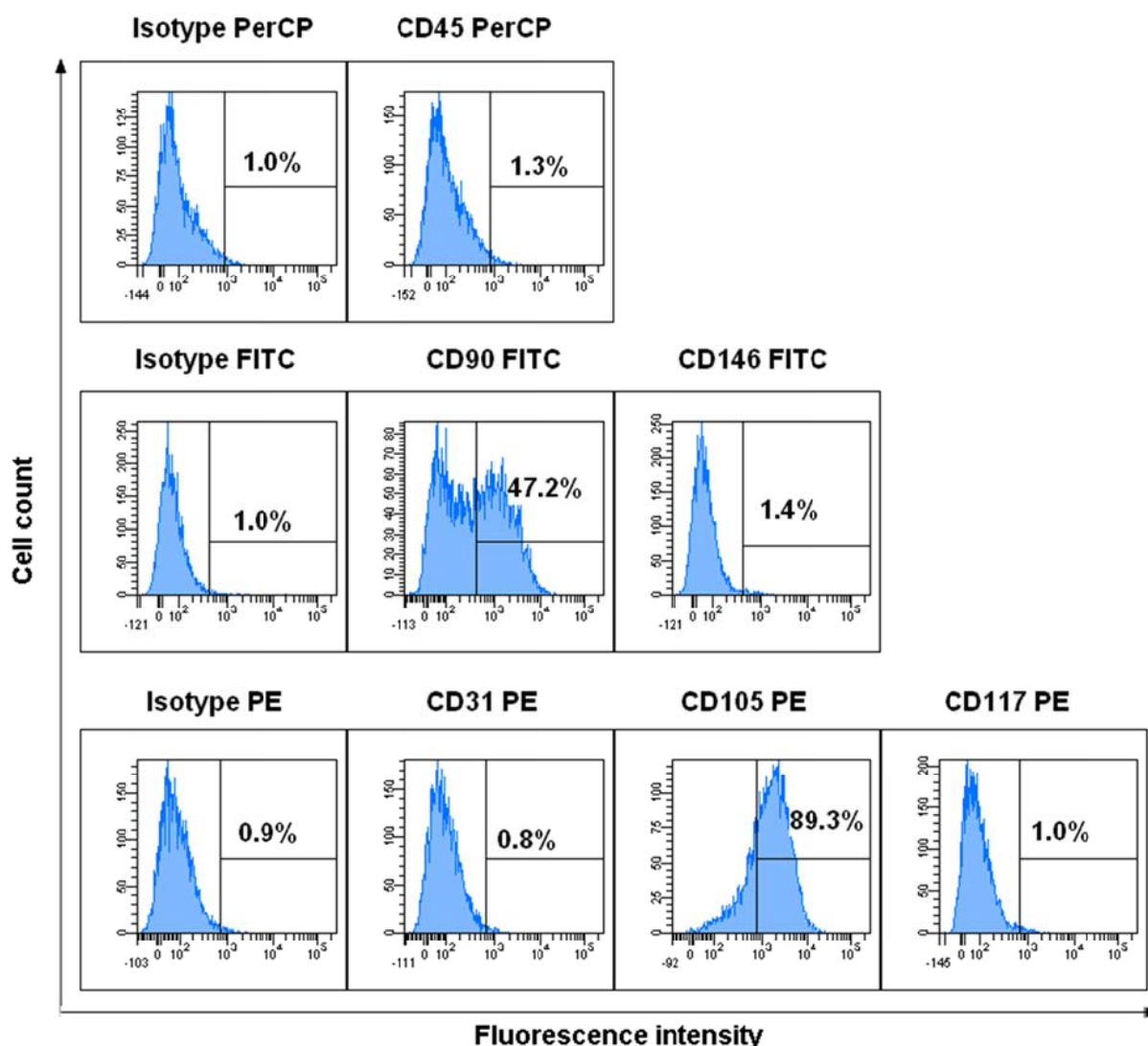
### ***3.3.3 Phenotypic characterisation of CDCs***

Flow cytometric analysis was used to determine the expression of cell surface markers in CDC samples in comparison with BMSC. The CDC population was a highly heterogeneous group of cells, which could hardly be defined by the forward- and side-scattering of light alone (Figure 3.3A). The viable cell population of interest was gated according to the forward-scatter versus the side-scatter dot-plots excluding the non-viable cells and debris as shown in figure 3.3A. Non-viable cells stained by To-pro3 had high fluorescence intensity in the APC channel whilst debris had a low forward scatter (see Figure 3.3). The non-viable cells represented typically less than 10% of the total cell population (see Figure 3.3B). Similar gating strategy was used for BMSC.

CDCs expressed high levels of mesenchymal cell surface markers including CD90 (43% with Median Fluorescence Intensity (MFI) of 1431) and CD105 (88%; MFI= 2036) as the BMSC control cells do, although the MFI of CD90 and CD105 was significantly lower in CDCs than in BMSC (see Table 4.3). In contrast, the expression of the stem cell marker CD117/c-kit appeared to be very low (only present in 1% of the CDC population). Similarly, the endothelial markers CD31 and CD146 and the haematopoietic marker CD45 were detected in only 0.9%, 1.4% and 1.0% of CDCs with MFI of 766, 506 and 754, respectively. Thus, the expression of cell surface markers confirmed the mesenchymal origin of CDCs.



**Figure 3.3 Event definition and live cell gating in four CDC samples.** The CDC population is highly heterogeneous thus did not show distinction in cell size or granularity. (A) Representative scatter plots from four CDCs samples. The entire cell population on the forward scatter (FSC)-side scatter (SSC) plots was gated for analysis excluding the cell debris which showed a low FSC (in black). (B) Representative corresponding histograms from four CDC samples. Live cells were stained with To-pro3 and detected in the APC channel. Typically, live cells (To-pro3-negative cells) have a significant lower fluorescent intensity and represent approximately 90% of the entire population. The non-viable cells (To-pro3-positive) were excluded from the analysis.



**Figure 3.4 Immunophenotype of CDCs.** Representative histograms of cell surface marker expression in CDCs determined by flow cytometry. Cells were stained with either the relevant isotype control or test antibodies. The threshold of the isotype control antibodies was set up at approximately 1%. Strong expression of the mesenchymal cell marker CD105 was detected in around  $87.8 \pm 9.2\%$  of CDCs from patients with ischaemic heart disease with a median fluorescence intensity of  $2036 \pm 442$ , whereas CD90 was expressed in  $42.8 \pm 18.0\%$  of cell population with a moderate MFI of  $1431 \pm 367$ . In contrast, low level (MFI less than 1000) of endothelial cell markers (CD31, CD146), the haematopoietic cell marker CD45 and stem cell marker CD117/c-kit expression was found on very small proportions of CDCs (approximately 1%).



The comparison between test antibodies and their corresponding isotype controls on the gated live cells were performed for each fluoro-chrome and each sample. Representative histograms are shown in Figure 3.4 and a summary of all the data is detailed in Table 3.3.

**Table 3.3 Summary of cell surface marker expression on CDCs analysed by flow cytometry.**

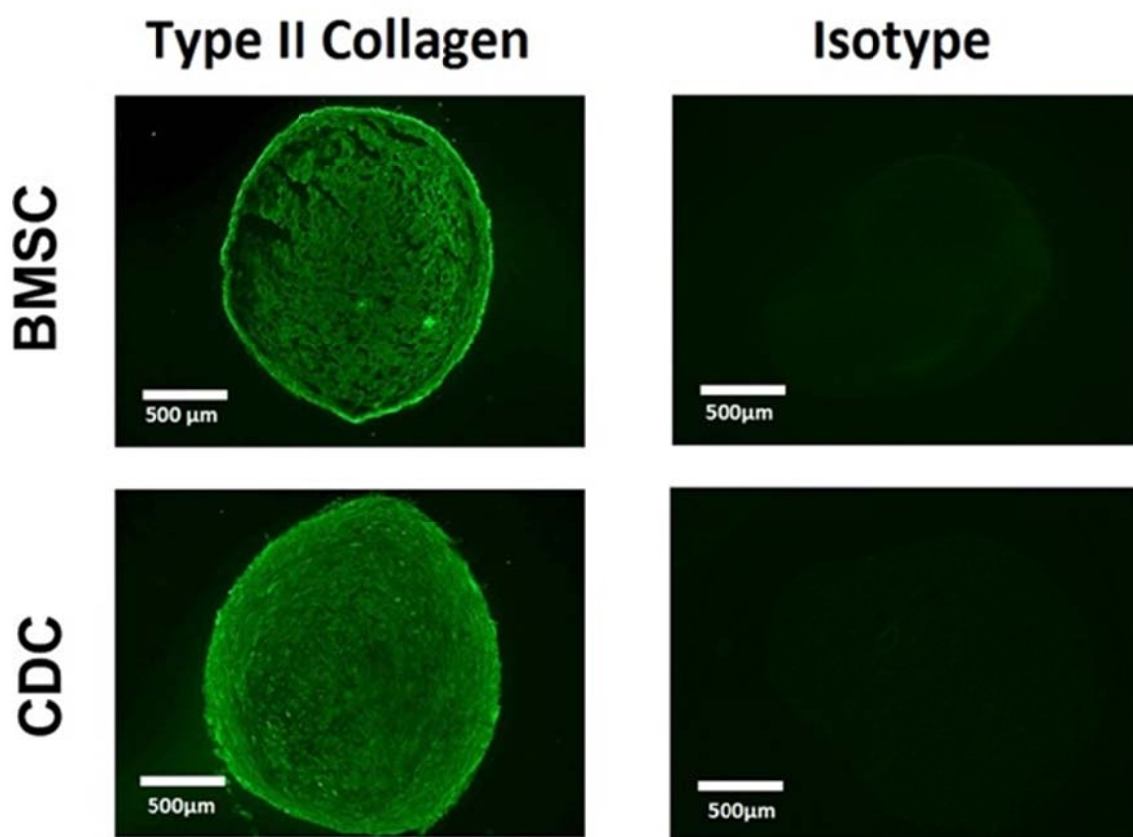
| Surface Marker | Definition     | CDC (n=4) |           | BMSC (n=3) |           |
|----------------|----------------|-----------|-----------|------------|-----------|
|                |                | MFI       | %         | MFI        | %         |
| CD31           | endothelial    | 798±136   | 0.9±0.4   | 727±57     | 1.5±0.5   |
| CD45           | haematopoietic | 754±503   | 1.0±0.3   | 665±194    | 1.2±0.3   |
| CD90           | mesenchymal    | 1431±367  | 42.8±18.0 | 10018±2571 | 92.8±7.2  |
| CD105          | mesenchymal    | 2036±442  | 87.8±9.2  | 8228±4928  | 93.8±4.6  |
| CD117          | haematopoietic | 702±286   | 0.9±0.3   | 564±150    | 0.8±0.8   |
| CD146          | endothelial    | 506±47.4  | 1.4±0.0   | 1006 ±352  | 28.3±27.6 |

### **3.3.4 *In vitro* differentiation assays**

BMSC can differentiate into the three mesenchymal cell lineages *in vitro*, and this is one of the criteria used to define mesenchymal stem/progenitor cells [148]. Therefore, differentiation of CDCs into the chondrogenic, adipogenic and osteogenic lineages was performed and compared to that of BMSC.

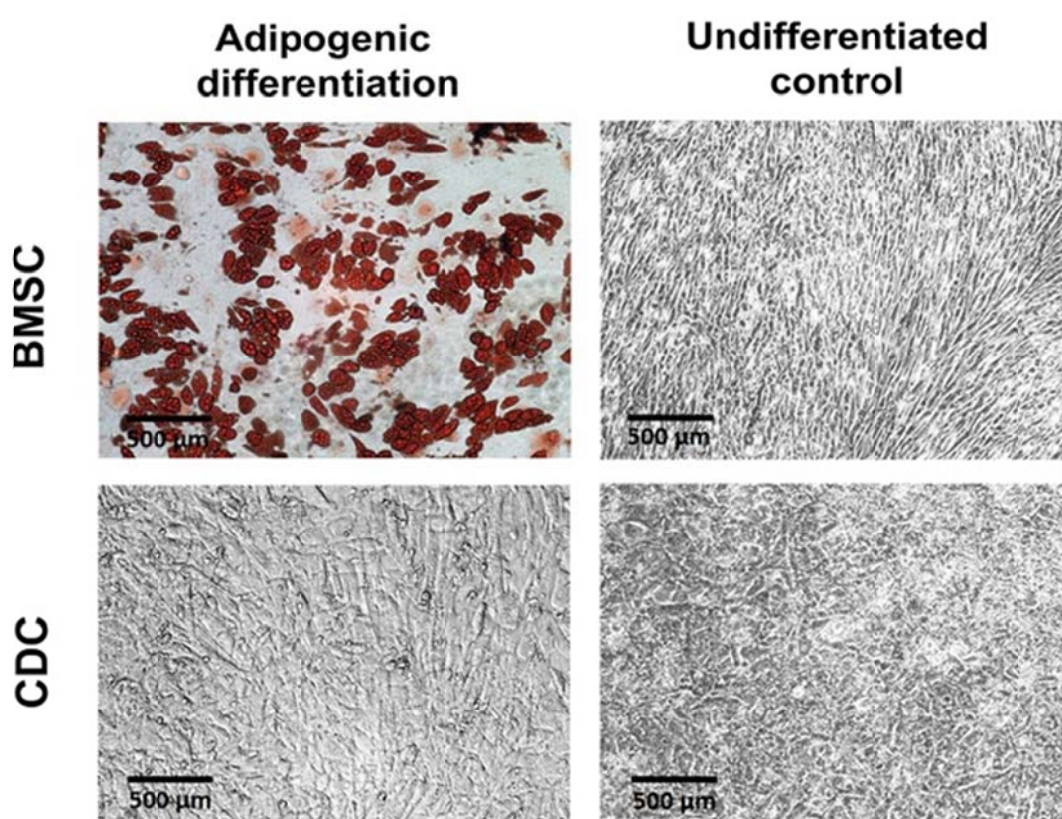
For chondrogenic differentiation, cell pellets (n=8) were incubated *in vitro* with complete chondrogenic medium supplemented with TGF-β3 following the methods described in chapter 2 (section 2.5.3), with BMSC as positive control. Following differentiation, the snap-frozen cell pellets were sectioned and stained for Type II Collagen, as a marker of chondrogenesis. Like BMSC, all CDC samples consistently showed positive

immunoreactivity for type II collagen (Figure 3.5). This confirms that CDCs can differentiate into chondrocytes *in vitro*.



**Figure 3.5 Type II collagen staining following chondrogenic differentiation (4× magnification).** BMSCs (n=3) and CDCs (n=8) were induced to differentiate into the chondrogenic lineage *in vitro*. Cells were harvested by centrifugation and cell pellets incubated in complete chondrogenic medium containing TGF- $\beta$ 3. Following induction, cell pellets were frozen, sectioned and the sections incubated with goat anti-collagen type II IgG or goat IgG isotype control. The bound antibody was detected by incubation with a rabbit anti-goat AlexaFluor<sup>®</sup> 488-conjugated secondary antibody. BMSC and CDC cell pellets stimulated by TGF- $\beta$ 3 supplemented medium showed strong green fluorescence compared to the isotype controls, suggesting that CDCs, like BMSC, could differentiate into chondrocytes and form type II collagen rich cartilage tissue given chondrogenic differentiation signals (scale bar=500μm).

CDCs (n=4) and BMSC (n=3), were cultured under adipogenic induction conditions according to the methods detailed in chapter 2 (section 2.5.1). Following induction, cells were stained with Oil red O to assess the presence of lipids in the cells. BMSC became confluent and most cells showed accumulation of lipid vacuoles stained in red under the light microscope. In contrast, CDCs became confluent but no lipid droplets were formed in the cells following differentiation and staining (Figure 3.6).



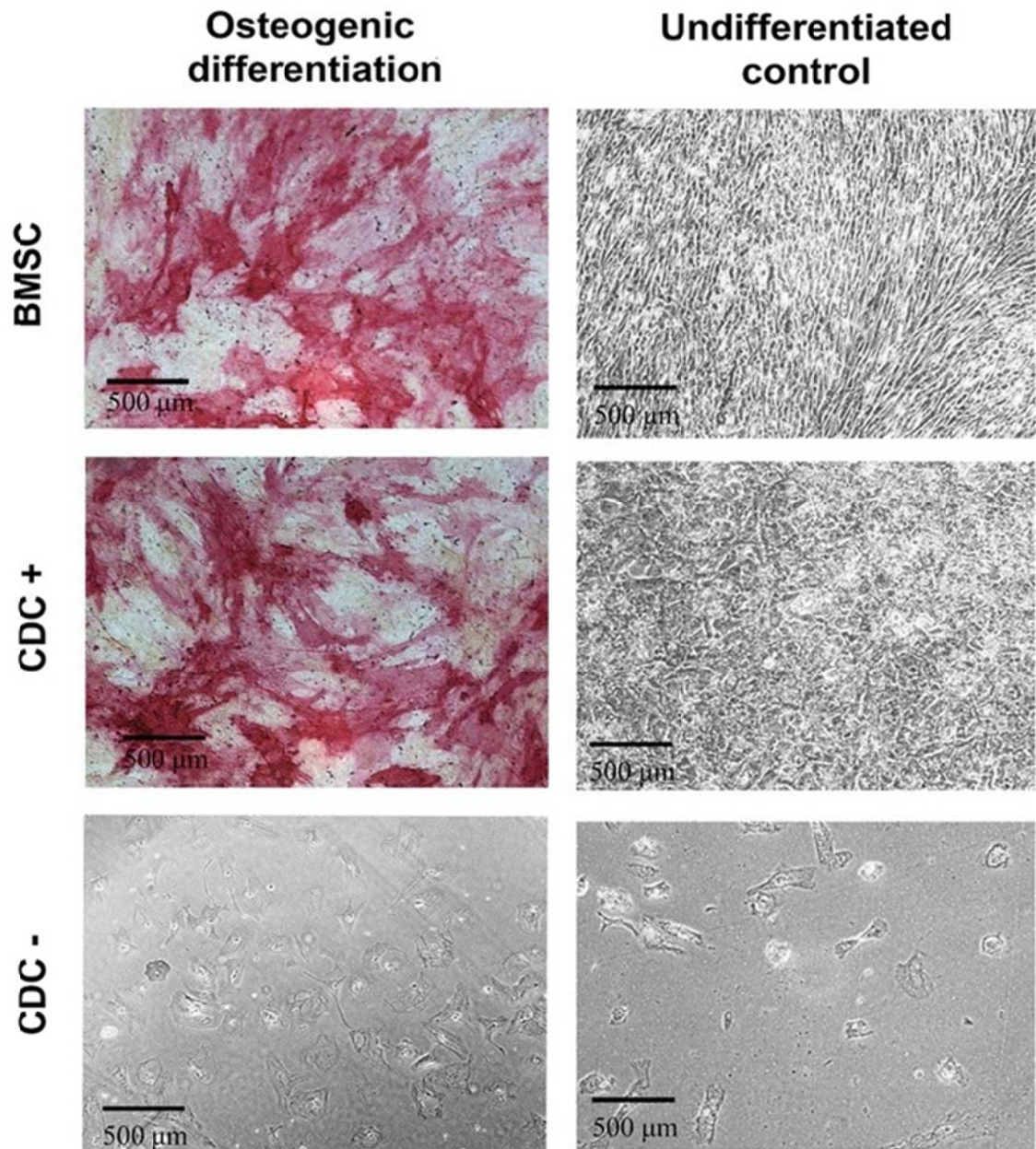
**Figure 3.6 Lipid staining following adipogenic differentiation (4× magnification).** BMSCs (n=3) and CDCs (n=4) were subjected to three cycles (18 days) of adipogenic differentiation in vitro. Following differentiation, cells were visually checked using light microscope and stained by Oil Red O to detect the formation of lipid droplets. BMSCs stimulated by adipogenic induction medium showed different cell morphology and red-stained lipid vacuoles whereas CDCs showed little difference from the non-stimulated control and lack of adipogenic differentiation potential (Scale bar=500µm).

The osteogenic differentiation potential of CDCs (n=10), with BMSC (n=3) as control, was also tested as described in chapter 2 (section 2.5.2). BMSC adhered easily and proliferated well in the induction media. Cells started to change morphology from spindle-shape to cubical-shape when they became confluent, usually happening at the beginning of the second week of induction, and cell delamination occurred as they became over confluent. The staining for alkaline phosphatase showed extensive pink dye under the light microscope, suggesting that a majority of BMSC differentiated into osteoblasts *in vitro* under the conditions tested (Figure 3.7).

Interestingly, CDCs demonstrated a varied potential of osteogenic differentiation. Several of the CDCs showed similar pattern to BMSC, adhering, growing and differentiating into the shape of osteoblasts and were positive for alkaline phosphatase staining. Whereas other samples showed poor adhesion and growth leading to premature delamination and little evidence of osteogenic differentiation (negative for alkaline phosphatase staining) on the few cells that remain in the dish (Figure 3.7).

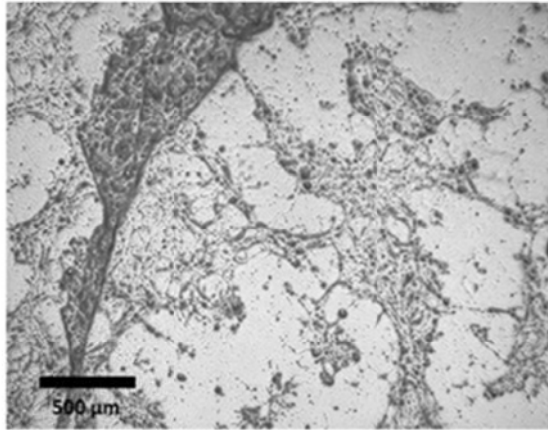
As the osteogenic differentiation conditions may be too harsh for CDCs, modifications to the original protocol were made to improve the osteogenic differentiation of the latter group of CDCs. First, cells were seeded on fibronectin-coated dishes to resemble the standard culture conditions of CDCs, and second, the CDCs seeding density was doubled in the osteogenic assay. Unfortunately, none of these modifications tested on their own or combined, resulted in a better osteogenic differentiation of CDCs (See Figure3.8). Thus, a conclusion can be drawn that a proportion of CDC samples have lost their osteogenic differentiation potential.



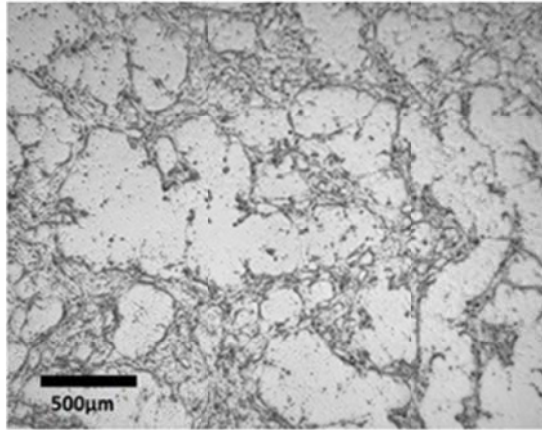


**Figure 3.7 Staining for alkaline phosphatase following osteogenic induction (magnification  $\times 4$ ).** Representative images of BMSCs ( $n=3$ ) and CDCs ( $n=10$ ) following osteogenic differentiation *in vitro*. Alkaline phosphatase staining (in pink) was used to detect the formation of osteoblasts in the cultures following differentiation. BMSC stained strongly positive for alkaline phosphatase following differentiation, compared to the undifferentiated control cultures. The osteogenic potential varied among individual CDC samples. Several samples (CDC+) showed strong positive staining for alkaline phosphatase as observed in BMSCs, whilst others (CDC-) showed significantly poor ALP staining with little pink staining (scale bar= $500\mu\text{m}$ ).

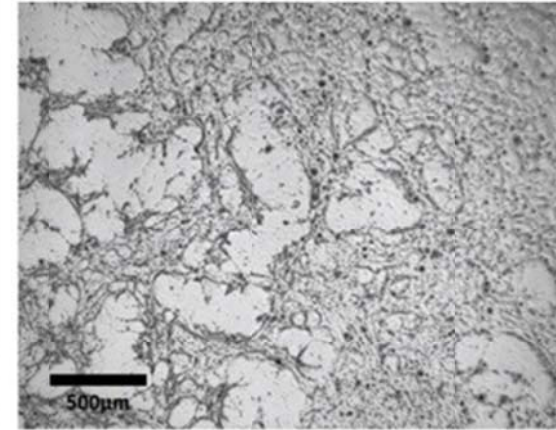
(A) Increased cell seeding density



(B) Fibronectin coated well



(C) Increased density on fibronectin coated well



**Figure 3.8 Modification of culture conditions for osteogenic differentiation (magnification  $\times 4$ ) and the outcome of ALP staining.** (A) CDCs with poor osteogenic potential were seeded at  $6 \times 10^4$  cells per well (double the standard seeding density) under osteogenic inducing conditions. (B) CDCs with poor osteogenic potential were seeded at  $3 \times 10^4$  cells per well on fibronectin coated plates. (C) CDCs with poor osteogenic potential were seeded at  $6 \times 10^4$  cells per well on fibronectin coated plates. Following differentiation all cultures were stained with alkaline phosphatase. No alkaline phosphatase staining was observed under these conditions at the time of delamination. Modifications of the culturing conditions either increasing the cell density, coating the plates with fibronectin or combining both did not improve osteogenic differentiation of CDC(-).

### ***3.3.5 Clonogenic capacity***

CDCs were seeded as described in Chapter 2 (section 2.6). All CDCs tested (n=6) had a low clonogenic capacity, with less than 1% of the cell population being able to grow into small colonies. The expression of Ki67, a mitotic marker, was also below 1% in P2 CDCs (data not shown).

## **3.4 Discussion**

This chapter demonstrated that CDCs isolated from IHD patients resemble BMSC. However, the adipogenic differentiation of these cells was not proven, and some of CDCs seem to have also lost their ability to differentiate into osteoblasts *in vitro* from our preliminary results.

### ***3.4.1 Isolation and expansion of CDCs from patients with IHD***

The cohort of patients recruited to this study was highly representative of the current population suffering from severe IHD in the UK and Europe. Therapies that involve manipulating resident cardiac stem cells would further boost the benefits of revascularisation on cardiac viability, both anatomically and functionally [149].

This study confirms that cardiospheres and CDCs can be efficiently and consistently generated from human myocardial biopsies *in vitro* according to previous protocols [88–90]. CDCs were successfully isolated from 68 patients suffering from IHD undergoing cardiac surgery. CDCs have now been isolated and expanded from the endocardium, as well as the epicardium, and from the atrium, from patients with transplanted hearts, chronic IHD or

congenital heart disease (Table 6.1) regardless of age, gender, severity of disease, co-morbidities and the presence of cardiovascular risk factors.

Our previous study showed that CDCs can be routinely expanded from full thickness atrial biopsies from this cohort of patients, whilst only 36% of the left ventricular epicardial biopsies yielded CDCs [90]. A similar observation has been reported by Smith RR *et al.* [89]. Taking this into consideration, all 68 biopsies in the present study were taken from the right atrial appendage. Firstly, right atrial (RA) biopsies were technically easy to obtain by a specialist cardiac surgeon from every single patient with relatively low rate of complications or minor ethical concerns, regardless of the cardiac procedure performed (e.g. aortic valve replacement or CABG). Secondly, the quality of the myocardial sample was consistent compared to the ventricular biopsies as the human ventricular wall is considerably thicker than the atrial wall to be able to sample precisely. Gross connective and adipose tissue underneath the epicardium was removed, leaving mainly myocardial tissue. Finally, all RA biopsies yielded OCs, cardiospheres and CDCs.

In conclusion, the RA appendage seems to be a reservoir of CDCs that could be routinely isolated and expanded for future clinical applications.

#### ***3.4.2 Cell surface marker expression and differentiation potential of CDC from IHD patients***

The phenotype of CDCs isolated from the RA appendage of patients with IHD is consistent with a heterogeneous population of mesenchymal progenitors of cardiac origin. CDCs are positive for CD90 and CD105 and negative for endothelial and haematopoietic markers.



This is in agreement with previous data obtained by us and others [90], [92], [150]. These cells also express the cardiac transcription factor GATA-4 and low levels of MEF2C, [147].

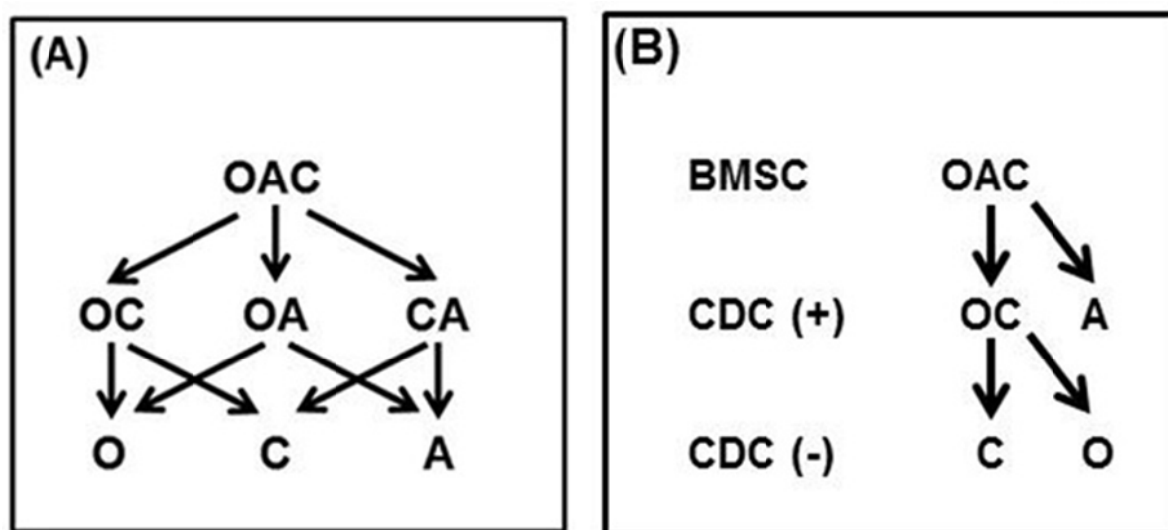
Whereas the high proportion of CD105+ cells in the CDC population is not disputed, the CD90+ and CD117/c-kit+ fractions varied significantly between studies (Table 6.1). The expression of CD90 in CDC seems to be dependent on cardiovascular risk factors such as diabetes [90]. Here we also show that there are fewer CD117/c-kit+ cells ( $0.9 \pm 0.3\%$ ) than previously described (10%-30%), [89], [93]. CD117/c-kit expression in rat OCs has been shown to vary during culture, with a peak of expression around 21 days following plating [95]. We observed a decrease in the number of CD117/c-kit+ cells between the OCs and CDCs [90] which is consistent with the CD117/c-kit marker being lost during the expansion of CDCs as shown previously [91]. In addition, Mishra *et al.* described the decrease of CD117/c-kit+ CDC generated from RA with age among young patients suffering from congenital heart disease [92]. In contrast, Itzhaki-Alfia *et al.* showed a particularly high level of CD117/c-kit+ CDCs ( $31 \pm 4\%$ ) originated from LV biopsies from a mixed group of adult patients undergoing cardiac surgery in Israel. These patients were suffering from end-stage heart failure and had severely compromised LVEF ( $< 15\%$ ) [93]. In conclusion, CD117/c-kit expression seems to be reduced in culture and with age and is increased in the presence of heart failure symptoms.

The mesenchymal nature of the CDC population isolated in this study encouraged assessment of their differentiation potential. CDCs were induced to differentiate into the three mesenchymal cell lineages *in vitro* and were compared to BMSC. Clearly, CDCs have lost the potential to give rise to adipocytes. This is in agreement with a previous study by R. Koninckx *et al.* who described that CDCs were able form chondrocytes *in vitro*, but not

adipocytes or osteocytes [91]. Although present in almost 90% of CDCs, CD105 fluorescence intensity was lower in CDCs than in BMSC, suggesting a lower expression of the receptor on the cell surface. CD105 expression level has been reported to be an indicator of adipogenic ability [151], so the findings in the current study are consistent with CDCs losing their adipogenic differentiation potential. In the present study, all CDC samples could give rise to chondrocytes *in vitro*, but some of them were not capable of forming osteoblasts.

BMSC from young healthy donors represent a heterogeneous population containing stem/progenitor cells with trilineage differentiation potential: A, adipocytic; O, osteoblastic and C, chondrocytic differentiation potential (see Figure 3.9). We hypothesised that the CDC population may lose their adipogenic and osteogenic differentiation potential gradually and become bipotent and subsequently unipotent progenitors and that this process may be influenced by age and/or disease. In the present study, CDCs from patients with IHD may represent these bipotent (OC) and unipotent (C) cell sub-populations in the proposed hierarchy of mesenchymal stem/progenitor cells, suggesting that they have a more limited differentiation potential. It remains to be demonstrated whether this limited differentiation potential *in vitro* may affect their therapeutic potential *in vivo* if used as treatment for IHD. Meanwhile, it is still worth comparing the limited ability of CDC differentiation between individuals, and also between the CDC and BMSC from the same patient by using quantitative methods, such as qPCR, to confirm these preliminary findings and ascertain that the differentiation potential would be associated with difference in cell origins more than individuals.

In conclusion, CDCs from IHD patients are a population of cardiac mesenchymal progenitors with variable mesenchymal lineage differentiation potential. In addition, this study confirms that CDCs from this patient cohort had a low clonogenic capacity (<1%) and slow proliferation. The difference in the mesenchymal lineage differentiation potential did not seem to be caused by the cells' clonogenic or proliferative capacity.



**Figure 3.9 Hierarchy of mesenchymal stem/progenitor cells.** (A) Proposed hierarchy of mesenchymal stem/progenitor cells, where a tri-lineage progenitor population would give rise to bipotent and unipotent subpopulations of cells. MSC such as bone marrow-derived mesenchymal stem/progenitor cells at the top of hierarchy have tri-lineage differentiation potential (OAC) *in vitro*. By losing differentiation potential of any one mesenchymal lineage, mesenchymal progenitors become bipotent progenitors (OC, OA or CA) and may further lose differentiation potential of another lineage, which turns them into unipotent progenitors (O, C or A). (B) Schematic representation of the proposed loss of differentiation potential by CDCs. BMSC has OAC tri-lineage differentiation potential and ranks highest in the hierarchy. CDC (osteo+) loses adipogenic potential but still possesses OC bi-lineage differentiation potential, which is superior than the unipotent CDC (osteo-) population that loses both adipogenic and osteogenic potentials and ranks lowest in the hierarchy.

## **Chapter 4 Pro-angiogenic and therapeutic potential of cardiosphere-derived cells from patients with chronic ischaemic heart disease**

## 4.1 Background

CDCs are a novel type of resident cardiac derived stem/progenitor cells that have recently emerged as a cell-based therapy to treat MI [59]. They are thought to exert their regenerative function in the ischaemic heart tissue through at least four possible mechanisms including: 1) a paracrine effect to preserve cell viability and prevent apoptosis, 2) a pro-angiogenic mechanism to achieve revascularisation and optimal reperfusion of the ischaemic myocardium, 3) a beneficial impact on remodelling the extracellular matrix decreasing scarring and providing a favourable regenerative environment, and 4) a cardiomyogenic mechanism to generate contractile cardiomyocytes to compensate for ischaemic injury.

At the start of this study, the cardiogenic potential of CDCs was considered to be their main mode of action, and the one responsible for the improvement in cardiac function in a rodent model of heart disease [89]. During the course of this thesis, evidence has become available to suggest that CDCs exert their function via an indirect paracrine fashion. In mice, transplanted CDCs have been shown to release factors that mediate protection against the death of cardiomyocytes, the promotion of cardiomyocyte proliferation and contractility and the regeneration of new blood vessels [21], [152–156]. Li *et al.* recently reported that CDCs may have a greater therapeutic potential than other stem/progenitor cell populations, including BMSC, adipose tissue MSC (AMSC), bone marrow mononuclear cells (BM-MNC) and a purified CD117/c-kit<sup>+</sup> subpopulation of CDCs [98].

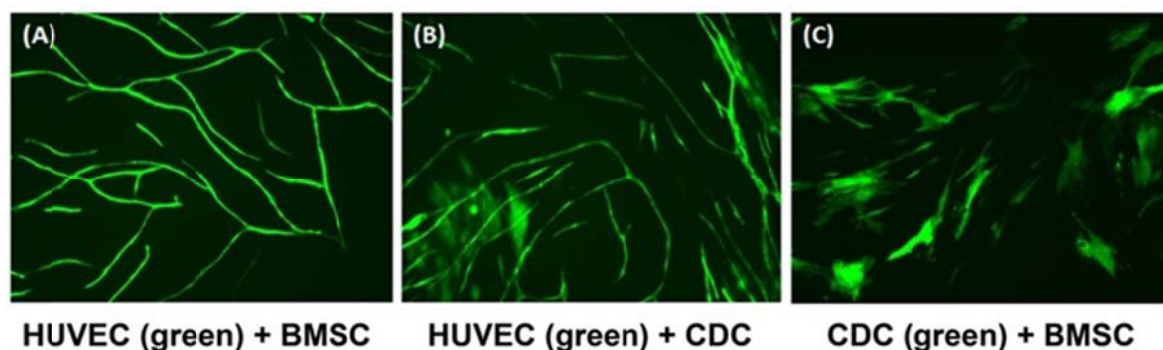
Chapter 3 of this Thesis showed that CDCs from patients with IHD expressed mesenchymal cell surface markers similar to BMSC and had bipotent or unipotent

mesenchymal lineage differentiation ability. *In vitro*, BMSC have been shown to support a robust network of blood vessels by endothelial cells [157], [158], raising the possibility that CDCs might also foster angiogenesis to the same extent as BMSC. In animal models of acute and chronic MI, an increased capillary density following BMSC transplantation has been associated with improvement in heart function [17], [23], [159], [160]. In addition, Yoon *et al.* demonstrated very elegantly in mice that the depletion of perivascular supportive cells, but not the endothelial cell lineage, negatively affected the regenerative potential of bone marrow stem cell populations and the reversal of LV dysfunction [161].

There is growing evidence that stromal supportive cells play an essential role in the generation of new blood vessels. Currently, a wide range of *in vitro* and *in vivo* models are available to assess angiogenesis. The most commonly used methods include the tubule-forming assay using Matrigel<sup>TM</sup>, the rat aortic ring and co-cultures of endothelial and stromal cells *in vitro*, the chick chorioallantoic membrane, the rabbit cornea model, the zebrafish or the skin fold chamber *in vivo* (For review, see [162]). CDCs do not express endothelial markers, and unlike HUVEC, are not able to form tubular structures when supported by BMSC [147]. However, similar to BMSCs, CDCs can support blood vessel formation as shown previously by others in this laboratory (Figure 4.1).

Although reliable and quick, the classical Matrigel<sup>TM</sup> assay mainly designed to assess the endothelial cell performance in angiogenesis was not entirely suitable for this study; hence we developed a more relevant assay based on the co-culture of endothelial cells and CDCs modifying previous protocols [163]. *In vitro* assays are relative easy to conduct and quantification of vessel network is straightforward. However, caution has to be taken to select the appropriate assays and to interpret the results as to whether they would reflect the

*in vivo* situation [164]. The aim of this study was to investigate the pro-angiogenic potential of CDCs from different patients. Consequently it was important to establish a robust *in vitro* angiogenesis assay to assess potential angiogenic potency and to predict their therapeutic potential *in vivo*.



**Figure 4.1 Vascular supportive function of mesenchymal stem/progenitor cells.** Like BMSC (A), CDCs can support GFP-labelled HUVEC (green) to form a robust tubular network *in vitro* in co-cultures (B), but they are unable to form vascular networks when co-cultured with BMSC (C) (Courtesy of G.N. Thomas).

The vascular supportive ability of CDCs therefore also needs to be tested *in vivo*. Standard experimental models of MI in mice, rats, rabbits, dogs and pigs, involve the induction of MI by surgical occlusion of the left coronary artery [165]. Recently, chronic coronary artery occlusion models such as implanting ameroid constrictors to dogs [159], and gradual occlusion of coronary arteries in coronary atherosclerosis-prone WHHL rabbits have also been developed successfully [166]. Transplantation of human cells requires that the animals are immunodeficient, in order to retain administered human cells for the duration of the study. The rodent (rat) model of ischaemic cardiomyopathy used in this thesis had been previously established by Dr Pilar Sepulveda's group with the aim of testing the therapeutic potential of human BMSC and CD34+ BMMNC [141], [143]. In this model, MI was induced by permanent ligation of the left anterior descending (LAD) coronary artery in adult athymic nude rats (*HHH-Foxn1<sup>rnu</sup>*) as described elsewhere [141], [143]. No primary

coronary revascularisation was performed in the MI rats. Seven days post-MI, cells were transplanted in the peri-infarct region at several sites. Increase in vessel density and improvement in heart function were the primary outcomes measured in the transplanted animals.

## **4.2 Aims and objectives**

The last chapter suggested that CDC resembled BMSC in phenotype, and were likely to be cardiac MSC; however, CDC samples from different individual patients might have different differentiation potential into the mesenchymal lineages. We hypothesised that CDCs from IHD patients could support neovascularisation but variation among the patient population would probably exist, which might be predicted by individual patient's clinical background and disease status, and more importantly, be associated with the therapeutic outcome. Therefore, the aims of this chapter were to determine the pro-angiogenic and therapeutic potential of CDCs from patients with IHD isolated and expanded as described in chapter 3 and to compare it with BMSC. The specific objectives are as follows:

- 1) To design and establish a simple, but efficient, *in vitro* angiogenesis model that would allow medium-to-high throughput testing of the pro-angiogenic ability of CDCs,
- 2) To establish the best parameters to quantitate angiogenesis in the above established model,
- 3) To characterise the therapeutic potential of CDCs from IHD patients in an experimental model of MI,
- 4) To design a statistical model to identify independent clinical predictors of angiogenesis supported by CDCs.



## 4.3 Results

### 4.3.1 Establishment of the *in vitro* model of angiogenesis

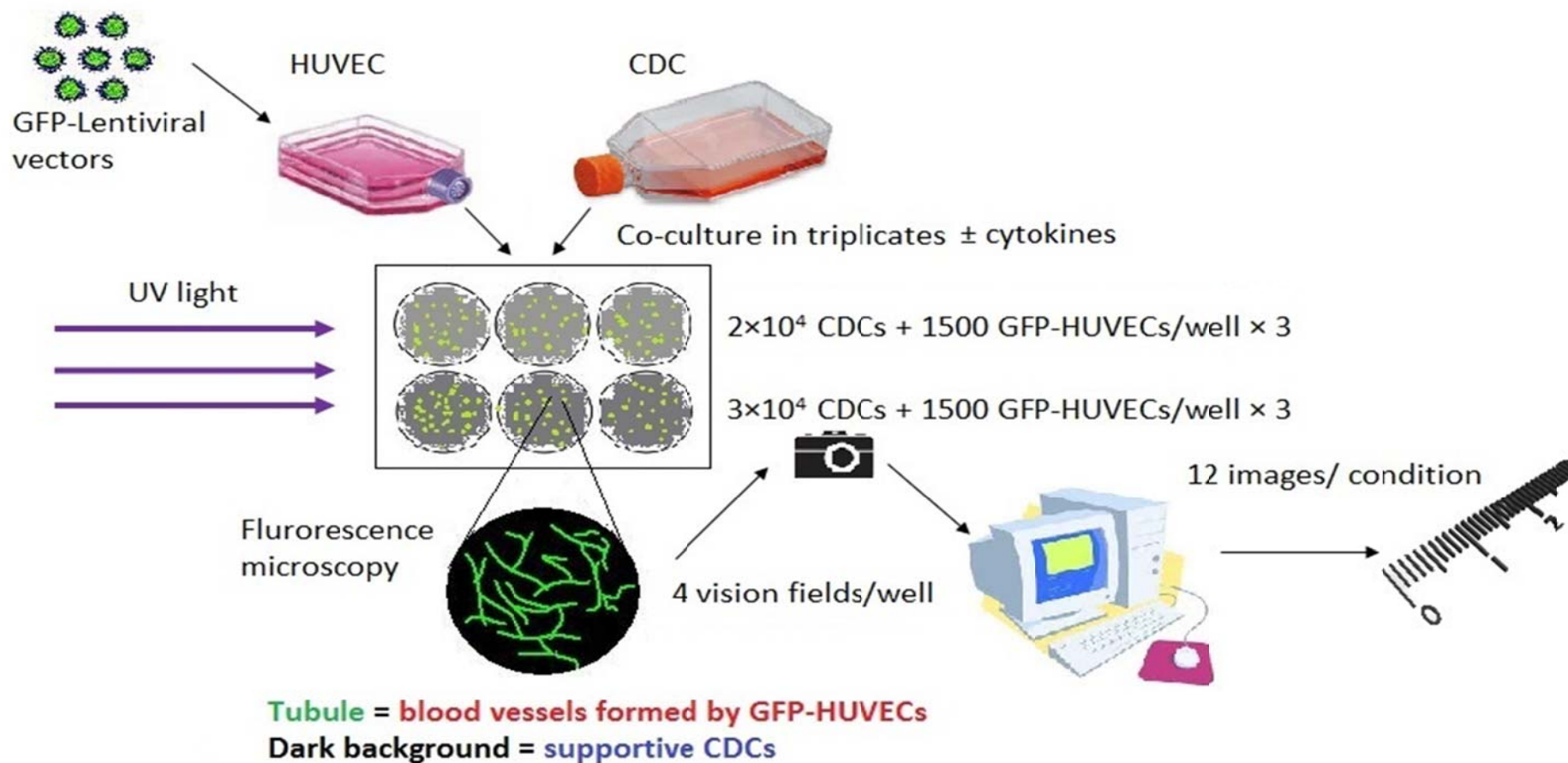
Detailed characterisation of human CDCs in Chapter 3 of this thesis have demonstrated that these cells do not express markers of endothelial cells. Additionally, and consistent with the results presented in chapter 3, preliminary experiments by George Thomas *et al.* from this laboratory [147] have shown that human CDCs are not able to form tubular structures in an *in vitro* model of angiogenesis (Figure 4.1). Because of the similarities with BMSC, we hypothesised at the beginning of this study that CDCs would be able to support the formation of tubular structures by endothelial cells such as HUVEC.

A relevant angiogenesis assay was established in this study to assess the pro-angiogenic potential of human CDCs (as described in section 2.8 of Materials and methods). The assay was based on previously described protocols [163] but in this study modifications had to be made in order to optimise the culturing conditions for the tubular growth supported by CDCs. First, the co-cultures were seeded on fibronectin-coated plates in order to maintain them for 14 days. Coating the plates with other extracellular matrix component, such as collagen I, did not produce optimal vessel network formation and the cultures were not stable after 7-10 days, as the monolayer of cells would ‘peel off’, making it impossible to monitor tubule formation beyond that time. Second, seeding densities and cell ratios had to be optimised for BMSC and CDCs, as the previously described model used human dermal fibroblasts [163]. Third, HUVEC were genetically marked to express GFP to allow the monitoring of tubule formation over time with live cells.

For this purpose a bank of GFP-labelled HUVEC was established by transducing three batches of P2 HUVEC from single donors with lentiviral vector particles expressing GFP (LV-GFP) at an MOI of 3. Typical titers of the LV-GFP stocks were in the range of  $6 \times 10^6$  to  $2 \times 10^7$  transducing units per mL. At the MOI used, over 98% of HUVEC were transduced with no detriment to cell viability or proliferation. Additionally, four batches of P4 BMSC were used to test the ability of the GFP-labelled HUVEC to form tubule networks and to use as controls. In all subsequent experiments with CDCs, the co-culture of GFP-HUVEC and BMSC was used as an internal positive control in the experiment. The assay was optimised to seed  $1 \times 10^4$ ,  $2 \times 10^4$  or  $3 \times 10^4$  stromal cells (BMSC or CDC) and  $1.5 \times 10^3$  GFP-HUVEC and three wells were seeded for each cell density (see Figure 4.2).

Monitoring of the co-cultures under the fluorescent microscope was originally carried out at day 4, 7, 10 and 14. A total of 12 images ( $4 \times$  images/well) were recorded for each condition. Adobe Photoshop CS package was used to process the images according to section 2.8.3 before the processed images were finally analysed by the Angiosys v1.0 software [167]. Quantitation of parameters such as number of junctions, number of tubules and total tubule length (TTL) were performed from each set of 12 images. Generally these parameters were proportional to each other and the decision was made to take TTL as the best parameter to reflect overall angiogenesis as it measures all tubules formed as part of the network. The TTL relative to the control co-cultures of GFP-HUVEC and BMSC (represented as 100%) was also determined and defined as relative tubule length or RTL.

