

**THE POTENTIAL UTILITY OF STEM CELLS  
IN THE TREATMENT OF CONGENITAL HEART DISEASE**

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**Thesis submitted to the University of Nottingham  
for the degree of Doctor of Philosophy**

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*"The heart of creatures is the foundation of life, the Prince of all, the sun of their microcosm, from where all vigour and strength does flow"*

William Harvey

*Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus*

1628

## **Abstract**

Non-ischaemic right ventricular dysfunction and cardiac failure is a source of considerable morbidity in children with congenital heart disease. Despite an increasing body of evidence suggesting that the intrinsic regenerative capacity of the heart can be encouraged by stimulation of resident cardiac stem cells or the transplantation of extracardiac progenitor cells, cell transplantation has not previously been studied in the paediatric setting where enhancing the function of the ventricle in response to supraphysiological workloads might be beneficial.

Firstly I studied extra-cellular matrix composition, myocyte homeostasis and gene expression in right ventricular biopsies obtained from patients with Hypoplastic Left Heart Syndrome (HLHS) undergoing neonatal surgical palliation and from patients undergoing neonatal truncus arteriosus repair in order to investigate potential differences in the myocardial substrate which could have implications for adaptive growth potential and haemodynamic performance in HLHS.

Simultaneous to these activities, I collected, isolated and analysed umbilical cord blood stem cells from children born with either structurally-normal hearts or HLHS to investigate whether such cell populations might be useful in cardiac augmentation.

We then transplanted human cord blood stem cells from normal cord blood donors into an immunosuppressed neonatal sheep model of right ventricular training, taking load-independent functional measurements at baseline and again after one month.

Transplanted human cells were detected in the myocardium, spleen, kidney and bone marrow up to 6 weeks after transplantation. Human cells expressed the haematopoietic marker CD45 and in the bone marrow and spleen, also the mature B cell marker CD23. Significant functional improvements were seen in the group receiving human cord blood stem cells compared to placebo. Data demonstrated that lineage negative-enriched cord blood stem cells engraft and adopt traditional haematopoietic cell fates in both myocardium as well as natural niches such as bone marrow and spleen.

## **Publications**

1. Davies B, d'Udekem Y, Ukoumunne OC, Algar EM, Newgreen DF, Brizard CP. Differences in structure and myocyte homeostasis between the neonatal morphological right ventricle in hypoplastic left heart syndrome and truncus arteriosus.  
*Eur J Cardiothorac Surg* **34**, 738-744 (2008)
2. Davies B, Li S, Cullinane F, Edwards GA, Newgreen DF, Elwood N, Brizard CP. Human cord blood stem cells improve right ventricular function in the pressure-loaded neonatal ovine right ventricle (under review)

## **Poster Presentations**

1. Davies B, Li S, Oppido G, Rebel S, Setty S, d'Udekem Y, Cullinane F, Edwards GA, Newgreen DF, Elwood N, Brizard CP.  
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## **Declaration**

Except where acknowledged, I declare this to be my own work undertaken at and funded by the Australia and New Zealand Children's Heart Research Centre, a division of the Murdoch Childrens Research Institute, Melbourne, Australia between July 2005 and August 2007.

A handwritten signature in black ink, appearing to read 'Ben Davies', with a stylized, flowing script.

**Ben Davies**

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## **1.0 Background**

### **1.1 Contemporary challenges in human congenital heart disease**

Congenital heart disease affects 1 in 125 children and is the leading non-infectious cause of death in children.

One of the most therapeutically challenging cardiac defects is hypoplastic left heart syndrome (HLHS) in which left-sided cardiac underdevelopment co-exists with aortic hypoplasia<sup>1</sup>. Considered uniformly lethal until 1980, it composes 7 to 9% of neonatal congenital heart disease and is responsible for 25% of cardiac deaths in the first week of life. Untreated, most children die within a month of birth. The principal surgical treatment offered to parents consists of repair over three stages performed at approximately 1 week, 3 months and 2 years of age respectively, culminating in a univentricular circulation in which the morphological right ventricle supports the systemic circulation whilst blood returns passively to the lungs via a Fontan circuit.