The majority of CDC samples tested were unable to support tubule formation at a seeding density of  $1 \times 10^4$  CDC/well. GFP-HUVECs grew randomly in large clusters. In contrast,

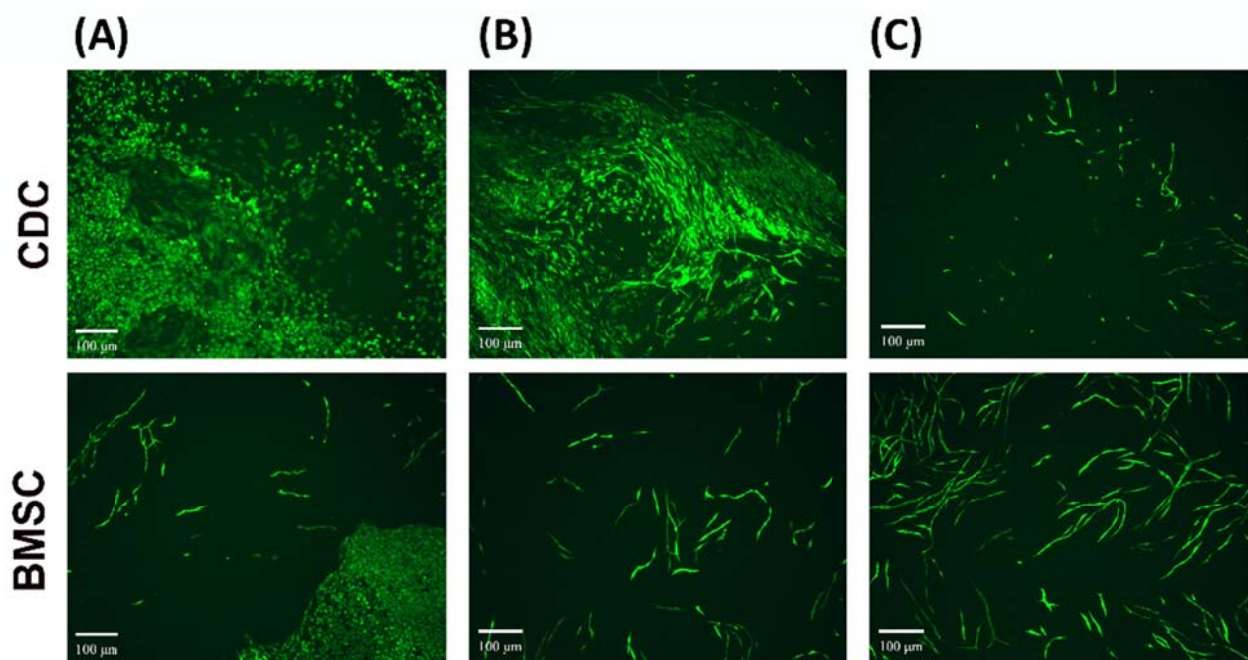


**Figure 4.2 Angiogenesis assay using the co-culture of GFP-HUVEC and CDCs.** Lentiviral vector (LV) particles expressing GFP were produced by transient transfection in HEK293T cells. Typically LV particles at  $5 \times 10^6$  to  $2 \times 10^7$  transducing units/mL were used to transduce HUVEC at a multiplicity of infection (MOI) of 3. GFP-labelled HUVECs were co-cultured with increasing densities of CDCs in triplicate. After 2 weeks in culture, the capillary tubule network was visualised under the fluorescence microscope and images taken and processed using the Angiosys software.

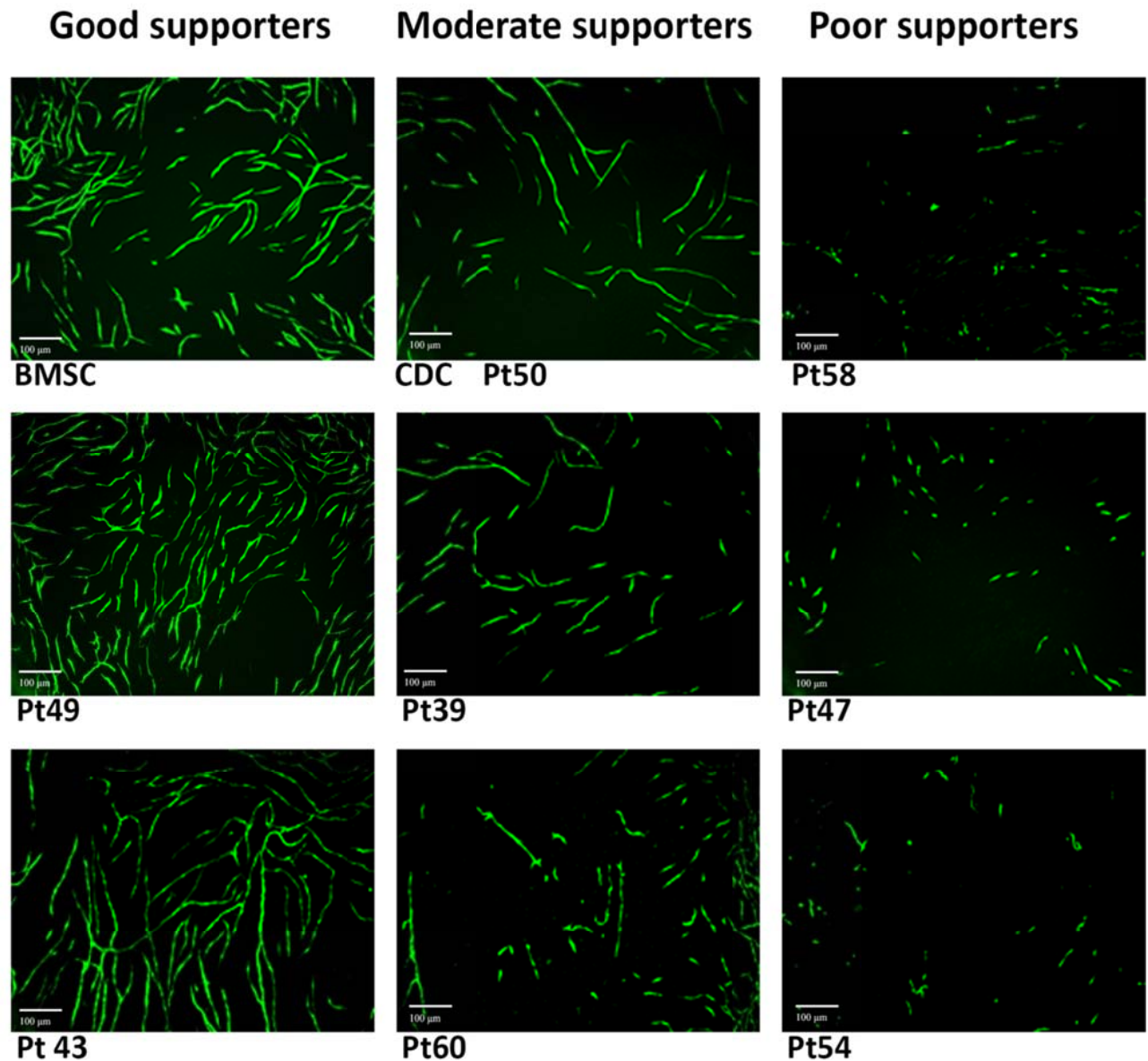
BMSCs could support the formation of some tubular structures at this seeding density, but not always a well-defined tubule network, as HUVEC clusters could also be seen (see Figure 4.3A). As the seeding density doubled to  $2 \times 10^4$  CDCs, some CDC samples were able to support GFP-HUVECs vessel network. The same trend was observed with the control BMSC, where the total tubule length (TTL) formed by GFP-HUVEC increased at this seeding density (see Figure 4.3B). At  $3 \times 10^4$  of either CDCs or BMSCs, clusters of GFP-HUVECs were not observed. Instead, a network of tubular structures was formed with both CDCs and control BMSC (Figure 4.3C and Figure 4.4). Therefore it was selected as the best stromal cell density to co-culture with  $1.5 \times 10^3$  HUVEC to obtain optimal vessel network in the established angiogenesis assay. Interestingly, CDCs' pro-angiogenic potential varied and this was evident even from the first 5 CDCs samples tested in the angiogenesis assay (Figure 4.4).

#### ***4.3.2 Pro-angiogenic ability of CDCs from patients with IHD.***

Once the angiogenesis assay was optimised, the ability of CDCs isolated from 43 patients to support the formation of tubular structures reminiscent of blood vessels was assessed. For each patient sample, TTL and RTL were recorded as a mean value (see Table 4.1). In brief, these results indicate that different patient samples support angiogenesis to a different degree (Figure 4.4). CDCs were grouped in tertiles according to their ability to support GFP-HUVEC tubule formation in vitro. Those categories were based on TTL and RTL: Good supporters of angiogenesis (1<sup>st</sup> tertile), Moderate supporters (2<sup>nd</sup> tertile) and Poor supporters (3<sup>rd</sup> tertile) (Table 4.1). CDCs from 14 patients could support angiogenesis with RTL over 60%, whilst 14 could support a moderate network of tubular structures and 15 CDC samples showed poor support of angiogenesis in vitro with RTL less than 11% (see Table 4.2, Figure 4.5A).



**Figure 4.3 Establishing the angiogenesis assay.** Representative images of  $1.5 \times 10^3$  of GFP- labelled HUVEC co-cultured with  $1 \times 10^4$  cells per well (A),  $2 \times 10^4$  cells per well (B) or  $3 \times 10^4$  cells per well (C) of stromal/supportive cells. At lower densities of stromal cells (BMSC or CDCs), HUVEC did not form a robust tubule network. Instead extensive monolayers of endothelial cells grew in the wells, which made accurate quantification difficult. However, at  $3 \times 10^4$  cells per well, both CDCs and BMSC were able to support a significant tubular network for precise automatic quantification by using the AngioSys Software. (scale bar = 100  $\mu\text{m}$ ).



**Figure 4.4 Pro-angiogenic ability of human CDCs from patients with IHD (4× magnification).** Representative images of tubule formation of  $3 \times 10^4$  human CDCs from different patients co-cultured with  $1.5 \times 10^3$  GFP-labelled HUVEC in endothelial cell growth media for 14 days. The total tubule length (TTL) compared with control supportive cells BMSC was recorded as relative tubule length (RTL). CDCs were grouped in three categories (Good, Moderate and Poor) according to their ability to support GFP-HUVEC tubule formation represented by TTL. Pt, represents patient identification number given in this study. (scale bar = 100  $\mu$ m)

**Table 4.1 Total and relative tubule length estimated for all CDCs samples included in this study.**

| Cell Type | ID                   | TTL   | RTL  | Group                               |
|-----------|----------------------|-------|------|-------------------------------------|
| BMSC      | 8F3543<br>23/11/2010 | 6894  | 1.00 | control                             |
| CDC       | Pt49                 | 8701  | 1.62 | 1st tertile<br>(good supporter)     |
| CDC       | Pt46                 | 6906  | 1.28 |                                     |
| CDC       | Pt23                 | 6420  | 1.14 |                                     |
| CDC       | Pt08                 | 13500 | 1.00 |                                     |
| CDC       | Pt10                 | 14000 | 0.98 |                                     |
| CDC       | Pt43                 | 9873  | 0.95 |                                     |
| CDC       | Pt48                 | 5072  | 0.94 |                                     |
| CDC       | Pt56                 | 8559  | 0.88 |                                     |
| CDC       | Pt13                 | 5500  | 0.80 |                                     |
| CDC       | Pt64                 | 6475  | 0.98 |                                     |
| CDC       | Pt68                 | 6401  | 0.97 |                                     |
| CDC       | Pt57                 | 7554  | 0.78 |                                     |
| CDC       | Pt41                 | 4561  | 0.66 |                                     |
| CDC       | Pt39                 | 4180  | 0.61 |                                     |
| CDC       | Pt26                 | 3118  | 0.56 | 2nd tertile<br>(moderate supporter) |
| CDC       | Pt25                 | 2039  | 0.36 |                                     |
| CDC       | Pt47                 | 1600  | 0.30 |                                     |
| CDC       | Pt33                 | 1160  | 0.28 |                                     |
| CDC       | Pt50                 | 1567  | 0.24 |                                     |
| CDC       | Pt60                 | 2052  | 0.23 |                                     |
| CDC       | Pt54                 | 2093  | 0.22 |                                     |
| CDC       | Pt72                 | 1405  | 0.21 |                                     |
| CDC       | Pt58                 | 1285  | 0.18 |                                     |
| CDC       | Pt71                 | 1077  | 0.17 |                                     |
| CDC       | Pt32                 | 689   | 0.17 |                                     |
| CDC       | Pt30                 | 652   | 0.16 |                                     |
| CDC       | Pt44                 | 1584  | 0.14 |                                     |
| CDC       | Pt59                 | 727   | 0.11 |                                     |
| CDC       | Pt28                 | 621   | 0.11 | 3rd tertile<br>(poor supporter)     |
| CDC       | Pt45                 | 1192  | 0.11 |                                     |
| CDC       | Pt63                 | 704   | 0.11 |                                     |
| CDC       | Pt61                 | 654   | 0.10 |                                     |
| CDC       | Pt14                 | 600   | 0.10 |                                     |
| CDC       | Pt35                 | 880   | 0.09 |                                     |
| CDC       | Pt40                 | 855   | 0.08 |                                     |
| CDC       | Pt27                 | 396   | 0.07 |                                     |
| CDC       | Pt34                 | 69    | 0.02 |                                     |
| CDC       | Pt29                 | 57    | 0.01 |                                     |
| CDC       | Pt55                 | 121   | 0.01 |                                     |
| CDC       | Pt37                 | 62    | 0.01 |                                     |
| CDC       | Pt42                 | 34    | 0.00 |                                     |
| CDC       | Pt51                 | 15    | 0.00 |                                     |
| CDC       | Pt52                 | 116   | 0.02 |                                     |

ID, identification; Pt, patient identification followed by number; CDC, cardiosphere-derived cells; BMSC, bone marrow-derived stem cells; TTL, total tubule length; RTL, relative tubule length according to the control in each batch.

Table 4.2 shows that CDCs samples in the 1<sup>st</sup> tertile (good supporters) displayed a mean TTL of  $7693 \pm 3029\mu\text{m}$  ranging from 4180 to 14000 $\mu\text{m}$ , which is very similar to that of BMSC (mean TTL  $7568 \pm 3300\mu\text{m}$  and range 4168-10967 $\mu\text{m}$ ). The mean TTL for CDC samples grouped in the 2<sup>nd</sup> tertile (moderate supporters) was  $1429 \pm 668\mu\text{m}$  ranging from 621 to 3118 $\mu\text{m}$ , whilst the 3<sup>rd</sup> tertile of CDCs samples presented a mean TTL of  $321 \pm 337\mu\text{m}$  and a range of 15 to 880 $\mu\text{m}$  (poor supporters). Figure 4.5.B shows that the three groups (1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> tertile, respectively) are significantly different when TTLs were compared by using the Kruskal–Wallis one-way ANOVA. The same results were obtained when comparing RTLs of each group (Figure 4.5C). In addition, the mean TTL of good supporters is not significantly different from that of BMSC (Figure 4.5B), thus indicating that CDCs grouped in the 1<sup>st</sup> tertile are as good as BMSC in supporting vessel network formation *in vitro*.

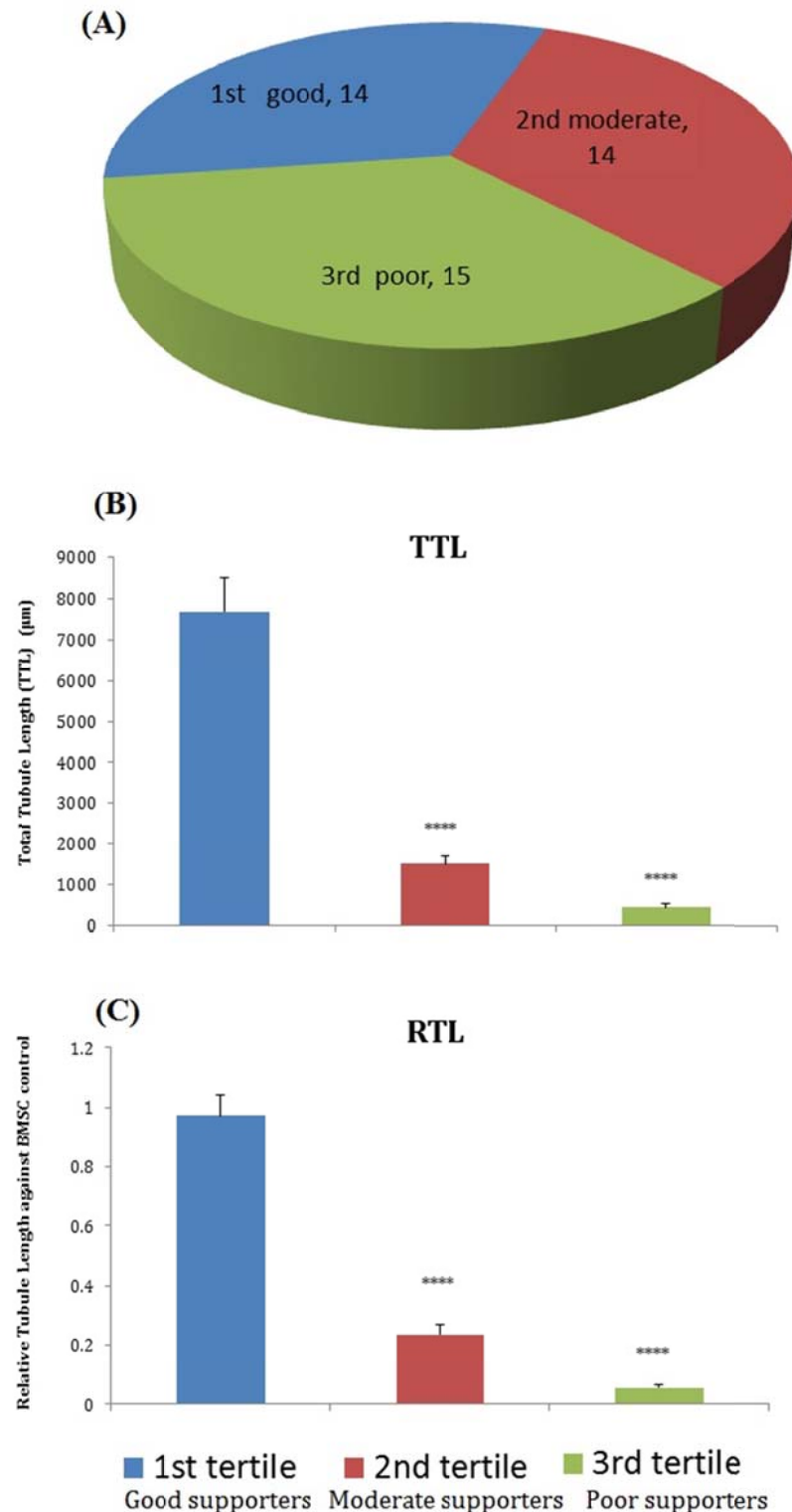
**Table 4.2 Descriptive statistics of the CDC classes: good, moderate and poor supporters of angiogenesis.**

|      | Tertile | Number | Group    | TTL  |      |            | RTL       |
|------|---------|--------|----------|------|------|------------|-----------|
|      |         |        |          | mean | SD   | range      | range     |
| BMSC | n/a     | 4      | control  | 7568 | 3300 | 4168-10967 | 1         |
| CDCs | 1st     | 14     | good     | 7693 | 3029 | 4180-14000 | 0.61-1.62 |
| CDCs | 2nd     | 14     | moderate | 1429 | 668  | 621-3118   | 0.11-0.56 |
| CDCs | 3rd     | 15     | poor     | 321  | 337  | 15-880     | 0.00-0.11 |

BMSC, bone marrow mesenchymal/stromal cells; CDCs, cardiosphere-derived cells; n/a, not applicable; RTL, relative tubule length; SD, standard deviation; TTL, total tubule length.

In conclusion, CDCs from IHD patients had a varied pro-angiogenic potential. Approximately 1/3 of the samples tested supported approximately 10% of the TTL that BMSC from the healthy donors could support.





**Figure 4.5 Pro-angiogenic ability of CDCs.** (A) CDC samples from a total of 43 IHD patients with different pro-angiogenic potential were subgrouped into three tertiles. The 1<sup>st</sup> tertile was defined as good supporters (n=14), the second as moderate supporters (n=14) and the 3<sup>rd</sup> as poor supporters (n=15). (B and C) The total tubule length (TTL) and relative tubule length (RTL) of the three tertiles, respectively. Data is presented as mean and standard deviation. The average TTL and RTL were highly significantly different between the three groups. (\*\*\*\*,  $p < 0.0001$ ).

### ***4.3.3 Therapeutic potential of CDCs from IHD patients***

In order to confirm the results obtained *in vitro* and to assess the therapeutic potential of CDCs from good and poor supporters of angiogenesis, an experimental model of MI was established with the help of Dr P. Sepulveda's group (University of Valencia, Spain) as described elsewhere [141], [142]. Similar to human BMSC, the injection of human CDCs required the use of immune-compromised rats (HHH-*Foxn1<sup>rmu</sup>*) that were purchased from Charles River Laboratories (as described in section 2.8). The weight and baseline heart function parameters of the animals used in this study are presented in Table 4.3. Animals with fractional shortening (FS) above 35% following ligation were excluded from the study.

A total of 25 adult athymic rats were included. Baseline LV chamber parameters at systolic and diastolic phases were measured by echocardiography and functional values, such as fractional area change (FAC), fractional shortening (FS) and anterior wall thickness (AWT), were derived accordingly before surgery. The baseline values were similar for all animals included in the study ( $p>0.05$ ). Animals were then divided in three treatment groups: (i) CDCs from good supporters, (ii) CDCs from poor supporters and (iii) saline (control). One week post-LAD artery ligation, the chest of the animals was re-opened and a total of  $1 \times 10^6$  cells/ animal in 5 $\mu$ L PBS, or saline only, were transplanted into five sites of the infarct border zone. Animals were monitored for one month after cell transplantation. On day 15 and day 30 post-transplant, echocardiography was performed to monitor changes in heart function and heart anatomy. Table 4.4 shows the results of the *in vivo* experiments.

**Table 4.3 Summary of heart anatomical measurements and functional parameters in rats transplanted with either CDCs or saline.**

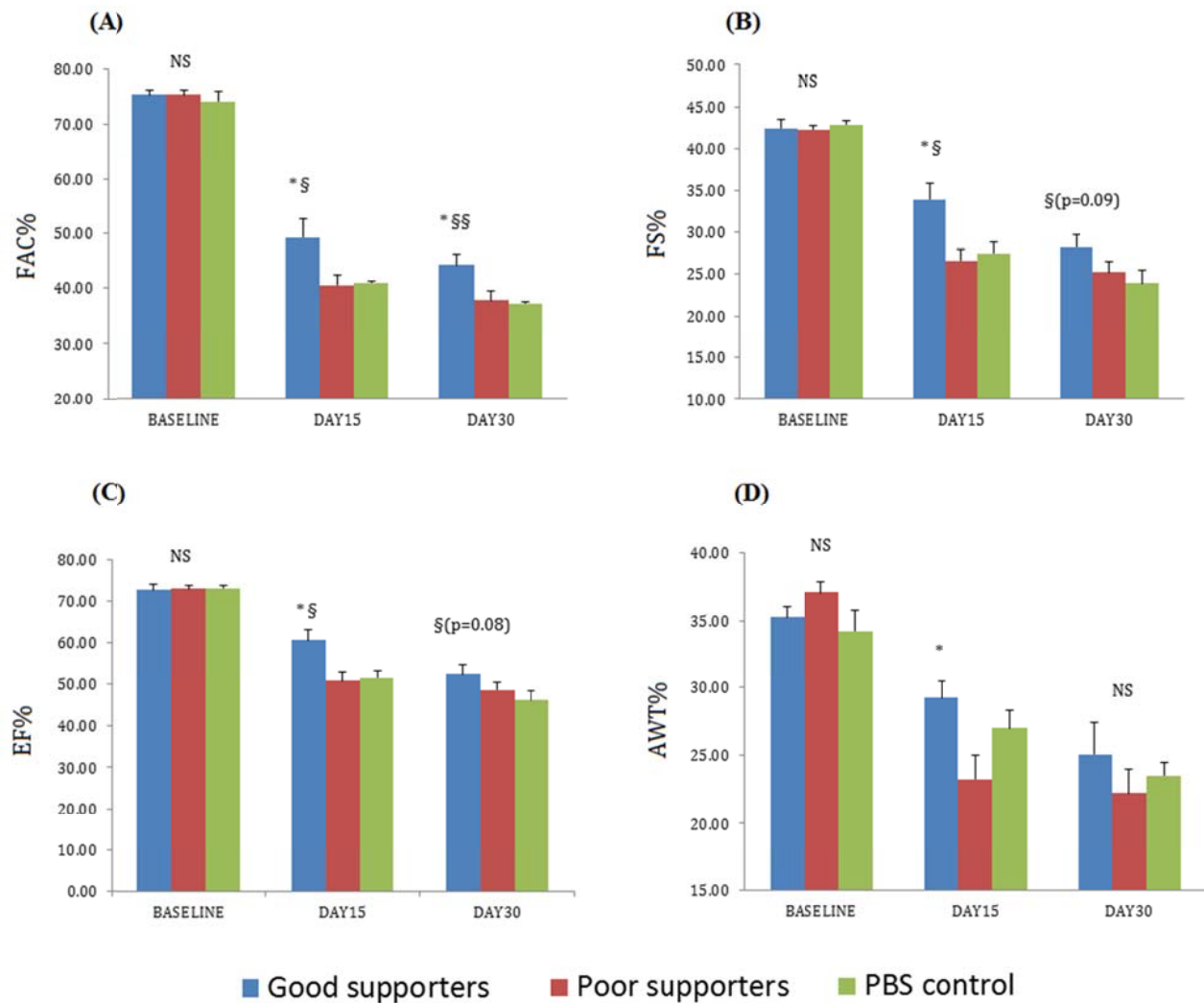
|           | Good supporters (n=8) |           |             |           |             |           | Poor supporters (n=9) |           |             |           |             |           | PBS control (n=8) |           |             |           |             |           |
|-----------|-----------------------|-----------|-------------|-----------|-------------|-----------|-----------------------|-----------|-------------|-----------|-------------|-----------|-------------------|-----------|-------------|-----------|-------------|-----------|
|           | Baseline              |           | DAY15       |           | DAY30       |           | Baseline              |           | DAY15       |           | DAY30       |           | Baseline          |           | DAY15       |           | DAY30       |           |
|           | <u>Mean</u>           | <u>SD</u> | <u>Mean</u> | <u>SD</u> | <u>Mean</u> | <u>SD</u> | <u>Mean</u>           | <u>SD</u> | <u>Mean</u> | <u>SD</u> | <u>Mean</u> | <u>SD</u> | <u>Mean</u>       | <u>SD</u> | <u>Mean</u> | <u>SD</u> | <u>Mean</u> | <u>SD</u> |
| LVAWd     | 1.44                  | 0.05      | 1.17        | 0.09      | 1.12        | 0.12      | 1.45                  | 0.03      | 1.11        | 0.06      | 1.05        | 0.06      | 1.56              | 0.06      | 1.09        | 0.11      | 1.04        | 0.09      |
| LVd       | 5.43                  | 0.24      | 6.85        | 0.80      | 7.33        | 0.75      | 5.35                  | 0.44      | 6.92        | 0.78      | 7.19        | 0.78      | 5.90              | 0.08      | 7.47        | 0.56      | 7.64        | 0.76      |
| IVSd      | 1.20                  | 0.16      | 1.37        | 0.23      | 1.34        | 0.09      | 1.29                  | 0.15      | 1.25        | 0.18      | 1.33        | 0.17      | 1.44              | 0.14      | 1.56        | 0.24      | 1.54        | 0.24      |
| LVAWs     | 2.23                  | 0.15      | 1.66        | 0.11      | 1.50        | 0.21      | 2.31                  | 0.09      | 1.45        | 0.14      | 1.36        | 0.15      | 2.37              | 0.12      | 1.50        | 0.20      | 1.36        | 0.14      |
| LVs       | 3.14                  | 0.23      | 4.57        | 0.77      | 5.28        | 0.62      | 3.09                  | 0.28      | 5.10        | 0.77      | 5.37        | 0.61      | 3.38              | 0.11      | 5.43        | 0.48      | 5.83        | 0.77      |
| IVSs      | 2.03                  | 0.23      | 2.06        | 0.20      | 1.94        | 0.20      | 1.86                  | 0.29      | 1.82        | 0.28      | 1.99        | 0.44      | 2.21              | 0.28      | 2.31        | 0.23      | 2.08        | 0.46      |
| LV Area d | 28.31                 | 2.72      | 36.26       | 5.65      | 39.76       | 5.55      | 27.67                 | 3.16      | 38.55       | 5.63      | 41.08       | 5.71      | 31.45             | 2.53      | 40.32       | 4.72      | 45.05       | 4.12      |
| LV Area s | 7.15                  | 1.09      | 18.50       | 4.77      | 22.28       | 4.74      | 6.82                  | 0.98      | 22.84       | 3.82      | 25.59       | 4.75      | 8.24              | 1.68      | 24.16       | 2.48      | 28.96       | 4.47      |
| FAC(%)    | 74.78                 | 2.43      | 49.29       | 9.76      | 44.30       | 5.52      | 75.30                 | 2.83      | 40.63       | 5.33      | 37.88       | 5.42      | 73.83             | 4.54      | 39.91       | 3.55      | 35.97       | 4.03      |
| FS (%)    | 41.73                 | 3.36      | 33.45       | 5.35      | 27.93       | 4.32      | 42.33                 | 1.56      | 26.60       | 3.83      | 25.25       | 3.43      | 42.71             | 1.38      | 27.24       | 3.35      | 23.86       | 3.95      |
| AWT(%)    | 35.26                 | 2.96      | 29.24       | 3.58      | 25.05       | 6.74      | 37.08                 | 2.38      | 23.26       | 4.98      | 22.22       | 5.14      | 34.12             | 3.89      | 26.71       | 5.47      | 23.37       | 2.28      |
| EF(%)     | 72.53                 | 4.03      | 60.53       | 7.15      | 52.41       | 6.24      | 72.83                 | 1.98      | 50.59       | 6.25      | 48.36       | 5.43      | 72.98             | 1.59      | 51.37       | 5.26      | 45.92       | 6.49      |

LVAWd = left ventricle anterior wall end-diastolic thickness, LVd = left ventricle end-diastolic diameter, IVSd=interventricular septum diastolic thickness, LVAWs = left ventricle anterior wall end-systolic thickness, LVs = left ventricle end-systolic diameter, IVSs=interventricular septum systolic thickness, LVArea d = left ventricle end-diastolic area, LVArea s = left ventricle end-systolic area, FAC% = left ventricle per cent fractional area change, FS% = left ventricle per cent fractional shortening, AWT% = left ventricle anterior wall per cent thickening, EF% = left ventricle per cent ejection fraction.

On Day 15, the percentage of FAC (Figure 4.6A) was significantly higher in animals injected with good supporters than with CDC from poor supporters (49% vs. 41%,  $p<0.05$ ) or in saline injected animals (49% vs. 40%,  $p<0.05$ ), whilst there was no significant difference between animals injected with either CDC from poor supporters or saline. On Day 30 similar results were observed between good and poor supporters (44% vs. 38%;  $p<0.05$ ), good supporters and control (44% vs. 36%,  $p<0.01$ ) and poor supporters and control (38% vs. 36%,  $p>0.05$ ).

Fractional shortening (FS%) was significantly better in animals injected with CDCs from good supporters than poor supporters (34% vs. 27%,  $p<0.05$ ) or saline (34% vs. 27%,  $p<0.05$ ) on Day 15 (Figure 4.6B). There was no differences between animals injected with poor supporters or saline (27% vs. 27%,  $p>0.05$ ). Although there was a trend that advantage was still observed in FS% on Day 30 between animals injected with good supporters and saline (28% vs. 24%,  $p=0.09$ ), there were no significant differences between the good and poor supporter groups (28% vs. 25%,  $p=0.19$ ).

Changes in the anterior wall of the left ventricle, measured as percentage of AWT, were also seen on Day 15 (Figure 4.6C). There was a significant difference between animals injected with CDCs from good or poor supporters (29% vs. 23%,  $p<0.05$ ), but the differences between the good supporters and saline treatment groups (29% vs. 27%,  $p>0.05$ ) were not significant. Although the difference was not significant, regional wall motion in the rats treated with CDCs from the poor supporters seemed to perform worse than the saline (control) groups (23% vs. 27%,  $p>0.05$ ). On Day 30 differences between groups had disappeared (Figure 4.6C).

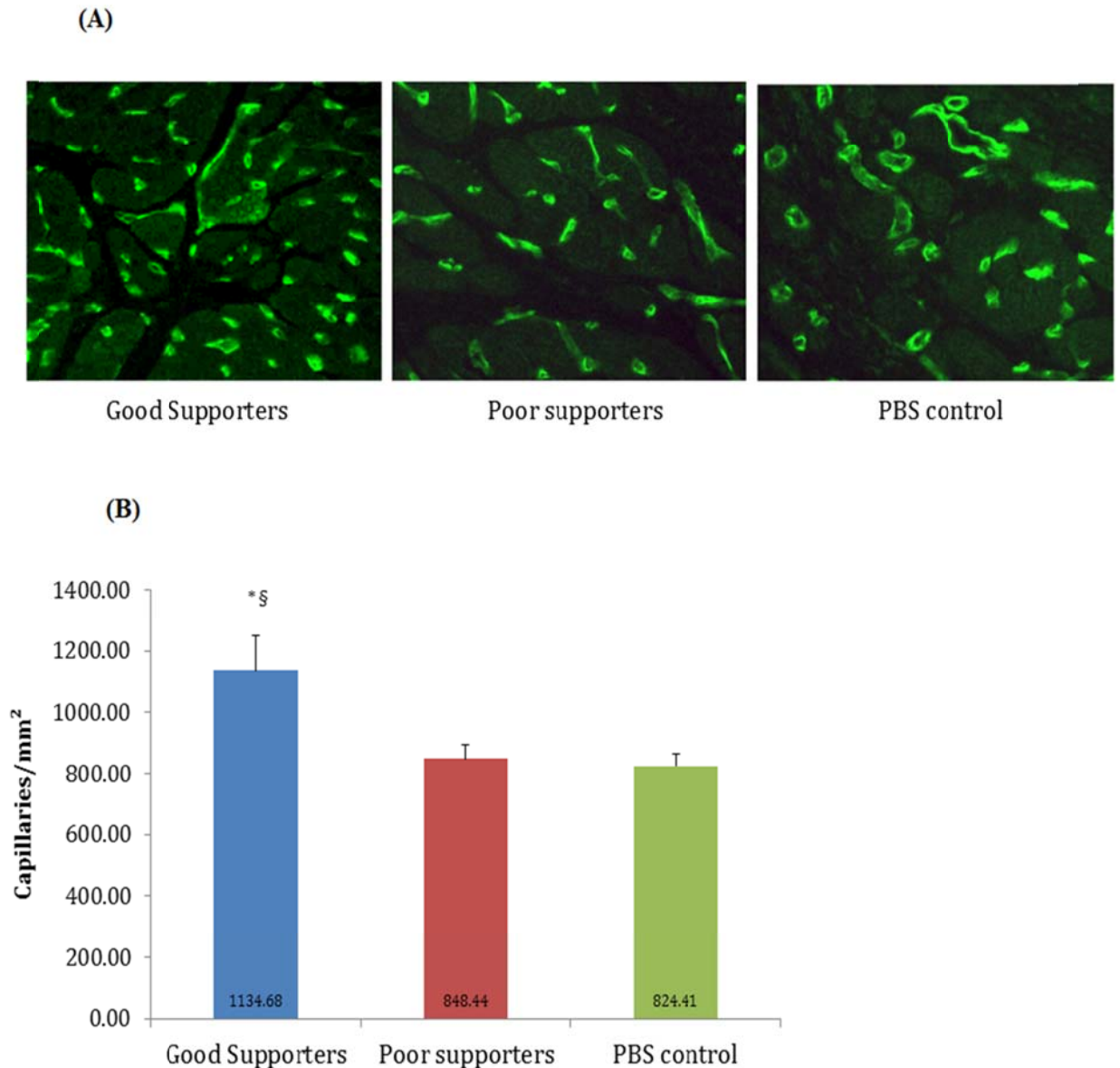


**Figure 4.6 Effect of CDC transplantation on LV function.** Adult athymic (HIH-*Foxn1<sup>rmu</sup>*) rats had the left anterior descending (LAD) artery ligated. Animals were split into three groups which received either CDCs from good supporters (n=8), CDC from poor supporters (n=9) or saline (n=8) 7 days post-LAD artery ligation. Animals were monitored for 30 days. Echocardiography was used to measure heart function at baseline and two week intervals. (A) Percentage of fractional area change (FAC%). (B) Percentage of fractional shortening (FS%) change. (C) Percentage of anterior wall thickening (AWT%). (D) Change in left ventricular ejection fraction (EF%). ANOVA and post-hoc *t*-test were used. P values <0.05 and <0.01 were considered significant and highly significant respectively. NS= not significant. \*= significant between animals transplanted with good supporters and poor supporters, §= significant or trend to become significant between the good supporters and saline injected animals, §§= highly significant between the good supporters and saline groups. Animals treated with CDCs from good supporters showed significant improvement in FAC% over the poor supporters and the placebo groups at Day15 and Day30. Similarly FS% and LVEF% improved significantly in animals transplanted with good supporters over poor supporters or control animals at Day 15. At Day 30 this trend persisted but did not reach statistical significance. There was a trend suggesting that CDC from poor supporters had a detrimental effect on AWT% compared to CDCs from good supporters.

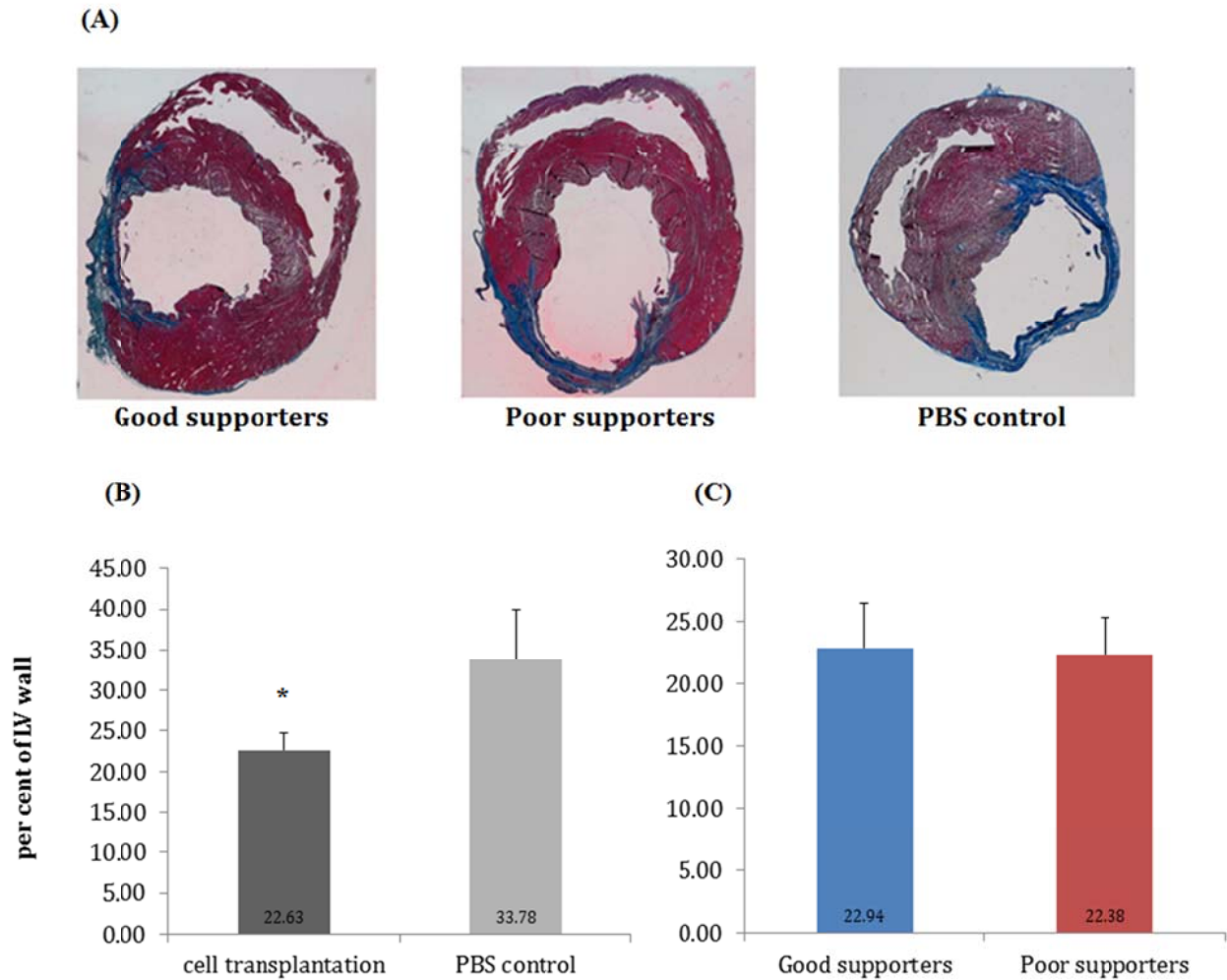
Similarly, changes in ejection fraction (EF%) were deemed to be significantly different between animals injected with CDCs from good than poor supporters (61% vs. 52%,  $p<0.05$ ) and good supporters than saline (61% vs. 51%,  $p<0.05$ ) on Day 15 (Figure 4.6D), which confirmed the relevance of the parameters above in assessing heart function while validated the previous findings. No significant differences were observed between poor supporters and saline injected animals (52% vs. 51%,  $p>0.05$ ). On Day 30, as in FS, changes in EF only had a trend to become significant when comparing animals injected with good supporters and saline (53% vs 46%,  $p=0.08$ ), but not when comparing good and poor supporters (52% vs. 48%;  $p=0.19$ ) or poor supporters and saline injected animals (48% vs. 46%;  $p>0.05$ ).

In summary, heart function does not deteriorate as much when CDCs from good supporters of angiogenesis were injected into the peri-infarct area of animals with permanent LAD artery ligation, whereas CDCs from poor supporters do not have a beneficial effect on heart function compared to control. However, this difference between the good and poor supporters diminished at Day 30, suggesting an early paracrine effect was helpful in preventing the progressive deterioration of heart function.

Immunohistological examinations of the rat hearts showed that blood vessel density was significantly different between the three groups of animals ( $p=0.01$ ) (Figure 4.6A). The group of rats transplanted with CDC from good supporters had increased vessel density in the infarct border zone ( $1135 \pm 117$  capillaries/mm<sup>2</sup>) compared to animals injected with CDC from the poor supporters ( $848 \pm 94$  capillaries/mm<sup>2</sup>,  $p=0.02$ ) or simply saline ( $824 \pm 111$  capillaries/mm<sup>2</sup>,  $p=0.01$ ). No significant differences were observed between poor supporters or saline injected animals ( $p=0.82$ ). (Figure 4.7B)



**Figure 4.7 Effect of CDC transplantation on neovascularisation at the infarct border zone.** (A) The representative images of *in vivo* neovascularisation at the infarct border zone 30 days after infusion of CDC from good supporters (n=8), poor supporters (n=9) or saline (n=8). Four weeks following transplantation of CDCs or saline (PBS control) into animals with permanent ligation of the left anterior descending artery, the hearts were excised, sectioned and stained with rat anti-CD31 antibody. The antibody bound to the tissue was detected with goat anti-rat Alexa 488-conjugated antibody. (B) Capillary density in the heart of animals infused with good supporters, poor supporters or PBS control. Vessels were counted in 10 fields in the peri-infarct zone at 200 $\times$  and referred as number of vessels per unit area (mm<sup>2</sup>) using a light microscope and the Image Proplus 7.1 software. ANOVA and post-hoc *t*-test were used. P values <0.05 were considered significant. \*= significant between animals transplanted with good supporters and poor supporters, §= significant between the good supporters and saline injected animals. Capillary density was significantly increased in animals injected with CDCs from good supporters compared to poor supporters and control.



**Figure 4.8 Effect of CDC transplantation on scar size.** (A) Masson's trichrome staining of rat hearts following infusion of CDCs from good supporters (n=8), poor supporters (n=9) or PBS control (n=8). The fibrotic tissue around the infarct stains blue against the healthy myocardium in red. (B) Infarct size in animals transplanted with CDCs or control animals. The infarct size in left ventricles was measured in 8-12 transverse 7 $\mu$ m sections (1 slice every 200 $\mu$ m of tissue) from the apex to the base with 2% paraformaldehyde and stained with Masson's trichrome. The fibrotic zone was determined using the Proplus 7.1 software and infarct size expressed as a percentage of total left ventricular area and as a mean of all slices from each heart. (C) Comparison between animals infused with CDCs from good or poor supporters of angiogenesis. Student's t-test was used to determine differences between groups and  $p < 0.05$  was considered statistically significant (\*). CDC transplantation had a significant beneficial effect on reducing infarct size, however, no differences were observed between good and poor supporters of angiogenesis.

The average scar size following LAD artery ligation was 34% of the left ventricular wall.

However, the MI rats that received CDC treatment had a significant reduction of scar size



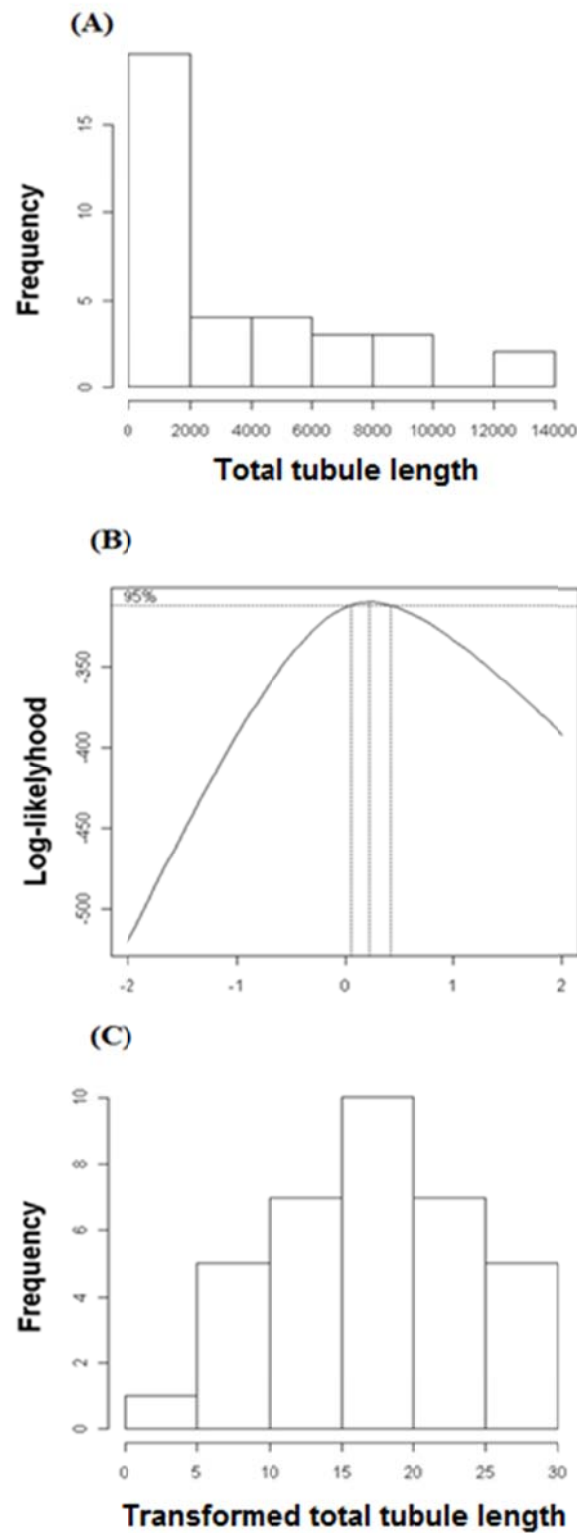
after 4 weeks ( $p < 0.05$ ). The non-viable scar tissue only comprised 23% of the left ventricular wall which was 11% smaller in size than that of the saline treated group (Figure 4.8B). Interestingly, no significant difference in scar size was observed between the good and poor supporters injected animal (Figure 4.8C).

To conclude, these results confirmed our hypothesis that CDCs with varied functional capacities observed *in vitro* possessed different therapeutic effect in an *in vivo* model of MI, which is directly attributed to *in vivo* blood vessel density. Therefore, our *in vitro* angiogenesis assay could be developed as a potency assay to determine CDC's therapeutic potential.

#### ***4.3.4 Cardiovascular risk factors as predictors of CDC's pro-angiogenic potential***

In this study, data including the total tubule length (TTL) supported by  $3 \times 10^4$  CDCs was used to assess whether there was any independent variable among the correspondent cardiovascular risk factors to predict their pro-angiogenic ability in a multiple linear regression model. TTL was used as the dependent variable in the model, whereas variables such as age, sex, type of disease, NYHA class, co-morbidity of type 2 diabetes mellitus, hypertension and hypercholesterolaemia, cigarette smoking and family history were included as independent variables.