The enormous improvement in survival for the 1<sup>st</sup> stage procedure since it was first described by Norwood<sup>2</sup> in the 1980s ranks amongst the greatest achievements in congenital cardiac surgery in the past 20 years. However, despite advances in surgical technique and intensive care<sup>3-5</sup>, there remains a persistent and frustrating 5-15% risk of sudden death before the 2<sup>nd</sup> stage can be completed. Contributing factors include the presence of inefficient parallel circulations in this period combined with the inherently inferior

pumping capability of the morphological right ventricle. Later on in life there is a steady attrition attributable to the long-term complications of a Fontan circulation.

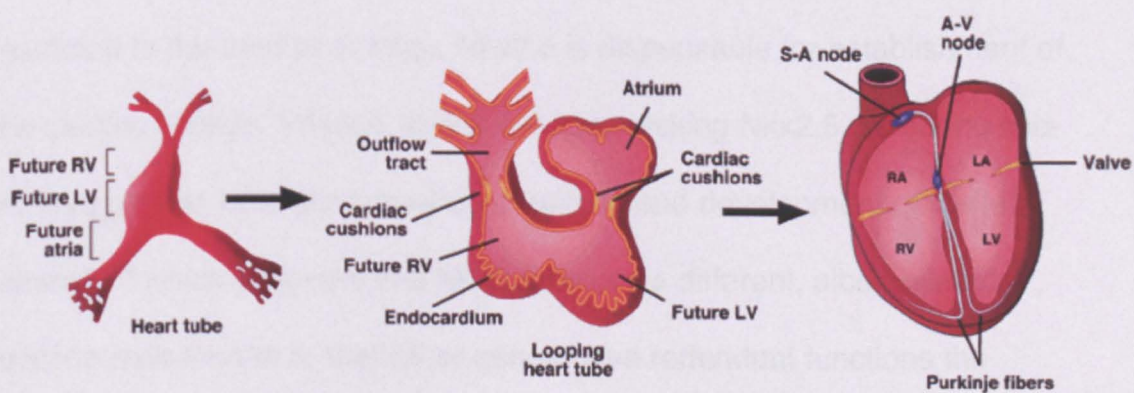
It is now increasingly apparent that several congenital heart defects once attributed to multifactorial aetiologies are actually due to mutations or defects in developmental control genes, transcriptional programs and cell signalling. Greater insight into normal cardiac development and the understanding of congenital heart disease has the potential for impacts on human disease in several ways. The identification of cardiac control genes and their associated transcription factors in both health and disease<sup>6-8</sup> could be exploited to design therapies for the improvement or prevention of congenital heart defects. The involvement of developmental control mechanisms in the pathogenesis of adult heart disease also indicates therapeutic potential of such strategies.

Already a number of novel haemodynamic therapeutic strategies concentrating on improving epigenetic factors for cardiac development, such as hydraulic flow<sup>9,10</sup>, have been developed. For example, the attempts to modulate the natural history of congenital aortic valve stenosis to a more favourable one by fetal aortic balloon valvuloplasty.<sup>11</sup>

1.2 Cardiac developmental biology

The heart is the first organ to form in the embryo and all subsequent events in the development of the organism depend on its function (figure 1.2.1). Inherited mutations in cardiac regulatory genes give rise to congenital heart disease, the commonest form of human birth defect, affecting 1 in 125 live births and their frequency in spontaneously aborted pregnancies is estimated to be ten times higher.<sup>12</sup> From initial morphological foundations, work has evolved through physiological and functional studies towards gaining a greater appreciation at genetic and molecular levels. Together, the increased insight this affords has provided new therapeutic approaches for prevention and palliation of cardiac disease.

Figure 1.2.1: Synopsis of cardiac development



Reproduced with permission from Olson EN *Nature Medicine* 2004; 10:467

### 1.2.1 Heart Development & Congenital Heart Disease

The initial commitment of mesodermal progenitor cells to a cardiac fate is dependent on signalling between adjacent tissues. Members of bone morphogenetic protein (BMP) family have positive roles and wingless proteins have positive and negative roles in establishment of the cardiac lineage<sup>13-16</sup>. Entry and maintenance of cells into the cardiac lineage in response to appropriate signals is coupled to expression of transcription factors that initiate the program for cardiac gene expression and drives the morphogenic events involved in formation of the multi-chambered heart.

It was not until the early 1990s that the genetic underpinnings of cardiac morphogenesis began to emerge. In particular, the discovery of the homeobox gene *tinman* (*tin*), required the formation of the primitive heart in the fruit fly *Drosophila melanogaster*.<sup>17,18</sup> A mammalian ortholog of *tinman* called *Nkx2.5* or *Csx*, is expressed in cardiac muscle cells from the onset of embryonic heart formation until adulthood<sup>6,19</sup>. Although highly conserved and restricted to the cardiac lineage, *Nkx2.5* is dispensable for establishment of the cardiac lineage. Instead, knock-out mice lacking *Nkx2.5* die during mid-embryogenesis from abnormalities of growth and development of the left ventricle<sup>20</sup> which suggests that *Nkx2.5* serves a different, albeit related, function than *tinman* or that other genes have redundant functions the specification and subsequent development of cardiac cell fate.

In contrast to skeletal muscle, in which a single transcription factor MyoD is sufficient to activate the full program of muscle differentiation, cardiac muscle depends on a combination of transcription factors. In mammals as well as fruit flies, the MADS-box factor myocyte enhancer factor-2 (MEF-2), in concert with other transcription factors, directly activates the expression of genes encoding myofibrillar proteins.<sup>21,22</sup> Likewise, serum response factor (SRF), a related MADS-box factor, associates with an array of transcription factors including Nkx2.5, GATA4 and myocardin to control the expression of muscle structural genes whose products such as actin, myosin and troponins<sup>23,24</sup> are incorporated into the contractile apparatus. The homeodomain protein Irx4 also activates ventricular genes whilst suppressing atrial genes in the ventricular chambers<sup>25</sup>. Analysis of regulatory DNA sequences responsible for cardiac transcription revealed a complex regulation whereby individual genes are often controlled by multiple, independent enhancers that direct expression in highly regionally-restricted patterns in the developing heart<sup>26</sup>. Cardiac transcription factors, in addition to activating subordinate genes involved in cardiac function, also amplify and maintain their expression within mutually-reinforcing transcriptional circuits involving positive feedback loops and protein-protein interactions.

Two populations of cardiac progenitors termed primary and secondary heart fields contribute to the developing heart in vertebrates<sup>27</sup>. Cardiac myocytes are organised into a linear tube that then undergoes right-ward looping in response to an axial signalling system that establishes asymmetry across the left-right axis of the embryo<sup>28</sup>. Subsequent balloon-like growth of the looped

heart tube and septation gives rise to the multichambered heart. The right and left ventricular and atrial chambers of the heart each have distinct functions and are separated by interatrial and interventricular septae so blood can be delivered to the lungs and thence the body, in series.

Examples of congenital heart disease in which one ventricle is underdeveloped, for example hypoplastic left heart syndrome or pulmonary atresia with intact ventricular septum, may suggest that the two ventricles might form independently according to mutually exclusive growth programs in regionally distinct areas in the embryologic heart tube. Insight into the genetic circuitry of ventricular development was provided by the discovery of two chamber-restricted basic helix-loop-helix transcription factors, HAND1 and HAND2, that are expressed in left and right ventricles, respectively<sup>29</sup>.