First, the TTL variable was clearly non-normal and displayed a considerable left-hand 'skewness' on the histogram (Figure 4.9A). The dependent variable was then transformed using a Box-Cox transformation with parameter  $\lambda = 1/5$  (95% confidence interval 0.04 to 0.4), (see Figure 4.9B) identified by fitting the profile likelihood function and this provided an acceptable normal distribution of the dataset (Figure 4.9C).



**Figure 4.9 Transformation of total tubule length dataset.** The observed total tubule length (TTL) obtained from 35 CDC samples collected and analysed blindly shows a significant bias (to the left) characteristic of a non-normal distribution (A). The data was transformed using a Box-Cox transformation with parameter  $\lambda = 0.2$  (95% confidence interval 0.04 to 0.4) (B). The resulting transformed data achieved a normal distribution (C).

Second, the transformed TTL data and step-wise methods were used to eliminate non-significant predictors and arrive at a final multiple regression model. The concise process of generating the model is demonstrated step by step as programme scripts with symbol # marked as notes in Table 4.4. The model refining process with other models attempted to fit the data from this study and the corresponding results are listed in Appendix 5.

**Table 4.4 R scripts of the generalised linear regression model**

```

hj<- read.csv("C:/destop/hj.csv", header = T)
attach(hj)
#load MASS package to calculate lambda
bcxcox(Tubule ~ Age + Sex + NYHA + Smoker + Procedure + Vessles + FHx + DM + HBP
+ HBChol)
# This gets a power transformation lambda = 1/5
bcTubule <- (Tubule^(1/5) - 1)/(1/5)
# Create variable for never smoked/smoked - first load nnet package
sm <- class.ind(Smoker)
# Fitting model with Smoker = 1 as reference gives p-values of 0.8586 for Smoker
= 2 and 0.2440 for Smoker = 3, so no sig difference: therefore pool smoking
status 1, 2 and 3 (i.e cols 2,3,4 of sm).
Smoke <- sm[,2] + sm[,3] + sm[,4]
summary(lm(bcTubule ~ Age + Sex + NYHA + Smoke + Procedure + Vessles + FHx + DM
+ HBP + HBChol))
lm(formula = bcTubule ~ Age + Sex + NYHA + Smoke + Procedure + Vessles + FHx + DM
+ HBP + HBChol)
# step wise method start here and more parsimonious model gives, e.g.
summary(lm(bcTubule ~ Age + NYHA + Smoke + Vessles + FHx + HBP))
lm(formula = bcTubule ~ Age + NYHA + Smoke + Vessles + FHx + HBP)
# diagnostics on model
out <- lm(bcTubule ~ Age + NYHA + Smoke + Vessles + FHx + HBP)
res <- studres(out)
qqnorm(res)
fit <- fitted(out)
plot(fit,res)
plot(fit,bcTubule,ylim = c(0,30),xlim = c(0,30),main = "Plot of Box-Cox
transformed tubule length against fitted model values")
plot(fit,bcTubule,ylim = c(0,30),xlim = c(0,30),main = "Plot of Box-Cox
transformed tubule length against fitted model values", ylab = "Box-Cox
transformed tubule length", xlab = "Fitted values")
lines(c(0,30),c(0,30),col="blue")
# Finally selected model
summary(lm(bcTubule ~ Age + NYHA + Smoke + factor(Procedure) + FHx + DM+ HBP))
lm(formula = bcTubule ~ Age + NYHA + Smoke + factor(Procedure) + FHx + DM+ HBP )
out <- lm(bcTubule ~ Age + NYHA + Smoke + factor(Procedure) + FHx + DM+ HBP )
res <- studres(out) # studentised residuals
qqnorm(res)
res <- stdres(out) # standerised residuals
qqnorm(res)
fit <- fitted(out)
plot(fit,res)
ypred <- (fit/5 + 1)^5
plot(ypred,Tubule,ylim = c(0,14000),xlim = c(0,14000),main = "Plot of tubule
length against model values", ylab = "Tubule length", xlab = "Fitted values")
lines(c(0,14000),c(0,14000),col="blue")

```

Finally, following the step-wise method described in Table 4.4 a final model was established which included age, NYHA class, history of smoking, type of disease, diseased right coronary artery, family history of heart disease, diabetes mellitus and hypertension. This model identified several factors, such as history of cigarette smoking, NYHA class, aortic stenosis and diseased right coronary artery, as significant independent positive predictors of angiogenesis. By contrast, hypertension seems to be a significant independent negative predictor of angiogenesis (see Table 4.5). Interestingly, factors such as age, family history of heart disease or co-morbidity of hypercholesterolaemia or type 2 diabetes mellitus, and number of diseased coronary arteries in patients with CAD did not manifest significant effects on total tubule length (Table 4.5).

**Table 4.5 The predictors of the pro-angiogenic potential of CDCs**

| Coefficients                 |                 | Estimate   | Std. Error | t value | p-value  |
|------------------------------|-----------------|------------|------------|---------|----------|
| <b>(Intercept)</b>           |                 | 9.2        | 6.8        | 1.4     | 0.19     |
| <b>Age</b>                   |                 | -0.1       | 0.1        | -1.2    | 0.23     |
| <b>NYHA class</b>            |                 | 2.6        | 1.1        | 2.4     | 0.03 *   |
| <b>Smoking history</b>       |                 | 5.3        | 2.0        | 2.7     | 0.01 *   |
|                              | Aortic stenosis | 7.5        | 3.6        | 2.1     | 0.05 *   |
| <b>Type of disease</b>       | CAD             | 6.7        | 3.6        | 1.8     | 0.08.    |
|                              | Others          | as control |            |         |          |
| <b>Right coronary artery</b> |                 | 6.6        | 2.5        | 2.6     | 0.01 *   |
| <b>Family history</b>        |                 | 1.9        | 1.2        | 1.5     | 0.15     |
| <b>Diabetes mellitus</b>     |                 | -3.4       | 2.3        | -1.5    | 0.16     |
| <b>Hypertension</b>          |                 | -7.3       | 2.2        | -3.4    | <0.01 ** |

(\*)  $p < 0.05$ ; (\*\*)  $p < 0.01$ .

Residuals:

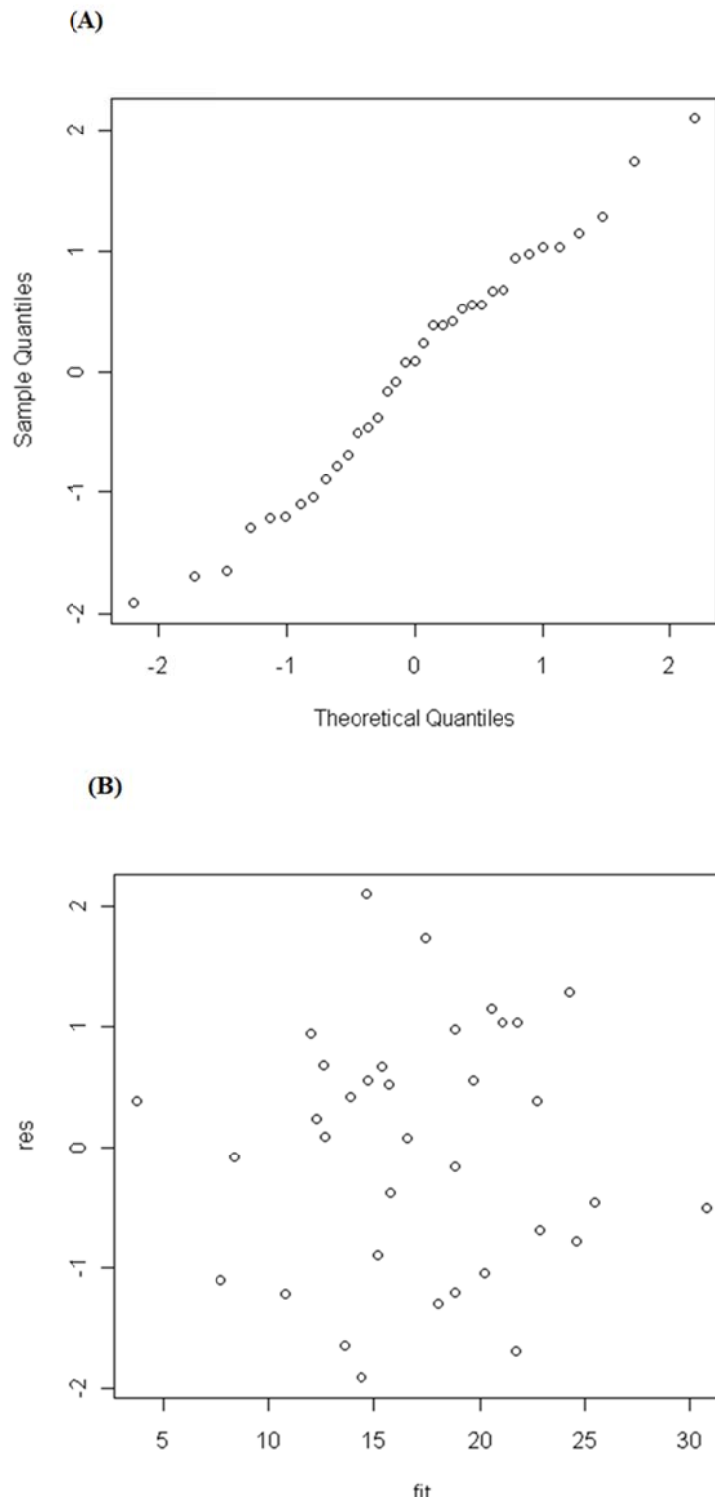
|      |      |        |     |     |
|------|------|--------|-----|-----|
| Min  | 1Q   | Median | 3Q  | Max |
| -8.2 | -3.3 | 0.3    | 2.7 | 7.9 |

Residual standard error: 4.8 on 25 degrees of freedom

Multiple  $R^2$ : 0.64, Adjusted  $R^2$ : 0.51

F-statistic: 5.0 on 9 and 25 DF, p-value: <0.001

The validation of the final best fitted model was carried out by performing the standard diagnostics on the residuals of the regression model. A normal Q-Q plot of residuals and a plot of residuals against fitted values are presented in Figures 4.10 A and B, respectively. The plots show excellent normality and stability of variance and no outliers were detected. The final model accounted for over 51% of the variability in the data ( $R^2 = 0.51$ ), which is considered high for data of this kind.



**Figure 4.10 Diagnostic tests of the multi-linear regression model.** (A) Normal Q-Q plot of residuals showed a linear curve without outliers and (B) plot of fitted values against residuals did not manifest a specific shape, suggesting a validated model without co-linearity between independent variables.

## 4.4 Discussion

This chapter demonstrated that CDCs from patients with IHD, (i) can support angiogenesis to a varying degree *in vitro* and *in vivo*, (ii) have beneficial effects on cardiac function when transplanted in a rodent model of MI, providing the patient samples are stratified according to their pro-angiogenic potential, and (iii) their pro-angiogenic potential can be predicted by cardiovascular risk factors such as hypertension and history of smoking, as well as NYHA class and type of disease.

### 4.4.1 Establishing a model of *in vitro* angiogenesis

*In vitro*, blood vessel formation can be modelled using a variety of assays including the rat aortic ring angiogenesis assay and the short-term culture of endothelial cells in Matrigel<sup>TM</sup>. Whereas the former is used mainly to test pro- and anti-angiogenic drugs that affect the sprouting and growth of endothelial cells from the rat aorta *in vitro*, the latter allows the modelling of endothelial cell behaviour and the steps leading to capillary formation and growth. Matrigel<sup>TM</sup>, the gelatinous protein mixture obtained from the extracellular matrix of mouse sarcoma cells [168], represents a quick and easy method to test endothelial cells from different sources and to investigate the effect of different compounds on cell survival, proliferation, tubule formation and expansion. But both of these methods are limited to the study of endothelial cells. They are not appropriate to assess the function of mural/supportive cells or the interactions between supportive and endothelial cells. Co-culture of GFP-labelled HUVECs with stromal cells, like CDCs, allows assessment of the influence of stromal cells on the behaviour of endothelial cells, and it resembles better the *in vivo* situation where endothelial cells form neovasculature with the support of neighbouring stromal cells.

Mural/supportive cells, such as mesenchymal stromal cells, are essential in the recruitment of endothelial cells to form blood vessels and the maintenance of vascular stability once formed *in vivo*. It has been shown previously that blood vessel networks are formed more robustly when stromal cells are present in the cultures [169].

Therefore, in order to assess the angiogenic potential of CDCs, a suitable *in vitro* angiogenesis assay was required in the present study. The assay consists of a 2D co-culture of endothelial and stromal supportive cells (CDCs) and has the added advantage of quantitating blood vessel network formation.

#### ***4.4.2 Angiogenesis in vitro as a potency test for CDCs' function***

The original hypothesis was that CDCs could be developed as a cell therapy to enhance therapeutic angiogenesis in the ischaemic heart. In this chapter, it is confirmed that CDCs' function resembles that of mesenchymal/stromal cells in supporting blood vessel formation. Optimal revascularisation takes place in the presence of endothelial and stromal cells [169], such as vascular smooth muscle cells or pericytes. BMSC, and in this instance CDCs, are able to perform this function as they are mesenchymal progenitors. Notably, CDCs can promote vessel network formation to a varying degree (see Figure 4.3), with some very striking differences (e.g. comparison between good and poor supporter groups). Thus, the *in vitro* angiogenesis model established in this chapter could be used as a potency test for CDCs, and in the future for microvascular endothelial cells from a variety of patients. To our knowledge, this is the first time that the pro-angiogenic function of CDCs obtained from patients with IHD is systematically assessed. Approximately 1/3 of the CDCs samples tested in this study showed poor angiogenic support, representing <10% of the TTL of BMSC, whilst 1/3 showed good support of angiogenesis (>70% TTL compared with



BMSC). Nonetheless, the data reflecting the pro-angiogenic potential of CDCs from IHD patients was severely left-skewed, suggesting that the difference between moderate supporters and poor supporters were smaller than that between moderate supporters and good supporters. In other words, nearly half of the CDC samples tended to foster relatively small number of tubules (<20% RTL) whilst only a quarter of the patient samples could support as good length of capillaries as BMSC (>80% RTL).

#### ***4.4.3 In vivo angiogenesis and therapeutic potential of CDCs***

Although generally correlated, care must be taken to interpret results obtained *in vitro* before achieving proof-of-principle in a relevant *in vivo* model.

In this study, we have successfully transplanted in rats CDCs from two clearly distinct groups of patients that have very different phenotype *in vitro*: good and poor supporters of angiogenesis. Results from these *in vivo* experiments confirmed the results obtained *in vitro*. In addition, CDCs from good supporters provided significant protection against the decline of LV function by attenuating the deterioration of FAC ( $\Delta$ FAC% = 41% in PBS treated animals vs. 26% in CDC good supporter-treated animals from baseline), FS (16% in PBS-treated vs. 9% in CDC good supporter-treated animals), AWT (9% vs. 6% in PBS and CDC good supporter-treated animals, respectively) and EF (22% in PBS-treated vs. 12% in CDC good supporter-treated animals) compared to control. Interestingly, CDCs from poor supporters did not attenuate LV remodelling compared to control. The beneficial effect of CDCs from good supporters seems to diminish after 4 weeks following cell transplantation and after two weeks for FS and AWT. This has been observed before by our collaborators when transplanting human BMSC in the same animal model (Dr P. Sepulveda, personal communication). The presence of cells injected in the heart has been shown to decline over

time even in syngeneic animals [170]. However, it is not surprising that human cells may be lost perhaps quicker during the sub-acute phase of MI, as the nude rats used in this study have T-cells depleted from the thymus, but they still possess NK cells and B-cells in their bone marrow. Therefore, it is plausible that engraftment and survival of human cells in the transplanted animals may be only temporary due to cell clearance by the remaining immune system in the recipient animals. Another explanation is that, given that the good supporters can revascularise the ischaemic myocardium by the end of the first month, evident functional restoration of the ischaemic heart would take much longer period of time to appear. It might be too early to observe such effect by the end of the first month in this study. These are some of the limitations of the *in vivo* model used in this study. The efficacy of CDCs from different patients with chronic IHD may still have to be tested in larger animal species, and perhaps with more relevant animal models. For those patients with poor support of angiogenesis, it may be necessary to combine CDCs and BMSC to enhance the improvement of cardiac function, as previously suggested with cardiac c-kit positive cells and BMSC [171].

Human CDCs isolated from transplanted (healthy) hearts have been shown to have a therapeutic effect on heart function and contractility in a mouse model of MI [89], [98], [152]. However, to my knowledge this is the first time that the therapeutic potential of CDCs isolated from patients with chronic ischaemia and undergoing heart surgery has been demonstrated in an experimental model of MI. It is also the first time that variation in their ability to support neovascularisation *in vivo* and attenuate LV adverse remodelling following myocardial infarction is described.

Li *et al.* [98] compared human CDC with mesenchymal progenitors derived from bone marrow and fat and concluded that CDC had a superior reparative ability than bone marrow and fat MSC. The preclinical development of CDCs as therapy for heart disease has led to the development of the first Phase I clinical trial using these cells as treatment in an autologous transplantation setting [59], [60]. The results of the trial are promising, but the limitation is that there are only a handful of patients treated and that the treatment is given to those with recent MI. CDCs did not improve left ventricular EF over control in this trial, but the treatment reduced scar size significantly [59]. The results of this trial will need to be confirmed in a larger trial. Stem cell therapy still might be a potential new treatment for patients suffering from IHD, but the pool of patients is still very heterogeneous and patient stratification may need to be incorporated in the design of new clinical trials with either CDC or BMSC.

#### ***4.4.4 Clinical predictors of angiogenesis***

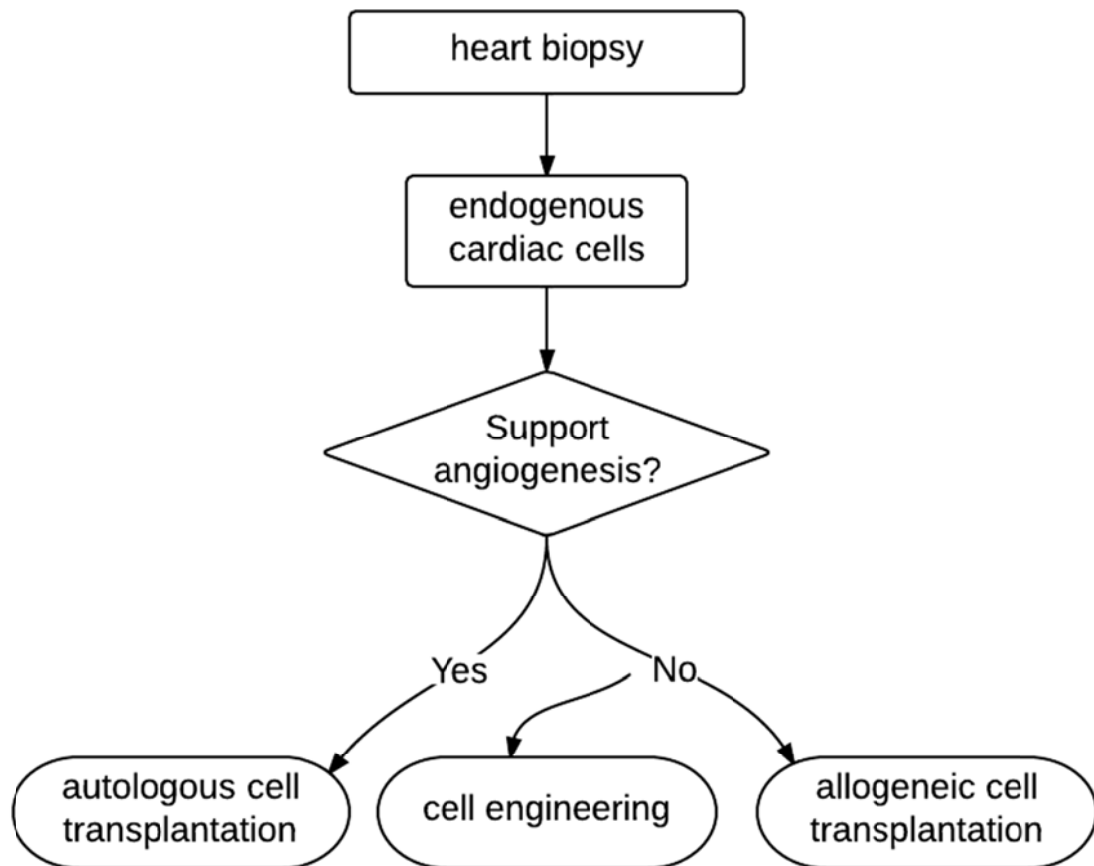
In order to understand the differences observed between patients and to determine whether there are any clinical parameters that may predict the angiogenic potential of CDCs, a regression model that included multiple variables was developed. Total tubule length (TTL) was used as a quantitative measure of angiogenesis and the model was fitted using the demographic characteristics of the patient cohort (see Table 3.1). On building up the multi-linear regression model, a Box-Cox transformation of the original TTL dataset was applied ( $\lambda=0.2$ ) to achieve a normal distribution. The primary model suggested that not all demographic factors played a role in predicting the pro-angiogenic ability of CDCs. Therefore, factors such as sex and hypercholesterolaemia were excluded from the model through a step-wise approach. A multi-linear regression model, with statistical significance, was finally established with the first 35 patients (adjusted  $R^2=0.51$ ,  $p<0.001$ ) using blinded

methods. In this model, parameters such as NYHA Class ( $p=0.03$ ), type of disease ( $p=0.05$ ), including the culprit coronary artery, i.e. right coronary artery in our study ( $p=0.02$ ) and history of cigarette smoking ( $p=0.01$ ) are positive independent predictors of the pro-angiogenic potential of CDCs. In contrast, hypertension ( $p=0.002$ ) is a negative predictor of CDCs' ability to foster angiogenesis.

This is the first time that heart function was noted to considerably affect the pro-angiogenic potential of CDCs. NYHA class reflects the scale of mismatch between alveolar ventilation and perfusion, suggesting an impairment of cardiac output. The regression model suggested that CDCs from patients with NYHA class III and IV tended to have better pro-angiogenic ability than those patients with less severe symptoms.

It is obvious from this study that not all patients will be (i) able to support new blood vessel formation in the damaged heart tissue and (ii) suitable candidates for autologous CDCs transplantation if these cells were to be used in the clinic. Nearly half of the patients' samples from this cohort have impaired vascular supportive function, suggesting that these are the patients who would benefit from either allogeneic cell therapy or would require the function of their CDCs to be restored (see Figure 4.11).

In order to understand how the function of some of these cells may be restored, we need to first understand what the differences are between them. For this purpose, the classification of CDCs into different groups (good, moderate and poor) according to their pro-angiogenic ability will help dissect the molecular mechanisms involved in these processes. Further characterization of these phenotypically and functionally different groups is performed in the following chapters of this study.



**Figure 4.11 Proposed strategies on choice of CDC therapies.** IHD patients with endogenous CDCs that are able to foster angiogenesis would be suitable for autologous transplantation. Patients whose CDCs are poor in supporting angiogenesis will probably benefit from either allogeneic cell transplantation or transplantation with autologous cells that have undergone modification by cell engineering.

## **Chapter 5    Transcriptional and post-transcriptional regulation   of   human   cardiosphere-derived   cell function**

## 5.1 Background

The recent clinical trial where autologous CDCs were administered to patients who suffered from a recent MI, the CADUCEUS trial, showed that the treatment is safe and that CDC transplantation could reduce scar size and improve myocardial viability [59]. Despite these encouraging results, the reduction of infarct size was not accompanied by an improvement in heart function. In the previous chapters of this thesis, I demonstrated that CDCs isolated from approximately 2/3 of IHD patients are functionally less competent than BMSC and the remaining CDCs. Their mesenchymal differentiation and pro-angiogenic potential *in vitro* are indicative of their therapeutic potential *in vivo* and only CDCs that possess a pro-angiogenic phenotype, where endothelial cells could be supported to form robust vascular network *in vitro* and *in vivo*, are capable of improving systolic heart function (chapter 4 of this thesis). Therefore, it is crucial that the differences between CDCs samples are understood in order to (i) identify biomarkers that correlate with cell function, and (ii) stratify patients prior to treatment with the view of improving efficacy in future clinical trials.

Neovascularisation would enhance heart tissue viability through anti-apoptosis, proliferation, endogenous progenitor mobilisation, and attenuation of the metabolic disorder caused by oxidative stress following ischaemic injury [119], [172–176]. A large number of molecules, including inflammatory cytokines, growth factors and receptors and signalling pathway elements have been identified to be involved in angiogenesis following ischemic insult to myocardium [120].

As this is the first time that differences in the pro-angiogenic and therapeutic potential of CDCs are described, there are no previous studies defining molecular differences

between such cells. There are, however, numerous studies in which the genetic profiles of mesenchymal stromal cells (MSC) from tissues such as bone marrow, fat and umbilical cord blood are analysed [177–179] compared the genomic profiles of bone marrow-, adipose tissue- and umbilical cord blood-derived MSC to non multipotent fibroblasts. The study identified 16 genes highly up-regulated and 30 genes significantly down-regulated in MSC compared with fibroblasts. They concluded that a panel of markers, rather than a single one, is essential in the identification of multipotent MSC.

Although in heart disease a great deal of attention has focused on the role of cardiomyocytes in cardiac dysfunction, there is growing evidence that stromal cells play a major role in supporting both endothelial cells and cardiomyocytes. Mesenchymal/stromal cells are responsible for the synthesis and deposition of ECM components during homeostasis and in disease [180], [181]. The expression of ECM components and their modulators seem to be altered in pathological or stress conditions and during cardiac remodelling and fibrosis [182], [183]. In our laboratory, we have observed that CDCs with good pro-angiogenic potential have a reduced level of Type I collagen compared with CDCs with poor angiogenic potential (Sweeney D *et al.*, unpublished results).

Type I Collagen, a major component of the ECM, is regulated by a number of factors through transforming growth factor-beta (TGF- $\beta$ ), interleukin-1 (IL-1), interferon-gamma (IFN- $\gamma$ ) and tumour necrosis factor-alpha (TNF- $\alpha$ ) signalling pathways. The TGF- $\beta$  superfamily, which includes TGF- $\beta$ , bone morphogenetic proteins (BMPs) and activins, induces the expression of ECM proteins such as fibronectin, laminin,



tenascin, besides collagen and inhibits ECM degradation. In contrast, IFN- $\gamma$  and TNF- $\alpha$  signalling inhibit collagen synthesis [184]. However, a dual role of TGF- $\beta$  has also been reported [185–188]. A recent study by Fragiadaki M *et al* [189] described how a high dose of TGF- $\beta$  (10ng/mL) could inhibit collagen expression through the induction of the transcription factor Cut-like Homeobox 1, CUX-1, in renal mesangial cells.

Another important group of ECM proteins are matrix metalloproteinases (MMPs) which contribute more to angiogenesis than simply degrading ECM components. MMPs enhance angiogenesis by cleaving endothelial cell-cell adhesion molecules to release ECM-bound angiogenic growth factors, exposing cryptic pro-angiogenic integrin binding sites, and generating ECM component fragments to enable cell migration [190]. BMP-1/mTLD, or procollagen C endo-peptidase (PCP), is one of the MMPs which cleaves pro-collagen I, II and III, and plays a critical role in bone formation and possibly in angiogenesis [191–194]. Previous results showed that CDCs were good supporters of angiogenesis and had osteogenic differentiation capacity had a higher expression level of BMP-1 (Sweeney D *et al.*, unpublished results).

During the course of this study, data have become available which suggest that both angiogenesis and fibrosis are extensively regulated at a post-transcriptional level and miRNAs have been identified that protect against ischaemic injury and heart failure. Recent studies have demonstrated that the expression of ECM components such as collagens and their regulators (e.g. miRNAs) is altered during chronic oxidative stress [195] and cardiac remodelling [196]. One example of this is the miR-29b family

which controls collagen and collagen metabolic enzyme gene expression in trabecular meshwork cells [195], [197] and is associated with cardiac fibrosis [196]. The expression of miR-29b is significantly reduced in diseased compared to normal human heart tissue [196], [198].

## **5.2 Aims and objectives**

We hypothesised that the cardiosphere method was an effective way to isolate and derive the cardiac MSC population from autologous biopsy, and the variation of the pro-angiogenic ability of CDCs from IHD patients was regulated by certain genes or microRNAs at transcriptional or post-transcriptional level, or even both, to influence multiple processes and signalling pathways involved in angiogenesis and fibrosis. Besides, as we had been having particular interests in the BMP-1/COL1A2 pathway in angiogenesis, we also hypothesised that it might be one of the main targets for the molecular regulation. The aim of this chapter was to identify the molecular mechanisms that play critical roles in modulating the pro-angiogenic function of CDC from IHD patients. The specific objectives are as follows:

- 1) To obtain and compare gene expression profiles of CDCs from functionally different groups (e.g. good and poor supporters of angiogenesis) and identify genes that are differentially expressed in those groups.
- 2) To confirm and validate the differential expression of candidate genes in CDC from different functional groups.
- 3) To select a list of miRNAs from the current databases that may regulate certain extracellular matrix components and to confirm the inhibitory potency of the selected miRNAs *in vitro*.

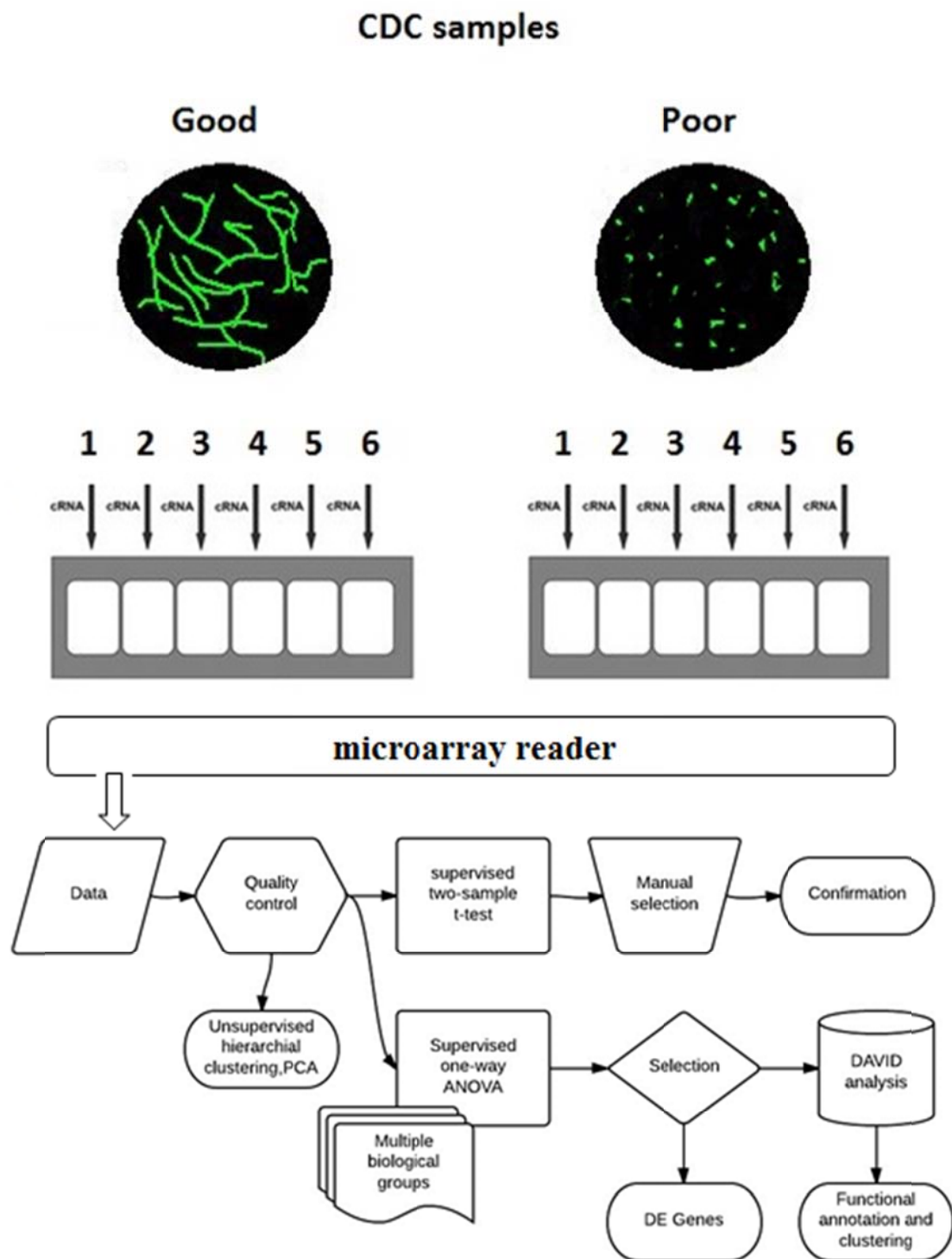
- 4) To determine the endogenous levels of the specific miRNAs in CDCs from different groups of CDCs.

## 5.3 Results

In chapter 4 of this thesis differences in the pro-angiogenic and therapeutic potential of CDCs were observed. In order to understand the molecular mechanisms underlying those phenotypic and therapeutic differences a global gene expression analysis was conducted using cDNA array technology. The experimental design is detailed in Figure 5.1.

### *5.3.1 Summary of the selected patient CDC samples and RNA quality*

CDCs were cultured as described in Materials and Methods (section 2.3.4). High quality total RNA was successfully isolated from 12 CDC samples, 6 samples from good supporters of angiogenesis and 6 samples from poor supporters (see Table 5.1). The RNA samples were sent to the gene profiling service at the Department of Cardiovascular Medicine, University of Oxford (see flow chart in Figure 5.1). The total RNA was diluted to a concentration of 1µg/ µL and a final amount of 50ng was taken for amplification and labelling of cRNA as described in section 2.9. Labelled cRNA from each sample was hybridised to a Sentrix Human-6 v3 expression Illumina Beadchip containing 48,804 probe sets and developed using Streptavidin-Cy3 as described in section 2.13. Data was collected and analysed to yield the transcript abundance of each gene present in the array.



**Figure 5.1 Gene expression analysis comparing functionally different CDC samples.** The diagram depicts the protocol followed to assess differentially gene expression between two groups of CDCs with different vascular supportive ability. CDC samples were tested in the *in vitro* angiogenesis assay and samples classified as ‘good’ (n=6) and ‘poor’ supporters (n=6) which were then selected for gene profiling analysis using cDNA array technology. The flow chart corresponds to steps taken during data analysis. DE; differentially expressed genes.

**Table 5.1 Clinical characteristics of the CDC samples selected for gene expression profiling.**

| Subgroup | Pt ID | Sex | Procedure | NYHA | AS | CAD | No. diseased vessels | LAD | LCX | RCA | DM | HBP | HBChol | Smoker  | FHx | TTL( $\mu$ m) |
|----------|-------|-----|-----------|------|----|-----|----------------------|-----|-----|-----|----|-----|--------|---------|-----|---------------|
| Good     | GT05  | F   | CABG      | I    | N  | Y   | 3                    | Y   | Y   | Y   | Y  | N   | N      | current | Y   | 13500         |
| Good     | GT07  | M   | CABG      | III  | N  | Y   | 3                    | Y   | Y   | Y   | N  | N   | Y      | Ex      | Y   | 14000         |
| Good     | HZ43  | M   | CABG      | III  | N  | Y   | 1                    | N   | N   | Y   | N  | Y   | Y      | Ex      | N   | 9873          |
| Good     | HZ46  | M   | CABG      | IV   | N  | Y   | 3                    | Y   | Y   | Y   | Y  | Y   | Y      | Ex      | N   | 6906          |
| Good     | HZ48  | M   | CABG      | II   | N  | Y   | 2                    | Y   | Y   | N   | N  | N   | Y      | Non     | N   | 5072          |
| Good     | HZ49  | M   | CABG      | III  | N  | Y   | 3                    | Y   | Y   | Y   | N  | Y   | Y      | Ex      | N   | 8701          |
| Poor     | HZ32  | M   | CABG      | II   | N  | Y   | 3                    | Y   | Y   | Y   | N  | Y   | Y      | Ex      | N   | 689           |
| Poor     | HZ35  | M   | CABG      | II   | N  | Y   | 3                    | Y   | Y   | Y   | N  | Y   | Y      | Ex      | Y   | 880           |
| Poor     | HZ37  | M   | AVR       | III  | Y  | N   | 0                    | N   | N   | N   | Y  | Y   | Y      | Ex      | N   | 62            |
| Poor     | HZ44  | M   | Others    | II   | N  | N   | 0                    | N   | N   | N   | N  | N   | Y      | Ex      | Y   | 1584          |
| Poor     | HZ45  | F   | CABG      | II   | N  | Y   | 2                    | Y   | N   | Y   | N  | Y   | Y      | Non     | Y   | 1192          |
| Poor     | HZ51  | F   | CABG      | III  | N  | Y   | 2                    | Y   | Y   | N   | Y  | Y   | Y      | Non     | Y   | 15            |

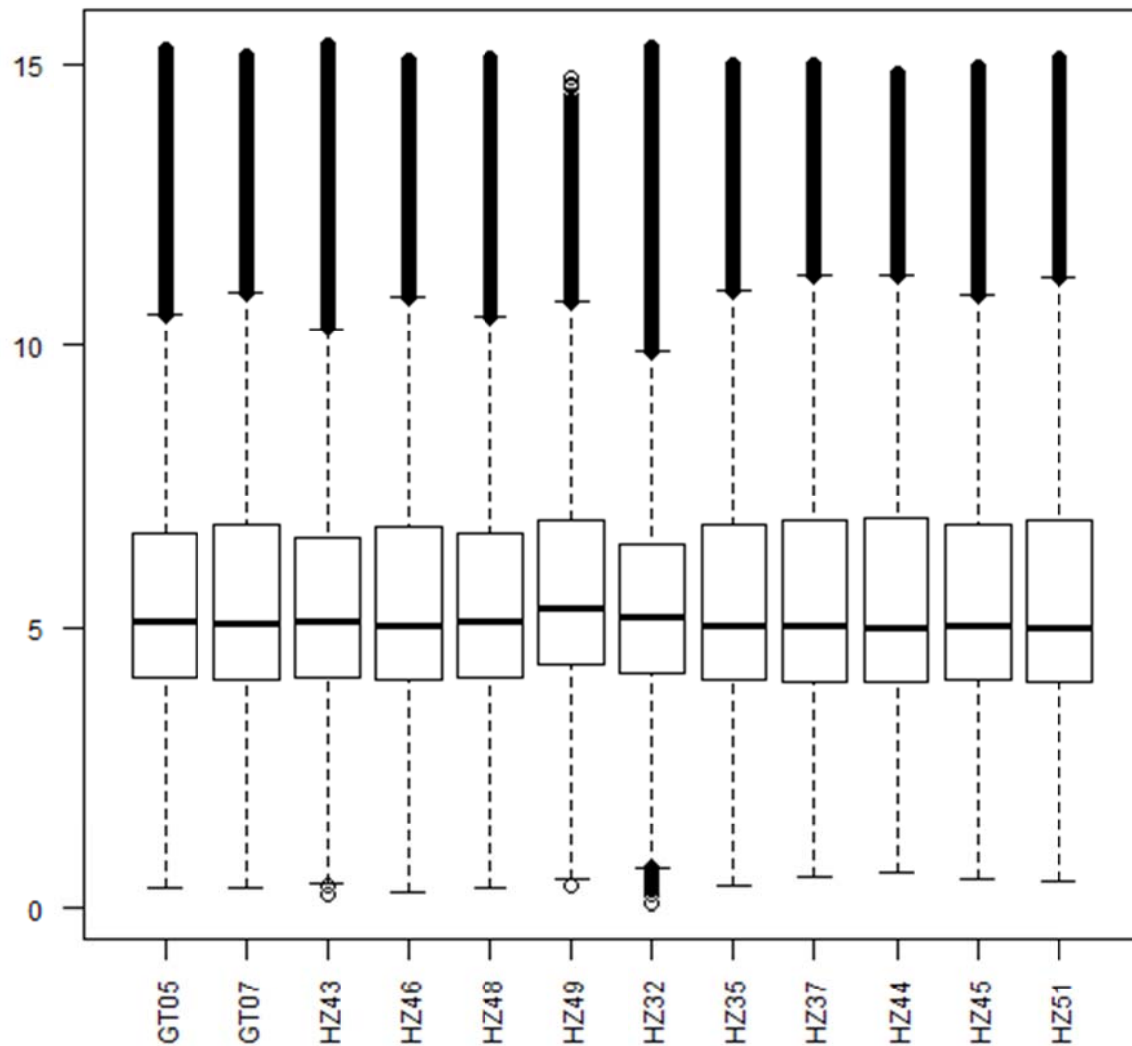
AS, aortic stenosis; CABG, coronary artery bypass graft; CAD, coronary artery disease; DM, diabetes mellitus; F, female; FHx, family history of CAD; HBChol; high blood cholesterol; HBP, hypertension; LAD, left anterior descending artery; LCX, left circumflex artery; M, male; NYHA, New York Heart Association; Pt ID, patient identification; RCA, right coronary artery; TTL, total tubule length.

### ***5.3.2 Quality control of gene expression***

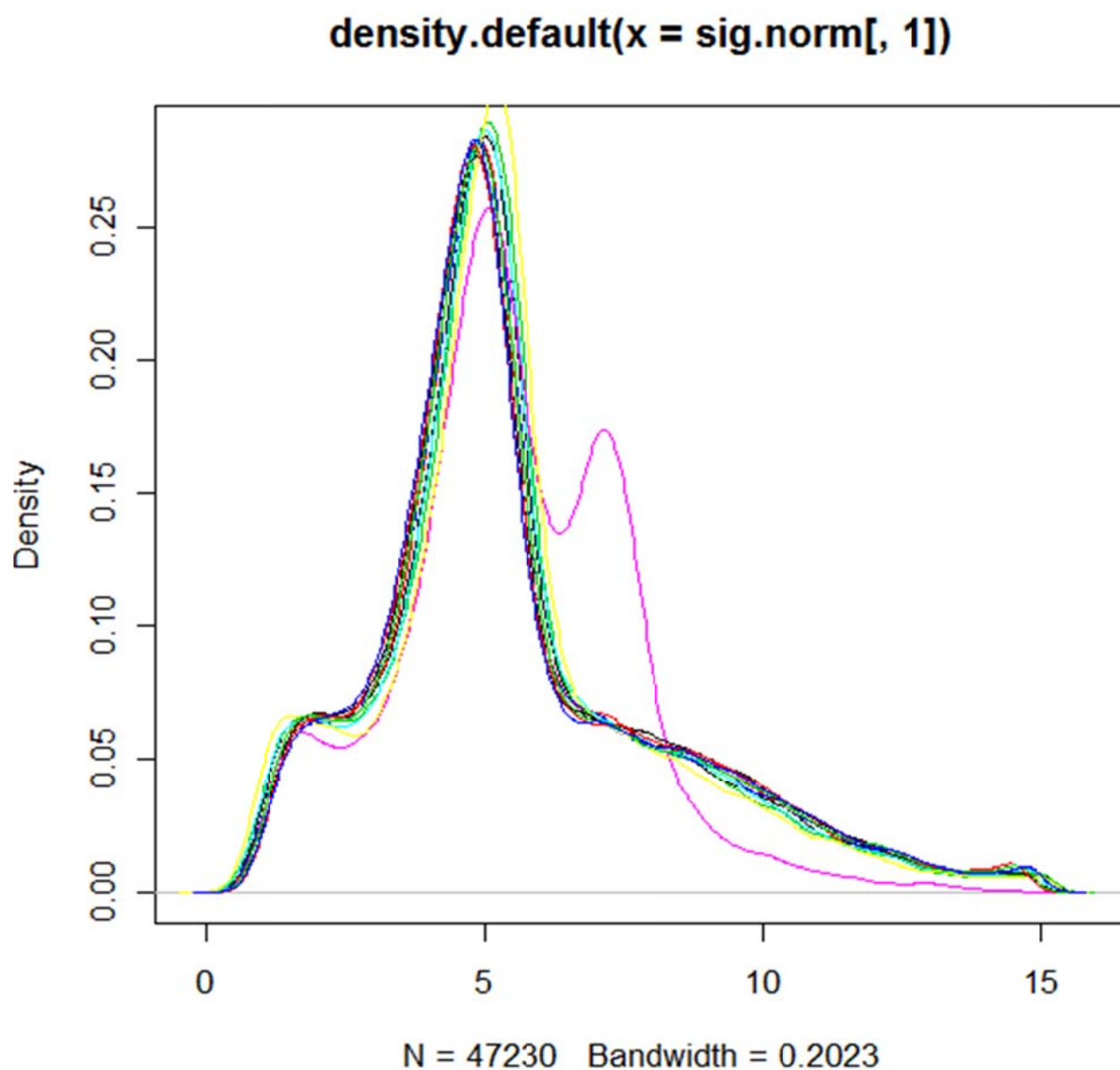
Quality control of microarray data is essential to minimise systematic errors and disqualify outliers in the data set prior to performing any comparisons. Batch differences in the beadchip, background fluorescence and imbalanced levels of hybridisation are the most common systematic errors. Since all samples in this study were hybridised on the same batch of beadchip (Batch number 5957979009), normalisation for batch difference was not required. Background fluorescence errors and random variation during signal detection were eliminated by normalisation of the raw data with a custom script written in R v2.14.1 using the vsn Bioconductor software package (as described in section 2.13). Following normalisation, a total of 47,230 hybridised probes showed significant fluorescent signal on the Illumina beadchip for data analysis. Imbalances in the level of hybridisation were also tested. Figure 5.2 shows the Boxplots of the raw data set, after  $\log_2$  transformation, where range, upper, median and lower quartile of the fluorescence intensity for each sample are summarised. The data demonstrate similar fluorescence intensity amongst all samples tested and hence no imbalanced hybridisation to the beadchip.

Besides the above systematic errors, variation caused by measurement error was minimised by analysing the probability density curve of gene expression generated using the Partek R Genomics SuiteT v6.11.0701 software. The density curves depicted the overall signal distribution of all probes for each RNA sample. Figure 5.3 shows the presence of an outlier amongst the samples. The probability distribution of the transformed fluorescence intensity for one sample (Pt49) did not conform to the distribution of the remaining samples. It reflected the occurrence of measurement error for this specific sample; hence CDC sample Pt49 was removed from the analysis. A total of 11 samples were qualified for direct comparison of gene

expression. The raw data was imported into the Partek Genomics Suite and analysed in two groups: (1) good supporters (n=5) and (2) poor supporters (n=6) of angiogenesis respectively.



**Figure 5.2 Quality control: level of hybridisation.** Boxplots of the log<sub>2</sub> transformed fluorescence intensity, with range, upper and lower quartile (clear rectangle) and median (black horizontal line) values given for each sample used in the cDNA array. All samples had similar readings of fluorescence intensities with the medians approximately around  $2^5$  and range at comparable level among samples.



**Figure 5.3 Quality control: probability density plots of gene expression.** All samples except one (Pt 49) show similar probability density plots. Pt 49 had a significantly biased probability density curve (pink) in comparison to the others. This abnormal pattern was most likely to be caused by errors during measurement.