Knockout mice lacking HAND2 do not form a right ventricle and mice lacking both genes form neither ventricle<sup>30</sup>. These mice were the first to demonstrate that a single gene mutation could result in the ablation of an entire segment of the heart and therefore supported the concept that the heart is assembled in a modular fashion with each compartment governed by unique genetic programs. The segmental pattern of heart development is also exemplified by the phenotype of mice lacking the homeobox gene *Is/1*, in which entire regions of the heart may be absent<sup>31</sup>. Mutations in other cardiac control genes in mice and zebrafish further substantiate such a model<sup>32</sup>.

Further growth and maturation of cardiac myocytes within the developing ventricular chambers depends on the interplay of signalling events between adjacent cell layers and epigenetic factors such as flow and shear stress<sup>10,33</sup>. Signalling by neuregulins from the endocardium, a thin layer of cells lining the cardiac chambers, to the ErbB receptor in the myocardium is required for growth of the mesocardium/ventricular layer<sup>34</sup>. Mutations that alter this signalling interaction result in thin-walled myocardium and embryonic death. Interference with neuregulin signalling in the adult heart by antibodies to ErbB, as used in breast cancer therapy, can also lead to contractile dysfunction<sup>35</sup>. The epicardium also provides a rich source of signals stimulating cardiac growth, for example retinoic acid and peptide growth factors<sup>36</sup>.

Heart valve formation requires signalling from the myocardium to localised swellings of the endocardium known as cardiac cushions and occurs by TGF- $\beta$  family members. Disruption of this mechanism in knockout mice lacking cardiac expression of BMPs or BMP receptors result in valve abnormalities and VSDs which account for a majority of congenital heart defects<sup>37,38</sup>.

Neural crest cells from the pharyngeal arches also populate the developing heart and are important in the formation of valves, interventricular septum and patterning of the outflow tract and great arteries<sup>39,40</sup>.

Each cardiac cycle of contraction and relaxation is controlled and propagated throughout the heart by specialised conduction tissue and by direct cell-cell coupling of cardiac myocytes. Until recently, the embryonic origins of the cell lineages that form the cardiac conduction system were unclear; it has now

been demonstrated that Purkinje cells are derived from a subpopulation of ventricular cardiomyocytes in response to signalling by endothelin-1 and neuregulins<sup>41,42</sup>. Fate mapping of cardiac conduction lineages in mice has yielded insights into the transcriptional pathways responsible for the formation of this specialised cardiac structure and the molecular basis for cardiac conduction defects<sup>43</sup>.

Mutations in cardiac transcription factors, their regulatory genes and the genes they in turn regulate, have been identified in humans with congenital heart disease<sup>7,44-46</sup>. Humans and mice heterozygous for missense mutations in Nkx2.5 show a spectrum of structural and functional defects including atrial-septal and ventricular-septal anomalies and conduction defects<sup>47</sup>. The molecular basis for these defects is unclear but it may be that subtle defects in growth or patterning of cardiac myocytes during embryogenesis occur. Holt-Oram syndrome, characterised by heart and limb defects, has been attributed to mutations in the T-box gene TBX5. Missense mutations in GATA4 that impair its interaction with TBX5 have also been shown to cause VSDs in humans<sup>48</sup>. Despite such descriptions of abnormal cardiac phenotypes, important gaps in our understanding of developmental control mechanisms remain. Relatively few target genes regulated by cardiac transcription factors have been identified and how they ultimately relate to cardiac malformations is unclear. It must be considered that most of the conclusions regarding the functions of cardiac control genes have been based on phenotypes resulting from homozygous gene deletion in model organisms, whereas most mutations in humans producing survivable

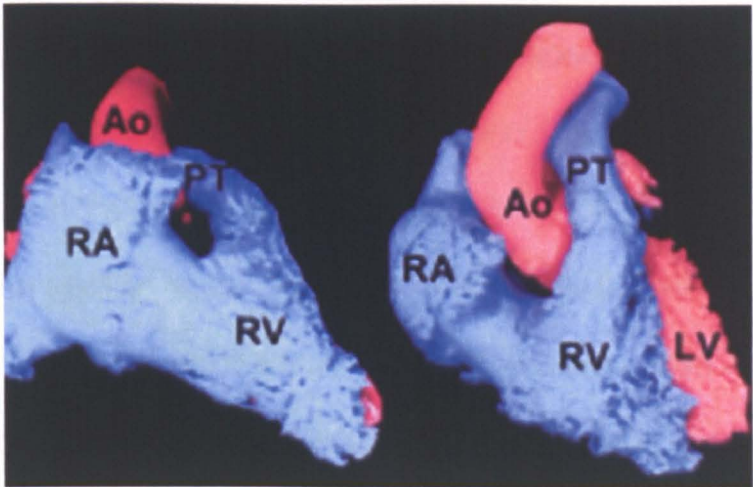
disease, are heterozygous. Congenital heart defects in human also show remarkable variability in penetrance and expressivity, indicative of modifier genes and environmental influences in determining disease phenotypes. Much remains to be learned about the identities and mechanisms of action of such modifiers.

### 1.3 The morphological right ventricle

#### *Anatomy*

The normal morphological right ventricle lies anteriorly in the chest, is wrapped around the left ventricle and has a geometrically complex shape unlike the ellipsoid shape of the left ventricle which is more conical in shape and has a wall thickness 3 to 4 times greater than the RV free wall. RV trabeculations are coarse compared to the finely trabeculated left ventricle and the RV outflow tract is muscular and elongated (figure 2). These differences in ventricular morphology reflect differences in genetically-determined developmental patterning and their differing physiological roles.

**Figure 1.3.1:** Endocast of heart showing cardiac chambers and great vessels: RA, right atrium; RV, right ventricle; LV, left ventricle; Ao, aorta; PT, main pulmonary artery/pulmonary trunk



Ho, S. et al. *Heart* 2001;86:3ii-11ii

## *Physiology*

Once considered haemodynamically unimportant<sup>49</sup> and essentially a conduit to the pulmonary circulation, the right ventricle actually performs several essential functions:

- Maintain adequate pulmonary perfusion pressure under varying circulatory and loading conditions in order to deliver desaturated venous blood to the membranes of the lungs for gas exchange
- Maintain a low systemic venous pressure to prevent tissue and organ congestion

Although there is some beat-to-beat variability, in the absence of shunting, the average stroke volume of left and right ventricles must be equal. The normal right ventricle has a lower ejection fraction than the left, consequently its end diastolic volume is a little larger. The contractile mechanics by which blood is ejected from the right ventricle differs compared to the left ventricle whose pressure-volume relationship shows an efficient square wave pump with well-defined period of isovolumic contraction, ejection, relaxation and diastolic filling. In the normal right ventricle with normal pulmonary vascular resistance, the ejection phase is markedly different to the left ventricle. Ejection from the RV begins early in the rise in pressure, often with a poorly defined period of isovolumic contraction. Equally important, ejection continues after right ventricular pressure has peaked; indeed up to 50% of the stroke volume is ejected during the decline in RV pressure, a period that

can last as long as 80ms. Although variable, the right ventricular isovolumic relaxation period may also be poorly defined. Although the cyclical efficiency is low, the energy efficiency is high.

Right ventricular function may be impaired by primary right-sided heart disease, or secondary to left-sided cardiomyopathy or valvular disease<sup>50</sup>.

Right ventricular dysfunction may likewise affect left ventricular function, not only by limiting left ventricular preload, but also by adverse systolic and diastolic interaction via the interventricular septum and the pericardium, a well-characterised phenomenon known as ventricular interdependence.<sup>51</sup>

## **1.4 Functional assessment of the right ventricle**

Although LV function and dysfunction and its relationship to clinical prognosis has been studied extensively, the role of RV morphology and its function in cardiovascular disease has not until recently attracted a similar degree of interest, largely due to the fact that the majority of clinical workload comprises of acquired cardiovascular disease predominantly affecting the left ventricle.