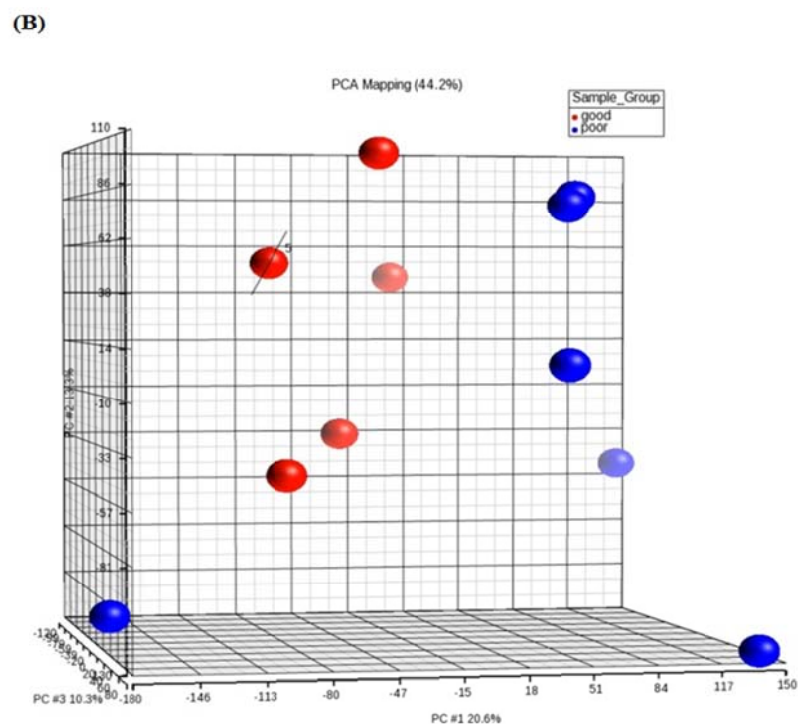
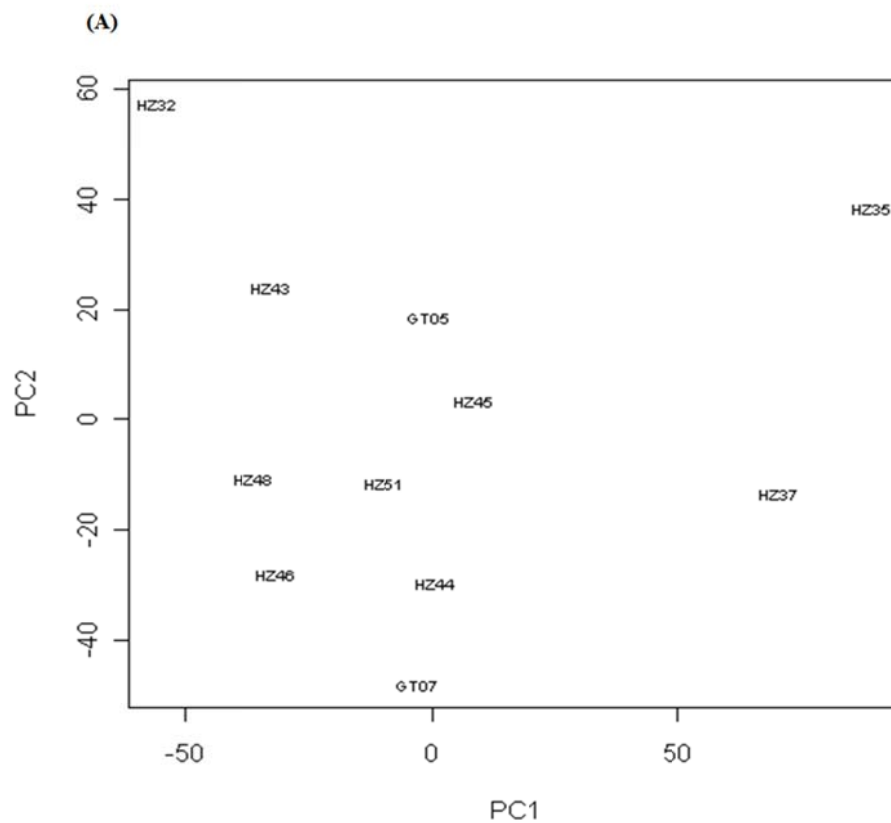


### ***5.3.3 Principal component analysis (PCA) and hierarchical clustering (HCL)***

PCA and HCL to detect the differentially expressed (DE) genes between the two groups were performed. This allowed the data to be categorised according to the similarities of gene expression over 47,000 gene probes and to cluster them. First, PCA was conducted. Through complex mathematical processing, PCA provided a projection of complex data sets onto a reduced dimensional space by mapping the experimental samples to facilitate visualisation. Two- (2D) and three-dimensional (3D) maps are shown in Figure 5.4 A and B respectively. The 2D mapping of PCA (Figure 5.4A) did not show evident clustering of the samples, whilst on the 3D mapping, good (in red) and poor (in blue) supporters of angiogenesis were grouped in two clearly distinct clusters (with the exception of one sample), as shown in Figure 5.4B.

Second, a HCL algorithm was also used to group the experimental samples according to their similarities in gene expression. HCL produced a tree-like dendrogram with clear group differences between good and poor supporters (Figure 5.5A). The poor supporters group assembled on the left-hand side of the dendrogram, whereas the good supporters did on the right.

Taken together, the results from the PCA and HCL analyses confirmed the functional grouping strategy for CDC samples into good and poor supporters of angiogenesis that has been described in chapter 4 of this thesis, with the exception of one sample.



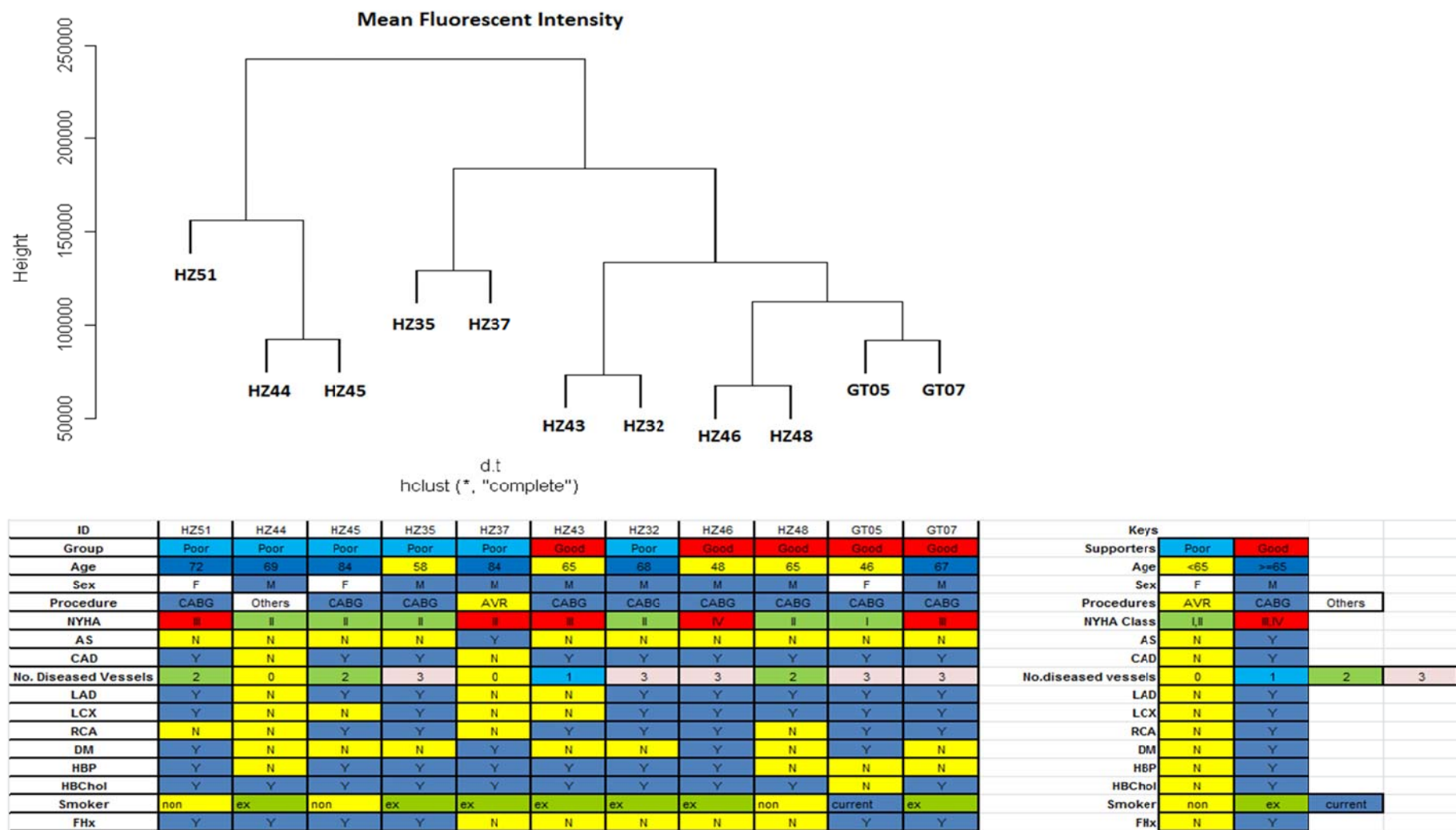
**Figure 5.4 Principal component analysis.**(A) Two-dimensional (2D), and (B) three-dimensional (3D) mapping. Whilst on the 2D mapping there was no evidence of clustering, the 3D mapping showed good supporters (red) and poor supporters (blue) grouped in two distinct clusters. The first three principle components explained 44.2% of the sample variance.

The HCL result was reviewed by matching the clinical parameters of the samples tested including, age, sex, procedures (whether CABG or others), NYHA class, aortic stenosis, coronary artery disease, number of diseased vessels, LAD, LCX, RCA diseased vessel, diabetes mellitus, hypertension, high cholesterol, smoking history and family history. Figure 5.5B shows that support of angiogenesis remains as the best grouping strategy. Interestingly, the number of diseased vessels (whether none, 1, 2 or 3 coronary disease vessels) seems to correlate with the clustering observed as a result of HCL analysis.

#### ***5.3.4 Differentially expressed genes in good vs. poor supporters of angiogenesis***

Although good and poor supporters clustered together using PCA and HCL analyses, the heat map of gene expression profiles did not reveal dramatic differences between the two groups (see Figure 5.6).

To identify genes with altered gene expression between good and poor supporters, a one-way ANOVA was performed, which revealed >2,000 genes with significantly differential expression values between the two groups. Applying a false discovery rate (FDR) lower than 0.05 and accounting for multiple testing using the standard Bonferroni's correction, only one gene, *nucleolar complex associated 4 homolog (NOC4L)*, showed significant differential expression between the two groups ( $p=8.73 \times 10^{-7}$ ). The analysis was repeated grouping the samples of hypertensive vs. non-hypertensive patients, as hypertension was found to be a significant negative predictor of angiogenesis (chapter 4 of this thesis). Lowering the FDR to 0.20 and accounting for multiple testing, only one gene, *CUX1*, was found to be differentially expressed between these two groups. Table 5.2 shows the fold-change, adjusted p values and FDR for *NOC4L* and *CUX1* genes.



**Figure 5.5 Hierarchical clustering.** (A) Dendrogram of the unsupervised clustering results showing similarities within the functional groups of good and poor supporters. (B) Classification of patient samples according to their clinical characteristics. Most of the poor supporters were clustered on the left-hand side of the dendrogram whilst the good supporters were mainly on the right-hand side.

{good supporters} { poor supporter }



**Figure 5.6 Partial heatmap comparing the gene expression profiles of CDC from good and poor supporters.** The heatmap showed a high degree of homogeneity in gene expression between CDCs from good and poor supporters of angiogenesis. The colours are artificial, green=0.0, red=7.6, and black=5.4 (logarithm scale of signal).

**Table 5.2 List of differentially expressed genes according to the primary data analysis criteria.**

| Primary selection | Symbol       | Adj. p-value          | Fold Change | FDR  |
|-------------------|--------------|-----------------------|-------------|------|
| Good vs. Poor     | <i>NOC4L</i> | $8.73 \times 10^{-7}$ | 1.56        | 0.04 |
| non-HBP vs. HBP   | <i>CUX1</i>  | $1.99 \times 10^{-6}$ | 1.36        | 0.20 |

FDR, false discovery rate; HBP, hypertensive.

Taken together the data suggest that although good and poor supporters cluster in two distinct groups according to differences in gene expression, those differences are relatively small to detect significant changes. This may be an indication that although the vascular supportive function of CDCs might be regulated at the transcriptional level, other post-transcriptional regulatory mechanisms of gene expression may also be involved.

In a secondary analysis, Student's *t*-test was used and the differentially expressed (DE) genes ( $p < 0.01$ ) were selected with a cut-off of 2-fold change in gene expression between the good and the poor supporter groups. A total of 44 genes were included in this category (see Table 5.3), among which, 19 genes were up-regulated in the good supporters whilst 25 genes were up-regulated in poor supporters.

**Table 5.3 List of differentially expressed genes according to the secondary data analysis criteria.**

| Symbol                                 | Probe ID | Good |      | Poor  |      | p-value | FDR  | Fold change |
|----------------------------------------|----------|------|------|-------|------|---------|------|-------------|
|                                        |          | mean | SD   | mean  | SD   |         |      |             |
| (A) upregulated in CDC poor supporters |          |      |      |       |      |         |      |             |
| HAPLN1                                 | 2060202  | 7    | 1.88 | 10.06 | 0.92 | 0.0064  | 0.72 | 8.36        |
| HAPLN1                                 | 1850397  | 6.84 | 1.23 | 9.15  | 0.72 | 0.0036  | 0.71 | 4.97        |

|                 |         |       |      |       |      |        |      |      |
|-----------------|---------|-------|------|-------|------|--------|------|------|
| <i>PALMD</i>    | 4290494 | 6.04  | 0.75 | 8.2   | 1    | 0.0033 | 0.74 | 4.45 |
| <i>ZNF365</i>   | 4180301 | 6.25  | 0.62 | 8.22  | 0.44 | 0.0002 | 0.62 | 3.92 |
| <i>HECW2</i>    | 6290475 | 7.11  | 0.93 | 8.84  | 0.55 | 0.004  | 0.73 | 3.31 |
| <i>GPC4</i>     | 6330270 | 6.34  | 0.63 | 8.06  | 0.73 | 0.0025 | 0.69 | 3.3  |
| <i>ARSB</i>     | 270215  | 8.18  | 0.55 | 9.8   | 0.84 | 0.0051 | 0.72 | 3.07 |
| <i>SFTA1P</i>   | 6400717 | 6.97  | 0.84 | 8.52  | 0.7  | 0.0084 | 0.73 | 2.93 |
| <i>CENTA1</i>   | 830735  | 9.65  | 0.71 | 11.1  | 0.37 | 0.0019 | 0.74 | 2.72 |
| <i>GALNT5</i>   | 4010138 | 6.83  | 0.29 | 8.27  | 0.9  | 0.0077 | 0.72 | 2.71 |
| <i>DOK1</i>     | 2030204 | 4.19  | 0.96 | 5.58  | 0.35 | 0.0088 | 0.71 | 2.62 |
| <i>SC5DL</i>    | 6220397 | 8.4   | 0.35 | 9.78  | 0.8  | 0.0058 | 0.71 | 2.62 |
| <i>KATNAL2</i>  | 3060482 | 3.15  | 0.3  | 4.5   | 0.37 | 0.0001 | 0.6  | 2.54 |
| <i>TLR4</i>     | 1500180 | 6.77  | 0.39 | 7.99  | 0.51 | 0.0018 | 0.78 | 2.32 |
| <i>BPGM</i>     | 3060682 | 7.26  | 0.36 | 8.46  | 0.6  | 0.0036 | 0.71 | 2.3  |
| <i>NOV</i>      | 4290037 | 5.58  | 0.4  | 6.78  | 0.58 | 0.0036 | 0.72 | 2.3  |
| <i>FAM13B</i>   | 3520324 | 7.26  | 0.36 | 8.44  | 0.48 | 0.0015 | 0.75 | 2.26 |
| <i>CKB</i>      | 4150066 | 5.87  | 0.4  | 7     | 0.65 | 0.0085 | 0.72 | 2.18 |
| <i>MGC16291</i> | 4180669 | 3.91  | 0.33 | 5.01  | 0.57 | 0.0042 | 0.71 | 2.16 |
| <i>SGK3</i>     | 6510500 | 4.34  | 0.37 | 5.44  | 0.43 | 0.0015 | 0.77 | 2.15 |
| <i>STAT1</i>    | 4810187 | 10.91 | 0.49 | 11.99 | 0.31 | 0.0016 | 0.72 | 2.11 |
| <i>TMEM140</i>  | 4670414 | 6.01  | 0.58 | 7.07  | 0.37 | 0.005  | 0.71 | 2.08 |
| <i>N4BP2L2</i>  | 7200097 | 5.84  | 0.42 | 6.86  | 0.5  | 0.0053 | 0.73 | 2.04 |
| <i>RNU1F1</i>   | 5670753 | 6.19  | 0.33 | 7.2   | 0.54 | 0.0054 | 0.72 | 2.01 |
| <i>HSF2BP</i>   | 7610079 | 6.07  | 0.42 | 7.08  | 0.49 | 0.0056 | 0.7  | 2.01 |

**(B) upregulated in CDC good supporters**

|                 |         |       |      |      |      |        |      |      |
|-----------------|---------|-------|------|------|------|--------|------|------|
| <i>SNORA79</i>  | 1090196 | 7.56  | 0.49 | 6.09 | 0.74 | 0.0042 | 0.72 | 2.78 |
| <i>LOC91461</i> | 4590561 | 7.7   | 0.66 | 6.39 | 0.54 | 0.0056 | 0.7  | 2.48 |
| <i>CDC20</i>    | 1500010 | 11.06 | 0.56 | 9.75 | 0.67 | 0.0073 | 0.72 | 2.47 |
| <i>SNCAIP</i>   | 1660019 | 7.03  | 0.7  | 5.72 | 0.35 | 0.0031 | 0.74 | 2.47 |
| <i>KIFC1</i>    | 7330674 | 7.02  | 0.4  | 5.79 | 0.24 | 0.0001 | 0.61 | 2.36 |
| <i>TK1</i>      | 4730196 | 9.42  | 0.27 | 8.2  | 0.72 | 0.0064 | 0.71 | 2.32 |

|                     |         |       |      |       |      |        |      |      |
|---------------------|---------|-------|------|-------|------|--------|------|------|
| <i>SPC24</i>        | 5910349 | 8.89  | 0.26 | 7.68  | 0.57 | 0.0019 | 0.75 | 2.31 |
| <i>DHDH</i>         | 6770630 | 5.28  | 0.54 | 4.1   | 0.35 | 0.0018 | 0.76 | 2.26 |
| <i>PKDCC</i>        | 3990494 | 8.2   | 0.67 | 7.04  | 0.5  | 0.0093 | 0.71 | 2.24 |
| <i>PTTGI</i>        | 10414   | 10.42 | 0.44 | 9.27  | 0.53 | 0.0037 | 0.72 | 2.22 |
| <i>LOC653820</i>    | 3840358 | 9.47  | 0.25 | 8.32  | 0.62 | 0.004  | 0.73 | 2.22 |
| <i>LOC100130009</i> | 2060750 | 7.04  | 0.35 | 5.9   | 0.49 | 0.0019 | 0.74 | 2.2  |
| <i>PTTG3P</i>       | 5960224 | 10.85 | 0.52 | 9.74  | 0.59 | 0.0095 | 0.7  | 2.16 |
| <i>FSD1</i>         | 1510176 | 5.46  | 0.38 | 4.37  | 0.35 | 0.0008 | 0.77 | 2.13 |
| <i>PTTGI</i>        | 1510291 | 11.44 | 0.46 | 10.35 | 0.54 | 0.0063 | 0.71 | 2.12 |
| <i>FAM64A</i>       | 5870193 | 7.96  | 0.39 | 6.9   | 0.63 | 0.0099 | 0.71 | 2.09 |
| <i>APOBEC3B</i>     | 3170068 | 5.99  | 0.45 | 4.93  | 0.58 | 0.0089 | 0.71 | 2.08 |
| <i>IER5L</i>        | 2710142 | 8.83  | 0.29 | 7.78  | 0.55 | 0.0043 | 0.71 | 2.07 |
| <i>HMGAI</i>        | 7400286 | 12.15 | 0.22 | 11.11 | 0.35 | 0.0003 | 0.72 | 2.06 |

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FDR, false discovery rate; ID, identification; SD, standard deviation.

### 5.3.5 Gene annotation analysis

Although it is tempting to place emphasis on individual genes, the list of those DE following the primary analysis was, in this case, reduced to two genes. In addition, it is also informative to use a statistical approach to identify biological processes or pathways which might be enriched in good vs. poor supporters. For this purpose, the online DAVID v6.7 tool was used [144], [199]. Since the number of DE genes was small using the very stringent selection (FDR<0.05 and corrected  $p<10^{-6}$ ), enrichment analysis in DAVID was conducted using the uncorrected p values ( $p<0.05$ ) as the only inclusion criteria. Two lists, containing 1,674 up-regulated and 1,682 down-regulated genes in good versus supporters respectively, were separately uploaded onto DAVID to assess the biological pathways with the highest enrichment score (ES). The top three annotation clusters for the list of up-regulated genes were (1) alternative



splicing (ES=4.02), (2) Cell cycle: mitotic and G1 phase (ES=2.15) and (3) Cell cycle: M phase (ES=2.13) (Table 5.4). For the list of down-regulated genes, the top three clusters were (1) alternative splicing (ES=1.49), (2) lysosome and vacuolar organisation (ES=1.1) and (3) ubiquitin interacting motif (ES=1.03) (Table 5.5).

### **5.3.6 Confirmation of differences in gene expression**

In order to confirm the above results, the two individual genes obtained in the primary analysis, were selected for further gene expression analysis. *NOC4L* is the human homologue of *Sacharomyces cerevisiae* *NOC4*, a nucleolar associated factor that has been involved in ribosomal RNA processing [200–202]. The *CUX1* gene encodes for a member of the homeodomain family of DNA binding factors which regulate gene expression, morphogenesis, differentiation and cell cycle progression [203–207]. *CUX1* has recently been reported to control the transcription of the type I collagen gene *COL1A2* [189]. In addition to those two genes, four genes from the secondary analysis were also selected. *Signal transducer and activator of transcription 1* (*STAT1*) is an important gene for cell viability and apoptosis [208–210]. *Glypican 4* (*GPC4*) codes for a protein which is a heparan sulfate proteoglycan (HSPG) and acts as co-receptor for growth factor to influence cells proliferation. *Toll-like receptor 4* (*TLR-4*) is involved in pathogen recognition and activation of the innate immune system [211], [212] and *hyaluronan and proteoglycan link protein 1* (*HAPLN-1*) is involved in stabilising aggregates of proteoglycan monomers with hyaluronic acid in the extracellular matrix [213]. These genes were selected on the basis of their association with ECM remodelling, cell proliferation and cell division, cell survival, cell migration and oxidative stress. Significant differential gene expression between

Table 5.4 Enriched clusters of genes in CDC from good supporters of angiogenesis according to DAVID

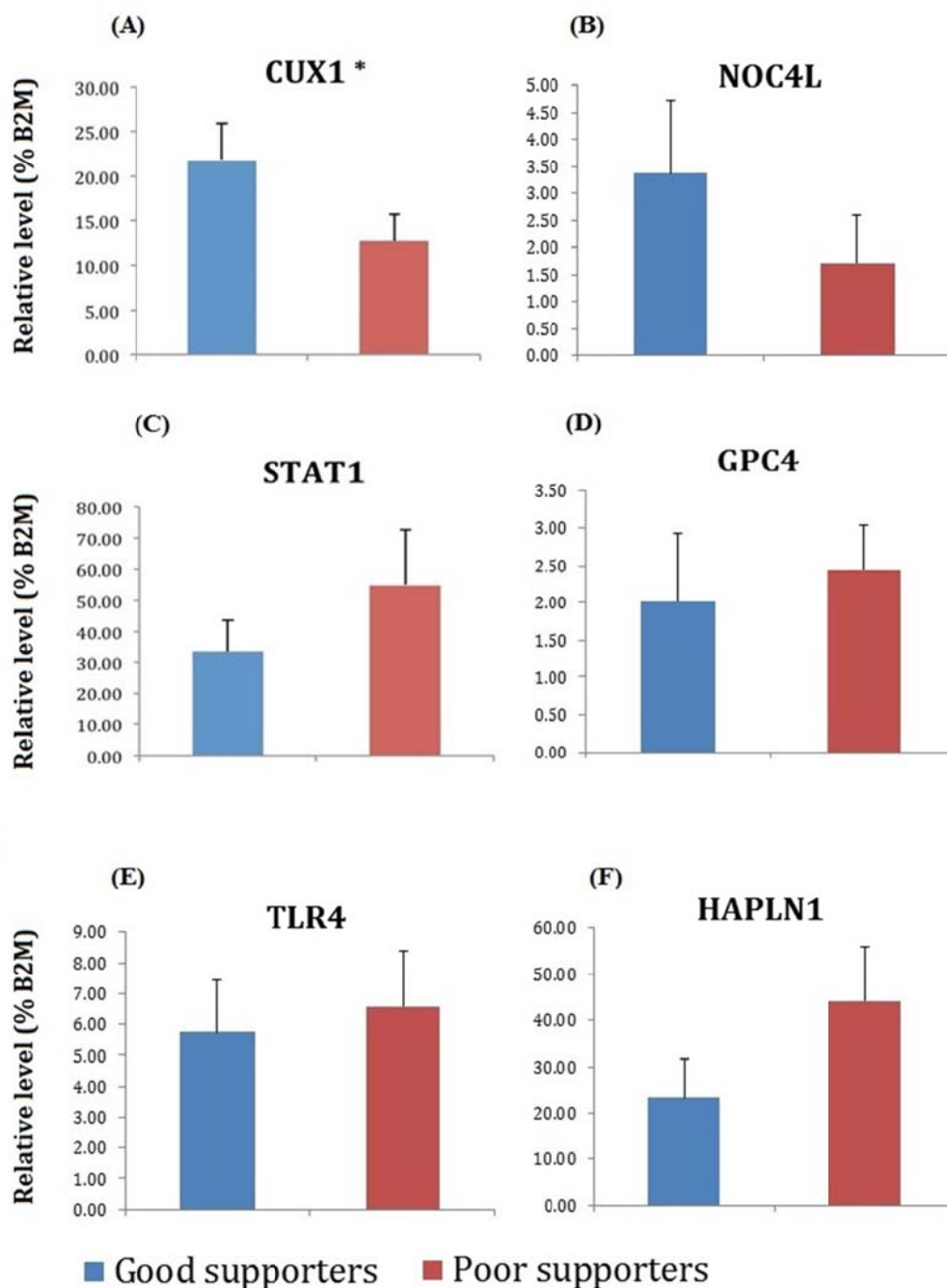
| Annotation Cluster 1     |                 | Enrichment Score: 4.02                                |  |    | Count | P_Value | Benjamini |
|--------------------------|-----------------|-------------------------------------------------------|-------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|-------|---------|-----------|
| <input type="checkbox"/> | UP_SEQ_FEATURE  | splice variant                                        | RT                                                                                  |    | 438   | 1.9E-8  | 4.9E-5    |
| <input type="checkbox"/> | SP_PIR_KEYWORDS | <a href="#">alternative splicing</a>                  | RT                                                                                  |    | 437   | 2.9E-8  | 4.0E-6    |
| <input type="checkbox"/> | UP_SEQ_FEATURE  | sequence variant                                      | RT                                                                                  |    | 581   | 3.4E-1  | 1.0E0     |
| <input type="checkbox"/> | SP_PIR_KEYWORDS | <a href="#">polymorphism</a>                          | RT                                                                                  |    | 554   | 4.3E-1  | 9.3E-1    |
| Annotation Cluster 2     |                 | Enrichment Score: 2.15                                |  |    | Count | P_Value | Benjamini |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">mitotic cell cycle</a>                    | RT                                                                                  |    | 40    | 1.0E-5  | 5.4E-3    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">cell cycle phase</a>                      | RT                                                                                  |    | 42    | 2.6E-5  | 1.2E-2    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">cell cycle process</a>                    | RT                                                                                  |    | 49    | 2.6E-4  | 8.4E-2    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">G1 phase of mitotic cell cycle</a>        | RT                                                                                  |    | 3     | 2.3E-1  | 9.8E-1    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">G1 phase</a>                              | RT                                                                                  |    | 3     | 2.9E-1  | 9.8E-1    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">interphase of mitotic cell cycle</a>      | RT                                                                                  |    | 7     | 4.2E-1  | 9.9E-1    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">interphase</a>                            | RT                                                                                  |    | 7     | 4.4E-1  | 9.9E-1    |
| Annotation Cluster 3     |                 | Enrichment Score: 2.13                                |  |    | Count | P_Value | Benjamini |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">M phase</a>                               | RT                                                                                  |    | 37    | 9.6E-6  | 6.4E-3    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">mitotic cell cycle</a>                    | RT                                                                                  |    | 40    | 1.0E-5  | 5.4E-3    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">cell cycle phase</a>                      | RT                                                                                  |    | 42    | 2.6E-5  | 1.2E-2    |
| <input type="checkbox"/> | INTERPRO        | <a href="#">Tubulin/FtsZ, 2-layer sandwich domain</a> | RT                                                                                  |    | 8     | 5.8E-5  | 7.3E-2    |
| <input type="checkbox"/> | KEGG_PATHWAY    | <a href="#">Pathogenic Escherichia coli infection</a> | RT                                                                                  |    | 13    | 7.0E-5  | 1.2E-2    |
| <input type="checkbox"/> | INTERPRO        | <a href="#">Tubulin, conserved site</a>               | RT                                                                                  |    | 8     | 7.9E-5  | 5.1E-2    |
| <input type="checkbox"/> | PIR_SUPERFAMILY | PIRSF002306:tubulin                                   | RT                                                                                  |    | 8     | 8.9E-5  | 3.7E-2    |
| <input type="checkbox"/> | INTERPRO        | <a href="#">Tubulin</a>                               | RT                                                                                  |   | 8     | 1.1E-4  | 4.6E-2    |
| <input type="checkbox"/> | INTERPRO        | <a href="#">Tubulin/FtsZ, GTPase domain</a>           | RT                                                                                  |  | 8     | 1.1E-4  | 4.6E-2    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">cell cycle</a>                            | RT                                                                                  |  | 63    | 1.9E-4  | 7.0E-2    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">cell cycle process</a>                    | RT                                                                                  |  | 49    | 2.6E-4  | 8.4E-2    |
| <input type="checkbox"/> | UP_SEQ_FEATURE  | site:Involved in polymerization                       | RT                                                                                  |  | 4     | 1.0E-3  | 2.3E-1    |
| <input type="checkbox"/> | GOTERM_CC_FAT   | <a href="#">microtubule cytoskeleton</a>              | RT                                                                                  |  | 45    | 1.2E-3  | 5.5E-2    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">protein polymerization</a>                | RT                                                                                  |  | 9     | 2.9E-3  | 3.3E-1    |
| <input type="checkbox"/> | GOTERM_MF_FAT   | <a href="#">structural molecule activity</a>          | RT                                                                                  |  | 49    | 3.6E-3  | 3.6E-1    |

Table 5.5 Enriched clusters of genes in CDC from poor supporters of angiogenesis according to DAVID

| Annotation Cluster 1     |                 | Enrichment Score: 1.49                      |    |    | Count | P_Value | Benjamini |
|--------------------------|-----------------|---------------------------------------------|---------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------|-------|---------|-----------|
| <input type="checkbox"/> | UP_SEQ_FEATURE  | splice variant                              | RT                                                                                    |    | 509   | 9.7E-4  | 9.4E-1    |
| <input type="checkbox"/> | SP_PIR_KEYWORDS | <a href="#">alternative splicing</a>        | RT                                                                                    |    | 508   | 1.2E-3  | 4.8E-1    |
| <input type="checkbox"/> | UP_SEQ_FEATURE  | sequence variant                            | RT                                                                                    |    | 709   | 9.6E-1  | 1.0E0     |
| <input type="checkbox"/> | SP_PIR_KEYWORDS | <a href="#">polymorphism</a>                | RT                                                                                    |    | 675   | 9.7E-1  | 1.0E0     |
| Annotation Cluster 2     |                 | Enrichment Score: 1.1                       |    |    | Count | P_Value | Benjamini |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">vacuole organization</a>        | RT                                                                                    |    | 9     | 2.4E-3  | 1.0E0     |
| <input type="checkbox"/> | KEGG_PATHWAY    | <a href="#">Lysosome</a>                    | RT                                                                                    |    | 16    | 2.6E-3  | 3.6E-1    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">lysosome organization</a>       | RT                                                                                    |    | 6     | 1.6E-2  | 1.0E0     |
| <input type="checkbox"/> | GOTERM_CC_FAT   | <a href="#">vacuolar part</a>               | RT                                                                                    |    | 9     | 4.0E-2  | 8.5E-1    |
| <input type="checkbox"/> | GOTERM_CC_FAT   | <a href="#">vacuolar membrane</a>           | RT                                                                                    |    | 8     | 5.3E-2  | 8.6E-1    |
| <input type="checkbox"/> | GOTERM_CC_FAT   | <a href="#">vacuole</a>                     | RT                                                                                    |    | 22    | 9.1E-2  | 9.2E-1    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">vacuolar transport</a>          | RT                                                                                    |    | 5     | 1.3E-1  | 9.9E-1    |
| <input type="checkbox"/> | GOTERM_CC_FAT   | <a href="#">HOPS complex</a>                | RT                                                                                    |    | 3     | 1.6E-1  | 9.0E-1    |
| <input type="checkbox"/> | SP_PIR_KEYWORDS | <a href="#">lysosome</a>                    | RT                                                                                    |    | 13    | 2.0E-1  | 9.5E-1    |
| <input type="checkbox"/> | GOTERM_CC_FAT   | <a href="#">endosome</a>                    | RT                                                                                    |    | 24    | 2.1E-1  | 9.0E-1    |
| <input type="checkbox"/> | GOTERM_CC_FAT   | <a href="#">lytic vacuole</a>               | RT                                                                                    |    | 17    | 2.2E-1  | 9.0E-1    |
| <input type="checkbox"/> | GOTERM_CC_FAT   | <a href="#">lysosome</a>                    | RT                                                                                    |    | 17    | 2.2E-1  | 9.0E-1    |
| <input type="checkbox"/> | GOTERM_CC_FAT   | <a href="#">lysosomal membrane</a>          | RT                                                                                    |    | 4     | 2.3E-1  | 8.9E-1    |
| <input type="checkbox"/> | GOTERM_CC_FAT   | <a href="#">late endosome</a>               | RT                                                                                    |    | 5     | 4.5E-1  | 9.3E-1    |
| <input type="checkbox"/> | GOTERM_BP_FAT   | <a href="#">lysosomal transport</a>         | RT                                                                                    |    | 3     | 4.5E-1  | 1.0E0     |
| Annotation Cluster 3     |                 | Enrichment Score: 1.03                      |  |  | Count | P_Value | Benjamini |
| <input type="checkbox"/> | UP_SEQ_FEATURE  | repeat:UIM 1                                | RT                                                                                    |  | 4     | 6.0E-2  | 1.0E0     |
| <input type="checkbox"/> | UP_SEQ_FEATURE  | repeat:UIM 2                                | RT                                                                                    |  | 4     | 6.0E-2  | 1.0E0     |
| <input type="checkbox"/> | SMART           | <a href="#">UIM</a>                         | RT                                                                                    |  | 4     | 1.3E-1  | 9.9E-1    |
| <input type="checkbox"/> | INTERPRO        | <a href="#">Ubiquitin interacting motif</a> | RT                                                                                    |  | 4     | 1.5E-1  | 1.0E0     |

good and poor supporters of angiogenesis could only be confirmed by qRT-PCR for *CUX1* ( $p=0.03$ ) (Figure 5.7A) although the differential expression was not significant between the hypertensive and non-hypertensive subgroups ( $p=0.36$ , data not shown). The expression of *NOC4L* ( $p=0.42$ ), *STAT1* ( $p=0.16$ ), *GPC4* ( $p=0.35$ ) was not significantly different between good and poor supporters of angiogenesis (Figure 5.7B-D) whilst there was a trend for *TLR4* and *HAPLN1* genes to be differentially expressed ( $p=0.08$ ) (Figure 5.7E and F).

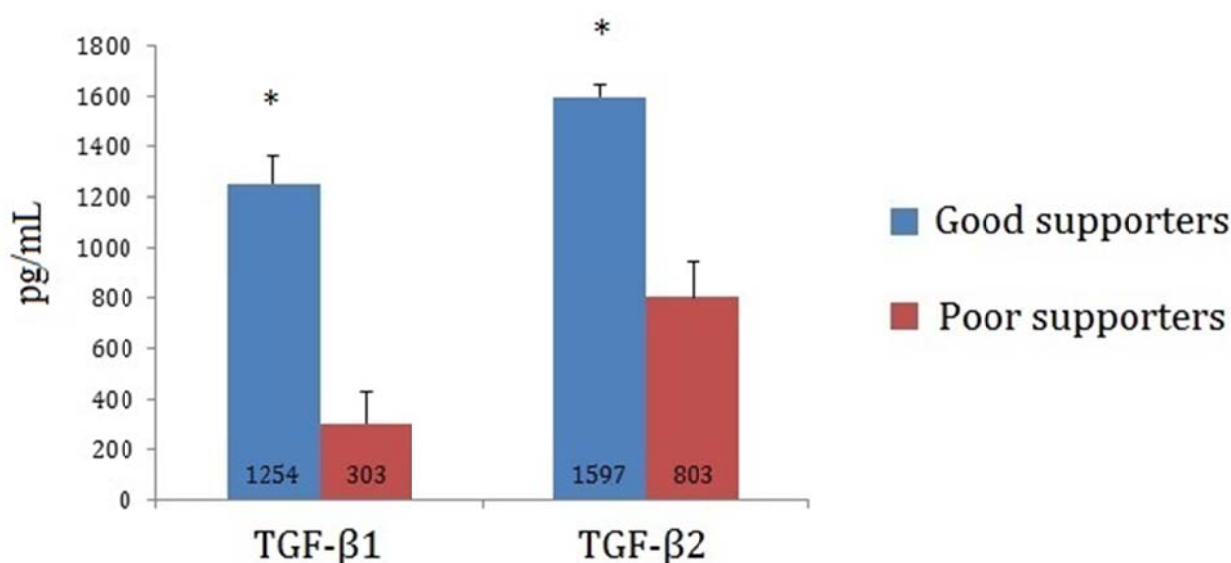
In conclusion, the difference in gene expression between good and poor supporters of angiogenesis could only be confirmed in one (*CUX1*) out of six of the selected genes (Figure 5.7). The results of the qRT-PCR analysis validated the preliminary cDNA array result by applying the highly stringent rule with the outcome that no drastic changes in gene transcription could be observed between the two functionally distinct groups of CDCs. Although the differences in gene expression for individual genes (*HAPLN-1*, *NOC4L* and *TRL4*) were not significant according to q-PCR results, it might be due to a small number of samples being analysed in this instance. However, these results also indicate that support of angiogenesis by CDCs is a complex process with a polygenic trait, and regulation of angiogenesis may also take place both at transcriptional and post-transcriptional level.



**Figure 5.7 Validation of gene expression by qRT-PCR.** qRT-PCR was used to assess changes in gene expression from a selection of genes. RNA from a replicate used in the cDNA array was treated with DNase and cDNA prepared. The cDNA was used as template in quantitative PCR reactions in triplicate for each gene-specific primer set. The mean average raw Ct value was normalised to  $\beta$ 2-microglobulin (B2M) and analysed using the  $\Delta$ Ct method. Differential gene expression between good and poor supporters of angiogenesis was determined for *CUX1* (A), *NOC4L* (B), *STAT1* (C), *GPC4* (D), *TLR4* (E) and *HAPLN1* (F) genes. Bar graphs represent means and the standard errors (SE) of the mRNA abundance as a percentage of the B2M mRNA in CDCs from good (n=9) and poor (n=10) supporters of angiogenesis. Student's t-test was used to determine differences between the two groups with  $p < 0.05$  (\*) considered to be statistically significant.

### 5.3.7 The effect of TGF- $\beta$ 1 treatment on the CUX1/COL1A2 pathway

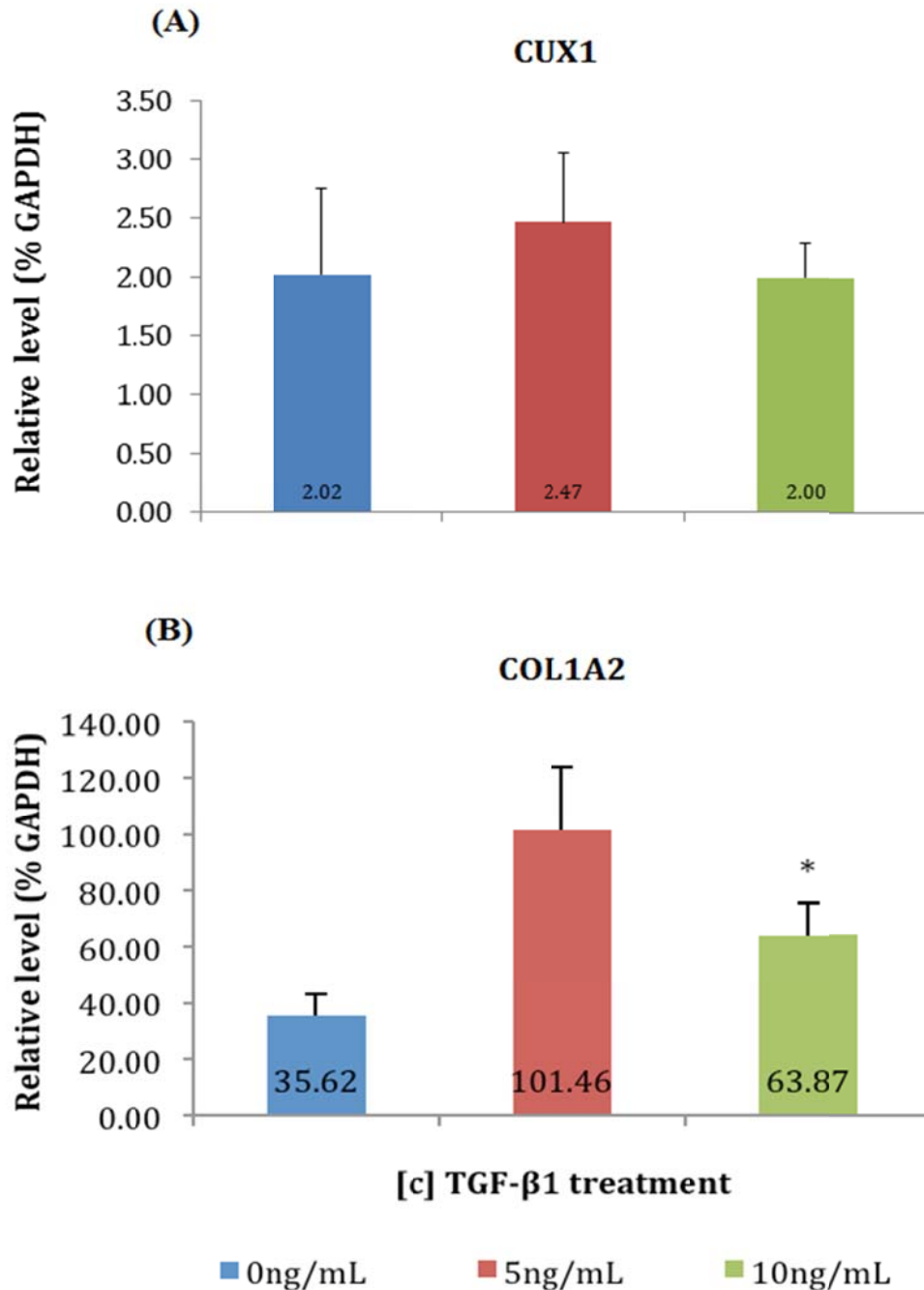
The *CUX1* gene modulates the expression of *COL1A2*, and its own transcription is induced by TGF- $\beta$ 1 [189]. As demonstrated above, *CUX1* is differentially expressed in CDCs from good and poor supporters of angiogenesis. In order to determine whether there was a correlation between *CUX1* expression and TGF- $\beta$ 1 or TGF- $\beta$ 2 in these two groups, an ELISA was used and the amount of these two factors produced by the cells determined. As shown in Figure 5.8, TGF- $\beta$ 1 and TGF- $\beta$ 2 were expressed at significantly higher levels ( $p < 0.05$ ) in good supporters of angiogenesis than in poor supporters.



**Figure 5.8 TGF- $\beta$  level in the cell culture supernatant of CDCs from good and poor supporters.** Approximately  $5 \times 10^5$  cells were cultured in EGM-2 for 48h in triplicate prior to harvesting the conditioned media. The conditioned media from good ( $n=5$ ) and poor ( $n=5$ ) supporters of angiogenesis was diluted 1:2 and the amount of TGF- $\beta$ 1 or - $\beta$ 2 measured using an ELISA kit. Student's *t*-test was used to determine differences between the groups, with  $p < 0.05$  considered statistically significant (\*). CDCs from good supporters of angiogenesis produce a significant higher amount of TGF- $\beta$ 1 and - $\beta$ 2 than CDCs from poor supporters.

In the renal system, *CUX1* gene expression is induced with low (5ng/mL) and high (10ng/mL) doses of TGF- $\beta$ . [189]. However, at low dose, CUX-1 increases the expression of *COL1A2*, whilst at high dose, CUX-1 functions as a repressor of gene transcription, reducing the expression of the type I collagen gene [189]. CDCs from poor supporters expressed significantly higher level of Type I collagen protein than good supporters (Sweeney *et al.*, unpublished results). Therefore, the hypothesis was that in poor supporters of angiogenesis, the expression of *CUX1* could be enhanced by treating the cells with TGF- $\beta$ 1 and hence the transcription of *COL1A2* would be inhibited. In brief, five CDCs samples from poor supporters were treated with low and high doses of TGF- $\beta$ 1 and the levels of *CUX1* and *COL1A2* mRNA was determined by qRT-PCR. Interestingly, neither 5ng/mL nor of 10ng/mL of TGF- $\beta$ 1 changed *CUX1* mRNA levels significantly (Figure 5.9B). However, the *COL1A2* gene expression was significantly increased by 45% when cells were treated with the high dose of TGF- $\beta$ 1 (Figure 5.9B), but not significantly affected by the low dose. In conclusion, treatment with a high dose of TGF- $\beta$ 1 had the opposite effect than expected according to the original hypothesis. Therefore, the role of CUX-1 and its interaction with TGF- $\beta$  and Type I collagen would require further assessment in these cells.

In summary, *COL1A2* increased expression following TGF- $\beta$  treatment does not seem to be CUX-1-dependent in CDCs. However, the reduced expression of type I collagen in good supporters compared to poor supporters might be modulated post-transcriptionally (e.g., by miRNAs).



**Figure 5.9 Effect of TGF-β1 treatment on *CUX1* and *COL1A2* gene expression in CDCs from poor supporters of angiogenesis.** CDCs from poor supporters of angiogenesis were cultured for 72h in the presence of low dose (5ng/mL) or high dose (10ng/mL) of TGF-β1 in triplicate. Following this, total RNA was isolated and the expression of *CUX1* and *COL1A2* genes assessed by qRT-PCR. The abundance of *CUX1* (A) or *COL1A2* (B) mRNAs relative to the *GAPDH* gene was determined using the  $\Delta C_t$  method. Data represent the average with the standard error (SE) of the mRNAs as percentage of *GAPDH*. ANOVA and post-hoc tests were used to assess the differences between treatment groups and p values <0.05 (\*) were considered statistically significant.



### 5.3.8 The role of miRNAs on the post-transcriptional regulation of type I collagen

During the course of this study, it has been reported that regulation of collagen expression can be modulated by miRNAs such as the miR-29 family of miRNAs in trabecular meshwork cells, which plays critical role in the aqueous humour circulation and the dysfunction of trabecular meshwork cells is believed to cause ECM deposition and is directly associated with primary open-angle glaucoma [195]. In order to investigate whether miRNAs had a significant role in the differences in gene expression observed between good and poor supporters, we interrogated the literature and the current databases for the following target genes: *COL1A2*, *bone morphogenetic protein 1 (BMP1)*/*mammalian tolloid (mTLD)* and *chordin (CHRD)*. *BMP1/mTLD* encodes for a metalloproteinase, also called procollagen C endopeptidase, which cleaves collagen. *BMP1* and *mTLD* represent two transcripts (variant 1 and variant 3, respectively) of the same genetic locus. *CHRD* encodes for chordin, an antagonist of BMP signalling, which was used as control as chordin, like Type I collagen, is another substrate of BMP-1. PicTar and TargetScan databases were interrogated in June 2012 and together with reports found in the literature, we selected five miRNAs to test our hypothesis: hsa-miR-15a, hsa-miR-29a and b, hsa-miR-105 and hsa-miR-195 miRNAs. The miR-15a family, and in particular miR-195, is involved in cell cycle progression and apoptosis [214]. The miR-29 family of miRNAs targets over sixteen ECM-related genes [215]. miR-29b has been reported before to target *COL1A2* and *BMP1* in trabecular meshwork cells [195] and to be down-regulated in the heart following MI in mice [196]. miR-105 has been implicated in the regulation of *Toll-like Receptor 2* and 4, *TLR2* and *TLR4* genes, and suggested to have a role in inflammation [216]. However, to our knowledge and with

the exception of miR-29b, none of these genes have been shown to be targets for hsa-miR-15a, hsa-miR-105 or hsa-miR-195 (Table 5.6).