Any form of RV assessment is hampered by load-dependent changes in geometry, the unusual physiology ejection reflecting low pulmonary vascular resistance, ventricular interaction and the effect of respiration, to which congenital abnormalities of size, position and structure that may be superimposed. An appreciation of RV function is however essential in the management of congenital heart disease which is of increasingly relevance to clinical practice. Many patients have become adolescents and adults thanks to the major advances of paediatric cardiology and cardiac surgery in the latter half of the 20<sup>th</sup> century. In particular, there are a substantial number of patients with single-ventricle physiology, systemic right ventricles (RVs), or complex intracardiac baffles who are now entering adult life, creating a patient population in whom the RV is often a key prognostic factor<sup>52</sup>.

In everyday practice, clinicians largely rely on non-invasive two-dimensional echocardiography supplemented by alternative techniques including tissue Doppler imaging, three dimensional echocardiography, cardiac magnetic resonance imaging and invasive assessment with pressure–volume loops, which permit accurate assessment of RV volume, mass and function.<sup>53-59</sup>

### *Pressure-volume loops*

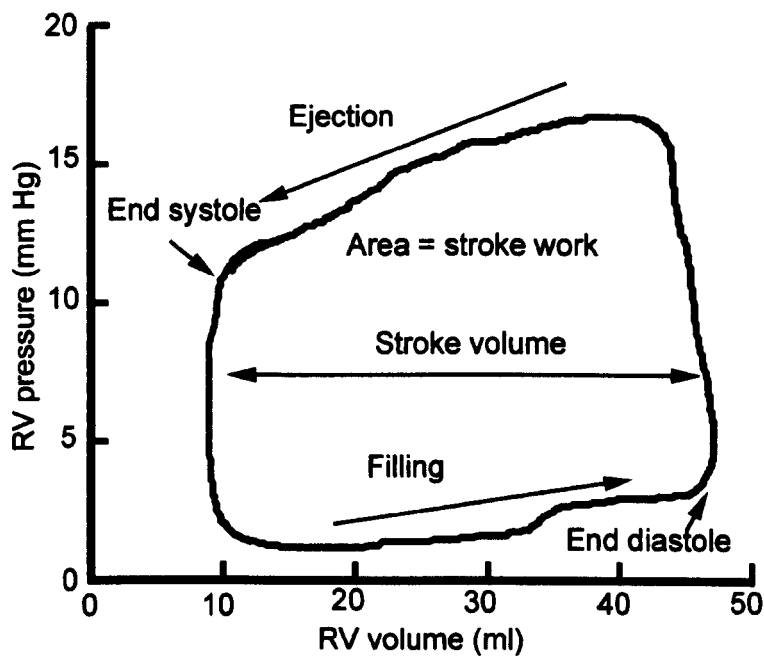
Analysis of RV function by pressure–volume loops is attractive because it simultaneously quantifies key determinants of ventricular function in a relatively independent fashion.<sup>60,61</sup> Conventional indices such as end-systolic and end-diastolic pressures and volumes, stroke volume, stroke work and ejection fraction can be directly derived from the loops (Figure 1.4.1).

Differentiation of the volume signal ( $dV/dt$ ) yields RV inflow and outflow signals, from which early and late filling rates and ejection rate can be obtained. Analysis of the pressure decay during relaxation yields the relaxation time constant  $\tau$ , whereas differentiation of the pressure signal yields  $dP/dt_{\text{MIN}}$  and  $dP/dt_{\text{MAX}}$ . Whilst revealing important information on systolic and diastolic right ventricular function, these however also reflect prevailing loading conditions.

Pressure–volume relations, which may be derived from pressure–volume loops collected during a loading intervention, provide indices of right ventricular systolic and diastolic function largely independent of loading conditions and therefore better reflect intrinsic myocardial function. The position and slope (end systolic elastance) of the end systolic pressure–volume relation are sensitive indicators of right ventricular systolic function.

Additional systolic indices include the slope of the relation between stroke work and end diastolic volume, the preload recruitable stroke work (PRSW), and the slope of the relation between  $dP/dt_{MAX}$  and end diastolic volume (dPdt-EDV).<sup>62</sup>

**Figure 1.4.1:** Steady state pressure-volume loops obtained from an adult sheep



Diastolic function may be derived from the end diastolic pressure–volume points. The slope of this relation represents diastolic stiffness or diastolic compliance (1/stiffness). When obtained over a wider range, the end diastolic pressure–volume relation is generally non-linear and better approximated by an exponential fit, such as:

$EDP = A \cdot \exp(k \cdot EDV)$  where  $k$  is the diastolic stiffness constant.<sup>63</sup>

Construction of pressure–volume relations requires pressure and volume data obtained during various loading conditions, preferably induced by rapid, mechanical interventions that minimally disturb intrinsic myocardial function. This can be achieved by temporary occlusion of the inferior vena caval inflow either with an endoluminal balloon or direct occlusion at open surgery. The conductance catheter is the most commonly used experimental instrument to acquire ventricular pressure–volume loops. The catheter contains a high fidelity pressure sensor and electrodes to measure right ventricular electrical conductance from which instantaneous ventricular cavity volume can be determined.<sup>64</sup> Pressure–volume loop analysis is regarded as the optimal way to quantify RV function, has been widely used in experimental animal studies<sup>65–68</sup> and although validated and used in humans,<sup>69–71</sup> its clinical use is hampered by its invasive nature, cumbersome logistics and sterilisation issues of the expensive, fragile catheters.

Alternative methods have been proposed that could provide estimates of systolic pressure–volume relationships based on steady state and single-beat data which whilst conceptually attractive by obviating the need for a loading intervention, their accuracy remains to be determined.<sup>72</sup>

## **1.5 Stem Cells**

Stem cells are defined by their capacity for self-renewal, transformation into dedicated progenitor cells and differentiation into specialised progeny.<sup>73,74</sup>

They can be further classified by morphological and functional criteria or by their surface markers. Their discovery and importance to science in general is potentially great, offering a tool to help unravel early molecular events in organ development or disease and potentially therapeutic hope for patients requiring repair or replacement of damaged, degenerative or genetically-deficient tissues and organs.

### **1.5.1 Current status of cardiovascular cell therapy**

The possibility of using stem cells in the repair, regeneration or repopulation of damaged or diseased adult tissue has prompted cardiovascular researchers to engage in innovative work to explore ways in which cardiac function may be improved.

In the developed world, contemporary management of acute cardiovascular events such as acute myocardial infarction (AMI), thrombotic cerebral infarction and peripheral arterial disease has reduced mortality but increased overall morbidity. Chronic heart failure has emerged as a major worldwide epidemic and has experienced a shift in aetiology towards long-term survivors of myocardial infarction as opposed to hypertension or valvular disease.<sup>75</sup> Owing to this burden of disease, research work to date has

necessarily been focussed on improving left ventricular function in chronic ischaemic heart disease by stem cell transplantation. Other work has explored the production of tissue-engineered vascular conduits and valves using related cell culture and matrix scaffold techniques.<sup>76-78</sup>

### **1.5.2 Cardiac homeostasis**

The long-standing dogma of heart development, one of a terminally-differentiated organ incapable of self-renewal or regeneration, has recently been challenged. Low levels of myocyte proliferation compared to other tissues such as liver, was previously taken to mean that the post-natal heart could only react to increases in physiological workload through hypertrophy as opposed to hyperplasia. Difficulties in identifying mitotic figures or DNA synthesis within myocytes further reinforced the notion that the heart functions throughout life with the same terminally-differentiated cells.<sup>79</sup> Yet the only way this paradigm could be realised would be if cardiac myocytes were somehow immortal or otherwise exempt from the normal ageing process affecting the rest of the body.