**Table 5.6 MicroRNAs selected by searching Pictar and TargetScan databases and PubMed**

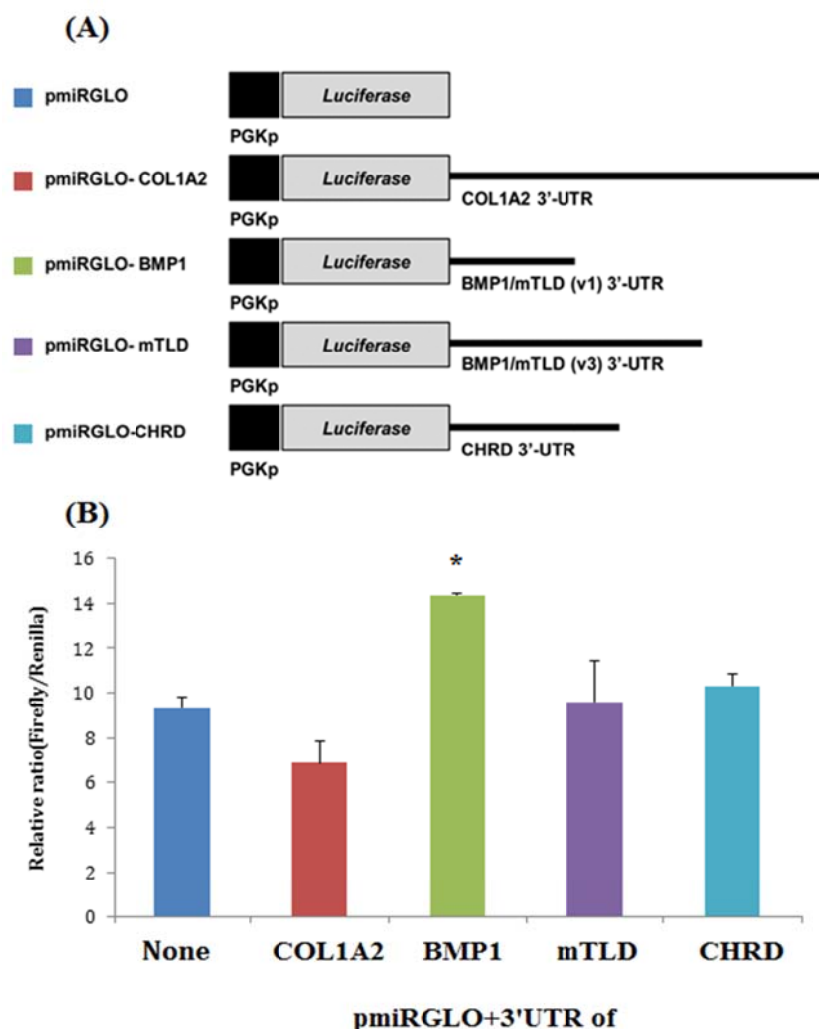
| MicroRNA | Targets                          |                                           |
|----------|----------------------------------|-------------------------------------------|
|          | Report in literature<br>(PubMed) | Interrogation of Databases<br>(June 2012) |
| miR-15a  | unknown                          | <i>CHRD</i> (Top score)                   |
| miR-29b  | <i>BMP1</i> , <i>COL1A2</i>      | <i>BMP1</i>                               |
| miR-105  | unknown                          | <i>BMP1</i> (Top score)                   |
| miR-195  | <i>COL1A2</i> *                  | <i>CHRD</i>                               |

(\*) from a prediction, not confirmed by experimental data [217].

### 5.3.9 The inhibitory effect of miRNAs on the targeted 3'-UTRs

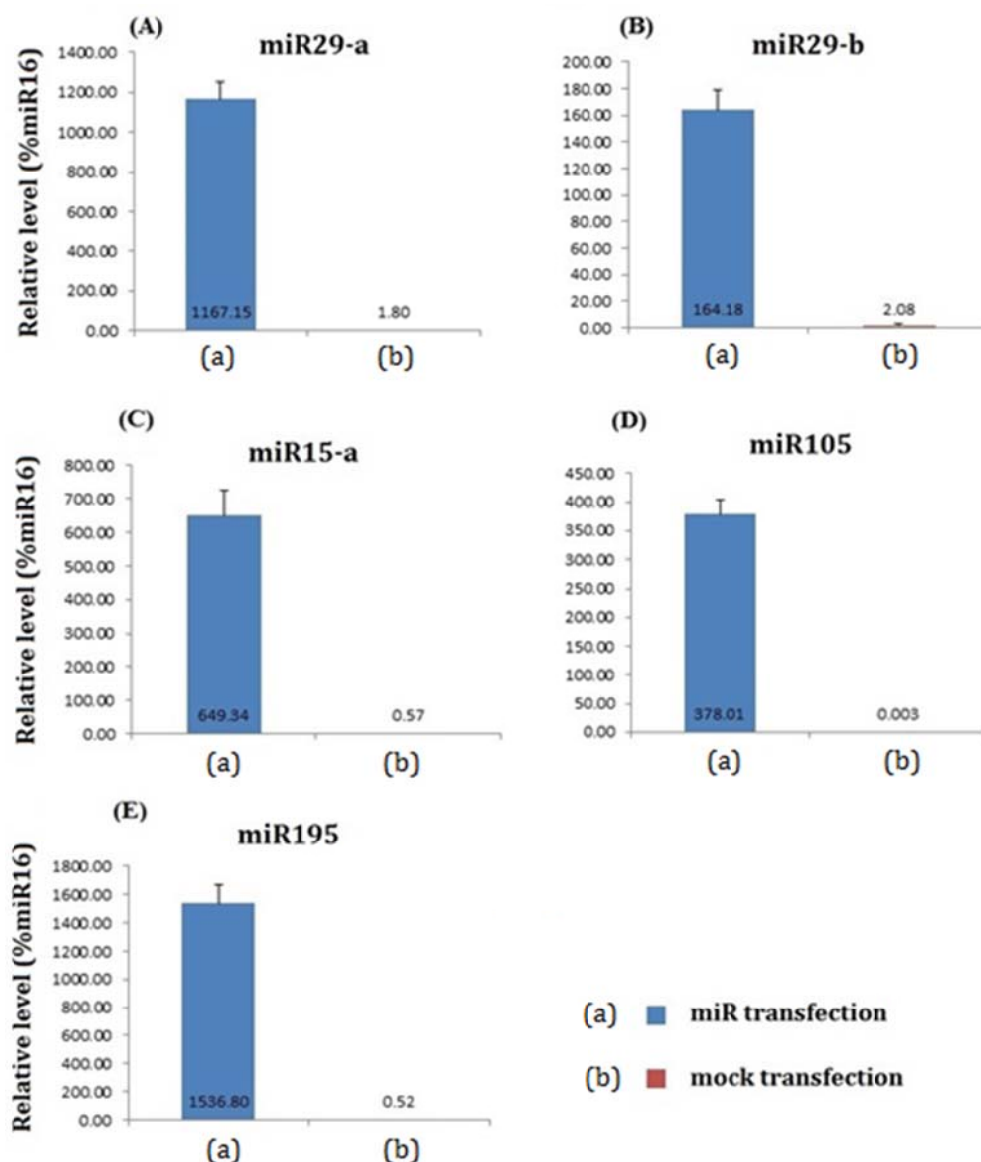
The 3'UTR regions of the human *COL1A2*, *BMP1*, *mTLD* and *CHRD* mRNA transcripts were cloned into the pmiRGLO plasmid as described in section 2.7 to generate the relevant constructs (Figure 5.10A). In order to confirm the expression of firefly luciferase from the recombinant plasmids, a heterologous system was used. HEK293T cells were transiently transfected with the luciferase expressing constructs and the relative ratio of firefly/Renilla luciferase activity measured in the cell extracts as described in section 2.14. The constructs containing the 3'-UTR of *COL1A2*, *mTLD* and *CHRD* displayed relative levels of firefly/Renilla luciferase activities similar to that of the parental control plasmid (pmiRGLO plasmid, Figure 5.10B). Interestingly, a significant increased relative level in firefly luciferase/Renilla activity

was observed when the 3'-UTR of *BMP1* was inserted downstream the firefly luciferase reporter gene (Figure 5.10B), suggesting that there might be elements in the *BMP1* 3'-UTR that could enhance firefly luciferase expression or activity.



**Figure 5.10 The relative level of luciferase activity expressed from the plasmids used in this study.** (A) Schematic gene structures of the luciferase plasmids. The 3'untranslated regions (UTR) of the *COL1A2*, *BMP1*, *mTLD* and *CHRD* genes were cloned downstream the firefly luciferase gene in the parental plasmid pmiRGLO. The expression of the firefly luciferase constructs was controlled by the *PGK* gene promoter (PGKp). (B) Relative ratios of luciferase activity. The above plasmids were transiently transfected into HEK293T cells using DharmaFECT<sup>®</sup> DUO transfection reagent. Approximately 48h post-transfection the cells were lysed and the luciferase activity in the cell lysates measured as a ratio of firefly luciferase (reporter gene) versus Renilla luciferase (internal control gene). The data represent means with the standard errors (SE) of five experiments. Statistical analysis using Student's *t*-test and  $p < 0.05$  showed that the addition of the 3'UTR from the *BMP1* gene to the parental plasmid significantly enhanced the levels of firefly luciferase activity.

The expression levels of miR-29a, miR-29b, miR-15a, miR-105, and miR-195 were significantly increased after transfection of miRNA mimics into HEK293FT cells. (see Figure 5.11)

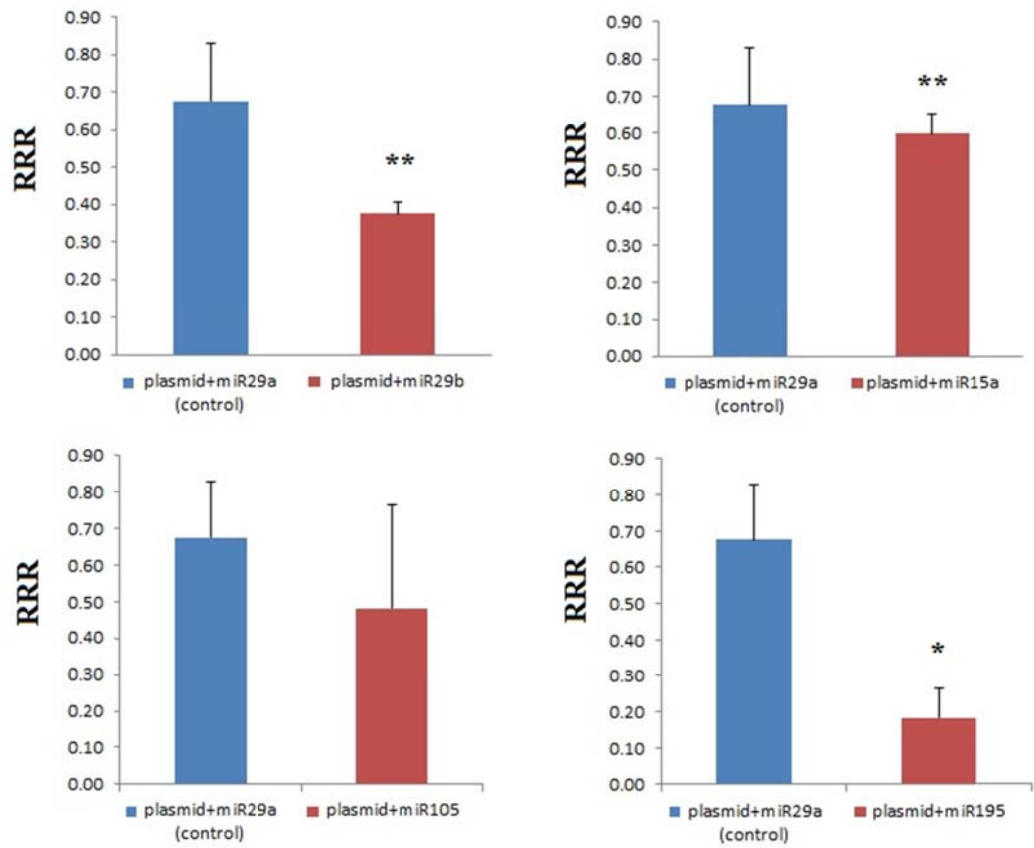


**Figure 5.11 Transfection efficiency of miRNAs into HEK293FT cells.** Cells were transiently co-transfected with 1.2nM of the respective miRNAs mimics (miR) or no miRNA (mock) and the relevant plasmids using DharmaFECT® DUO transfection reagent. Approximately 48h post-transfection, total RNA was isolated from the transfected cells and the relative expression levels of miR-29a (A), miR-29b (B), miR-15a (C), miR-105 (D) and miR-195 (E) measured by qRT-PCR. The mean average raw Ct value was normalised to miR-16 and analysed using the  $\Delta\text{Ct}$  method. miRNA expressions in transfected and mock transfected cells are represented in means and the standard errors (SE) as a percentage of miR-16. Data was obtained from five independent experiments. Student's *t*-test was used to determine differences between the two groups with  $p < 0.05$  (\*) considered to be statistically significant.

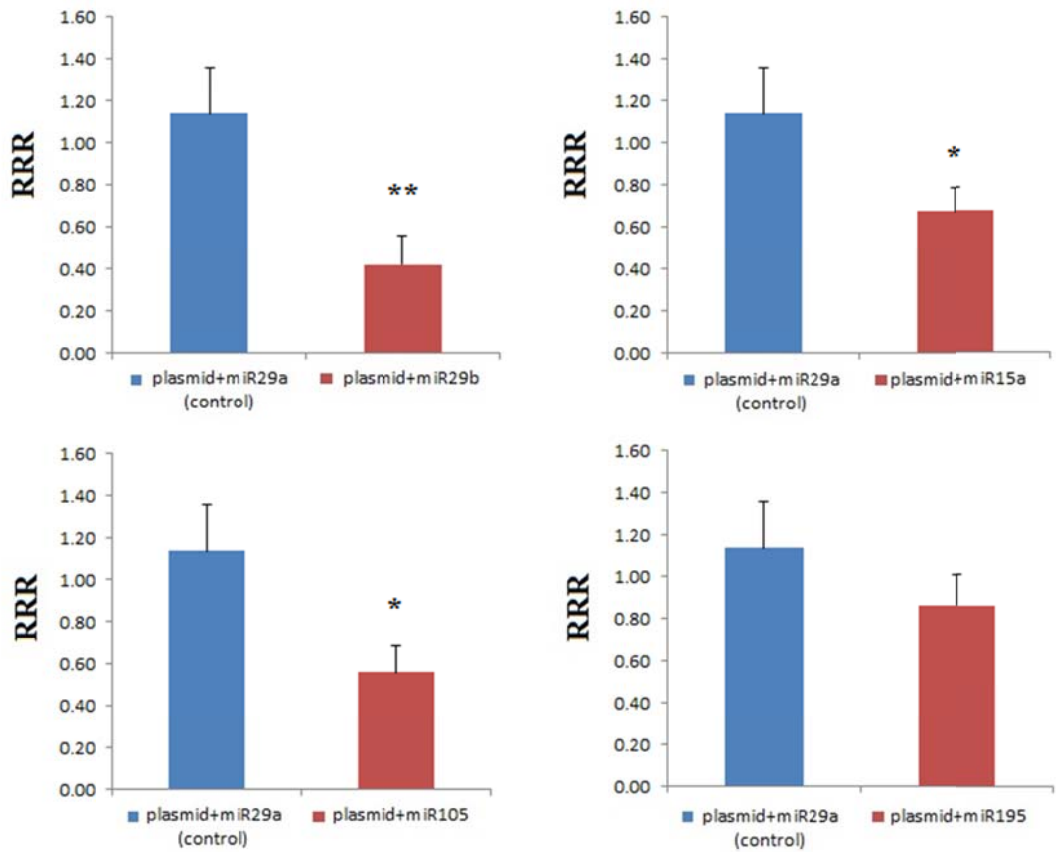
The inhibitory effect of the selected miRNAs on firefly luciferase containing the different 3'UTRs was assessed by co-transfecting HEK293T cells with the plasmids (Figure 5.10A) and the miRNA mimics and comparing those to a non-targeting control (NTC) mimic transfected with has-miR-29a. Relative firefly luciferase activity from the construct containing the human *COL1A2* 3'-UTR was significantly reduced in the presence of hsa-miR-29b (to 38%), hsa-miR-15a (to 60%) and hsa-miR-195 (to 18%) mimics, whilst hsa-miR-105 mimic did not have a significant effect on the reporter gene expression (Figure 5.12A). Firefly luciferase expression was significantly reduced by hsa-miR-29b (to 42%), hsa-miR-15a (to 67%) and hsa-miR-105 (to 56%) mimics from the construct containing the 3'-UTR of the *BMP1* transcript (Figure 5.12B). In contrast, only hsa-miR-15a mimic had an inhibitory effect on luciferase activity from the constructs containing the 3'-UTR of *mTLD* and *CHRD* transcripts, reducing the relative expression of firefly luciferase to around 40% (Figure 5.12C and D, respectively).

Taken together these results suggested that the human *COL1A2* 3'-UTR possessed binding sites for miR-29b, miR-15a and miR-195, whereas *BMP1* 3'-UTR contains binding sites for miR-29b, miR-105 and miR-15a. The binding site for miR-15a is also shared by the 3'-UTRs of the human *mTLD* and *CHRD* genes. To our knowledge, this is the first time that *COL1A2*, *BMP1*, *mTLD* and *CHRD* are described as potential target genes for miR-195, miR-105 and miR-15a, respectively (Figure 5.13) and these genes may be co-regulated by multiple miRNAs, such as miR-29b, miR-15a, miR-105 and miR-195.

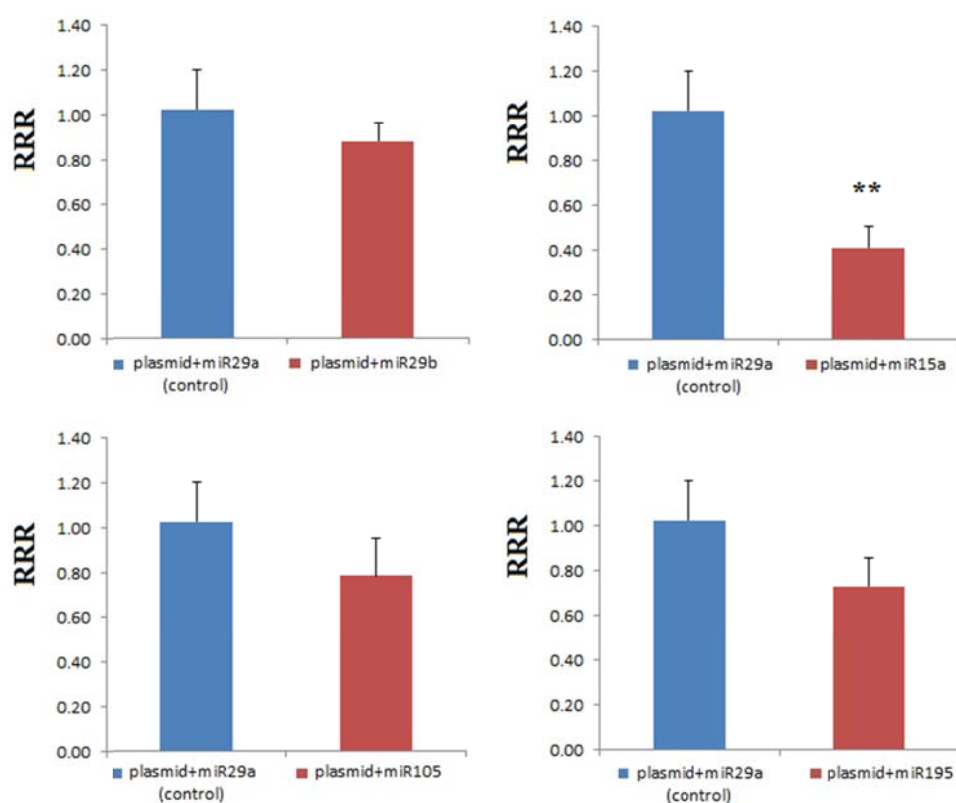
**(A) COL1A2 3'UTR**



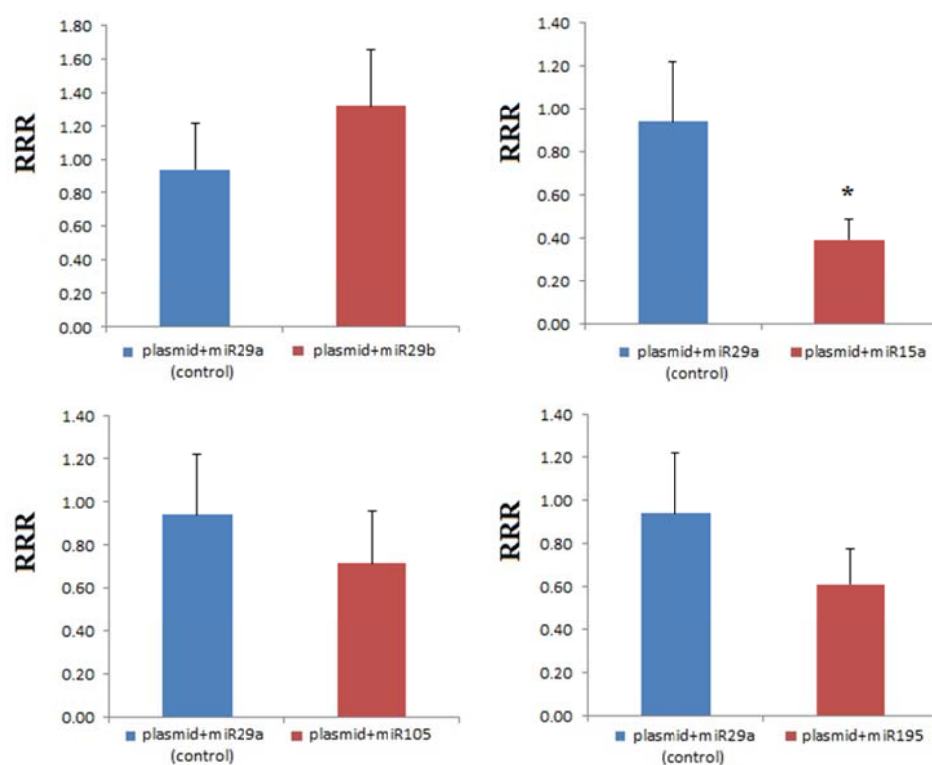
**(B) BMP1 3'UTR**



### (C) mTLD 3'UTR

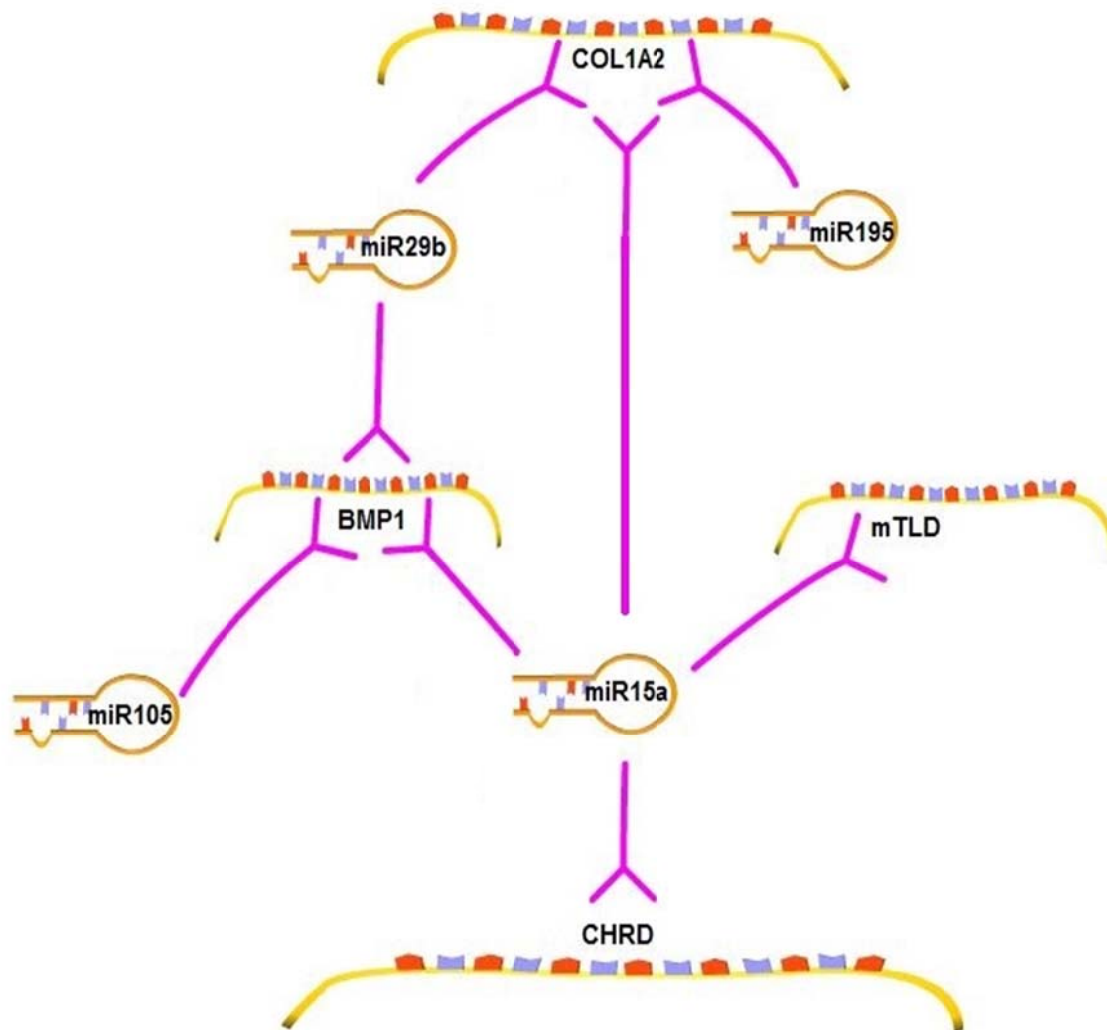


### (D) CHR1 3'UTR



**Figure 5.12 Effect of selected miRNAs on the expression of *COL1A2*, *BMP1*, *mTLD* and *CHRD* genes.** Cells were transiently co-transfected with 1.2nM of the respective miRNAs (miR) mimics or no targeting control mimic (miR-29a) and the relevant luciferase plasmids using DharmaFECT<sup>®</sup> DUO transfection reagent. Approximately 48h post-transfection the cells were lysed and the luciferase activity in the cell lysates measured as a ratio of firefly luciferase (reporter gene) versus Renilla luciferase (internal control gene). Relative luciferase activity (RRR) measured in cells transfected with miR-29a (control), miR-29b, miR-15a, miR-105 or miR-195 and pmiRGLO-3'UTR *COL1A2* (A), pmiRGLO-3'UTR *BMP1* (B), pmiRGLO-3'UTR *mTLD* (C) or pmiRGLO-3'UTR *CHRD* (D). The data represent means and the standard errors (SE) of five experiments. Statistical analysis using student's t-test with  $p < 0.05$  (\*) and  $p < 0.01$  (\*\*) was considered statistically significant.



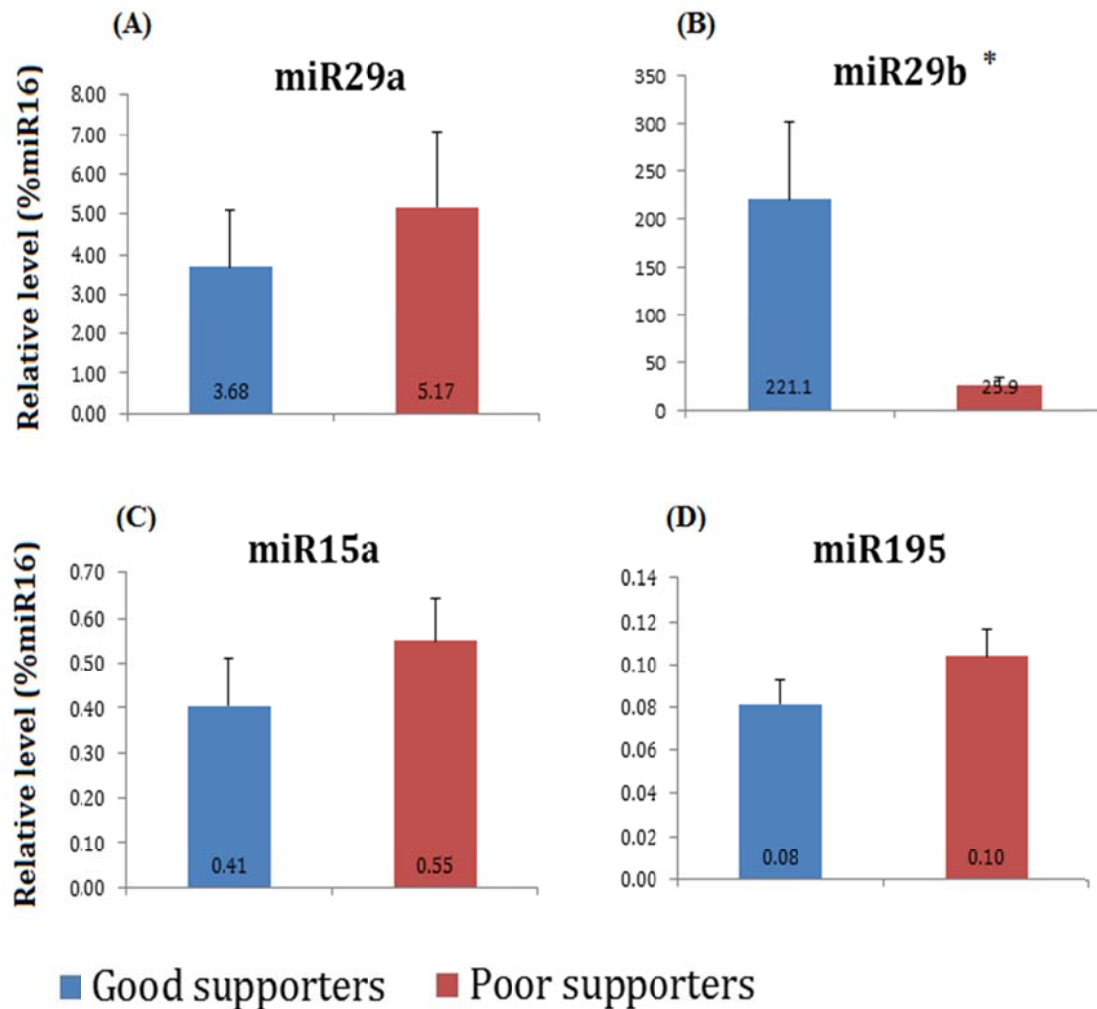


**Figure 5.13 MicroRNA regulation of gene expression.** The diagram represents the proposed regulation of *COL1A2*, *BMP1*, *mTLD* and *CHRD* genes by miR-15a, miR-29b, miR-105 and miR-195.

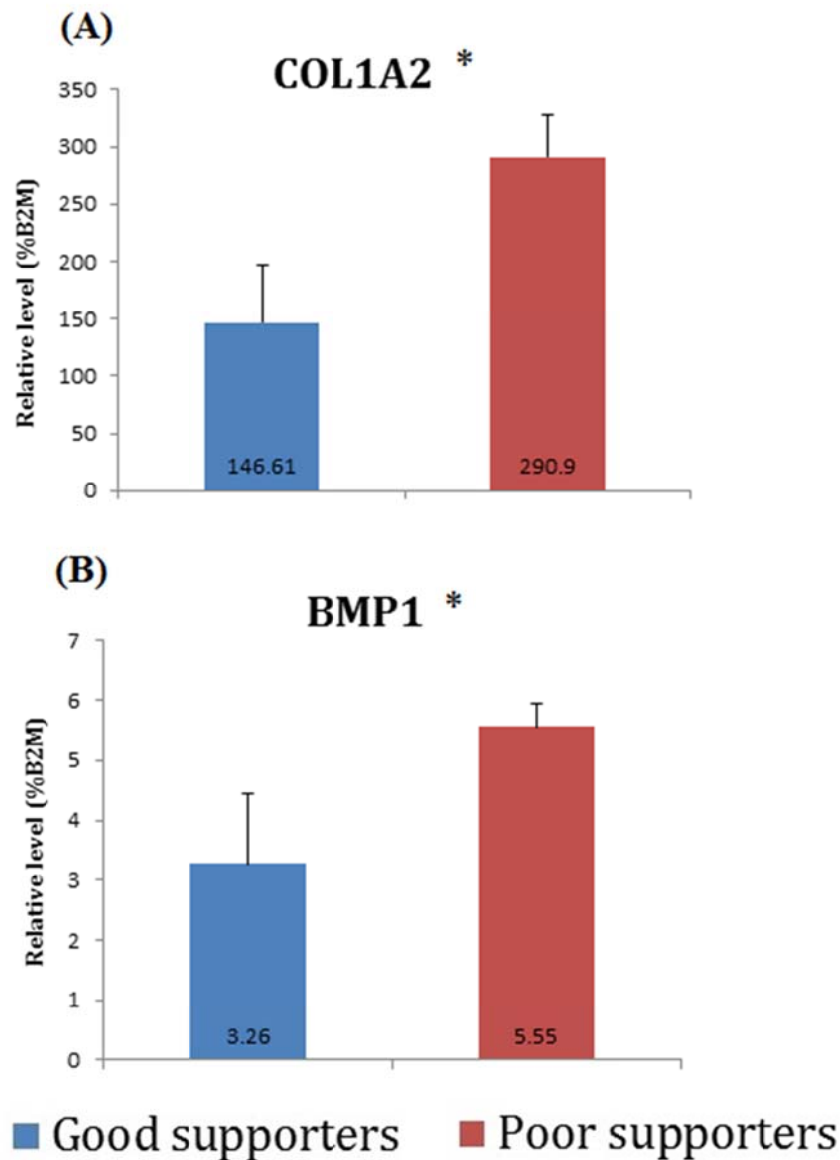
### 5.3.10 The levels of selected miRNAs in CDCs

In order to understand the relevance of miRNA expression in CDCs and their role in modulating gene expression, the endogenous levels of miR-29a (NTC), miR-15a, miR-29b, miR-105 and miR-195 were determined in CDCs from good and poor supporters of angiogenesis by qRT-PCR (Figure 5.14). The level of miR-105 was undetectable in the samples included in this study (Ct values  $\geq 40$ , data not shown). The expression of miR-29a (Figure 5.14A), miR-15a (Figure 5.14C) and miR-195 (Figure 5.14D) was relatively low and not significantly different between good and poor supporters. Interestingly, a significant differential expression of miR-29b ( $P=0.04$ ) was observed between CDCs samples (Figure 5.14B). In brief, CDCs from good supporters of angiogenesis had significantly higher levels of endogenous miR-29b than CDCs from poor supporters (approximately 8.5-fold difference). In addition, the expression of miR-29b ( $10^1 \sim 10^2$  percent of endogenous control miR-16) was observed to be higher than miR-15a ( $10^{-1}$  percent of endogenous control miR-16), miR-105 (undetected) or miR-195 ( $10^{-1} \sim 10^{-2}$  percent of endogenous control miR-16) in CDCs.

The level of *COL1A2* and *BMPI* mRNA in good (n=6) vs. poor (n=9) supporters was also determined (Figure 5.15). As predicted, a negative correlation between miR-29b and *COL1A2* and *BMPI* mRNA was observed, confirming that miR-29b might be modulating the expression of type I collagen and BMP-1 in these cells.



**Figure 5.14 Relative endogenous levels of the selected miRNAs in CDCs.** CDCs from good (n=8) and poor (n=8) of angiogenesis were maintained in culture and total RNA isolated from each samples. The endogenous levels of miR-29a (A), miR-29b (B), miR-15a (C), miR-105 (D) and miR-195 (E) in the cells were assessed by qRT-PCR. The mean average raw Ct value was normalised to miR-16 and analysed using the  $\Delta\text{Ct}$  method. The data represent the means and standard errors (SE) of the specific miRNAs abundance as a percentage of miR-16 in CDCs from good and poor supporters of angiogenesis. Student's *t*-test was used to determine differences between the two groups with  $p < 0.05$  (\*) considered to be statistically significant. The expression of miR-105 was negligible in CDCs. Significant differential expression between good and poor supporters was only observed for miR-29b.



**Figure 5.15 Endogenous *COL1A2* and *BMP1* mRNA levels in CDCs.** CDCs from good (n=6) and poor (n=9) of angiogenesis were maintained in culture and total RNA isolated from each samples. The endogenous levels the *COL1A2* (A) and *BMP1* (B) genes in the cells were assessed by qRT-PCR. The mean average raw Ct value was normalised to  $\beta$ 2-microglobulin (B2M) and analysed using the  $\Delta$ Ct method. The data represent the means with standard errors (SE) of the specific mRNAs abundance as a percentage of B2M mRNA abundance. Student's *t*-test was used to determine differences between the two groups with  $p < 0.05$  (\*) considered to be statistically significant. The expression of miR-105 was negligible in CDCs. Significant differential expression between good and poor supporters was observed for *COL1A2* ( $p=0.03$ ) and *BMP1* ( $p=0.05$ ) mRNA.

## 5.4 Discussion

This chapter describes the global gene expression profiles of CDCs from patients with IHD and compared gene changes between good and poor supporters of angiogenesis at the transcriptional and post-transcriptional levels.

### *5.4.1 Differentially expressed genes in good vs. poor supporters of angiogenesis*

By using cDNA array technology, which is a robust and reliable method, I was able to compare the global gene expression profile of multiple CDC samples in parallel. Principal component analysis (PCA) and hierarchical clustering (HCL) convincingly showed that the gene expression profiles of good and poor supporters clustered in two distinct groups. However, there were no major significant differences between the two clusters. As a result, only one gene, *NOC4L*, was differentially expressed between the two groups of CDCs at a false discovery rate (FDR) of 0.05 and p values corrected for multiple testing (Table 5.1). Similarly, raising the FDR to 0.20 and grouping the samples according to their pro-angiogenic support and the co-morbidity of hypertension, only one gene, *CUX1*, was differentially expressed (Table 5.1). These results show that although functionally distinguishable, CDC samples from good and poor supporters of angiogenesis have a high degree of similarity in global gene expression. These results represent evidence that CDCs from good and poor supporters are the same type of cells despite their different pro-angiogenic potential and their ability to differentiate into mesenchymal cell lineages (section 4.3 Figure 4.4; section 3.3 Figure 3.7). However, the cDNA array data confirmed our previous results obtained by phenotypic analysis of these cells using flow cytometry.

A secondary analysis was carried out to select differentially expressed (DE) genes with a  $p < 0.01$  and more than 2-fold change in gene expression. This analysis yielded a list of 44 DE genes in good supporters compared to poor supporters.

Currently, there is no ‘gold standard’ to quantitate gene expression; therefore, it is important to consider the reliability of the array results by validating a selection of the identified genes experimentally [218], [219]. This study used qRT-PCR to validate the gene expression data from the cDNA array. qRT-PCR was chosen as the validation method due to its high degree of target specificity and its ability to detect small differences in fold change. Six genes were selected from the primary and secondary analyses: *NOC4L*, *CUX1*, *STAT1*, *GPC4*, *TLR4* and *HAPLN1*. The difference in gene expression between good and poor supporters of angiogenesis could only be confirmed for *CUX1*. The results of the qRT-PCR analysis confirmed the cDNA array results in that no drastic changes in gene transcription could be observed between the two functional groups of CDCs. These results may be due to the small number of samples being analysed in this study (good supporters  $n=6$  and poor supporters  $n=6$ ). However, they may also indicate that support of angiogenesis by CDCs is a complex process with a polygenic trait or that regulation of angiogenesis may take place both at transcriptional and post-transcriptional levels.

#### ***5.4.2 Genes and pathways enriched in good and poor supporters of angiogenesis***

As the list of DE genes was extremely short using the stringent selection of the primary analysis, gene enrichment analysis using DAVID was conducted using uncorrected  $p$  values as a single criterion. Interestingly, these genes were moderately enriched for genes involved in alternative splicing and cell cycle/cell division in the

good supporters (Table 5.2), and alternative splicing, lysosome and vacuolar organisation and ubiquitin interacting motif in the poor supporters (Tables 5.3). The enrichment analysis supported the results obtained from the cDNA array in that differences in gene expression between good and poor supporters of angiogenesis may be modulated by alternative splicing or post-transcriptional mechanisms. Alternative splicing is a mechanism by which a single gene codes for multiple mRNAs and hence multiple proteins. In a gene expression profiling experiment, the abundance of different mRNA isoforms from a gene may vary but its total gene expression may remain unchanged. In order to determine whether alternative splicing is the underlying mechanism behind the differences between good and poor supporters of angiogenesis, exon arrays or RNA sequencing should be conducted, and this may warrant further research. In an exon array, the probes are specifically designed around expected or potential splice sites of predicted exons on a gene. RNA sequencing or ‘next generation sequencing’ can provide information on differential gene expression including alternative spliced variants, non-coding RNAs, gene alleles and even gene fusion.

#### ***5.4.3 Transcriptional regulation of type I collagen by TGF- $\beta$***

In this chapter I have presented evidence that *CUX1* is the only DE gene between good and poor supporters of angiogenesis. I have also presented evidence that TGF- $\beta$  is produced at significantly higher levels in good supporters than in poor supporters. Modulation of *COL1A2* expression at the transcriptional level is regulated by TGF- $\beta$  via mechanisms that involve both the canonical and non-canonical signalling pathways [220], [221]. The promoter of *COL1A2* contains sequences that promote the

positive regulation of transcription, but at the same time possesses regions that have been implicated in negative transcriptional regulation. These negative regulatory elements (NREs) include a methylation-responsive CpG island which is recognised by Regulatory Factor X protein [222], a TCC-rich box which has been associated with Fli1 protein binding [223], [224] and a CCAAT region where CUX-1 competes for binding with CBF and exert its repressor activity [189]. Additionally, CUX-1 has been reported to carry CCAAT displacement in the human thymidine kinase [225] and sperm H2B gene transcription [226]. Fragiadaki *et al.* suggested that CUX-1 acts as a repressor of type I collagen transcription in response to high doses of TGF- $\beta$ 1 [189]. Their findings suggest that there is a TGF- $\beta$ 1 negative feedback loop that modulates the production of fibrosis-related genes. I hypothesised that treating CDCs from poor supporters with TGF- $\beta$ , the transcription of the *CUX1* gene would be enhanced whilst *COL1A2* gene transcription would be inhibited. However, in this Chapter I present evidence that in CDCs from poor supporters TGF- $\beta$ 1 does not enhance the transcription of *CUX1* but increases the levels of *COL1A2* mRNA. Overall, these data suggest that the TGF- $\beta$  negative feedback loop proposed by Fragiadaki *et al.* [189] may not be functional in CDCs from poor supporters, or that this loop might be activated at even higher doses of TGF- $\beta$ .

#### ***5.4.4 Post-transcriptional regulation of type I collagen by miR-29b***

The data presented in this thesis suggest that post-transcriptional regulation of gene expression may be another mechanism which controls CDCs' function.



MiRNAs, or small non-coding RNAs, are molecules that function via base-pairing with complementary sequences within mRNAs and are involved in post-transcriptional regulation of gene expression [227]. Binding of the miRNA to specific core sequences in the 3'UTR of the target genes results in gene silencing by mRNA degradation or translational repression [228], [229].

*COL1A2* has been reported to be a target for the miR-29 family of miRNAs in trabecular meshwork cells [195]. miR-29a, -b, and -c expressions were also regulated by TGF- $\beta$ s. [197] In addition, Ott CE *et al.*, have predicted that *COL1A2* may be a target of miR-195 [217]. However, to my knowledge, this prediction has not yet been confirmed experimentally. Following interrogation of PicTar and TargetScan databases, miR-15a, miR-29a and b, miR-105 and miR-195 were selected on the basis of the prediction that they would target *COL1A2*, *BMP1*/*mTLD* or *CHRD* mRNAs. Using a luciferase reporter gene assay, I have confirmed that miR-15a binds to the 3'UTR of *COL1A2*, *BMP1*, *mTLD* and *CHRD*, whilst miR-29b binds to *COL1A2* and *BMP1* 3'UTRs only. I have also shown that *BMP1* and *COL1A2* 3'UTRs are targets for miR-105 and miR-195 respectively. To my knowledge and with the exception of miR-29b, this is the first time that *COL1A2*, *BMP1*, *mTLD* and *CHRD* are shown to be targets of miR-15a, miR-105 and miR-195.

The use of a heterologous system such as HEK293T is very valuable in that the inhibitory effect of a particular miRNA on a predicted target can be quantitatively determined using a reporter gene assay. However, it is important to measure the endogenous (or physiological) levels of such miRNAs in the cells of interest. Interestingly, the levels of miR-15a, miR-29a, miR-105 and miR-195 were low or

undetectable (e.g. miR-105) in CDCs and no significant differences were observed between good and poor supporters. In contrast, miR-29b levels were significantly higher in good than in poor supporters of angiogenesis and this correlated with a lower expression of *COL1A2* in good supporters compared to poor supporters. These results are in agreement with preliminary results obtained in our laboratory which indicated that good supporters expressed significantly lower levels of Type I collagen compared with poor supporters. Our data suggest that in these cells, miR-29b, but no other of the miRNAs tested, seem to be a master regulator of Type I collagen expression and its mechanism of action might be both inhibition of translation and RNA degradation.

## **Chapter 6    General discussion**

## 6.1 Review of this thesis

This thesis was designed to characterise CDCs from patients with chronic IHD undergoing cardiac surgery and their function in order to improve autologous cell-based therapies for heart disease. In summary this thesis aimed at (1) isolating and expanding cells from this patient cohort, (2) establishing a quantitative and robust assay to assess their vascular supportive function, (3) testing their therapeutic potential in a rodent model of MI, (4) determining whether disease and/or cardiovascular risk factors affect cell function and (5) identifying molecular mechanisms that control cell function.

Human CDCs had been obtained originally from ventricular endocardial biopsies from transplanted hearts [88], [89]. In this study, CDCs were successfully isolated and expanded using the ‘cardiosphere’ method from a cohort of patients (n=68) representative of the current trend in adult cardiovascular surgery [230], [231]. As reported by us and others, CDCs can be routinely cultured from atrial biopsies from this patient cohort [90] and from neonatal and young patients with congenital heart defects [92]. There are currently fourteen studies that have characterised the cell surface markers expression of human cardiospheres (CS) and CDCs (see Table 6.1). Whilst they all report consistently high expression of CD105 (80-100%) and negligible expression of CD31 and CD34 markers, the expression of CD117/c-kit and CD90 varies considerably. Previous studies isolated the cells from transplanted hearts [88], [89], [95], [98], [232] and found higher proportions of CD117/c-kit<sup>+</sup> cells (ranging from 5-17%). By contrast, CDCs obtained from this patient cohort seem to have a negligible number of c-kit<sup>+</sup> cells amongst them [90], [91].

**Table 6.1 Summary of major studies including the characterisation of cardiac progenitors following the ‘cardiosphere’ method**

| First Author    | Year | Cell type | Patient cohort             | Age          | Sample size | Biopsy site | Ckit      | CD90  | CD105 | CD31  | CD34  |
|-----------------|------|-----------|----------------------------|--------------|-------------|-------------|-----------|-------|-------|-------|-------|
| Messina [88]    | 2004 | CS        | Heart surgery              | 1m – 80 yrs  | N/A         | A/V         | +         | N/A   | N/A   | +     | +     |
| Smith [89]      | 2007 | CDC       | Transplantation            | 31 ± 2 yrs,  | 59          | V           | ~15%      | ~40%  | ~95%  | ~4%   | ~10%  |
|                 |      |           | Nontransplant              | 47 ± 4 yrs   | 11          |             |           |       |       |       |       |
| Tateishi [233]  | 2007 | CS        | Heart surgery              | 9d -77 yrs   | 18          | RA/OFT, LV  | 0.80%     | 68%   | 99%   | 0.40% | 0.50% |
| Barth [234]     | 2008 | CDC       | N/A                        | N/A          | 12          | V           | 13%       | 34%   | 99%   | 9%    | 7%    |
| Davis[95]       | 2009 | OC        | Transplantation            | 32 ± 12 yrs, | 59          | V           | ~12%      | ~32%  | N/A   | ~5%   | ~5%   |
|                 |      |           | Unexplained cardiomyopathy | 49 ± 15 yrs  | 12          |             |           |       |       |       |       |
|                 |      | CS        | As above                   |              |             |             | +         | +     | +     | +     | -     |
| Li [232]        | 2010 | CS        | Transplantation            | N/A          | 11          | V           | 16.90%    | N/A   | +     | N/A   | N/A   |
|                 |      | CDC       |                            |              |             |             | 5.20%     | 18%   | 100%  | N/A   | N/A   |
| Maxeiner [235]  | 2010 | OC/CDC    | Heart surgery              | 4m – 9 yrs   | 6           | N/A         | mRNA +    | N/A   | N/A   | N/A   | N/A   |
|                 |      |           |                            | 24 - 81 yrs  | 4           |             | Protein - |       |       |       |       |
| Zakharova [236] | 2010 | OC        | Chronic IHD                | 53 - 73 yrs  | 10          | A           | ~30%      | N/A   | N/A   | N/A   | N/A   |
| Koninx [91]     | 2010 | CDC       | Chronic IHD                | 68 ± 7 yrs   | 12          | RA          | -         | +     | N/A   | N/A   | -     |
| Mishra [92]     | 2011 | CDC       | CHD                        | <1m          | 27          | RA          | 9%        | 55%   | 85%   | N/A   | N/A   |
|                 |      |           |                            | 1m – 2yrs    | 47          |             | 7%        | 70%   | 80%   | N/A   | N/A   |
|                 |      |           |                            | 2 - 13 yrs   | 29          |             | 3%        | 65%   | 80%   | N/A   | N/A   |
| Li [98]         | 2012 | CDC       | Transplantation            | N/A          | N/A         | V           | 7%        | 18%   | 100%  | 0.6%  | 1%    |
| Makkar [59]     | 2012 | CDC       | MI after PCI               | 54 ± 3 yrs   | 17          | V           | N/A       | N/A   | >98%  | N/A   | N/A   |
| Chan [90]       | 2012 | CDC       | Chronic IHD                | 67± 2 yrs    | 22          | RA          | 0.2 %     | 55%   | 80%   | 2.10% | 1%    |
| This study      | 2013 | CDC       | Chronic IHD                | 68± 10 yrs   | 69          | RA          | 1.5 %     | 50.7% | 89.6% | 5.2%  | 0.7%  |

A= atrium, CHD= congenital heart disease, CS= cardiospheres, IHD= ischaemic heart disease, LV= left ventricle, MI= myocardial infarction, N/A= not applicable, OC= outgrowth cells, OFT= outflow tract, PCI= percutaneous coronary intervention, RA= right atrium, V= ventricular septum.

Zakharova *et al.* described approximately 30% of CD117/c-kit<sup>+</sup> in OCs in a patient cohort similar to the one included in this thesis [236]. Interestingly, the proportion of CD117/c-kit<sup>+</sup> cells amongst CDCs declined with age [92] and most probably during culture and expansion *in vitro* [90], [95] and the variation in CD90 expression has been associated with co-morbidities such as diabetes [90].

The cell population isolated and expanded in this study resembles a cardiac progenitor cell population of mesenchymal origin. Mesenchymal progenitors from bone marrow are able to differentiate into chondrocytes, adipocytes and osteoblasts *in vitro* under the appropriate inducing conditions. Consistent with previous reports [91], all CDC samples tested here differentiated into the chondrocytic lineage and lack the ability to give rise to the adipocytic lineage. Interestingly, differences were found during differentiation to osteoblasts *in vitro* and this is the first time that this phenomenon is described, suggesting that CDCs from patients with IHD are bipotent or unipotent progenitors. It is therefore plausible that mesenchymal progenitor cells lose their differentiation potential or that this potential is affected by age or disease.

In chapter 4, an *in vitro* co-culture assay was established to test the ability of CDCs to support vascular network formation. Consistent with their mesenchymal phenotype, CDCs cannot form tubule structures in the co-culture assay, but their function is defined by their vascular support. CDC samples were grouped into three tertiles according to their ability to support HUVEC tubule formation and termed ‘good’, ‘moderate’ and ‘poor’ supporters of angiogenesis. Although robust, *in vitro* assay results are not always confirmed *in vivo*. Remarkably, their support of angiogenesis *in vitro* correlated with their therapeutic potential in a rodent model of MI. As proof of

principle, CDCs were transplanted into the heart of rats that had the LAD artery permanently ligated. In this experimental model, transplantation of CDCs from good supporters of angiogenesis prevents ventricular remodelling and significantly improves systolic function compared to CDCs from poor supporters. Interestingly, animals transplanted with CDCs, whether from good or poor supporters of angiogenesis, showed a significant reduction of infarct size and scar tissue compared with control animals. The significant reduction of infarct size is in agreement with the results obtained in the CADUCEUS trial [59]. In this thesis, transplantation of CDCs from good supporters of angiogenesis significantly enhances blood vessels formation in the rodent heart and improves heart function. Taken together these results suggest that although all CDC may have an anti-fibrotic effect and may reduce infarct size, it is only a fraction of those CDC with pro-angiogenic ability that may ameliorate LV remodelling and improve systolic function. These results might be conceptually very important as they provide evidence that (i) there may be a correlation between blood vessel formation and LV function, (ii) the reduction of fibrotic tissue and the regeneration of blood vessels, might be exerted by different mechanisms, and (iii) patient stratification in future clinical trials might be necessary to improve autologous cell-based therapies for heart disease.

Risk factors associated with IHD are known to affect not only the numbers, but the mobilisation, homing and engraftment of cells, both resident in the bone marrow and mobilised into the peripheral circulation [67], [99]. The therapeutic potential of CDCs as supporters of new blood vessel formation in the heart is most likely affected by disease and/or cardiovascular risk factors. In a multiple regression model that can explain over 51% of the variation observed, factors such as history of cigarette

smoking, severe heart dysfunction measured by NYHA classification, global ischaemia caused by aortic stenosis, ischaemia due to coronary artery disease, particularly diseased RCA as the culprit vessel, were deemed to be significant independent positive predictors of angiogenic support, whilst hypertension appeared to be the only significant negative predictor. The former are all indicative of damage and hence these results suggest that increased angiogenesis may correlate with increased damage and/or increased repair of the heart tissue. In contrast, typical cardiovascular risk factors such as age and diabetes mellitus, which were also included in this model, did not become significant predictors of angiogenesis. One possible explanation is that factors such as diabetes affect specifically endothelial, but not mesenchymal/stromal cell function [237].

In order to understand the differences in the vascular supportive function of CDCs further molecular characterisation was performed comparing CDCs from good and poor supporters of angiogenesis. Often, a global gene expression analysis is conducted using cDNA array technology in the first instance. Chapter 5 aimed at identifying molecular markers that could correlate with cell function. Results obtained from the cDNA array showed that, although clustering was clearly observed between good and poor supporters following PCA, there was significant differential expression of only one gene. Further annotation analysis indicated that the two cell subgroups had expression enrichment of genes that function in alternative splicing hence the pro-angiogenic function of CDC might be also regulated post-transcriptionally. In parallel to carrying out the cDNA arrays, preliminary data from our laboratory showed a correlation between support of angiogenesis and expression of ECM components such as pro-collagen I and the metalloproteinase BMP-1. Based



on these results, it was hypothesised that post-transcriptional regulation of ECM components may correlate with angiogenesis support. In Chapter 5, the preliminary experiments with miRNAs that are potentially targeting ECM components demonstrated that functionally related genes are co-regulated by the same miRNAs and confirmed the hypothesis that CDC's vascular supportive function may be regulated post-transcriptionally by miRNAs such as miR-29b.

## **6.2 Limitations**

Due to time and technical restrictions, our study into the characterisation of cardiosphere derived cells from patients with ischaemic heart disease is still in its preliminary form and requires further refining and more evidence to support the results. This thesis has several main limitations. Firstly, and taking into consideration the variability in the population and the complexity of a common disease such as IHD, the study would benefit from a larger sample size. Data from a larger study will have more statistical power. The multiple regression model established to identify predictors of the cells' pro-angiogenic function is statistically significant with a relatively good fit, but a larger sample size may be required to refine the model and to ensure a more accurate prediction of the prognosis of cell transplantation.

Secondly, the rodent model used in this thesis to test the therapeutic potential of subgroups of CDCs is a model of MI without reperfusion and mimics post-infarct ischaemic cardiomyopathy in patients with no option for revascularisation. Although CDC transplantation showed therapeutic effect in this setting, it is not a one-suits-all model. Currently, most IHD patients benefit from coronary revascularisation regardless of previous MI. Therefore, the therapeutic effect observed in this study has

yet to be confirmed in a more relevant and sophisticated model of combined cell transplantation and coronary reperfusion, possibly in a large animal (e.g. pig) and in a phase I/II clinical trial.

Thirdly, although it is a consensus that collagen I deposition and fibrosis is closely related to heart remodelling and dysfunction [141], there is no clear evidence in this thesis that CDCs from good supporters of angiogenesis have superior ability to reduce collagen I deposition or fibrosis *in vivo* compared to CDCs from poor supporters.

Finally, the molecular characterisation of CDCs with different supporting ability concluded that the functional variation was mainly a result of post-transcriptional regulation. As above, the study could benefit from larger sample size and from more in-depth analyses using technologies such as exon arrays, miRNA arrays or next generation sequencing (RNA sequencing) and global DNA methylation status to better understand the molecular mechanisms governing CDCs' vascular supportive function.

### **6.3 Future directions**

Since CDCs from only 1/3 of the patient population would foster robust revascularisation and achieve favourable functional recovery in the ischaemic rat heart, further work would be required to enhance the potency of the CDCs from moderate and poor supporter groups. It is becoming more evident that autologous cell therapies for heart disease may have to preselect or stratify patients and/or would have to be supplemented with either other cells or biologics to enhance cell function.

It may be worthwhile to invest more time and efforts in identifying potential targets that correlate with cell function. Through this thesis and further work conducted in the laboratory, *COL1A2*, *BMP1*, *CUX1*, TGF- $\beta$  and miR-29b have been found to be differentially expressed in good and poor supporters of angiogenesis, emerging as potential biomarkers that correlate with CDC's function. miR-29b has been involved in cardiac fibrosis [196]. However, to my knowledge, the pro-angiogenic role of miR-29b has never been proven before. The demonstration that miR-29b may have a pro-angiogenic effect by reducing ECM components will highlight a single molecular pathway that could control fibrosis and angiogenesis, like the two sides of a coin. Reduction of fibrosis could be achieved by increasing the expression of a transcriptional repressor such as *CUX1* resulting in the reduction of collagen deposition in the ECM. Although this has been recently described in the renal system [189] results obtained in this thesis do not support this strategy in the cardiac cells. However, these experiments are preliminary and conducted only in poor supporters of angiogenesis. It is plausible that CDCs from poor supporters have become non-responsive to the TGF- $\beta$  induction of *CUX1* observed in renal cells. It would be important to ascertain whether this is a control mechanism unique to the renal system or whether in CDCs there is a difference in TGF- $\beta$  signalling between good and poor supporters of angiogenesis.

Although promising, the results of this thesis will need to be confirmed in a large animal model of MI. Based on this study and the results from the CADUCEUS trial, CDCs therapy seems to be an effective anti-remodelling treatment. However, more evidence is required to confirm CDC's potency in preserving heart function and long term benefits. Therefore, an MI model with coronary revascularisation would be

valuable to confirm the superiority of CDC good supporters as treatment post-MI *in vivo*.

Additionally, it would be important to compare the pro-angiogenic function and therapeutic potential of CDCs and BMSC, CDC and explant outgrowth cells from the same patients. Notably, the numbers and function of bone marrow derived cells is affected by age and cardiovascular risk factors [67], [238]. As CDC's support of angiogenesis seems to be affected by heart failure (NYHA functional class) and ischaemia, amongst other factors, it is important to determine whether mesenchymal progenitors from different tissues (e.g. bone marrow and heart) could be affected differently by disease state and/or co-morbidities. Recently, a great deal of heterogeneity between endothelial cells from different sources has been revealed [239] and this may be the case for mesenchymal progenitors too. To this end, the *in vitro* angiogenesis assay used in this thesis to diagnose or predict the angiogenic potential of CDCs could be modified to use autologous tissue specific endothelial cells in order to mimic more closely the cardiac vascular niche in the ischaemic heart.

## **6.4 Conclusion**

Human CDCs are a pool of heterogeneous resident mesenchymal cells of cardiac origin which resemble BMSC in their cell surface phenotype and vascular supportive function. The right atrial appendage is a rich source of CDCs in chronic IHD patients. Compared with BMSC from the healthy donors, CDCs from different patients support angiogenesis to a dramatically varying degree and have different ability to give rise to mesenchymal cell lineages. In addition, angiogenic support *in vitro* correlates with therapeutic potential *in vivo*. Those CDCs with well-preserved differentiation and pro-

angiogenic functions are likely to have dual reparative effects in the infarct heart by reducing scar size and improving heart function, which may be more efficient than autologous BMSC. The CDCs with impaired function are less effective in attenuating the functional deterioration after heart ischaemic injury. Functional divergence amongst human CDCs is most likely regulated at the transcriptional and post-transcriptional levels.

CDCs' function might be impaired partially due to the deposition of type I collagen as a pro-fibrotic factor. Type I collagen is expressed at lower level in CDCs from good supporters, which might probably be a result of the following mechanisms: (i) an autocrine mechanism by which a higher level of bio-available TGF- $\beta$  is produced by good supporters of angiogenesis resulting in an increase of *CUX1* transcription, activation of the negative regulatory elements in the *COL1A2* promoter, and subsequent reduction of *COL1A2* transcription; (ii) a higher level of miR-29b which targets and binds to the 3'-UTR of *COL1A2* mRNA inhibiting the translation of *COL1A2* and (iii) a higher level of BMP-1 which processes Type I collagen and reduces its synthesis. Clearly, these mechanisms might not be all active in CDCs from poor supporters of angiogenesis, thus better understanding of these molecular pathways in CDCs is required to restore their vascular supportive function.

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## Appendix 1 Plasmid maps

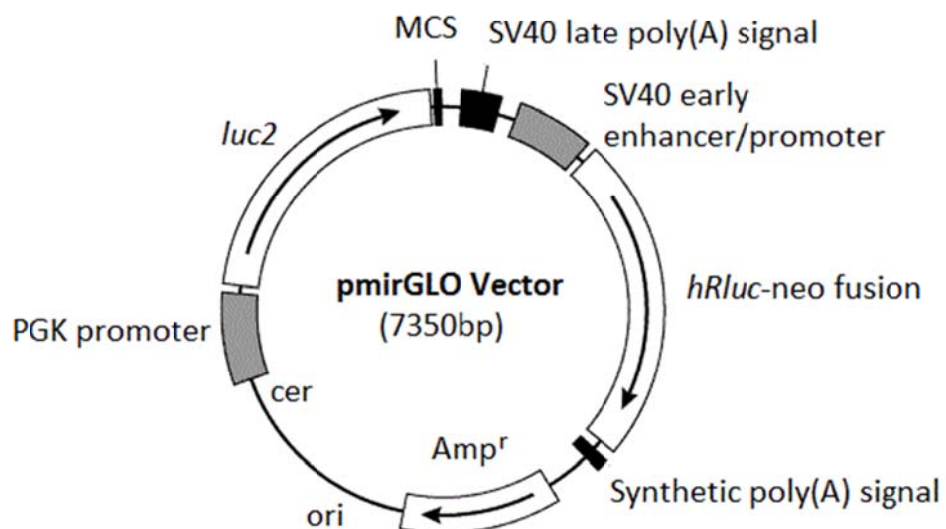
### pLV-GFP Vector



### pΔ8.91 Vector



### pVSV-G Vector



## Appendix 2 Reagent make-ups

### CEM

| component                                     | Volume(mL) |
|-----------------------------------------------|------------|
| IMDM                                          | 400        |
| Heat-inactivated defined FCS (100mL) 20%      | 100        |
| 100 U/mL penicillin and 100µg/mL streptomycin | 5          |
| 2mM L-glutamine                               | 5          |
| 0.1mM 2-mercaptoethanol                       | 0.5        |

### Medium supplement

| component                                     | Volume(mL) |
|-----------------------------------------------|------------|
| DMEM/F-12                                     | 490        |
| 100 U/mL penicillin and 100µg/mL streptomycin | 5          |
| 2mM L-glutamine                               | 5          |
| 0.1mM 2-mercaptoethanol                       | 0.5        |

### CGM

| component         | Volume(mL) | [C] (v/v) |
|-------------------|------------|-----------|
| CEM               | 7          |           |
| Medium supplement | 13         |           |
| B27               | 0.4        | 2%        |
| Cardiotrophin     | 0.005      | 25ng/mL   |
| FGF               | 0.004      | 20ng/mL   |
| EGF               | 0.02       | 10ng/mL   |
| Thrombin          | 0.05       | 25µg/mL   |

### HEK293FT complete culturing medium

| component   | Volume(mL) | [C] (v/v) |
|-------------|------------|-----------|
| DMEM/F12    | 440        | 88%       |
| FBS         | 50         | 10%       |
| L-glutamine | 5          | 1%        |
| NEAA        | 5          | 1%        |

### HEK293FT serum free medium

| component         | Volume(mL) | [C] (v/v) |
|-------------------|------------|-----------|
| DMEM high glucose | 490        | 98%       |
| L-glutamine       | 5          | 1%        |
| NEAA              | 5          | 1%        |

### **Appendix 3 Cytokine concentrations for conditioned angiogenesis assays**

| <b>cytokine</b> | <b>concentration</b> |
|-----------------|----------------------|
| FGF4            | 10ng/mL              |
|                 | 5ng/mL               |
| FGF7            | 10ng/mL              |
| TGFb1           | 10ng/mL              |

## **Appendix 4 Patient consent form**



**CONSENT FORM**  
**Culture and Characterisation of Human Cardiac Stem Cells**

**NRES Ref: 07/H0607/95, version 5, February 2012**

**Investigators:** Professor DP Taggart

Please initial box

I confirm that I have read and understand the information sheet (December 2011, version 4) for the above study and have had the opportunity to ask questions.

☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

☐

I understand that sections of any of my medical notes may be looked at by research staff and regulatory authorities where it is relevant to my taking part in research. I give my permission for these individuals to have access to my records.

☐

I understand that heart samples will be obtained during the procedure involved in this study. I give permission for this. I understand no original tissue will be stored but stem cells obtained from them may be stored till the end of the study. RNA or DNA may be taken from the cells. Cells may be transplanted into animal models of heart disease.

☐

I understand that my details and data will be stored for up to five years but will not be used for other purposes.

☐

I agree to take part in the above study.

☐

---

Name of Patient

Date

Signature

---

Name of Person taking consent  
(if different from investigator)

Date

Signature

---

Investigator

Date

Signature



## **Patient Information Sheet** **Culture and Characterisation of Human Cardiac Stem Cells**

**UNIVERSITY OF OXFORD**  
**NUFFIELD DEPARTMENT OF SURGERY**

**NRES: 07/H0607/95**  
**Version 5, February 2011.**

*You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.*

- *Part 1 tells you the purpose of this study and what will happen to you if you take part.*
- *Part 2 gives you more detailed information about the conduct of the study.*

*Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.*

### **Part 1**

#### **1. Why have I been chosen?**

You have been chosen because you are about to have heart surgery.

#### **2. What is the purpose of the study?**

Many treatments are being developed across the world that involves the use of stem cells (SC). They have the potential to regenerate and heal many types of damaged tissue, including damaged heart muscle. We now know that the human heart contains a small number of heart stem cells, which could potentially be used to regenerate human heart tissue. We



would like to improve the techniques for growing **human** adult heart stem cells, maybe for eventual use in patients with heart damage.

### **3. What does the study involve?**

During your operation we want to take two tiny pieces of heart muscle (each about the size of two grains of rice) from the two chambers of the heart. From these tissue 'biopsies' we will isolate cardiac stem cells.

### **4. Do I have to take part?**

No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

### **5. How do I say yes?**

If you decide to take part in the study, on the day you come into hospital, you will be approached by a member of the team to ask if you would participate in the study. Here you will be given the opportunity to ask questions. After this you will be asked to sign a consent form to be included in the study.

**If you have questions before your admission, write them down and bring them with you to hospital, we can answer any questions when you come in. You don't need to do anything before your admission.**

### **6. What will happen to me?**

After signing the consent form, you will have surgery as planned. The cardiac biopsies are taken during your operation whilst you are asleep. Otherwise the surgery is exactly the same as normal and we do not give you any other medicines or treatments. After you wake up there is nothing left to do and you will have finished the study. You will not need to be followed up or visit us again outside of what would normally happen after an operation.

**7. What are the possible disadvantages and risks of taking part?**

Taking very small tissue samples from the heart will add a few minutes to the operation and could theoretically cause some additional bleeding. However the risk of this is very low when done by an experienced surgeon.

**8. What are the possible benefits of taking part?**

The study is not beneficial to you, but the information we get might help improve the treatment of people with heart failure.

**9. What if there is a problem?**

If a problem arises during your hospital stay, then a member of the nursing staff, or the patient advice and liaison service (PALS) may be able to deal with your enquiry. In the unlikely event of a complaint arising after the study, you should contact Professor Taggart's Team, either in writing addressing the letter to Professor David Taggart, Department of Cardiothoracic Surgery, Level 1, John Radcliffe Hospital, Headley Way, Oxford, OX3 9DU or via his secretary on 01865 221111.

**10. Will my taking part in the study be kept confidential?**

Yes. All the information about your participation in this study will be kept confidential, see Part 2 for more details.

**This completes Part 1 of the Information Sheet.**



UNIVERSITY OF OXFORD  
NUFFIELD DEPARTMENT OF SURGERY

**Patient Information Sheet**  
**Isolation of Cardiac Stem Cells**  
**NRES: 07/H0607/95**  
**Version 5, February 2012.**

**If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.**

**Part 2**

Thank you for considering participation in the study. There are just a few more points that we are required to outline.

**11. What if there is a problem?**

As this study only occurs whilst you are asleep, by the time you wake up we have obtained all of the information and tissue that we require. All subsequent treatment will be standard medical care and problems thereafter are unlikely to be related to the study. If there is a complaint about your subsequent care or treatment, then this should be addressed via the normal NHS procedures, in the first instance to a member of the nursing staff, Professor Taggart's team or the patient liaison service. If there is a specific problem relating to the collection of the cardiac tissue samples or the study itself, then please feel free to contact Professor Taggart's address is given above.

**12. Complaints:**

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. They can be reached via Professor Taggart's

secretary. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

### **13. Harm:**

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against the University of Oxford, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

### **14. Will my taking part in this study be kept confidential?**

If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from Oxford University and NHS Blood and Transplant. As soon as you are included into the trial, you will be assigned a specific code, which is how Oxford University and NHS Blood and Transplant will identify you, access and store the information we obtain. This means you will be anonymised almost immediately. Samples and information about you (anonymised) will be passed to Oxford University and NHS Blood and Transplant to perform the research needed to develop any new technique. Details about you may also be looked at by authorised people from (the Trust or other NHS bodies) to check that the study is being (or has been) carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

### **In summary**

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it. All of our procedures for handling, processing, storage and destruction of their data are compliant with the Data Protection Act 1998.

## **15. What will happen to any samples I give?**

All samples will be labelled with a unique identifier, but your name and address will be removed. The samples will not be used for any other purpose, except those outlined in this information sheet. All samples will be processed to obtain heart stem cells and then the original tissue will be destroyed. We may take pieces of genes or genetic material, known as DNA and RNA, from these cells to make sure that they have not changed. Heart stem cells may be stored at NHS Blood and Transplant research labs during the course of the study and destroyed at the end of it. Heart stem cells may eventually be used to treat patients with heart problems. But before they can be used in patients, we'd need to test their ability to replace or heal damaged heart muscle. To do that, we'd transplant them into small animals such as laboratory rats or mice that also have heart damage. If the cells work in these animals, they may also be beneficial to humans. All information will be anonymised and stored in locked cabinets within locked offices. All electronic data will be held on secure databases, accessed only by authorised personnel who require passwords in order to gain access. The ownership of samples is held by Oxford University and NHS Blood and Transplant; the samples are gifted by yourself to Oxford University and NHS Blood and Transplant, and you have no legally binding rights to the samples or any subsequent commercial significance arising from the research.

## **16. What will happen to the results of the research study?**

The research may be published in scientific journals, presented at scientific meetings and may result in the development of a new technique for treating heart failure.

## **17. Who is organising and funding the research?**

The research is organised by University of Oxford and NHS Blood and Transplant. At present there is no specific funding for this project which will be done under other existing projects.

## **18. Who has reviewed the study?**

This study was given a favourable ethical opinion for conduct in the NHS by the Mid and South Buckinghamshire REC. NRES: 07/Q0607/95

***This completes Part 2. Thank you for considering taking part or taking time to read this sheet, someone will return to see if you wish to take part in the study and answer any questions you may have regarding the research.***

## **Appendix 5 R scripts for the establishment of linear regression model to predict the relationships between the pro-angiogenic potential of CDC from IHD patients and the clinical variables**

```
hj <- read.csv("C:/Documents and Settings/Administrator /2011 data analysis files/hj-HZ
most updated.csv", header = T)

> attach(hj)

> local({pkg <- select.list(sort(.packages(all.available = TRUE)),graphics=TRUE)
+ if(nchar(pkg)) library(pkg, character.only=TRUE)})

> local({pkg <- select.list(sort(.packages(all.available = TRUE)),graphics=TRUE)
+ if(nchar(pkg)) library(pkg, character.only=TRUE)})

> boxcox(Tubule ~ Age + Sex + NYHA + Smoker + Procedure + Scale + FHx + DM + HBP +
HBChol)

> boxcox(Tubule ~ Age + Sex + NYHA + Smoker + Scale + FHx + DM + HBP + HBChol)

> bcTubule <- (Tubule^(1/5) - 1)/(1/5)

> sm <- class.ind(Smoker)

> summary(lm(bcTubule ~ Age + Sex + NYHA + Smoker + Procedure + Scale + FHx + DM +
HBP + HBChol))
```

Call:

```
lm(formula = bcTubule ~ Age + Sex + NYHA + Smoker + Procedure +
    Scale + FHx + DM + HBP + HBChol)
```

Residuals:

| Min     | 1Q     | Median | 3Q    | Max   |
|---------|--------|--------|-------|-------|
| -10.741 | -2.834 | -1.169 | 4.144 | 9.497 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 17.8415  | 10.7957    | 1.653   | 0.1114   |
| Age         | -0.1697  | 0.1060     | -1.601  | 0.1224   |
| Sex         | 0.1872   | 3.1212     | 0.060   | 0.9527   |
| NYHA        | 2.6686   | 1.4694     | 1.816   | 0.0819 . |
| Smoker      | 2.6157   | 1.3732     | 1.905   | 0.0689 . |
| Procedure   | 0.3219   | 2.6679     | 0.121   | 0.9050   |
| Scale       | 0.8002   | 1.5672     | 0.511   | 0.6143   |
| FHx         | 1.3993   | 1.6242     | 0.862   | 0.3975   |
| DM          | -0.4377  | 3.1327     | -0.140  | 0.8901   |
| HBP         | -4.1485  | 2.6661     | -1.556  | 0.1328   |
| HBChol      | 0.4571   | 3.5095     | 0.130   | 0.8974   |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 6.338 on 24 degrees of freedom

Multiple R-squared: 0.4113, Adjusted R-squared: 0.166

F-statistic: 1.677 on 10 and 24 DF, p-value: 0.1447

```
> summary(lm(bcTubule ~ Age + Sex + NYHA + factor(Smoker) + Procedure + Scale + FHx +  
DM + HBP + HBChol))
```

Call:

```
lm(formula = bcTubule ~ Age + Sex + NYHA + factor(Smoker) + Procedure + Scale + FHx + DM  
+ HBP + HBChol)
```

Residuals:



| Min     | 1Q     | Median | 3Q    | Max   |
|---------|--------|--------|-------|-------|
| -11.384 | -2.806 | -1.469 | 3.948 | 9.072 |

Coefficients:

|                 | Estimate | Std. Error | t value | Pr(> t ) |
|-----------------|----------|------------|---------|----------|
| (Intercept)     | 13.5721  | 13.1644    | 1.031   | 0.3138   |
| Age             | -0.1434  | 0.1264     | -1.134  | 0.2689   |
| Sex             | -0.3253  | 3.3033     | -0.098  | 0.9225   |
| NYHA            | 2.8867   | 1.5456     | 1.868   | 0.0752 . |
| factor(Smoker)1 | 5.7931   | 5.1318     | 1.129   | 0.2711   |
| factor(Smoker)2 | 5.5462   | 2.8950     | 1.916   | 0.0685 . |
| factor(Smoker)3 | 5.4825   | 7.7871     | 0.704   | 0.4888   |
| Procedure       | 0.5323   | 2.7708     | 0.192   | 0.8494   |
| Scale           | 1.1094   | 1.6707     | 0.664   | 0.5136   |
| FHx             | 1.5742   | 1.7012     | 0.925   | 0.3648   |
| DM              | -1.3696  | 3.4810     | -0.393  | 0.6978   |
| HBP             | -3.7777  | 2.8000     | -1.349  | 0.1910   |
| HBChol          | 1.3978   | 3.8797     | 0.360   | 0.7221   |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 6.54 on 22 degrees of freedom

Multiple R-squared: 0.4255, Adjusted R-squared: 0.1122

F-statistic: 1.358 on 12 and 22 DF, p-value: 0.2573

```
> summary(lm(bcTubule ~ Age + NYHA + factor(Smoker) + Scale + FHx + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + NYHA + factor(Smoker) + Scale + FHx + HBP)
```

Residuals:

| Min     | 1Q     | Median | 3Q    | Max   |
|---------|--------|--------|-------|-------|
| -11.001 | -2.758 | -1.171 | 3.794 | 8.832 |

Coefficients:

|                 | Estimate | Std. Error | t value | Pr(> t ) |
|-----------------|----------|------------|---------|----------|
| (Intercept)     | 16.8164  | 8.9623     | 1.876   | 0.0719 . |
| Age             | -0.1514  | 0.1158     | -1.308  | 0.2024   |
| NYHA            | 2.6164   | 1.3027     | 2.008   | 0.0551 . |
| factor(Smoker)1 | 4.8836   | 4.3584     | 1.121   | 0.2727   |
| factor(Smoker)2 | 5.7970   | 2.3896     | 2.426   | 0.0225 * |
| factor(Smoker)3 | 6.2416   | 6.8069     | 0.917   | 0.3676   |
| Scale           | 0.7585   | 0.8756     | 0.866   | 0.3943   |
| FHx             | 1.2747   | 1.4145     | 0.901   | 0.3758   |
| HBP             | -3.7454  | 2.4461     | -1.531  | 0.1378   |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 6.052 on 26 degrees of freedom

Multiple R-squared: 0.4186, Adjusted R-squared: 0.2398

F-statistic: 2.34 on 8 and 26 DF, p-value: 0.04833

```
> hj <- read.csv("C:/Documents and Settings/Administrator /2011 data analysis files/hj-HZ  
most updated.csv", header = T)
```

```
> attach(hj)
```

The following object(s) are masked from 'hj (position 5)':

Age, CAD, DM, FHx, HbChol, HBP, ID, LAD, LCX, NYHA, Procedure,

RCA, Sex, Smoker, Tubule, valve, Vessles

```
> boxcox(Tubule ~ Age + CAD + RCA+ valve+ NYHA + Smoker + Procedure + Scale + FHx + DM  
+ HBP )
```

```
> summary(lm(bcTubule ~ Age + CAD + RCA+ valve+ NYHA + Smoker + Procedure + Scale +  
FHx + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoker + Procedure + Scale +  
FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -7.5074 | -2.8074 | 0.3849 | 2.6513 | 8.3491 |

Coefficients: (1 not defined because of singularities)

|             | Estimate | Std. Error | t value | Pr(> t )  |
|-------------|----------|------------|---------|-----------|
| (Intercept) | 12.37649 | 6.62096    | 1.869   | 0.07383 . |
| Age         | -0.12226 | 0.08126    | -1.505  | 0.14550   |
| CAD         | 8.05499  | 4.53964    | 1.774   | 0.08869 . |
| RCA         | 8.13413  | 3.49071    | 2.330   | 0.02853 * |
| valve       | 11.20309 | 8.14627    | 1.375   | 0.18176   |
| NYHA        | 2.27611  | 1.14662    | 1.985   | 0.05868 . |
| Smoker      | 2.60261  | 1.07263    | 2.426   | 0.02313 * |
| Procedure   | NA       | NA         | NA      | NA        |
| Scale       | -1.02833 | 1.69146    | -0.608  | 0.54893   |
| FHx         | 1.61256  | 1.26758    | 1.272   | 0.21551   |

|     |          |         |        |            |
|-----|----------|---------|--------|------------|
| DM  | -2.65940 | 2.40220 | -1.107 | 0.27924    |
| HBP | -8.07648 | 2.17278 | -3.717 | 0.00107 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.852 on 24 degrees of freedom

Multiple R-squared: 0.655, Adjusted R-squared: 0.5112

F-statistic: 4.556 on 10 and 24 DF, p-value: 0.001126

```
> summary(lm(bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoker + Procedure + Vessles +
  FHx + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoker + Procedure + Vessles +
  FHx + DM + HBP)
```

Residuals:

|         |         |        |        |        |
|---------|---------|--------|--------|--------|
| Min     | 1Q      | Median | 3Q     | Max    |
| -7.3755 | -3.0921 | 0.7108 | 2.3456 | 8.5739 |

Coefficients: (1 not defined because of singularities)

|             |          |            |         |            |
|-------------|----------|------------|---------|------------|
|             | Estimate | Std. Error | t value | Pr(> t )   |
| (Intercept) | 13.58316 | 6.70273    | 2.027   | 0.053963 . |
| Age         | -0.13543 | 0.08116    | -1.669  | 0.108151   |
| CAD         | 9.12462  | 4.49318    | 2.031   | 0.053498 . |
| RCA         | 8.93381  | 3.35061    | 2.666   | 0.013508 * |
| valve       | 7.23546  | 3.61590    | 2.001   | 0.056824 . |
| NYHA        | 2.13055  | 1.14435    | 1.862   | 0.074921 . |

|           |          |         |        |              |
|-----------|----------|---------|--------|--------------|
| Smoker    | 2.65346  | 1.01547 | 2.613  | 0.015248 *   |
| Procedure | NA       | NA      | NA     | NA           |
| Vessles   | -1.75883 | 1.74387 | -1.009 | 0.323237     |
| FHx       | 1.40803  | 1.27699 | 1.103  | 0.281129     |
| DM        | -2.30651 | 2.36580 | -0.975 | 0.339315     |
| HBP       | -8.19186 | 2.14800 | -3.814 | 0.000843 *** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.789 on 24 degrees of freedom

Multiple R-squared: 0.6639, Adjusted R-squared: 0.5239

F-statistic: 4.741 on 10 and 24 DF, p-value: 0.000862

```
> summary(lm(bcTubule ~ Age + CAD + RCA + NYHA + factor(Smoker) + factor(Procedure) +
Vessles + FHx + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + NYHA + factor(Smoker) + factor(Procedure) +
Vessles + FHx + DM + HBP)
```

Residuals:

|        |        |        |       |       |
|--------|--------|--------|-------|-------|
| Min    | 1Q     | Median | 3Q    | Max   |
| -7.129 | -2.831 | 0.000  | 2.365 | 8.944 |

Coefficients: (1 not defined because of singularities)

|             |          |            |         |           |
|-------------|----------|------------|---------|-----------|
|             | Estimate | Std. Error | t value | Pr(> t )  |
| (Intercept) | 19.2634  | 8.8084     | 2.187   | 0.03967 * |
| Age         | -0.1132  | 0.1017     | -1.114  | 0.27747   |

|                    |         |        |        |            |
|--------------------|---------|--------|--------|------------|
| CAD                | 2.0115  | 3.7738 | 0.533  | 0.59937    |
| RCA                | 8.9227  | 3.6155 | 2.468  | 0.02184 *  |
| NYHA               | 2.0953  | 1.2183 | 1.720  | 0.09950 .  |
| factor(Smoker)1    | 3.3924  | 3.8659 | 0.878  | 0.38969    |
| factor(Smoker)2    | 5.1557  | 2.1474 | 2.401  | 0.02525 *  |
| factor(Smoker)3    | 10.6804 | 6.0697 | 1.760  | 0.09237 .  |
| factor(Procedure)1 | NA      | NA     | NA     | NA         |
| factor(Procedure)3 | -7.1633 | 3.7789 | -1.896 | 0.07122 .  |
| Vessles            | -1.6962 | 1.9359 | -0.876 | 0.39042    |
| FHx                | 1.3459  | 1.3471 | 0.999  | 0.32864    |
| DM                 | -2.3695 | 2.5923 | -0.914 | 0.37058    |
| HBP                | -8.2425 | 2.3484 | -3.510 | 0.00198 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.967 on 22 degrees of freedom

Multiple R-squared: 0.6686, Adjusted R-squared: 0.4878

F-statistic: 3.698 on 12 and 22 DF, p-value: 0.003839

```
> summary(lm(bcTubule ~ Age + NYHA + factor(Smoker) + factor(Procedure) + Vessles + FHx + HBP))
```

Call:

```
lm(formula = bcTubule ~ Age + NYHA + factor(Smoker) + factor(Procedure) + Vessles + FHx + HBP)
```

Residuals:

| Min | 1Q | Median | 3Q | Max |
|-----|----|--------|----|-----|
|-----|----|--------|----|-----|

-10.2033 -2.2522 0.2385 2.7285 8.3612

Coefficients:

|                    | Estimate | Std. Error | t value | Pr(> t )   |
|--------------------|----------|------------|---------|------------|
| (Intercept)        | 12.80107 | 8.87236    | 1.443   | 0.16200    |
| Age                | -0.07824 | 0.10892    | -0.718  | 0.47952    |
| NYHA               | 2.48060  | 1.18388    | 2.095   | 0.04687 *  |
| factor(Smoker)1    | 5.25379  | 4.01124    | 1.310   | 0.20267    |
| factor(Smoker)2    | 6.63865  | 2.24452    | 2.958   | 0.00686 ** |
| factor(Smoker)3    | 11.95513 | 6.53750    | 1.829   | 0.07990 .  |
| factor(Procedure)1 | 1.27842  | 4.08488    | 0.313   | 0.75701    |
| factor(Procedure)3 | -4.89662 | 4.04219    | -1.211  | 0.23754    |
| Vessles            | 1.36080  | 1.49457    | 0.911   | 0.37161    |
| FHx                | 1.50492  | 1.29029    | 1.166   | 0.25494    |
| HBP                | -6.32912 | 2.42609    | -2.609  | 0.01540 *  |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.486 on 24 degrees of freedom

Multiple R-squared: 0.5589, Adjusted R-squared: 0.3751

F-statistic: 3.041 on 10 and 24 DF, p-value: 0.01241

```
> boxcox(Tubule ~ Age + CAD + RCA+ valve+ NYHA + Smoker + Procedure + Scale + FHx + DM + HBP )
```

```
> summary(lm(bcTubule ~ Age + CAD + RCA+ valve+ NYHA + Smoker + Vessles + FHx + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoker + Vessles + FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -7.3755 | -3.0921 | 0.7108 | 2.3456 | 8.5739 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )     |
|-------------|----------|------------|---------|--------------|
| (Intercept) | 13.58316 | 6.70273    | 2.027   | 0.053963 .   |
| Age         | -0.13543 | 0.08116    | -1.669  | 0.108151     |
| CAD         | 9.12462  | 4.49318    | 2.031   | 0.053498 .   |
| RCA         | 8.93381  | 3.35061    | 2.666   | 0.013508 *   |
| valve       | 7.23546  | 3.61590    | 2.001   | 0.056824 .   |
| NYHA        | 2.13055  | 1.14435    | 1.862   | 0.074921 .   |
| Smoker      | 2.65346  | 1.01547    | 2.613   | 0.015248 *   |
| Vessles     | -1.75883 | 1.74387    | -1.009  | 0.323237     |
| FHx         | 1.40803  | 1.27699    | 1.103   | 0.281129     |
| DM          | -2.30651 | 2.36580    | -0.975  | 0.339315     |
| HBP         | -8.19186 | 2.14800    | -3.814  | 0.000843 *** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.789 on 24 degrees of freedom

Multiple R-squared: 0.6639, Adjusted R-squared: 0.5239

F-statistic: 4.741 on 10 and 24 DF, p-value: 0.000862

```
> summary(lm(bcTubule ~ Age + CAD + RCA+ valve+ NYHA + Smoker + FHx + DM + HBP ))
```



Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoker +  
    FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -7.6281 | -2.7171 | 0.6845 | 2.3824 | 8.1569 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 11.84703 | 6.48016    | 1.828   | 0.07948 .  |
| Age         | -0.12288 | 0.08022    | -1.532  | 0.13815    |
| CAD         | 6.41037  | 3.59933    | 1.781   | 0.08707 .  |
| RCA         | 6.66047  | 2.47998    | 2.686   | 0.01267 *  |
| valve       | 6.77055  | 3.58764    | 1.887   | 0.07080 .  |
| NYHA        | 2.41423  | 1.10963    | 2.176   | 0.03924 *  |
| Smoker      | 2.81040  | 1.00382    | 2.800   | 0.00972 ** |
| FHx         | 1.73956  | 1.23439    | 1.409   | 0.17108    |
| DM          | -2.50493 | 2.35842    | -1.062  | 0.29833    |
| HBP         | -7.94897 | 2.13519    | -3.723  | 0.00101 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.791 on 25 degrees of freedom

Multiple R-squared: 0.6497, Adjusted R-squared: 0.5236

F-statistic: 5.151 on 9 and 25 DF, p-value: 0.0005422

```
> summary(lm(bcTubule ~ Age + RCA+ valve+ NYHA + Smoker + FHx + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + RCA + valve + NYHA + Smoker + FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max     |
|---------|---------|--------|--------|---------|
| -7.7766 | -3.1247 | 0.7983 | 2.5406 | 10.0145 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 16.20313 | 6.24645    | 2.594   | 0.01538 *  |
| Age         | -0.12590 | 0.08349    | -1.508  | 0.14362    |
| RCA         | 8.12710  | 2.43501    | 3.338   | 0.00256 ** |
| valve       | 2.09137  | 2.54303    | 0.822   | 0.41833    |
| NYHA        | 2.25420  | 1.15125    | 1.958   | 0.06104 .  |
| Smoker      | 2.93113  | 1.04252    | 2.812   | 0.00925 ** |
| FHx         | 1.46752  | 1.27503    | 1.151   | 0.26022    |
| DM          | -1.82329 | 2.42241    | -0.753  | 0.45840    |
| HBP         | -6.83438 | 2.12498    | -3.216  | 0.00346 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.987 on 26 degrees of freedom

Multiple R-squared: 0.6052, Adjusted R-squared: 0.4838

F-statistic: 4.983 on 8 and 26 DF, p-value: 0.0008093

```
> summary(lm(bcTubule ~ Age + Vessles + valve + NYHA + Smoker + FHx + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + Vessles + valve + NYHA + Smoker + FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median  | 3Q     | Max    |
|---------|---------|---------|--------|--------|
| -9.0863 | -2.3118 | -0.0568 | 3.3835 | 9.5735 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 11.64970 | 7.28454    | 1.599   | 0.12185    |
| Age         | -0.10496 | 0.09129    | -1.150  | 0.26069    |
| Vessles     | 2.61058  | 1.12891    | 2.312   | 0.02893 *  |
| valve       | 1.86389  | 3.07457    | 0.606   | 0.54962    |
| NYHA        | 2.94885  | 1.25539    | 2.349   | 0.02670 *  |
| Smoker      | 3.17076  | 1.13366    | 2.797   | 0.00958 ** |
| FHx         | 2.14559  | 1.41565    | 1.516   | 0.14168    |
| DM          | -1.90527 | 2.67288    | -0.713  | 0.48231    |
| HBP         | -5.73183 | 2.25177    | -2.545  | 0.01719 *  |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.428 on 26 degrees of freedom

Multiple R-squared: 0.5323, Adjusted R-squared: 0.3884

F-statistic: 3.699 on 8 and 26 DF, p-value: 0.005245

```
> summary(lm(bcTubule ~ Age + Vessles + NYHA + Smoker + FHx + DM + HBP))
```

Call:

```
lm(formula = bcTubule ~ Age + Vessles + NYHA + Smoker + FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median  | 3Q     | Max    |
|---------|---------|---------|--------|--------|
| -9.4388 | -2.6673 | -0.1258 | 3.3152 | 8.7098 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 12.26702 | 7.12803    | 1.721   | 0.09670 .  |
| Age         | -0.09914 | 0.08971    | -1.105  | 0.27885    |
| Vessles     | 2.12646  | 0.78853    | 2.697   | 0.01191 *  |
| NYHA        | 2.89267  | 1.23722    | 2.338   | 0.02704 *  |
| Smoker      | 3.27927  | 1.10625    | 2.964   | 0.00627 ** |
| FHx         | 2.07413  | 1.39411    | 1.488   | 0.14840    |
| DM          | -1.68451 | 2.61676    | -0.644  | 0.52518    |
| HBP         | -5.42802 | 2.16943    | -2.502  | 0.01871 *  |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.364 on 27 degrees of freedom

Multiple R-squared: 0.5257, Adjusted R-squared: 0.4027

F-statistic: 4.275 on 7 and 27 DF, p-value: 0.002707

```
> summary(lm(bcTubule ~ Age + RCA+ valve+ NYHA + Smoker + FHx + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + RCA + valve + NYHA + Smoker + FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max     |
|---------|---------|--------|--------|---------|
| -7.7766 | -3.1247 | 0.7983 | 2.5406 | 10.0145 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 16.20313 | 6.24645    | 2.594   | 0.01538 *  |
| Age         | -0.12590 | 0.08349    | -1.508  | 0.14362    |
| RCA         | 8.12710  | 2.43501    | 3.338   | 0.00256 ** |
| valve       | 2.09137  | 2.54303    | 0.822   | 0.41833    |
| NYHA        | 2.25420  | 1.15125    | 1.958   | 0.06104 .  |
| Smoker      | 2.93113  | 1.04252    | 2.812   | 0.00925 ** |
| FHx         | 1.46752  | 1.27503    | 1.151   | 0.26022    |
| DM          | -1.82329 | 2.42241    | -0.753  | 0.45840    |
| HBP         | -6.83438 | 2.12498    | -3.216  | 0.00346 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.987 on 26 degrees of freedom

Multiple R-squared: 0.6052, Adjusted R-squared: 0.4838

F-statistic: 4.983 on 8 and 26 DF, p-value: 0.0008093

```
> summary(lm(bcTubule ~ Age + CAD+ RCA+ valve+ NYHA + Smoker + FHx + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoker + FHx + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -7.2101 | -2.6683 | 0.2781 | 2.6091 | 8.9134 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 12.63182 | 6.45373    | 1.957   | 0.06113 .  |
| Age         | -0.12253 | 0.08042    | -1.524  | 0.13967    |
| CAD         | 5.78997  | 3.56036    | 1.626   | 0.11596    |
| RCA         | 6.39013  | 2.47295    | 2.584   | 0.01574 *  |
| valve       | 6.13389  | 3.54591    | 1.730   | 0.09551 .  |
| NYHA        | 2.00799  | 1.04419    | 1.923   | 0.06550 .  |
| Smoker      | 3.19031  | 0.94024    | 3.393   | 0.00222 ** |
| FHx         | 1.17825  | 1.11828    | 1.054   | 0.30175    |
| HBP         | -7.85783 | 2.13872    | -3.674  | 0.00109 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.802 on 26 degrees of freedom

Multiple R-squared: 0.6339, Adjusted R-squared: 0.5212

F-statistic: 5.627 on 8 and 26 DF, p-value: 0.0003435

```
> summary(lm(bcTubule ~ Age + CAD+ RCA+ valve+ NYHA + factor(Smoker) + FHx + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + factor(Smoker) + FHx + HBP)
```

Residuals:

| Min    | 1Q     | Median | 3Q    | Max   |
|--------|--------|--------|-------|-------|
| -6.957 | -2.459 | 0.000  | 2.429 | 9.314 |

Coefficients:

|                 | Estimate | Std. Error | t value | Pr(> t )   |
|-----------------|----------|------------|---------|------------|
| (Intercept)     | 11.10231 | 7.79808    | 1.424   | 0.16740    |
| Age             | -0.09930 | 0.09695    | -1.024  | 0.31592    |
| CAD             | 5.92947  | 3.68968    | 1.607   | 0.12112    |
| RCA             | 6.45068  | 2.56546    | 2.514   | 0.01903 *  |
| valve           | 6.07308  | 3.68884    | 1.646   | 0.11273    |
| NYHA            | 1.95301  | 1.08442    | 1.801   | 0.08429 .  |
| factor(Smoker)1 | 4.03901  | 3.53650    | 1.142   | 0.26468    |
| factor(Smoker)2 | 6.24191  | 1.99951    | 3.122   | 0.00464 ** |
| factor(Smoker)3 | 12.33146 | 5.90258    | 2.089   | 0.04747 *  |
| FHx             | 1.08756  | 1.16819    | 0.931   | 0.36113    |
| HBP             | -7.89985 | 2.29483    | -3.442  | 0.00212 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.965 on 24 degrees of freedom

Multiple R-squared: 0.6388, Adjusted R-squared: 0.4883

F-statistic: 4.245 on 10 and 24 DF, p-value: 0.001787

```
> summary(lm(bcTubule ~ Age + CAD+ RCA+ valve+ NYHA + factor(Smoker) + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + factor(Smoker) + HBP)
```

Residuals:

| Min    | 1Q     | Median | 3Q    | Max   |
|--------|--------|--------|-------|-------|
| -7.578 | -2.721 | 0.000  | 2.956 | 9.607 |

Coefficients:

|                 | Estimate | Std. Error | t value | Pr(> t )   |
|-----------------|----------|------------|---------|------------|
| (Intercept)     | 11.05243 | 7.77708    | 1.421   | 0.16763    |
| Age             | -0.08685 | 0.09577    | -0.907  | 0.37312    |
| CAD             | 5.75782  | 3.67524    | 1.567   | 0.12977    |
| RCA             | 6.69657  | 2.54502    | 2.631   | 0.01436 *  |
| valve           | 6.18105  | 3.67718    | 1.681   | 0.10523    |
| NYHA            | 1.93062  | 1.08126    | 1.786   | 0.08631 .  |
| factor(Smoker)1 | 4.29542  | 3.51635    | 1.222   | 0.23327    |
| factor(Smoker)2 | 6.09089  | 1.98760    | 3.064   | 0.00517 ** |
| factor(Smoker)3 | 12.64733 | 5.87709    | 2.152   | 0.04125 *  |
| HBP             | -7.81647 | 2.28696    | -3.418  | 0.00217 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.951 on 25 degrees of freedom



Multiple R-squared: 0.6258, Adjusted R-squared: 0.4911

F-statistic: 4.645 on 9 and 25 DF, p-value: 0.001102

```
> sm <- class.ind(Smoker)
```

```
> summary(lm(bcTubule ~ Age + CAD+ RCA+ valve+ NYHA + sm + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + sm + HBP)
```

Residuals:

| Min    | 1Q     | Median | 3Q    | Max   |
|--------|--------|--------|-------|-------|
| -7.578 | -2.721 | 0.000  | 2.956 | 9.607 |

Coefficients: (1 not defined because of singularities)

|             | Estimate  | Std. Error | t value | Pr(> t )   |
|-------------|-----------|------------|---------|------------|
| (Intercept) | 23.69976  | 8.44660    | 2.806   | 0.00958 ** |
| Age         | -0.08685  | 0.09577    | -0.907  | 0.37312    |
| CAD         | 5.75782   | 3.67524    | 1.567   | 0.12977    |
| RCA         | 6.69657   | 2.54502    | 2.631   | 0.01436 *  |
| valve       | 6.18105   | 3.67718    | 1.681   | 0.10523    |
| NYHA        | 1.93062   | 1.08126    | 1.786   | 0.08631 .  |
| sm0         | -12.64733 | 5.87709    | -2.152  | 0.04125 *  |
| sm1         | -8.35191  | 6.18059    | -1.351  | 0.18870    |
| sm2         | -6.55644  | 5.55370    | -1.181  | 0.24889    |
| sm3         | NA        | NA         | NA      | NA         |
| HBP         | -7.81647  | 2.28696    | -3.418  | 0.00217 ** |
| ---         |           |            |         |            |

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.951 on 25 degrees of freedom

Multiple R-squared: 0.6258, Adjusted R-squared: 0.4911

F-statistic: 4.645 on 9 and 25 DF, p-value: 0.001102

>

> Smoke <- sm[,2] + sm[,3] + sm[,4]

> summary(lm(bcTubule ~ Age + CAD+ RCA+ valve+ NYHA + Smoke + HBP ))

Call:

lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoke + HBP)

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -8.4629 | -3.0362 | 0.8594 | 3.1103 | 8.6279 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 10.66031 | 6.88429    | 1.548   | 0.13315    |
| Age         | -0.09358 | 0.08418    | -1.112  | 0.27606    |
| CAD         | 5.67243  | 3.65203    | 1.553   | 0.13201    |
| RCA         | 6.41606  | 2.52780    | 2.538   | 0.01722 *  |
| valve       | 6.90953  | 3.61937    | 1.909   | 0.06694 .  |
| NYHA        | 2.10832  | 1.06957    | 1.971   | 0.05903 .  |
| Smoke       | 5.88414  | 1.94953    | 3.018   | 0.00549 ** |
| HBP         | -7.02926 | 2.18323    | -3.220  | 0.00333 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.936 on 27 degrees of freedom

Multiple R-squared: 0.5984, Adjusted R-squared: 0.4943

F-statistic: 5.747 on 7 and 27 DF, p-value: 0.0003802

```
> hf <- class.ind(NYHA)
```

```
> summary(lm(bcTubule ~ Age + CAD+ RCA+ valve+ hf + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + hf + Smoke +  
    HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -8.7398 | -2.9964 | 0.3604 | 3.6562 | 8.8204 |

Coefficients: (1 not defined because of singularities)

|             | Estimate | Std. Error | t value | Pr(> t ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 18.89252 | 7.67199    | 2.463   | 0.0210 * |
| Age         | -0.09425 | 0.08709    | -1.082  | 0.2895   |
| CAD         | 5.44962  | 3.80428    | 1.432   | 0.1644   |
| RCA         | 6.63592  | 2.66053    | 2.494   | 0.0196 * |
| valve       | 6.65432  | 3.78808    | 1.757   | 0.0912 . |
| hf1         | -5.35528 | 4.61826    | -1.160  | 0.2572   |
| hf2         | -4.49327 | 4.04128    | -1.112  | 0.2768   |

hf3 -1.46109 4.18110 -0.349 0.7297

hf4 NA NA NA NA

Smoke 5.57924 2.12802 2.622 0.0147 \*

HBP -6.58186 2.43545 -2.703 0.0122 \*

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.105 on 25 degrees of freedom

Multiple R-squared: 0.6022, Adjusted R-squared: 0.459

F-statistic: 4.206 on 9 and 25 DF, p-value: 0.002102

> summary(lm(bcTubule ~ CAD+ RCA+ valve+ NYHA + Smoke + HBP ))

Call:

lm(formula = bcTubule ~ CAD + RCA + valve + NYHA + Smoke + HBP)

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -9.7603 | -2.8837 | 0.1875 | 3.1108 | 8.6407 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )  |
|-------------|----------|------------|---------|-----------|
| (Intercept) | 4.184    | 3.683      | 1.136   | 0.26566   |
| CAD         | 5.783    | 3.666      | 1.577   | 0.12592   |
| RCA         | 6.351    | 2.538      | 2.503   | 0.01844 * |
| valve       | 6.422    | 3.608      | 1.780   | 0.08593 . |
| NYHA        | 2.211    | 1.070      | 2.066   | 0.04821 * |

Smoke      6.449    1.890   3.412   0.00198 \*\*

HBP        -7.500    2.151 -3.487   0.00163 \*\*

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.956 on 28 degrees of freedom

Multiple R-squared: 0.58,    Adjusted R-squared: 0.49

F-statistic: 6.445 on 6 and 28 DF, p-value: 0.0002362

```
> summary(lm(bcTubule ~ CAD+ RCA+ valve+ NYHA + Smoke + HBP + FHx))
```

Call:

```
lm(formula = bcTubule ~ CAD + RCA + valve + NYHA + Smoke + HBP + FHx)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -8.9890 | -3.0437 | 0.4445 | 2.8392 | 8.7004 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 3.3954   | 3.8138     | 0.890   | 0.38117    |
| CAD         | 5.9835   | 3.6911     | 1.621   | 0.11663    |
| RCA         | 6.1186   | 2.5644     | 2.386   | 0.02431 *  |
| valve       | 6.3164   | 3.6273     | 1.741   | 0.09300 .  |
| NYHA        | 2.2317   | 1.0755     | 2.075   | 0.04764 *  |
| Smoke       | 6.5795   | 1.9053     | 3.453   | 0.00184 ** |
| HBP         | -7.5715  | 2.1627     | -3.501  | 0.00163 ** |

FHx      0.9884   1.1550   0.856   0.39964

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.98 on 27 degrees of freedom

Multiple R-squared: 0.5911,    Adjusted R-squared: 0.4851

F-statistic: 5.576 on 7 and 27 DF, p-value: 0.0004717

```
> summary(lm(bcTubule ~ RCA+ valve+ NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ RCA + valve + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q     | Median | 3Q    | Max    |
|---------|--------|--------|-------|--------|
| -10.040 | -3.151 | 0.225  | 3.618 | 10.442 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 7.756    | 2.978      | 2.604   | 0.01437 *  |
| RCA         | 7.755    | 2.437      | 3.183   | 0.00347 ** |
| valve       | 2.208    | 2.486      | 0.888   | 0.38187    |
| NYHA        | 2.172    | 1.097      | 1.980   | 0.05722 .  |
| Smoke       | 6.491    | 1.938      | 3.350   | 0.00226 ** |
| HBP         | -6.505   | 2.108      | -3.085  | 0.00444 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.082 on 29 degrees of freedom

Multiple R-squared: 0.5427, Adjusted R-squared: 0.4638

F-statistic: 6.883 on 5 and 29 DF, p-value: 0.000241

```
> op <- class.ind(Procedure)
```

```
> summary(lm(bcTubule ~ RCA+ op + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ RCA + op + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -9.7603 | -2.8837 | 0.1875 | 3.1108 | 8.6407 |

Coefficients: (1 not defined because of singularities)

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 4.184    | 3.683      | 1.136   | 0.26566    |
| RCA         | 6.351    | 2.538      | 2.503   | 0.01844 *  |
| op0         | 6.422    | 3.608      | 1.780   | 0.08593 .  |
| op1         | 5.783    | 3.666      | 1.577   | 0.12592    |
| op3         | NA       | NA         | NA      | NA         |
| NYHA        | 2.211    | 1.070      | 2.066   | 0.04821 *  |
| Smoke       | 6.449    | 1.890      | 3.412   | 0.00198 ** |
| HBP         | -7.500   | 2.151      | -3.487  | 0.00163 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.956 on 28 degrees of freedom

Multiple R-squared: 0.58, Adjusted R-squared: 0.49

F-statistic: 6.445 on 6 and 28 DF, p-value: 0.0002362

```
> summary(lm(bcTubule ~ LAD+ op + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ LAD + op + NYHA + Smoke + HBP)
```

Residuals:

| Min      | 1Q      | Median  | 3Q     | Max    |
|----------|---------|---------|--------|--------|
| -10.3014 | -2.6396 | -0.0891 | 3.8435 | 8.2305 |

Coefficients: (1 not defined because of singularities)

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 4.253    | 4.127      | 1.031   | 0.31159    |
| LAD         | -3.958   | 4.373      | -0.905  | 0.37319    |
| op0         | 5.446    | 3.908      | 1.393   | 0.17446    |
| op1         | 12.890   | 5.698      | 2.262   | 0.03164 *  |
| op3         | NA       | NA         | NA      | NA         |
| NYHA        | 2.259    | 1.224      | 1.846   | 0.07552 .  |
| Smoke       | 6.199    | 2.082      | 2.978   | 0.00593 ** |
| HBP         | -6.079   | 2.247      | -2.706  | 0.01147 *  |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1



Residual standard error: 5.404 on 28 degrees of freedom

Multiple R-squared: 0.5007, Adjusted R-squared: 0.3937

F-statistic: 4.679 on 6 and 28 DF, p-value: 0.002056

```
> summary(lm(bcTubule ~ Vessles + op + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Vessles + op + NYHA + Smoke + HBP)
```

Residuals:

| Min      | 1Q      | Median  | 3Q     | Max    |
|----------|---------|---------|--------|--------|
| -10.6138 | -2.5054 | -0.2768 | 3.7481 | 7.7871 |

Coefficients: (1 not defined because of singularities)

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 2.995    | 4.006      | 0.748   | 0.46086    |
| Vessles     | 1.380    | 1.429      | 0.965   | 0.34271    |
| op0         | 5.184    | 3.887      | 1.334   | 0.19306    |
| op1         | 5.889    | 4.935      | 1.193   | 0.24280    |
| op3         | NA       | NA         | NA      | NA         |
| NYHA        | 2.690    | 1.151      | 2.336   | 0.02690 *  |
| Smoke       | 6.795    | 2.085      | 3.259   | 0.00293 ** |
| HBP         | -6.003   | 2.234      | -2.687  | 0.01200 *  |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.394 on 28 degrees of freedom

Multiple R-squared: 0.5026, Adjusted R-squared: 0.396

F-statistic: 4.716 on 6 and 28 DF, p-value: 0.001960

```
> summary(lm(bcTubule ~ Age + op + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + op + NYHA + Smoke + HBP)
```

Residuals:

| Min      | 1Q      | Median | 3Q     | Max    |
|----------|---------|--------|--------|--------|
| -10.6377 | -3.0566 | 0.2117 | 4.6128 | 7.8308 |

Coefficients: (1 not defined because of singularities)

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 9.45366  | 7.50570    | 1.260   | 0.21824    |
| Age         | -0.08862 | 0.09197    | -0.964  | 0.34348    |
| op0         | 5.59609  | 3.91488    | 1.429   | 0.16394    |
| op1         | 8.92840  | 3.73689    | 2.389   | 0.02386 *  |
| op3         | NA       | NA         | NA      | NA         |
| NYHA        | 2.53982  | 1.15405    | 2.201   | 0.03616 *  |
| Smoke       | 5.93112  | 2.13050    | 2.784   | 0.00952 ** |
| HBP         | -5.40062 | 2.28062    | -2.368  | 0.02502 *  |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.394 on 28 degrees of freedom

Multiple R-squared: 0.5026, Adjusted R-squared: 0.396

F-statistic: 4.715 on 6 and 28 DF, p-value: 0.001963

```
> summary(lm(bcTubule ~ Age + op + RCA + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + op + RCA + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -8.4629 | -3.0362 | 0.8594 | 3.1103 | 8.6279 |

Coefficients: (1 not defined because of singularities)

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 10.66031 | 6.88429    | 1.548   | 0.13315    |
| Age         | -0.09358 | 0.08418    | -1.112  | 0.27606    |
| op0         | 6.90953  | 3.61937    | 1.909   | 0.06694 .  |
| op1         | 5.67243  | 3.65203    | 1.553   | 0.13201    |
| op3         | NA       | NA         | NA      | NA         |
| RCA         | 6.41606  | 2.52780    | 2.538   | 0.01722 *  |
| NYHA        | 2.10832  | 1.06957    | 1.971   | 0.05903 .  |
| Smoke       | 5.88414  | 1.94953    | 3.018   | 0.00549 ** |
| HBP         | -7.02926 | 2.18323    | -3.220  | 0.00333 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.936 on 27 degrees of freedom

Multiple R-squared: 0.5984, Adjusted R-squared: 0.4943

F-statistic: 5.747 on 7 and 27 DF, p-value: 0.0003802

```
> summary(lm(bcTubule ~ Age + RCA + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + RCA + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -8.1714 | -3.1907 | 0.4193 | 3.8069 | 9.4663 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 13.70823 | 6.60295    | 2.076   | 0.04686 *  |
| Age         | -0.07739 | 0.08470    | -0.914  | 0.36836    |
| RCA         | 6.11477  | 1.89813    | 3.221   | 0.00314 ** |
| NYHA        | 2.19944  | 1.09348    | 2.011   | 0.05366 .  |
| Smoke       | 6.22871  | 1.98388    | 3.140   | 0.00387 ** |
| HBP         | -5.43671 | 2.07768    | -2.617  | 0.01395 *  |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.078 on 29 degrees of freedom

Multiple R-squared: 0.5434, Adjusted R-squared: 0.4647

F-statistic: 6.902 on 5 and 29 DF, p-value: 0.0002360

```
> summary(lm(bcTubule ~ RCA + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ RCA + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q      | Median  | 3Q     | Max    |
|---------|---------|---------|--------|--------|
| -9.3877 | -3.5895 | -0.1578 | 3.4415 | 9.7211 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t ) |    |
|-------------|----------|------------|---------|----------|----|
| (Intercept) | 8.294    | 2.906      | 2.854   | 0.00775  | ** |
| RCA         | 6.377    | 1.871      | 3.408   | 0.00188  | ** |
| NYHA        | 2.264    | 1.088      | 2.080   | 0.04612  | *  |
| Smoke       | 6.660    | 1.921      | 3.466   | 0.00161  | ** |
| HBP         | -5.932   | 2.000      | -2.966  | 0.00587  | ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.064 on 30 degrees of freedom

Multiple R-squared: 0.5302, Adjusted R-squared: 0.4676

F-statistic: 8.466 on 4 and 30 DF, p-value: 0.0001072

```
> summary(lm(bcTubule ~ RCA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ RCA + Smoke + HBP)
```

Residuals:

| Min    | 1Q     | Median | 3Q    | Max    |
|--------|--------|--------|-------|--------|
| -8.081 | -3.256 | 1.624  | 2.915 | 10.540 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )     |
|-------------|----------|------------|---------|--------------|
| (Intercept) | 12.479   | 2.207      | 5.655   | 3.28e-06 *** |
| RCA         | 6.883    | 1.952      | 3.526   | 0.001338 **  |
| Smoke       | 7.288    | 1.997      | 3.650   | 0.000956 *** |
| HBP         | -5.481   | 2.092      | -2.619  | 0.013515 *   |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.329 on 31 degrees of freedom

Multiple R-squared: 0.4625, Adjusted R-squared: 0.4104

F-statistic: 8.89 on 3 and 31 DF, p-value: 0.0002120

```
> summary(lm(bcTubule ~ LAD+ LCX+ RCA + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ LAD + LCX + RCA + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -9.2817 | -3.7579 | 0.0229 | 3.6472 | 9.9771 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 8.3582   | 3.3913     | 2.465   | 0.02012 *  |
| LAD         | 0.5954   | 2.8487     | 0.209   | 0.83595    |
| LCX         | -0.9659  | 2.9463     | -0.328  | 0.74547    |
| RCA         | 6.6474   | 2.5230     | 2.635   | 0.01357 *  |
| NYHA        | 2.2890   | 1.1610     | 1.972   | 0.05861 .  |
| Smoke       | 6.5430   | 2.0349     | 3.215   | 0.00328 ** |
| HBP         | -6.0603  | 2.1054     | -2.879  | 0.00757 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.232 on 28 degrees of freedom

Multiple R-squared: 0.532, Adjusted R-squared: 0.4318

F-statistic: 5.306 on 6 and 28 DF, p-value: 0.0009218

```
> summary(lm(bcTubule ~ valve+ LAD+ LCX+ RCA + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ valve + LAD + LCX + RCA + NYHA + Smoke + HBP)
```

Residuals:

| Min      | 1Q      | Median  | 3Q     | Max     |
|----------|---------|---------|--------|---------|
| -10.0110 | -3.2650 | -0.1134 | 3.4148 | 10.2713 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t ) |
|-------------|----------|------------|---------|----------|
| (Intercept) | 6.079    | 3.880      | 1.567   | 0.12883  |

|       |        |       |        |            |
|-------|--------|-------|--------|------------|
| valve | 3.834  | 3.243 | 1.182  | 0.24735    |
| LAD   | 3.033  | 3.500 | 0.866  | 0.39386    |
| LCX   | -1.639 | 2.980 | -0.550 | 0.58687    |
| RCA   | 8.014  | 2.759 | 2.905  | 0.00725 ** |
| NYHA  | 2.399  | 1.157 | 2.075  | 0.04769 *  |
| Smoke | 6.393  | 2.025 | 3.158  | 0.00389 ** |
| HBP   | -7.068 | 2.257 | -3.131 | 0.00416 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.195 on 27 degrees of freedom

Multiple R-squared: 0.5551, Adjusted R-squared: 0.4397

F-statistic: 4.812 on 7 and 27 DF, p-value: 0.001284

```
> summary(lm(bcTubule ~ valve + CAD + RCA + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ valve + CAD + RCA + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -9.7603 | -2.8837 | 0.1875 | 3.1108 | 8.6407 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )  |
|-------------|----------|------------|---------|-----------|
| (Intercept) | 4.184    | 3.683      | 1.136   | 0.26566   |
| valve       | 6.422    | 3.608      | 1.780   | 0.08593 . |



|       |        |       |        |            |
|-------|--------|-------|--------|------------|
| CAD   | 5.783  | 3.666 | 1.577  | 0.12592    |
| RCA   | 6.351  | 2.538 | 2.503  | 0.01844 *  |
| NYHA  | 2.211  | 1.070 | 2.066  | 0.04821 *  |
| Smoke | 6.449  | 1.890 | 3.412  | 0.00198 ** |
| HBP   | -7.500 | 2.151 | -3.487 | 0.00163 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.956 on 28 degrees of freedom

Multiple R-squared: 0.58, Adjusted R-squared: 0.49

F-statistic: 6.445 on 6 and 28 DF, p-value: 0.0002362

```
> summary(lm(bcTubule ~ valve + RCA + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ valve + RCA + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q     | Median | 3Q    | Max    |
|---------|--------|--------|-------|--------|
| -10.040 | -3.151 | 0.225  | 3.618 | 10.442 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 7.756    | 2.978      | 2.604   | 0.01437 *  |
| valve       | 2.208    | 2.486      | 0.888   | 0.38187    |
| RCA         | 7.755    | 2.437      | 3.183   | 0.00347 ** |
| NYHA        | 2.172    | 1.097      | 1.980   | 0.05722 .  |

Smoke      6.491    1.938   3.350   0.00226 \*\*

HBP        -6.505    2.108   -3.085   0.00444 \*\*

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.082 on 29 degrees of freedom

Multiple R-squared: 0.5427,    Adjusted R-squared: 0.4638

F-statistic: 6.883 on 5 and 29 DF, p-value: 0.000241

```
> summary(lm(bcTubule ~ Age+ RCA + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + RCA + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -8.1714 | -3.1907 | 0.4193 | 3.8069 | 9.4663 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 13.70823 | 6.60295    | 2.076   | 0.04686 *  |
| Age         | -0.07739 | 0.08470    | -0.914  | 0.36836    |
| RCA         | 6.11477  | 1.89813    | 3.221   | 0.00314 ** |
| NYHA        | 2.19944  | 1.09348    | 2.011   | 0.05366 .  |
| Smoke       | 6.22871  | 1.98388    | 3.140   | 0.00387 ** |
| HBP         | -5.43671 | 2.07768    | -2.617  | 0.01395 *  |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.078 on 29 degrees of freedom

Multiple R-squared: 0.5434, Adjusted R-squared: 0.4647

F-statistic: 6.902 on 5 and 29 DF, p-value: 0.0002360

```
> summary(lm(bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoke + Procedure + Vessles +  
FHx + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoke + Procedure + Vessles +  
FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median  | 3Q     | Max    |
|---------|---------|---------|--------|--------|
| -7.9666 | -3.3369 | -0.2339 | 2.8365 | 8.1075 |

Coefficients: (1 not defined because of singularities)

|             | Estimate | Std. Error | t value | Pr(> t )  |
|-------------|----------|------------|---------|-----------|
| (Intercept) | 10.82288 | 7.21198    | 1.501   | 0.14648   |
| Age         | -0.11303 | 0.08522    | -1.326  | 0.19723   |
| CAD         | 8.74516  | 4.61988    | 1.893   | 0.07049 . |
| RCA         | 8.28196  | 3.46929    | 2.387   | 0.02520 * |
| valve       | 7.87877  | 3.64948    | 2.159   | 0.04108 * |
| NYHA        | 2.43046  | 1.15087    | 2.112   | 0.04530 * |
| Smoke       | 4.89528  | 2.06523    | 2.370   | 0.02615 * |
| Procedure   | NA       | NA         | NA      | NA        |
| Vessles     | -1.32331 | 1.82201    | -0.726  | 0.47469   |

|     |          |         |        |            |
|-----|----------|---------|--------|------------|
| FHx | 1.61675  | 1.30183 | 1.242  | 0.22627    |
| DM  | -3.22704 | 2.32444 | -1.388 | 0.17780    |
| HBP | -7.53834 | 2.18796 | -3.445 | 0.00211 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.886 on 24 degrees of freedom

Multiple R-squared: 0.6502, Adjusted R-squared: 0.5044

F-statistic: 4.461 on 10 and 24 DF, p-value: 0.001295

```
> summary(lm(bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoke + op + Vessles + FHx +
DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoke + op + Vessles + FHx + DM
+ HBP)
```

Residuals:

|         |         |         |        |        |
|---------|---------|---------|--------|--------|
| Min     | 1Q      | Median  | 3Q     | Max    |
| -7.9666 | -3.3369 | -0.2339 | 2.8365 | 8.1075 |

Coefficients: (3 not defined because of singularities)

|             |          |            |         |           |
|-------------|----------|------------|---------|-----------|
|             | Estimate | Std. Error | t value | Pr(> t )  |
| (Intercept) | 10.82288 | 7.21198    | 1.501   | 0.14648   |
| Age         | -0.11303 | 0.08522    | -1.326  | 0.19723   |
| CAD         | 8.74516  | 4.61988    | 1.893   | 0.07049 . |
| RCA         | 8.28196  | 3.46929    | 2.387   | 0.02520 * |
| valve       | 7.87877  | 3.64948    | 2.159   | 0.04108 * |

|         |          |         |        |            |
|---------|----------|---------|--------|------------|
| NYHA    | 2.43046  | 1.15087 | 2.112  | 0.04530 *  |
| Smoke   | 4.89528  | 2.06523 | 2.370  | 0.02615 *  |
| op0     | NA       | NA      | NA     | NA         |
| op1     | NA       | NA      | NA     | NA         |
| op3     | NA       | NA      | NA     | NA         |
| Vessles | -1.32331 | 1.82201 | -0.726 | 0.47469    |
| FHx     | 1.61675  | 1.30183 | 1.242  | 0.22627    |
| DM      | -3.22704 | 2.32444 | -1.388 | 0.17780    |
| HBP     | -7.53834 | 2.18796 | -3.445 | 0.00211 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.886 on 24 degrees of freedom

Multiple R-squared: 0.6502, Adjusted R-squared: 0.5044

F-statistic: 4.461 on 10 and 24 DF, p-value: 0.001295

```
> summary(lm(bcTubule ~ Age + CAD + LAD + LCX + RCA + valve + NYHA + Smoke + Vessles +
  FHx + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + LAD + LCX + RCA + valve + NYHA + Smoke + Vessles +
  FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median  | 3Q     | Max    |
|---------|---------|---------|--------|--------|
| -7.9662 | -3.3359 | -0.2364 | 2.8410 | 8.1110 |

Coefficients: (1 not defined because of singularities)

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 10.81887 | 7.45305    | 1.452   | 0.16012    |
| Age         | -0.11302 | 0.08717    | -1.297  | 0.20765    |
| CAD         | 8.73358  | 5.73176    | 1.524   | 0.14121    |
| LAD         | -1.30699 | 4.94555    | -0.264  | 0.79392    |
| LCX         | -1.33124 | 2.90278    | -0.459  | 0.65082    |
| RCA         | 6.96240  | 2.84734    | 2.445   | 0.02255 *  |
| valve       | 7.87906  | 3.72885    | 2.113   | 0.04566 *  |
| NYHA        | 2.43176  | 1.23155    | 1.975   | 0.06044 .  |
| Smoke       | 4.89505  | 2.11063    | 2.319   | 0.02962 *  |
| Vessles     | NA       | NA         | NA      | NA         |
| FHx         | 1.61760  | 1.35113    | 1.197   | 0.24342    |
| DM          | -3.22767 | 2.38101    | -1.356  | 0.18839    |
| HBP         | -7.53942 | 2.25555    | -3.343  | 0.00282 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.991 on 23 degrees of freedom

Multiple R-squared: 0.6502, Adjusted R-squared: 0.4829

F-statistic: 3.886 on 11 and 23 DF, p-value: 0.002923

```
> summary(lm(bcTubule ~ Age + CAD +LAD+ LCX+ RCA+ valve+ NYHA + Smoke + FHx + DM +
HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + LAD + LCX + RCA + valve + NYHA + Smoke + FHx + DM +
HBP)
```

Residuals:

| Min     | 1Q      | Median  | 3Q     | Max    |
|---------|---------|---------|--------|--------|
| -7.9662 | -3.3359 | -0.2364 | 2.8410 | 8.1110 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 10.81887 | 7.45305    | 1.452   | 0.16012    |
| Age         | -0.11302 | 0.08717    | -1.297  | 0.20765    |
| CAD         | 8.73358  | 5.73176    | 1.524   | 0.14121    |
| LAD         | -1.30699 | 4.94555    | -0.264  | 0.79392    |
| LCX         | -1.33124 | 2.90278    | -0.459  | 0.65082    |
| RCA         | 6.96240  | 2.84734    | 2.445   | 0.02255 *  |
| valve       | 7.87906  | 3.72885    | 2.113   | 0.04566 *  |
| NYHA        | 2.43176  | 1.23155    | 1.975   | 0.06044 .  |
| Smoke       | 4.89505  | 2.11063    | 2.319   | 0.02962 *  |
| FHx         | 1.61760  | 1.35113    | 1.197   | 0.24342    |
| DM          | -3.22767 | 2.38101    | -1.356  | 0.18839    |
| HBP         | -7.53942 | 2.25555    | -3.343  | 0.00282 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.991 on 23 degrees of freedom

Multiple R-squared: 0.6502, Adjusted R-squared: 0.4829

F-statistic: 3.886 on 11 and 23 DF, p-value: 0.002923

```
> summary(lm(bcTubule ~ Age + CAD +RCA+ valve+ NYHA + Smoke + FHx + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoke + FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -8.1658 | -3.2745 | 0.3377 | 2.7472 | 7.9075 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 9.21768  | 6.79979    | 1.356   | 0.18735    |
| Age         | -0.10086 | 0.08277    | -1.219  | 0.23436    |
| CAD         | 6.70271  | 3.63056    | 1.846   | 0.07674 .  |
| RCA         | 6.55833  | 2.50655    | 2.616   | 0.01486 *  |
| valve       | 7.51861  | 3.58130    | 2.099   | 0.04604 *  |
| NYHA        | 2.63545  | 1.10513    | 2.385   | 0.02499 *  |
| Smoke       | 5.28905  | 1.97387    | 2.680   | 0.01285 *  |
| FHx         | 1.86582  | 1.24392    | 1.500   | 0.14615    |
| DM          | -3.36396 | 2.29479    | -1.466  | 0.15514    |
| HBP         | -7.32713 | 2.14796    | -3.411  | 0.00220 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.839 on 25 degrees of freedom

Multiple R-squared: 0.6425, Adjusted R-squared: 0.5138

F-statistic: 4.992 on 9 and 25 DF, p-value: 0.000675

```
> summary(lm(bcTubule ~ Age + RCA+ NYHA + Smoke + FHx + DM + HBP ))
```



Call:

```
lm(formula = bcTubule ~ Age + RCA + NYHA + Smoke + FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -8.1420 | -3.3520 | 0.2898 | 3.2950 | 9.8473 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 13.08257 | 6.60376    | 1.981   | 0.05785 .  |
| Age         | -0.08605 | 0.08494    | -1.013  | 0.32003    |
| RCA         | 6.47623  | 1.93202    | 3.352   | 0.00238 ** |
| NYHA        | 2.60306  | 1.14894    | 2.266   | 0.03171 *  |
| Smoke       | 5.76892  | 2.04057    | 2.827   | 0.00874 ** |
| FHx         | 1.69815  | 1.28899    | 1.317   | 0.19877    |
| DM          | -2.64111 | 2.37369    | -1.113  | 0.27566    |
| HBP         | -5.56554 | 2.07545    | -2.682  | 0.01234 *  |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.068 on 27 degrees of freedom

Multiple R-squared: 0.5766, Adjusted R-squared: 0.4668

F-statistic: 5.252 on 7 and 27 DF, p-value: 0.0007152

```
> summary(lm(bcTubule ~ Age + CAD +RCA+ valve+ NYHA + Smoke + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoke + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -7.7913 | -3.5619 | 0.2315 | 2.9726 | 8.1768 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 10.28760 | 6.92290    | 1.486   | 0.14930    |
| Age         | -0.09194 | 0.08451    | -1.088  | 0.28665    |
| CAD         | 6.04694  | 3.68975    | 1.639   | 0.11329    |
| RCA         | 6.73961  | 2.56310    | 2.629   | 0.01417 *  |
| valve       | 7.33546  | 3.66424    | 2.002   | 0.05583 .  |
| NYHA        | 2.38951  | 1.11886    | 2.136   | 0.04229 *  |
| Smoke       | 5.44424  | 2.01797    | 2.698   | 0.01209 *  |
| DM          | -1.89762 | 2.12546    | -0.893  | 0.38015    |
| HBP         | -7.15013 | 2.19566    | -3.256  | 0.00313 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.954 on 26 degrees of freedom

Multiple R-squared: 0.6103, Adjusted R-squared: 0.4904

F-statistic: 5.09 on 8 and 26 DF, p-value: 0.0006985

```
> summary(lm(bcTubule ~ Age + CAD +RCA+ valve+ NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -8.4629 | -3.0362 | 0.8594 | 3.1103 | 8.6279 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 10.66031 | 6.88429    | 1.548   | 0.13315    |
| Age         | -0.09358 | 0.08418    | -1.112  | 0.27606    |
| CAD         | 5.67243  | 3.65203    | 1.553   | 0.13201    |
| RCA         | 6.41606  | 2.52780    | 2.538   | 0.01722 *  |
| valve       | 6.90953  | 3.61937    | 1.909   | 0.06694 .  |
| NYHA        | 2.10832  | 1.06957    | 1.971   | 0.05903 .  |
| Smoke       | 5.88414  | 1.94953    | 3.018   | 0.00549 ** |
| HBP         | -7.02926 | 2.18323    | -3.220  | 0.00333 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.936 on 27 degrees of freedom

Multiple R-squared: 0.5984, Adjusted R-squared: 0.4943

F-statistic: 5.747 on 7 and 27 DF, p-value: 0.0003802

```
> summary(lm(bcTubule ~ Age + CAD + RCA + valve + Smoke + FHx + DM + HBP))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + Smoke + FHx + DM + HBP)
```

Residuals:

| Min    | 1Q     | Median | 3Q    | Max   |
|--------|--------|--------|-------|-------|
| -8.819 | -2.330 | -0.506 | 2.235 | 9.338 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )  |
|-------------|----------|------------|---------|-----------|
| (Intercept) | 15.15763 | 6.87384    | 2.205   | 0.0365 *  |
| Age         | -0.11602 | 0.08965    | -1.294  | 0.2070    |
| CAD         | 6.07356  | 3.93382    | 1.544   | 0.1347    |
| RCA         | 7.25758  | 2.70442    | 2.684   | 0.0125 *  |
| valve       | 7.61833  | 3.89046    | 1.958   | 0.0610 .  |
| Smoke       | 6.15820  | 2.10754    | 2.922   | 0.0071 ** |
| FHx         | 1.42571  | 1.33644    | 1.067   | 0.2959    |
| DM          | -1.63979 | 2.36609    | -0.693  | 0.4944    |
| HBP         | -6.74985 | 2.31868    | -2.911  | 0.0073 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.258 on 26 degrees of freedom

Multiple R-squared: 0.5612, Adjusted R-squared: 0.4262

F-statistic: 4.156 on 8 and 26 DF, p-value: 0.002626

```
> summary(lm(bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoke + FHx + DM + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + CAD + RCA + valve + NYHA + Smoke + FHx + DM + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max    |
|---------|---------|--------|--------|--------|
| -8.1658 | -3.2745 | 0.3377 | 2.7472 | 7.9075 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 9.21768  | 6.79979    | 1.356   | 0.18735    |
| Age         | -0.10086 | 0.08277    | -1.219  | 0.23436    |
| CAD         | 6.70271  | 3.63056    | 1.846   | 0.07674 .  |
| RCA         | 6.55833  | 2.50655    | 2.616   | 0.01486 *  |
| valve       | 7.51861  | 3.58130    | 2.099   | 0.04604 *  |
| NYHA        | 2.63545  | 1.10513    | 2.385   | 0.02499 *  |
| Smoke       | 5.28905  | 1.97387    | 2.680   | 0.01285 *  |
| FHx         | 1.86582  | 1.24392    | 1.500   | 0.14615    |
| DM          | -3.36396 | 2.29479    | -1.466  | 0.15514    |
| HBP         | -7.32713 | 2.14796    | -3.411  | 0.00220 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.839 on 25 degrees of freedom

Multiple R-squared: 0.6425, Adjusted R-squared: 0.5138

F-statistic: 4.992 on 9 and 25 DF, p-value: 0.000675

```
> cor(cbind(valve,CAD,RCA))
```

| valve | CAD | RCA |
|-------|-----|-----|
|-------|-----|-----|

valve 1.0000000 -0.8227534 -0.5803810

CAD -0.8227534 1.0000000 0.7054131

RCA -0.5803810 0.7054131 1.0000000

```
> summary(lm(bcTubule ~ Age + RCA+ valve+ NYHA + Smoke + FHx + DM + HBP ))
```

Call:

lm(formula = bcTubule ~ Age + RCA + valve + NYHA + Smoke + FHx + DM + HBP)

Residuals:

| Min     | 1Q      | Median  | 3Q     | Max     |
|---------|---------|---------|--------|---------|
| -8.4020 | -2.5492 | -0.4082 | 2.8838 | 10.5593 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 13.7696  | 6.6240     | 2.079   | 0.04765 *  |
| Age         | -0.1040  | 0.0865     | -1.202  | 0.24002    |
| RCA         | 8.0967   | 2.4711     | 3.277   | 0.00298 ** |
| valve       | 2.6678   | 2.5437     | 1.049   | 0.30394    |
| NYHA        | 2.4872   | 1.1521     | 2.159   | 0.04028 *  |
| Smoke       | 5.4345   | 2.0616     | 2.636   | 0.01396 *  |
| FHx         | 1.5893   | 1.2908     | 1.231   | 0.22926    |
| DM          | -2.7140  | 2.3703     | -1.145  | 0.26264    |
| HBP         | -6.1315  | 2.1408     | -2.864  | 0.00816 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.059 on 26 degrees of freedom

Multiple R-squared: 0.5938, Adjusted R-squared: 0.4688

F-statistic: 4.75 on 8 and 26 DF, p-value: 0.001116

```
> cor(cbind( Age, CAD ,RCA, valve, NYHA , Smoke, FHx , DM , HBP ))
```

|       | Age         | CAD          | RCA         | valve       | NYHA         |  |  |  |  |
|-------|-------------|--------------|-------------|-------------|--------------|--|--|--|--|
| Age   | 1.00000000  | -0.13699542  | -0.05094403 | 0.21504032  | -0.078255415 |  |  |  |  |
| CAD   | -0.13699542 | 1.00000000   | 0.70541311  | -0.82275335 | 0.051941499  |  |  |  |  |
| RCA   | -0.05094403 | 0.70541311   | 1.00000000  | -0.58038100 | 0.171293003  |  |  |  |  |
| valve | 0.21504032  | -0.82275335  | -0.58038100 | 1.00000000  | 0.011111111  |  |  |  |  |
| NYHA  | -0.07825542 | 0.05194150   | 0.17129300  | 0.01111111  | 1.000000000  |  |  |  |  |
| Smoke | -0.23837803 | -0.09349470  | -0.05441072 | 0.12000000  | 0.144444444  |  |  |  |  |
| FHx   | 0.12168322  | -0.05711098  | 0.04431552  | 0.07330167  | -0.006787191 |  |  |  |  |
| DM    | 0.05289695  | 0.18167991   | 0.24745670  | -0.08268982 | 0.275632744  |  |  |  |  |
| HBP   | 0.21039636  | 0.24383151   | 0.37416840  | 0.01946247  | 0.162187280  |  |  |  |  |
|       | Smoke       | FHx          | DM          | HBP         |              |  |  |  |  |
| Age   | -0.23837803 | 0.121683223  | 0.05289695  | 0.21039636  |              |  |  |  |  |
| CAD   | -0.09349470 | -0.057110977 | 0.18167991  | 0.24383151  |              |  |  |  |  |
| RCA   | -0.05441072 | 0.044315515  | 0.24745670  | 0.37416840  |              |  |  |  |  |
| valve | 0.12000000  | 0.073301667  | -0.08268982 | 0.01946247  |              |  |  |  |  |
| NYHA  | 0.14444444  | -0.006787191 | 0.27563274  | 0.16218728  |              |  |  |  |  |
| Smoke | 1.00000000  | -0.073301667 | -0.20672456 | -0.01946247 |              |  |  |  |  |
| FHx   | -0.07330167 | 1.000000000  | 0.40408679  | 0.07133159  |              |  |  |  |  |
| DM    | -0.20672456 | 0.404086789  | 1.00000000  | 0.11667777  |              |  |  |  |  |
| HBP   | -0.01946247 | 0.071331588  | 0.11667777  | 1.00000000  |              |  |  |  |  |

```
> out <- lm(bcTubule ~ Age + CAD +RCA+ valve+ NYHA + Smoke + FHx + DM + HBP )
```

```
> res <- studres(out)
```

```
> qqnorm(res)
```

```

> fit <- fitted(out)

> plot(fit,res)

> ypred <- (fit/5 + 1)^5

> plot(ypred,Tubule,ylim = c(0,14000),xlim = c(0,14000),main = "Plot of tubule length against
model values", ylab = "Tubule length", xlab = "Fitted values")

> plot(fit,bcTubule,ylim = c(0,30),xlim = c(0,30),main = "Plot of Box-Cox transformed tubule
length against fitted model values")

> summary(lm(bcTubule ~ Age + RCA+ valve+ NYHA + Smoke + FHx + HBP ))

```

Call:

```
lm(formula = bcTubule ~ Age + RCA + valve + NYHA + Smoke + FHx + HBP)
```

Residuals:

| Min    | 1Q     | Median | 3Q    | Max    |
|--------|--------|--------|-------|--------|
| -8.002 | -2.586 | -0.254 | 2.866 | 10.924 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 14.12638 | 6.65472    | 2.123   | 0.04309 *  |
| Age         | -0.10259 | 0.08698    | -1.179  | 0.24852    |
| RCA         | 7.61629  | 2.44918    | 3.110   | 0.00438 ** |
| valve       | 2.58233  | 2.55724    | 1.010   | 0.32155    |
| NYHA        | 2.08039  | 1.10228    | 1.887   | 0.06991 .  |
| Smoke       | 6.00058  | 2.01295    | 2.981   | 0.00602 ** |
| FHx         | 0.97527  | 1.18088    | 0.826   | 0.41611    |
| HBP         | -6.04603 | 2.15174    | -2.810  | 0.00911 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1



Residual standard error: 5.088 on 27 degrees of freedom

Multiple R-squared: 0.5733, Adjusted R-squared: 0.4627

F-statistic: 5.182 on 7 and 27 DF, p-value: 0.0007843

```
> summary(lm(bcTubule ~ Age + RCA+ valve+ NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ Age + RCA + valve + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q      | Median | 3Q     | Max     |
|---------|---------|--------|--------|---------|
| -8.6880 | -3.0546 | 0.6014 | 3.4341 | 10.3148 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 14.40855 | 6.60810    | 2.180   | 0.03779 *  |
| Age         | -0.09714 | 0.08624    | -1.126  | 0.26955    |
| RCA         | 7.79517  | 2.42569    | 3.214   | 0.00329 ** |
| valve       | 2.79746  | 2.52946    | 1.106   | 0.27816    |
| NYHA        | 2.06681  | 1.09588    | 1.886   | 0.06971 .  |
| Smoke       | 5.90338  | 1.99806    | 2.955   | 0.00628 ** |
| HBP         | -6.03586 | 2.13946    | -2.821  | 0.00870 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.059 on 28 degrees of freedom

Multiple R-squared: 0.5625, Adjusted R-squared: 0.4688

F-statistic: 6 on 6 and 28 DF, p-value: 0.0003962

```
> summary(lm(bcTubule ~ + RCA+ valve+ NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ +RCA + valve + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q     | Median | 3Q    | Max    |
|---------|--------|--------|-------|--------|
| -10.040 | -3.151 | 0.225  | 3.618 | 10.442 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t )   |
|-------------|----------|------------|---------|------------|
| (Intercept) | 7.756    | 2.978      | 2.604   | 0.01437 *  |
| RCA         | 7.755    | 2.437      | 3.183   | 0.00347 ** |
| valve       | 2.208    | 2.486      | 0.888   | 0.38187    |
| NYHA        | 2.172    | 1.097      | 1.980   | 0.05722 .  |
| Smoke       | 6.491    | 1.938      | 3.350   | 0.00226 ** |
| HBP         | -6.505   | 2.108      | -3.085  | 0.00444 ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.082 on 29 degrees of freedom

Multiple R-squared: 0.5427, Adjusted R-squared: 0.4638

F-statistic: 6.883 on 5 and 29 DF, p-value: 0.000241

```
> summary(lm(bcTubule ~ + RCA + NYHA + Smoke + HBP ))
```

Call:

```
lm(formula = bcTubule ~ +RCA + NYHA + Smoke + HBP)
```

Residuals:

| Min     | 1Q      | Median  | 3Q     | Max    |
|---------|---------|---------|--------|--------|
| -9.3877 | -3.5895 | -0.1578 | 3.4415 | 9.7211 |

Coefficients:

|             | Estimate | Std. Error | t value | Pr(> t ) |    |
|-------------|----------|------------|---------|----------|----|
| (Intercept) | 8.294    | 2.906      | 2.854   | 0.00775  | ** |
| RCA         | 6.377    | 1.871      | 3.408   | 0.00188  | ** |
| NYHA        | 2.264    | 1.088      | 2.080   | 0.04612  | *  |
| Smoke       | 6.660    | 1.921      | 3.466   | 0.00161  | ** |
| HBP         | -5.932   | 2.000      | -2.966  | 0.00587  | ** |

---

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.064 on 30 degrees of freedom

Multiple R-squared: 0.5302, Adjusted R-squared: 0.4676

F-statistic: 8.466 on 4 and 30 DF, p-value: 0.0001072

## **Appendix 6 Publication generated from this thesis**

# openheart Blood vessel repair and regeneration in the ischaemic heart

Huajun Zhang,<sup>1,2</sup> Casper van Olden,<sup>1,2</sup> Dominic Sweeney,<sup>2,3</sup>  
Enca Martin-Rendon<sup>2,3</sup>

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## ABSTRACT

The term 'therapeutic angiogenesis' originated almost two decades ago, following evidence that factors that promote blood vessel formation could be delivered to ischaemic tissues and restore blood flow. Following this proof-of-principle, safety and efficacy of the best-studied angiogenic factors (eg, vascular endothelial growth factor) were demonstrated in early clinical studies. Promising results led to the development of larger controlled trials that, unfortunately, have failed to satisfy the initial expectations of therapeutic angiogenesis for ischaemic heart disease. As the quest to delay the progression to heart failure secondary to ischaemic heart disease continues, alternative therapies have emerged as potential novel treatments to improve myocardial reperfusion and long-term heart function. The disappointing results of the clinical studies using angiogenic factors were followed by mixed results from the cell therapy trials. This review reflects the current angiogenic strategies for the ischaemic heart, their limitations and discusses future perspectives in the light of recent scientific and clinical evidence. It is proposed that combination therapies may be a new direction to advance therapeutic repair and regeneration of blood vessels in the ischaemic heart.

## INTRODUCTION

An inadequate supply of blood to the myocardium characterises ischaemic heart disease (IHD), resulting in an imbalance between myocardial oxygen supply and demand. The major causes of IHD are atherosclerosis, thrombosis or embolus in the coronary arteries that lead to low perfusion in the region supplied by the culprit vessel. Coronary artery disease (CAD) remains the primary cause of mortality worldwide.<sup>1</sup> In addition to CAD, pathologies such as aortic stenosis and hypertrophic cardiomyopathy can also result in ischaemic damage to the myocardium.<sup>2–3</sup> During the past decade, treatment for IHD has advanced into an inspiring new era. Improved regimes of medical therapy, which combine statins, antiplatelets agents, ACE inhibitors,  $\beta$ -blockers, and so on, offer the optimal treatment and

prevention for CAD. Furthermore, percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG) restore blood flow in the ischaemic territory and provide substantial gains for patients surviving ischaemic episodes. Revascularisation decreases the subsequent effect of acute haemodynamic instability and chronic unfavourable left ventricular (LV) remodeling, and it has contributed to a remarkable decrease in mortality.<sup>1–6</sup> However, even treatments meeting today's highest standard of medical care are unable to halt the progression of IHD to chronic heart failure (HF), adding to its rising incidence.<sup>7</sup> Therapeutic angiogenesis to promote myocardial perfusion and improve LV function has been the focus of extensive preclinical and clinical studies. Here, we review the main trials aimed at targeting blood vessel repair and regeneration in the ischaemic heart, and discuss the limitations of the current therapies.

## REVASCULARISATION IN HEART REPAIR

Following revascularisation (eg, PCI or CABG), up to 90% of the patients suffering from occluded coronary artery would achieve almost immediate patency. Hence, the 5-year cardiac event-free survival expectancy of patients with CAD receiving primary revascularisation has increased considerably from ~30% to >85%.<sup>8</sup> However, it is estimated that a quarter to one-third of the patients would experience inadequate myocardial reperfusion and would face higher risks of developing ventricular remodelling and early death.<sup>8</sup> In the last decade, no better treatment option has become available for the patients who are either not suitable for revascularisation or have not achieved a complete revascularisation. Importantly, even in patients who successfully undergo primary standard CABG, restenosis of the vein grafts is commonly seen after several years, followed by a decreased survival and recurrence of

<sup>1</sup>Nuffield Department of Surgical Sciences, University of Oxford, Oxford, UK  
<sup>2</sup>Stem Cell Research Laboratory, NHS Blood and Transplant, John Radcliffe Hospital, Oxford, UK  
<sup>3</sup>Radcliffe Department of Medicine, University of Oxford, Oxford, UK

**Correspondence to**  
Dr Enca Martin-Rendon;  
[enca.rendon@ndcls.ox.ac.uk](mailto:enca.rendon@ndcls.ox.ac.uk)

symptoms. They generally require secondary revascularisation, regardless of the normal patency of the grafted conduit. These patients, therefore, continue to experience residual myocardial ischaemia (MI) despite optimal therapy, and are in need of an alternative or supplementary revascularisation strategy to bring further attenuation or halt the advance of myocardial damage.<sup>9–10</sup> As it is important to achieve appropriate myocardial collateral blood flow and microvascular perfusion, repair and/or regeneration of blood vessels has emerged as a therapeutic approach for IHD.

Blood vessel formation is characterised by (1) the sprouting of new blood vessels from the existing ones (angiogenesis) and endothelial cells are responsible for the capillary growth, migration and organisation of the vessel lumen, (2) the increase in the size of the lumen of pre-existing arterioles by remodelling and growth (arteriogenesis), a process that is controlled by perivascular mural cells or (3) de novo vessel formation involving the migration, differentiation and incorporation of endothelial progenitors, normally from the bone marrow, into the damaged vessels (vasculogenesis;<sup>11</sup> [figure 1](#)). During adulthood, the quiescent vasculature is maintained in a paracrine fashion by angiopoietin-1 (Ang1) expressed by stromal and perivascular cells. Ang1 binds to its receptor Tie2 on the endothelial cells where angiopoietin 2 (Ang2) is stored ([figure 1A](#)). Neovascularisation is induced by ischaemia, cytokines and shear stress of blood flow, which causes the autocrine release of Ang2 competing with Ang1 to bind its receptor (Tie2). Such activation enables the endothelial cells to respond to exogenous growth factors ([figure 1B](#)). During angiogenesis, cytokine receptors on the surface of endothelial cells are activated by exogenous vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin growth factor (IGF) and transforming growth factor (TGF), inducing signalling pathways that modulate cell survival, proliferation and migration as well as vascular permeability. Activated endothelial cells produce platelet-derived growth factor (PDGF), which induces proliferation of the stromal cells via the Erk1/2 signalling pathway and further release of angiogenic factors ([figure 1C](#)). In addition to producing angiogenic growth factors, perivascular stromal cells are responsible for the extracellular matrix (ECM) remodelling. These cells produce metalloproteinases (MMPs), plasminogen activator (PA) and collagenases to cleave ECM components and to facilitate the migration of endothelial cells. This process is balanced by the secretion of protease inhibitors such as tissue inhibitor of metalloproteinases (TIMP) and PA inhibitor by stromal cells. Vessel maturation is controlled by the perivascular stromal cells ([figure 1C](#)), allowing the enlargement of vessel lumen. The increment in blood flow restores the paracrine effect of Ang1-Tie2 signalling followed by the maintenance of vascular quiescence ([figure 1D](#)). Recruitment of bone marrow progenitor cells to the ischaemic tissue, or vasculogenesis, is also observed, and it is another mechanism of neovascularisation in

response to ischaemia ([figure 1E](#)). Therapeutic angiogenesis aims at restoring the microcirculation in the ischaemic myocardium by delivering proangiogenic factors. The first promising results in humans were reported in severely symptomatic patients with critical limb ischaemia where VEGF was administered directly into the skeletal muscle, increasing the formation of collateral vessels.<sup>12</sup> This followed experimental proof of the concept in rabbits with induced unilateral limb ischaemia which received intra-arterial infusion of an acidic fibroblastic growth factor (aFGF or FGF1) encoding vector.<sup>13</sup> Soon after, clinical trials were also extended to patients with advanced symptomatic CAD<sup>14</sup> and those not eligible for standard revascularisation strategies.<sup>15</sup> In the first instance, patients with three vessel disease received human recombinant FGF1 (hrFGF1). Formation of capillaries could be observed in all patients around the site of injection.<sup>14</sup> In the second trial, plasmid DNA expressing VEGF165 directly injected into the myocardium was demonstrated to be safe and led to reduced symptoms and improved myocardial perfusion in some patients.<sup>15</sup>

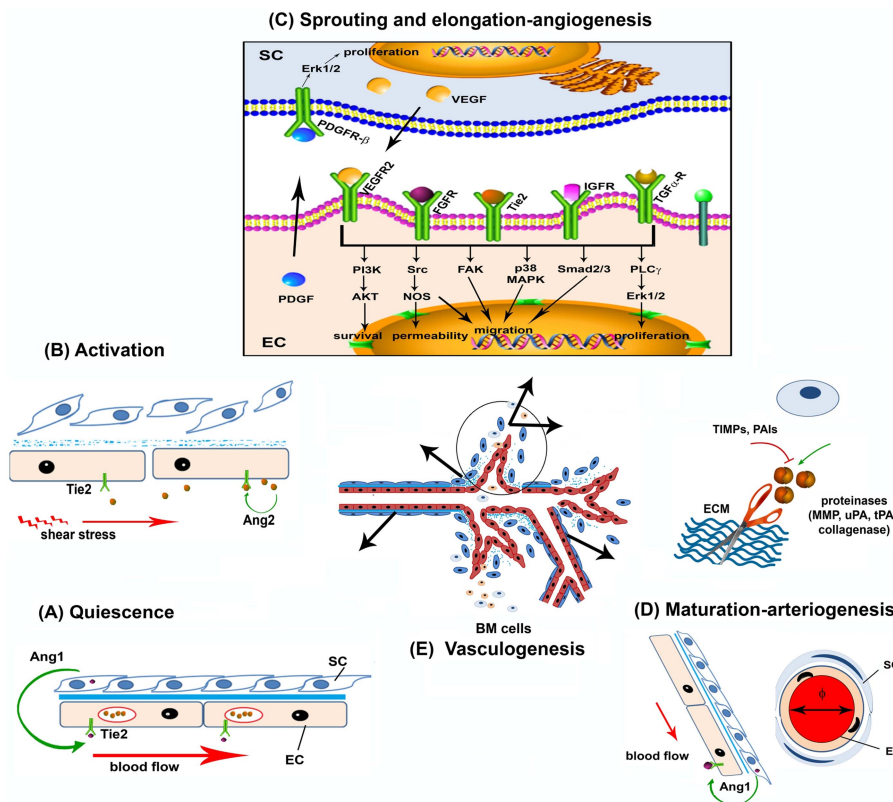
By contrast, antiangiogenic cancer therapies have resulted in cardiac toxicity. Drugs such as bevacizumab (a specific VEGF-blocking antibody), sorafenib and sunitinib (both tyrosine kinase inhibitors) have been used against tumour angiogenesis.<sup>16</sup> Although with different molecular targets and different specificity, the use of these approved drugs has been associated with cardiovascular side effects such as decrease in left ventricular ejection fraction (LVEF), increased incidence of HF, hypertension and MI.<sup>17–18</sup>

## ANGIOGENIC FACTORS FOR IHD

A number of proangiogenic factors are expressed in the ischaemic myocardium.<sup>19–20</sup> However, the VEGF and FGF families are the best-studied growth factors in angiogenesis following MI and the only ones that have been tested in the clinical setting (see [table 1](#)).

### The VEGF family

The VEGF family of angiogenic cytokines includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) in humans. They have high affinity for their receptors, namely VEGFR1 and VEGFR2, on endothelial cells which results in the activation of signalling cascades and the production of nitric oxide (NO) by these cells.<sup>21–22</sup> Two different isoforms of VEGF-A have been tested in clinical trials of VEGF-A<sub>165</sub> (containing 165 amino acids) and VEGF-A<sub>121</sub> (a shorter form of VEGF-A consisting of 121 amino acids). The role of VEGF-B and PlGF in angiogenesis still remains controversial and their clinical efficacy has not been assessed yet.<sup>23</sup> VEGF-A production is significantly upregulated by ischaemia in pigs and rats,<sup>24–25</sup> suggesting that VEGF-A is a likely mediator of ischaemia-induced myocardial neovascularisation. Preclinical studies showed that administration of an adenoviral vector expressing VEGF



**Figure 1** Process and mechanisms of blood vessel formation. (A) Vascular quiescence is maintained by the Ang1-Tie2 signalling pathway. Ang1 is expressed in perivascular SC and binds to the Tie2 receptor on EC in a paracrine fashion to stabilise the vasculature. EC in turn store Ang2. (B) Vascular activation is induced by multiple factors, such as hypoxia, VEGF and shear stress of blood flow on the vascular wall, and Ang2 is released from EC and competes with Ang1 to bind the Tie2 receptor. The autocrine antagonising effect of Ang2 on Ang1-Tie2 signalling activates the ECs by enabling them to react to growth factors, such as VEGF and FGF. EC that lack Ang2 production are likely to fail to respond to exogenous growth factors. (C) Angiogenesis is a crucial mechanism and process for neovascularisation where vascular sprouting and elongation take place. The angiogenesis process relies on the EC-SC interaction and it is fine-tuned through growth factor signalling pathways and remodelling of the ECM. Activated EC express cytokine receptors which respond to exogenous VEGF, FGF, IGF and TGF- $\alpha$  signalling to promote cell survival, cell migration, cell proliferation and vascular permeability. Simultaneously, activated EC produce PDGF which binds to PDGFR- $\beta$  on SC to promote their proliferation via the Erk1/2 signalling pathway. SC play a critical role in ECM remodelling during angiogenesis besides the secretion of angiogenic growth factors. Proteinases such as MMP, PA and collagenase are released from the SC to cleave ECM proteins to facilitate cell migration and vascular elongation. The process can be interrupted by TIMPs and PAIs, which target and antagonise MMP and PA. (D) Vascular maturation is seen as the neovasculature start sustaining a regular blood flow. The tissue reperfusion enables the overexpression of Ang1, and the restored Ang1-Tie2 signalling in turn suppresses Ang2 production and encourages Ang2 storage, stabilising the vascular cells while increasing the vascular diameter. (E) Vasculogenesis is another mechanism of neovascularisation. The mobilised progenitor cells from bone marrow penetrate the ischaemic tissue and incorporate with the newly formed local vascular network. Ang1, angiopoietin 1; Ang2, angiopoietin 2; EC, endothelial cells; ECM, extracellular matrix; FGF, fibroblast growth factor; IGF, insulin growth factor; MMP, metalloproteinase; PA, plasminogen activator; PAI, plasminogen activator inhibitor; PDGF, platelet-derived growth factor; PDGFR- $\beta$ , platelet-derived growth factor receptor  $\beta$ ; SC, stromal cells; TGF- $\alpha$ , transforming growth factor- $\alpha$  TIMP, tissue inhibitor of metalloproteinase; VEGF, vascular endothelial growth factor.

(AdVEGF) improved capillary density and LV function in a rodent model of acute MI (AMI).<sup>26</sup> In a large animal model of chronic MI, the injection of AdVEGF<sub>121</sub> into segments of stunned myocardium increased collateral vessel flow and improved cardiac function.<sup>27</sup> In addition to these experimental studies, early clinical trials with promising results led to the development of randomised controlled trials (RCTs) where recombinant protein and plasmid DNA or

adenoviral vectors expressing the angiogenic factors were used for treatment (table 1).

In the phase II VIVA trial, patients with stable CAD and no revascularisation option received an intracoronary infusion followed by intravenous injection of placebo or two different doses of recombinant human VEGF (rhVEGF-A<sub>165</sub>) protein.<sup>28</sup> There were no significant differences in exercise tolerance, quality of life (QoL) or myocardial perfusion at 60 days between the treated and

**Table 1** Randomised controlled trials with proangiogenic factors

| Family | Therapeutic factor                    | Trial name                       | Phase  | Administration                              | Patients with ICM cohort | Number of participants         | Main effect                                                                                            |
|--------|---------------------------------------|----------------------------------|--------|---------------------------------------------|--------------------------|--------------------------------|--------------------------------------------------------------------------------------------------------|
| VEGF   | VEGF-A165                             | VIVA <sup>28</sup>               | II     | IC and intravenous                          | CCS II–III               | 178                            | High-dose improved CCS class. Trend in exercise time and angina frequency but not myocardial perfusion |
|        | AdVEGF165 or plasmid/liposome VEGF165 | KAT <sup>33 34</sup>             | II     | IC                                          | CCS II–III for PCI       | 103                            | Improved myocardial perfusion at 6 months                                                              |
|        | AdVEGF121                             | REVASC <sup>31</sup>             | II     | IM minithx                                  | CCS II–IV                | 67                             | Improved time to 1 mm ST-segment depression on ECG at 26 weeks but not myocardial perfusion            |
|        | AdVEGF121                             | NOVA <sup>32</sup>               | I/II   | IM PC                                       | CCS II–IV                | 17/129 (premature termination) | Negative effect. Premature termination                                                                 |
|        | VEGF165 plasmid                       | EUROINJECT-ONE <sup>29</sup>     | II/III | IM PC                                       | CCS III–IV               | 74                             | Negative. No difference in myocardial perfusion                                                        |
| FGF    | VEGF165 plasmid                       | NORTHERN <sup>30</sup>           | II/III | IM PC                                       | CCS III–IV               | 120                            | Negative. No difference in myocardial perfusion                                                        |
|        | rFGF-2                                | Laham <i>et al</i> <sup>36</sup> | I/II   | Epicardial implantation in ungraftable area | CCS III–IV for CABG      | 24                             | Improvement in angina symptoms and myocardial perfusion at 3 years with high dose                      |
|        | rFGF-2                                | FIRST <sup>37</sup>              | II     | IC                                          | CCS III–IV               | 337                            | Trend towards 3 month improvement in angina. No effect on exercise time or myocardial perfusion        |
|        | Ad5-FGF4                              | AGENT <sup>38</sup>              | I/II   | IC                                          | CCS II–III               | 79                             | Improved exercise time                                                                                 |
|        | Ad5-FGF4                              | AGENT-2 <sup>39</sup>            | II     | IC                                          | CCS II–IV                | 52                             | Improved myocardial perfusion                                                                          |
|        | Ad5-FGF4                              | AGENT-3 <sup>40</sup>            | III    | IC                                          | CCS II–IV                | 416                            | Negative with a low dose                                                                               |
|        | Ad5-FGF4                              | AGENT-4 <sup>40</sup>            | III    | IC                                          | CCS II–IV                | 116                            | Improved exercise time and tolerance with high dose, only in women                                     |

CABG, coronary artery bypass grafting; CCS, Canadian Cardiovascular Society angina class (I–IV); IC, intracoronary; ICM, ischaemic cardiomyopathy; IM, intramyocardial; minithx, mini-invasive thoracotomy; PC, percutaneous; PCI, percutaneous coronary intervention.



placebo groups. At 120 days, a high dose of rhVEGF-A<sub>165</sub> improved the angina class only and, hence, the trial was terminated prematurely. In the EUROINJECT-ONE<sup>29</sup> and NORTHERN<sup>30</sup> trials, patients with severe stable IHD and no other treatment option received a percutaneous intramyocardial injection of plasmid DNA expressing VEGF<sub>165</sub> (phVEGF-A<sub>165</sub>). Neither of these trials found significant improvement in myocardial perfusion in the treated groups compared with the placebo groups despite some improvement in wall motion and LV function in the EUROINJECT-ONE trial.<sup>29 30</sup> Moreover, the REVASC study enrolled patients with intractable angina and no option of revascularisation to receive either AdVEGF-A<sub>121</sub> or maximal medical therapy. Interestingly, there were significant improvements in exercise tolerance and QoL in the AdVEGF-A<sub>121</sub>-treated patients compared with controls.<sup>31</sup> However, the placebo effect cannot be ruled out in this trial as patients in the control arm did not have a thoracotomy or receive placebo. The NOVA double-blinded, placebo-controlled, multicentre study investigated the safety of intramyocardial injection and the efficacy of BIOBYPASS (AdGVVEGF<sub>121.10NH</sub>) gene therapy in patients with refractory advanced CAD.<sup>32</sup> Injection of AdGVVEGF<sub>121</sub> did not improve exercise capacity or myocardial perfusion in a 52-week follow-up, and this study was also terminated prematurely. Finally, the 8-year follow-up of the Kuopio angiogenesis trial (KAT) showed that VEGF-A<sub>165</sub> expressed transiently in either an adenoviral vector or a plasmid was safe, did not increase the risk of mortality and it was well tolerated.<sup>33 34</sup> Nonetheless, no significant improvements were observed in the treated patients compared with the control patients. In the AdVEGF-A<sub>165</sub> group, myocardial perfusion improved compared with the plasmid VEGF-A<sub>165</sub> group at 6 months.<sup>34</sup>

### The FGF family

The FGF family comprises up to 22 ligands and four tyrosine kinase receptors, adding to the complexity of this system. Several of its members have been reported to promote angiogenesis in preclinical studies (for review, please see<sup>35</sup>). The best studied members of the family are FGF-1 and FGF-2, and together with them, FGF-4 has been tested in clinical trials to improve angiogenesis post-MI. The safety and feasibility of recombinant FGF-2 (rhFGF-2) in therapeutic angiogenesis have been evaluated in patients with chronic MI during CABG<sup>36</sup> and in the FIRST trial in participants with stable angina pectoris secondary to CAD.<sup>37</sup> While the phase I trial by Laham *et al*<sup>36</sup> delivered a single intracoronary injection of rhFGF-2 and showed signs of therapeutic efficacy, the larger phase II FIRST trial<sup>37</sup> that recruited 337 patients did not reveal any significant improvement in exercise tolerance or ischaemic areas in treated patients. Only a significant improvement in angina class and angina frequency was observed in the more affected patients.<sup>37</sup> The phase I KAT301 study is an ongoing trial designed to evaluate the safety and efficacy of intramyocardial injection of AdVEGF-D in patients

with chronic ischaemia and no option of revascularisation (<http://clinicaltrials.gov/show/NCT01002430>).

Like VEGF, FGF gene transfer was based on the use of adenoviral vectors as gene transfer vehicle. In the Angiogenic GENE Therapy (AGENT) double-blind RCT, the administration of five increasing doses of Ad5-FGF4 in patients with stable angina pectoris showed a trend towards clinical improvement in exercise tolerance. A single intracoronary administration of Ad5-FGF4 was safe and well tolerated with no immediate adverse events.<sup>38</sup> The efficacy of Ad5-FGF4 therapy was further examined in the AGENT 2, AGENT 3 and AGENT 4 studies. The AGENT 2 study showed an encouraging trend for improved myocardial perfusion in treated patients compared with the placebo group,<sup>39</sup> whereas the results of the AGENT 3 and AGENT 4 trials showed no significant differences between treatment and placebo in angina symptoms, angina class or exercise tolerance. However, when stratified by gender, the data indicated that in women, the placebo effect was small and the treatment had a significant effect.<sup>40</sup>

## THE REPARATIVE AND REGENERATIVE FUNCTION OF STEM CELL THERAPIES

Stem cell therapies have not been purely directed at stimulating blood vessel growth in the ischaemic heart but rather to repair and regenerate all cardiac tissues in their capacity of multipotent cells. The first evidence that stem/progenitor cells have the potential to be used as treatment for IHD was reported by Orlic *et al* in 2001. In this study, haematopoietic stem cells (HSC) mobilised into circulation and injected into infarcted myocardium of mice were able to improve heart function and regenerate heart tissue.<sup>41</sup> Since then a number of cell therapies have been tested in clinical trials. Here, we review trials that have administered cell therapies with the aim to improve a long-term heart function and myocardial perfusion.

### Bone marrow mononuclear cells

Unselected bone marrow mononuclear cells (BMNC) are clearly the most investigated cell-based therapy for IHD in clinical studies, with the longest follow-up lasting up to 5 years.<sup>42 43</sup> An attractive novel treatment for acute and chronic MI, BMNC are relatively easy to harvest, easy to process in a short time-frame using standardised techniques (eg, density gradient centrifugation and cell sorting) that usually yield large quantities of cells, ready to be administered to the patients in a matter of hours if required. This makes them extremely amenable to treat patients with AMI. In this patient cohort, BMNC have a beneficial but moderate effect on heart function.<sup>42 43</sup> Not surprisingly, following the expectations raised by the early-phase small clinical studies, several RCTs have generated mixed results (table 2).

The largest trial, the REPAIR-AMI, recruited patients post-AMI and showed an improvement of global LVEF in

**Table 2** Major cell-based therapy randomised controlled trials

| Origin | Cell type | Trial name                            | Phase | Administration | Patient cohort   | Number of participants | Main effect                                                                      |
|--------|-----------|---------------------------------------|-------|----------------|------------------|------------------------|----------------------------------------------------------------------------------|
| BM     | MNC       | REPAIR-AMI <sup>44</sup>              | II    | IC             | AMI + PCI        | 204                    | Improved LVEF, decreased mortality                                               |
|        |           | ASTAMI <sup>46</sup>                  | II    | IC             | AMI + PCI        | 97                     | Negative                                                                         |
|        |           | BELGIUM <sup>47</sup>                 | II    | IC             | AMI + PCI        | 67                     | Reduced scar size but no difference in myocardial perfusion                      |
|        |           | BOOST <sup>48</sup>                   | II    | IC             | AMI + PCI        | 60                     | Improved EF in short term with a single dose                                     |
|        |           | TIME/Late TIME <sup>53 54</sup>       | II    | IC             | AMI + PCI        | 120                    | Negative for LV function and infarct size                                        |
|        |           | Cao <i>et al</i> <sup>105</sup>       | I/II  | IC             | AMI + PCI        | 86                     | Safe, improved EF but similar myocardial perfusion to placebo                    |
|        |           | FINCELL <sup>52</sup>                 | I/II  | IC             | AMI + PCI        | 80                     | Safe, improved EF                                                                |
|        |           | HEBE <sup>50</sup>                    | II    | IC             | AMI + PCI        | 200                    | Negative                                                                         |
|        |           | BONAMI <sup>51</sup>                  | II    | IC             | AMI + PCI        | 101                    | Improved LV viability on SPECT imaging                                           |
|        |           | Hu <i>et al</i> <sup>56</sup>         | II    | IM             | ICM + CABG       | 60                     | Improved LV function and exercise tolerance                                      |
|        | CD34+     | FOCUS-CCTRN <sup>57</sup>             | II    | IM PC          | ICM no option    | 92                     | Negative                                                                         |
|        |           | Pokushalov <i>et al</i> <sup>58</sup> | II    | IM PC          | ICM no option    | 109                    | Improved mortality, LV function, HF symptoms at 12-month                         |
|        |           | CELLWAVE <sup>59</sup>                | I/II  | IC             | ICM no option    | 103                    | Improved LVEF and contractility, delivery of cells following shockwave           |
|        |           | Patel <i>et al</i> <sup>64</sup>      | I/II  | IM             | ICM + CABG       | 20                     | Improved LV function                                                             |
|        |           | REGENT <sup>62</sup>                  | II    | IC             | AMI + PCI        | 200                    | Negative                                                                         |
|        |           | Losordo <i>et al</i> <sup>66</sup>    | II    | IM PC          | Angina no option | 167                    | Improved exercise time, reduced CP frequency                                     |
|        |           | Perin <i>et al</i> <sup>63</sup>      | I/II  | IM PC          | ICM no option    | 20                     | Improved LVESV, potentially improved myocardial perfusion and oxygen consumption |
|        |           | Chen <i>et al</i> <sup>69</sup>       | I/II  | IC             | AMI + PCI        | 69                     | Improved LV function and myocardial perfusion                                    |
|        |           | Chen <i>et al</i> <sup>70</sup>       | I/II  | IC             | ICM + PCI        | 45                     | Improved LV function, exercise time, symptoms and myocardial perfusion           |
|        |           | Prochymal allogeneic <sup>71</sup>    | I/II  | Intravenous    | AMI + PCI        | 53                     | Improved EF                                                                      |
| Fat    | MSC       | POSEIDON <sup>72</sup>                | I/II  | IM PC          | ICM no option    | 30                     | Autologous cells improved exercise time, allogeneic cells reduced LVESV          |
|        |           | C-CURE                                | I/II  | IM PC          | ICM no option    | 47                     | Safe, improved LVEF, exercise tolerance, and performance                         |
|        |           | APOLLO <sup>75</sup>                  | I     | IC             | AMI + PCI        | 13                     | Improved perfusion and reduced scar size                                         |
|        |           | PRECISE <sup>76</sup>                 | I     | IC             | ICM no option    | 27                     | Safe. Improved oxygen consumption and reduced scar size.                         |
|        |           |                                       |       |                |                  |                        |                                                                                  |
|        |           |                                       |       |                |                  |                        |                                                                                  |
| Heart  | CDC c-kit | CADUCEUS <sup>85</sup>                | I     | IC             | Recent MI        | 25                     | Reduced scar size                                                                |
|        |           | SCIPIO <sup>84</sup>                  | I     | IC             | ICM no option    | 16                     | Improved LV function and reduced scar size                                       |

ALDH, alcohol dehydrogenase; AMI, acute myocardial ischaemia; BM, bone marrow; CABG, coronary artery bypass graft; CDC, cardiosphere-derived cells; EF, ejection fraction; HF, heart failure; IC, intracoronary; ICM, ischaemic cardiomyopathy; IM, intramyocardial; LV, left ventricular; LVESV, left ventricular end-systolic volume; MNC, mononuclear cells; MSC, mesenchymal stem cells; PC, percutaneous; PCI, percutaneous coronary intervention.

he treatment group compared with the control group ( $\Delta\text{LVEF}=2.9\%$ ), without significant changes of LV end-diastolic volumes 4 months following cell transplantation.<sup>44</sup> In addition, decreased mortality was observed in the treatment group compared with the control group at 2 years of follow-up.<sup>45</sup> In contrast, in other landmark studies, BMNC have not shown the alleged beneficial effect in the same patient cohort. The ASTAMI trial did not show a significant improvement in LV function or dimensions at 4–6 months of follow-up,<sup>46</sup> while the Belgium trial reported mixed results where there was no improvement on LVEF despite the significant reduction in infarct size,<sup>47</sup> and the BOOST trial showed a transient effect of BMNC on LVEF.<sup>48–49</sup> Moreover, RCTs such as the HEBE,<sup>50</sup> BONAMI,<sup>51</sup> FINCELL<sup>52</sup> and TIME<sup>53–54</sup> show no significant effect on heart function or contractility between treated and non-treated patients (table 2). Recent systematic reviews and meta-analysis, which also included smaller trials, have suggested that BMNC improve LVEF by 3–5%.<sup>42–43–55</sup> However, there is no significant reduction on the risk of mortality in patients treated with BMNC compared with controls.<sup>42–43</sup> The BAMI trial is so far the largest ongoing international multicentre RCT. It is designed to recruit 3000 patients to define the effect of single dose of intracoronary administration of BMNC on patients with AMI after successful primary revascularisation. The primary outcomes to be measured are long-term all-cause mortality, cardiac death, major associated cardiac events (MACE) and rehospitalisation between the cell therapy group and the placebo group (<http://clinicaltrials.gov/show/NCT01569178>).

Fewer data from RCTs are available in patients with chronic MI and HF (table 2). Intracoronary delivery of BMNC during CABG resulted in significant changes in LVEF and exercise tolerance in favour of the treatment.<sup>56</sup> Patients with HF, receiving optimal medical treatment and with no option of revascularisation, have been treated in two other trials. Following the promising results of the phase I trial, Perin *et al* treated patients with HF with BMNC in a phase II trial. Surprisingly, no significant improvement in left ventricular end-systolic volume or maximal oxygen consumption was observed in treated patients compared with controls.<sup>57</sup> The phase II FOCUS-CCTRN delivered BMNC by a percutaneous intramyocardial injection in patients with congestive HF with no option of revascularisation.<sup>57</sup> At 6 months of follow-up, no difference in LV systolic function, myocardial perfusion or myocardial viability was observed between treated and control patients. However, a modest improvement in LVEF was apparent in patients aged <62 years, although not in the treated group in its totality. In another trial, intramyocardial delivery of BMNC to patients with ischaemic HF improved HF symptoms, and LV function and even improved survival of these patients significantly.<sup>58</sup> In Pokushalov's study, patients suffer from a more severe stage of ischaemic HF (NYHA class III–IV) with lower rates of survival compared with Perin's study (NYHA class II–IV), which may be a

determinant of the decreased mortality observed in this patient cohort.

Recently, results from the CELLWAVE trial have been reported.<sup>59</sup> The phase I/II trial evaluated the effect of intracoronary infusion of BMNC in combination with extracorporeal shock wave in patients with HF due to ischaemic cardiomyopathy. The administration of BMNC aided by shock wave showed a modest but significant improvement in LVEF and regional wall thickening 4 months following treatment. In addition, the combination treatment seems to protect against major adverse cardiac events (MACE).<sup>59</sup>

Notably, BMNC are a heterogeneous cell population that contains haematopoietic stem and progenitor cells (HSC/HPC) and endothelial progenitor cells (EPC; around 2–4%), mesenchymal progenitor cells (MSC; 0.001–0.01% of the nucleated cells), committed progenitor cells and their differentiated progeny.<sup>60</sup> As a consequence, it is difficult to ascertain which cell types in the whole population are more potent or responsible for the therapeutic effect. This has led to the development of clinical trials which use selected cell populations such as HSC or MSC as treatment for IHD.

### Haematopoietic stem cells

Human HSC, identified by CD133, CD34 or aldehyde dehydrogenase (ALDH) markers, have been tested in clinical trials as treatment for IHD. CD133+ or CD34+ HSC can be enriched from mononuclear cells, either from the bone marrow or mobilised peripheral blood, by immunomagnetic cell separation procedures.<sup>61–62</sup> ALDH is a cytosolic enzyme used to identify stem cell populations. High ALDH activity has been found in the CD34+ lineage- (Lin-) HSC compartment and identifies primitive HSC. ALDH+ cells are isolated from the bone marrow by cell sorting.<sup>63</sup> Enriched CD133+ HSC were first injected into the infarct border zone of patients undergoing CABG.<sup>61</sup> Promising results from this small trial, with no adverse events and improved LV function and myocardial perfusion, led to the development of larger RCTs (table 2). Recently, the REGENT trial compared CD34+ CXCR4+ HSC with unfractionated BMNC or control in patients who suffered from AMI and received PCI as primary intervention. However, no significant improvement in LV function was observed between trial arms.<sup>62</sup> Patients undergoing off-pump CABG and presenting LV dysfunction (LVEF <35%) were treated with bone marrow CD34+ HSC in a small RCT.<sup>64</sup> The study showed a significant improvement in LVEF in treated patients compared with controls. In addition, CD34+ HSC have been tested in a phase I trial in patient with refractory angina and no option of revascularisation. In this study, the patients received G-CSF to mobilise bone marrow cells into circulation and CD34+ cells were enriched from peripheral blood mononuclear cells prior to injecting them into the myocardium using electromechanical mapping. Unlike in AMI trials, the primary outcome measured by this trial was change in

angina frequency.<sup>65</sup> Administration of CD34+ cells resulted in a significant reduction of angina frequency (the number of angina episodes per week). The results were confirmed in a dose escalation phase II trial<sup>66</sup> and this has been followed by the first phase III clinical trial with CD34+ cells as treatment for patients with refractory angina which is currently ongoing.<sup>67</sup> Recently, a newly identified HSC population expressing the ALDH enzyme has been tested in patients with chronic ischaemia and no option of revascularisation. Perin *et al*<sup>63</sup> provided preliminary evidence that treatment with ALDH+ cells is safe and may improve perfusion in those patients.

### Mesenchymal stem/stromal cells

MSC constitute another potential option for stem/progenitor cell-based therapy. MSC express cell surface markers such as CD90, CD105, CD44 and CD73, but not the haematopoietic markers CD45, CD34, CD14, CD19 and HLA-DR, and due to their immunomodulatory properties<sup>68</sup> they have attracted much commercial interest as they can be generated as an 'off-the-shelf' product for allogeneic cell transplantation. They have been identified in tissues such as bone marrow and adipose tissue. Autologous and allogeneic MSC transplantation is currently under investigation for IHD among other conditions; however, clinical data are scarce (table 2).

The first RCT using autologous BM-derived MSC treated patients who had suffered AMI and underwent PCI. The MSC treatment proved to be safe and significantly improved LV function.<sup>69</sup> Autologous BM MSC also improved exercise tolerance, NYHA class and LVEF significantly in patients with severe ischaemic cardiomyopathy.<sup>70</sup> A safety double-blinded phase I trial with intravenous administration of allogeneic BM MSC to treat patients with AMI showed a significant improvement in LVEF compared with controls.<sup>71</sup> Following this the authors compared autologous and allogeneic MSC in patients with ischaemic cardiomyopathy.<sup>72</sup> In the phase I/II POSEIDON trial, three doses of MSC were injected into patients. However, no control group was included in this trial. Autologous, but not allogeneic MSC, improved exercise capacity and QoL significantly. None of the injected cells improved maximal oxygen consumption or LVEF. In contrast, allogeneic MSC significantly reduced LV end-systolic volume. In this early study, no immunological reaction to MSC therapy was observed.<sup>72</sup> It would be interesting to see the results of the ongoing MSC-HF trial, a double blind, placebo-controlled trial administering BMSC to patients with chronic ischaemic HF.<sup>73</sup> Recently, BMSC preconditioned by exposure to a cardiogenic cocktail have been delivered by percutaneous intramyocardial injection to patients with HF and no option of revascularisation in the C-CURE phase I/II trial.<sup>74</sup> Compared with control patients, treated patients showed a significant improvement in LV systolic function and symptom relief, as well as a reduction in the rate of rehospitalisation and event-free survival.

Recently, adipose tissue MSC have been tested in the clinic in patients with IHD. The APOLLO and PRECISE trials showed that freshly isolated adipose tissue-derived MSC is a safe treatment in patients suffering from AMI and chronic MI (table 2). MSC infusion resulted in a significant improvement of perfusion defect and a 50% reduction of scar size in patients with AMI but there was no improvement in LV function after 6 months.<sup>75</sup> Similarly, in patients with no option of revascularisation, cell transplantation showed a moderate decrease in scar size at 6 months and a significant increase of peak oxygen consumption at 18 months, but no improvement of LV function was reported.<sup>76</sup>

### Cardiac stem/progenitor cells

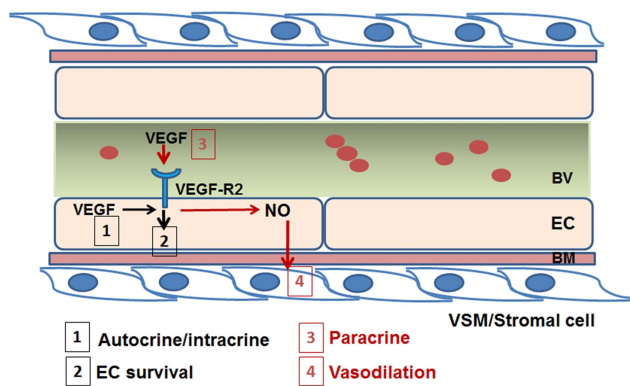
The human heart has been considered a terminally differentiated organ until recently, with no capability for self-repair. However, an elegant study by Bergmann *et al*<sup>77</sup> showed the first evidence of human cardiomyocyte renewal during adult life time, suggesting that the heart contains a population of stem/progenitor cells which may be involved in the steady-state tissue repair that occurs under normal and pathological conditions. These cardiac stem/progenitor cells (CSC) are characterised by the expression of c-kit,<sup>78</sup> Sca-1<sup>79</sup> or Islet-1<sup>80</sup> cell surface markers, the cardiac side population cells<sup>81</sup> or the formation of clusters called 'cardiospheres'.<sup>82 83</sup>

The first clinical trials using CSC as treatment for MI, the CADUCEUS and SCIPIO trials, have now been completed and have demonstrated that autologous transplantation of CSCs is safe.<sup>84 85</sup> In the CADUCEUS trial, patients who have suffered from a recent MI received an intracoronary injection of cardiosphere-derived cells or placebo. The results showed a significant improvement in myocardial viability and a reduced scar size 4–6 months following cell transplantation. Interestingly, the reduction in infarct size was not accompanied by an improvement in heart function and contractility.<sup>85</sup> In the SCIPIO trial, end-stage HF patients with LVEF <40% who did not respond to bypass surgery received an intracoronary infusion of c-kit+ CSC. The results confirmed a significant reduction in infarct size and increase in LVEF in the treated group compared with the control group.<sup>85</sup>

### LIMITATIONS OF THE CURRENT THERAPIES AND FUTURE PERSPECTIVES

The promising results obtained in preclinical models have not been replicated successfully in the clinic. The angiogenic response to ischaemia might be impaired in patients at multiple levels and influenced by a combination of cardiovascular risk factors and the hostile micro-environment in the heart, which are absent in the experimental models. In the ischaemic human heart, there might be a decreased production of factors that would be able to promote vessel sprouting and vessel maturation. There might also be a decreased number of cells with pro-angiogenic properties expressing receptors





**Figure 2** The role of vascular endothelial growth factor in vascular homeostasis. In the adult vasculature, VEGF produced by endothelial cells acts by an autocrine/intracrine mechanism (1) activating its receptor, VEGF-R2 and activating signalling pathways involved in endothelial cell survival (2). Moreover, VEGF produced by mural cells in the perivascular niche or circulating in blood vessels have a paracrine action (3) inducing the production of nitric oxide and causing vasodilation and inhibition of proliferation of vascular smooth muscle cells (4). BM, basal membrane; BV, blood vessel; EC, endothelial cells; NO, nitric oxide; VEGF, vascular endothelial growth factor; VEGF-R2, vascular endothelial growth factor receptor 2.

for those factors. Additionally, cells may have an impaired ability to home to ischaemic tissues and engraft, to promote vascular repair. Finally, if we believe that inflammation is, to some extent, beneficial for tissue repair and blood vessel formation, the current medical therapies, which include anti-inflammatory drugs, may have a detrimental effect on the process of angiogenesis and collateral vessel growth.

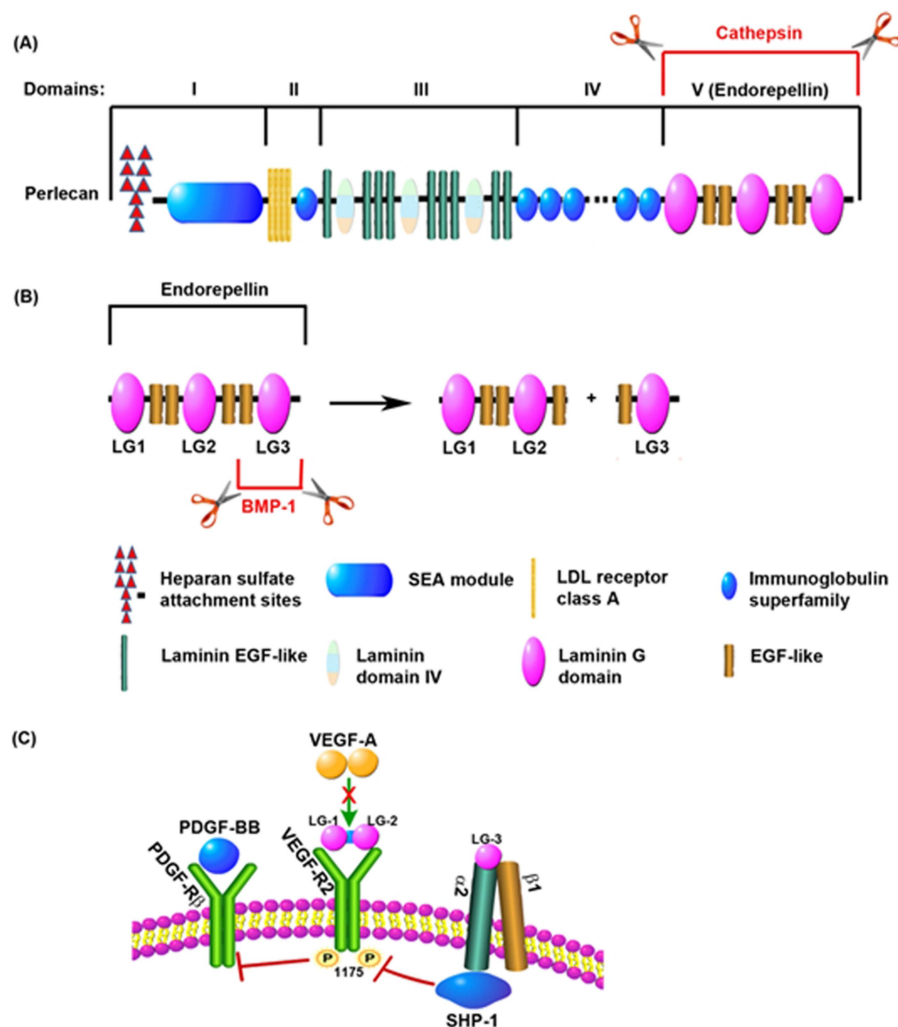
### Angiogenic therapies

The disappointing results of the pro-angiogenic factor trials might be explained by inadequacies in the current approaches as the main obstacle remains; an inability to deliver an effective angiogenic stimulus to the ischaemic human heart. Either recombinant protein with a relative short half-life or a transient expression in a plasmid or a viral vector that does not integrate into the host cell genomes and exert low transfection and transduction efficiencies in the target tissue and proangiogenic factor monotherapies have all failed to fulfil the early promises of therapeutic angiogenesis. Furthermore, an inadequate route of delivery and suboptimal doses may have diminished the efficacy of the treatment. Mechanistically, VEGF and FGF might not be the ideal therapeutic candidates to be used, or at least not on their own but in combination with arteriogenic factors. In addition to its pro-angiogenic action, VEGF has a major role in the maintenance of vascular integrity via an endothelial-specific autocrine mechanism that promotes cell survival<sup>86</sup> (figure 2). In mice, endothelial-specific deletion of VEGF results in vascular

degeneration that cannot be rescued by exogenous VEGF.<sup>86</sup> This may have major implications for patients with CAD and specially those suffering from diabetes, as it is a common cardiovascular risk factor characterised by endothelial cell dysfunction.<sup>87</sup> Angiogenic factor monotherapies might not be able to salvage the 'degenerating' endothelium in patients with CAD. Moreover, VEGF can also be arteriogenic and hence detrimental as increased microvessel density in the arteriosclerotic plaque is associated with disease progression.<sup>88–89</sup> Recently, a great deal of heterogeneity between endothelial cells from different sources has been revealed<sup>90</sup> and this may have to be taken into consideration when designing future trials targeting angiogenesis in IHD. It is also plausible that the effect of exogenous VEGF on its receptors is inhibited by endogenous antagonists released from the ECM by MMPs (figure 3). One of these antagonists is endorepellin, the C-terminal or V domain of perlecan, released by Cathepsin-L (figure 3A), which has an inhibitory effect on VEGF signalling.<sup>91</sup> Endorepellin contains three laminin G domains, named LG1, LG2 and LG3 (figure 3B). It is known that the metalloproteinase bone morphogenetic protein-1 cleaves the LG3 fragment of endorepellin<sup>92</sup> causing an increased activity of the molecule by making the two proteolytic products (LG1-LG2 and LG3) available to act separately. LG1-LG2 binds VEGF-R2 directly competing with VEGF for its receptor, while LG3 binds to  $\alpha 2\beta 1$  integrin, which also inhibits the VEGF-mediated activation of VEGF-R2<sup>93–94</sup> (figure 3C). Indirectly, high levels of these proteinases could have an antiangiogenic effect.

### Arteriogenic therapies

As arteriogenesis is also required to maintain functional collateral growth, mural cells such as vascular smooth muscle cells and pericytes may need to be targeted to promote vessel maturation and stabilise collateral networks. Angiogenic cytokines are not the only factors produced during ischaemic episodes. Following MI, there is an increase in the release of MMPs produced by neutrophil infiltration in the ischaemic heart. These MMPs play a major role in the tissue remodelling that takes place post-MI as they cleave components of the ECM (figure 1). The degradation of the ECM is followed by the deposition of a new basement membrane by the mural cells. During this shift, MMP inhibitors, such as TIMPs, compensate the action of MMPs by causing the deposition of de novo-synthesised ECM components, and the junctions between the endothelial cells are then re-established. We hypothesise that an imbalance in MMP and/or TIMP production may disrupt the process of angiogenesis in the ischaemic heart. It has been reported previously that plasma levels of MMP-9 correlate with LV dysfunction following MI, while MMP-2 levels are inversely correlated to LV volumes.<sup>95</sup> Additionally, the active form of MMP-9 and the MMP-9/



**Figure 3** Inhibition of VEGF-R2-mediated signalling cascade by endogenous antagonists. (A) Perlecan, an extracellular matrix component, is composed of several functional domains. The C-terminal domain, also named V domain or endorepellin, is released from the full length protein by proteolytic processing by cathepsin-L. (B) Endorepellin contains three laminin G domains (LG1, LG2 and LG3) separated by EGF domains. Metalloproteinases such as BMP-1 cleave endorepellin to release the LG3 domain from the LG1-LG2 fragment. (C) The LG1-LG2 fragment of endorepellin binds to VEGF-R2 inhibiting its downstream phosphorylation. LG3 binds to the  $\alpha 2$  domain of the  $\alpha 2 \beta 1$  integrin which can also inhibit VEGF-R2 activation through SHP-1. Inhibition of VEGF-R2 signalling enables PDGF-R $\beta$ -mediated signalling. BMP-1, bone morphogenetic protein-1; EGF, endothelial growth factor; PDGF-BB, platelet derived growth factor b; PDGF-R $\beta$ , platelet-derived growth factor receptor  $\beta$ ; SHP-1, SH2 domain-containing inositol-5'-phosphatase 1; VEGF-A, vascular endothelial growth factor a; VEGF-R2, vascular endothelial growth factor receptor 2. Adapted from Zoeller *et al.*<sup>94</sup>

TIMP ratio is significantly higher in patients with stable CAD than in healthy control individuals.<sup>96</sup>

Recently, mobilising agents such as granulocyte-macrophage colony stimulating factor and granulocyte colony stimulating factor (G-CSF) have been used in three controlled trials to promote arteriogenesis in patients with CAD.<sup>97–99</sup> It is proposed that G-CSF stimulate arteriogenesis by the release of monocytes and EPC.<sup>100</sup> These promising results may have a great impact in the design of future trials using combination therapies. G-CSF has been administered previously together with cell therapies in patients with refractory angina and no other treatment option.<sup>65 66</sup> However, in that setting, it is difficult to distinguish the effect of the cytokine from the effect of the cell

treatment, and the trials were not designed exclusively to improve blood vessel formation.

### Cell therapies

The divergent results of the cell-based therapy trials are not surprising either. Although the definite reasons for these mixed results remain largely elusive, differences in cell-related and patient-related factors are most likely to be the cause. Differences in study protocol and design, including time from reperfusion to cell injection, route of delivery, cell type, cell dose and cell isolation techniques are all factors that could influence the treatment outcome. In addition, the methods used for assessing outcomes (eg, echocardiography, MRI, LV angiography, etc)

and the relevant primary outcomes should be carefully selected as each of them have their own limitations. Indeed, the majority of the trials measured LVEF as a gold standard surrogate outcome for heart function. However, a closer correlate for collateral growth would be myocardial perfusion. Ten cell therapy trials measured myocardial perfusion, but only five of them reported a significant improvement,<sup>63 69 70 75 76</sup> and surprisingly four of these trials delivered MSC. Therefore, it is tempting to suggest that MSC might be potentially more proangiogenic and proarteriogenic than BMNC or CD34-enriched and CD133-enriched HSC. The mechanism of how cell therapies work is still not fully understood but it is suggested to be paracrine, and therefore supportive. MSC produce key factors such as Ang2, HGF, IGF-1, bFGF, SDF-1 and VEGF involved in both angiogenesis and arteriogenesis. The secretion of Ang2 by MSC and the binding to its receptor Tie2 on the EC would activate a number of receptors on the EC surface ready to respond to other angiogenic cytokines produced by MSC, thus promoting neovascularisation (figure 1). In addition, trials using cardiac progenitor cells have reported a large treatment effect on scar size,<sup>84</sup> an outcome that has not been widely used as a surrogate of heart function or blood vessel formation. In a head-to-head comparison with bone marrow mesenchymal/stromal cells (BMSC), adipose tissue MSC and BMNC, cardiac progenitors have shown a greater therapeutic ability in a rodent model of MI, as they produce large amounts of angiogenic cytokines and possess greater myogenic and angiogenic differentiation potentials.<sup>101</sup> Compared with the angiogenic monotherapies, cell therapies may be a more effective treatment for IHD because they provide a 'cocktail' of cytokines that would complement those endogenously produced to promote blood vessel formation and maturation, cell survival and/or activation of endogenous tissue repair and regeneration. However, the current cell therapies will still be facing the same challenges as angiogenic monotherapies if (1) the number of cells and cell function are affected by disease and cardiovascular risk factors leading to reduced homing capacity, (2) exogenous cytokines produced by the transplanted cells cannot rescue endothelial dysfunction (see figure 2) or (3) endogenous antagonists of angiogenic signalling pathways (figure 3) are still produced and not dampened down by cell transplantation.

Are we closer to achieving efficacy of therapeutic blood vessel regeneration and repair in IHD? Trials with angiogenic monotherapies have largely shown no effect, whereas arteriogenic factors have shown promising early results and cell therapies have shown a moderate effect on LVEF and scar size, myocardial viability and perfusion in acute and patients with chronic MI.<sup>42 43 102</sup> Furthermore, in patients receiving maximal medical care who were not eligible for revascularisation, cell therapies significantly reduce the risk of mortality.<sup>102</sup> Some trial results suggested that patients with a more severely impaired LV function may have a benefit from cell

transplantation, whereas patients with a rather preserved LV function post-MI may not.<sup>44</sup> Importantly, cell function is known to be affected by cardiovascular risk factors.<sup>103 104</sup>

Taken together, the current clinical evidence suggest that cell therapies may be a more effective treatment for IHD as they already provide a combination of cytokines that would promote blood vessel formation and maturation (angiogenesis and arteriogenesis), cell survival and/or activation of endogenous tissue repair and regeneration. However, in cases where cell function is impaired, a combination therapy (eg, cells and biologicals) that could promote arteriogenesis and/or vessel maturation rather than capillary formation only may be more suitable. Finally, patient stratification may be a prerequisite for successful therapeutic angiogenesis.

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# Blood vessel repair and regeneration in the ischaemic heart

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