Ventricular dysfunction was thought to intervene once the heart's hypertrophic capabilities were exhausted and the effects of myocardial infarction irreversible with any observed recovery of ventricular function attributed to hypertrophic and fibrosis remodelling.

Dispelling these myths has stimulated the clarification of myocardial homeostatic mechanisms that might then be exploited in an attempt to

favourably modulate clinically-relevant human disease processes. A therefore more rational explanation is that the heart, in keeping with other organs, experiences both cell growth and death and that the balance of these two processes achieves myocardial cell homeostasis, determining the overall functional efficacy of the organ and the survival of the organism as a whole. Any imbalance between cell growth and death in pathophysiological states might result in decompensation, overt heart failure and eventual death.

### **1.5.3 Evidence of post-natal myocardial proliferation and chimerism in solid-organ transplantation**

Irrespective of causation, cardiac failure has several common effector pathways including depressed contractility due to altered expression and function of calcium handling proteins and other sarcomeric proteins and enzymes, the development of interstitial fibrosis and a net loss of myocytes further reducing ventricular function. This has been borne out by studies of myocytes from patients dying from heart failure in whom a 10 to 60 fold increase in mitotic figures was noted,<sup>80</sup> albeit in a small and insignificant proportion of myocytes probably insufficient as an isolated, robust repair mechanism, but evidence of reparative intent at the very least. These concepts are supported by recent work suggesting large numbers of mitotic figures in adult hearts with aortic stenosis.<sup>80,81</sup> The discovery of male recipient cells in transplanted female donor hearts<sup>82</sup> raised the possibility that male cells colonised the donor heart, creating a chimera and differentiated into myocytes and blood vessels, a process consistent with the hypothesis

that extra-cardiac stem cell-like cells may migrate to a cardiac allograft and give rise to organ-specific progenies.

#### **1.5.4 Resident cardiac stem cells**

Although primarily comprised of terminally differentiated post-mitotic effector cells, many tissues retain smaller populations of stem cells capable of slowly replenishing cells that are lost through wear and tear, injury, and disease. An emerging body of evidence, including the isolation of stem cells from several different somatically-derived tissues and organs<sup>83-86</sup>, suggests that the homeostatic maintenance of most tissues is ultimately mediated by such tissue- or organ-specific stem cells that have the ability to self-perpetuate through a process known as self-renewal, in addition to being capable of giving rise to mature effector cell types in a sustained manner through differentiation.

The existence, origin and migratory properties of such dividing cells capable of attempts at cardiac renewal remains controversial. Possible sources include the bone marrow from where cells may be released as part of an ongoing process of renewal and also in response to injury. Others favour the existence of a local “resident” cardiac stem cell population, a theory supported by the isolation of cells expressing a progenitor ( $\text{lin}^-$ ,  $\text{c-Kit}^+$  and  $\text{Sca-1}^+$ ) phenotype.<sup>87</sup> The epicardium has also been postulated to harbour a subset of progenitor cell that migrates in the myocardium, differentiating into myocardial or vascular components.<sup>88</sup>

Akin to the brain, the heart seems to have reservoirs of progenitor cells that may not be sufficient to replace the acute loss of a large number of cells, but may be able to replace a slow apoptotic loss of cells over a lifetime. The presence of these cell populations, known as resident cardiac stem cells (CSC), capable of differentiating into cardiomyocyte or vascular lineages suggests that these cells could be used for cardiac tissue repair or a molecular target for stimulation.

The above concepts concerning cardiac regeneration may be unpalatable for some, but doubters need only look elsewhere in the animal kingdom where self-renewal is a well recognised phenomenon. Cardiac regeneration is well recognised in certain organisms such as the *Urodeles* species of amphibian<sup>89</sup> and zebrafish,<sup>90</sup> in whom total replacement of an amputated limb,<sup>91</sup> fin, or tail can also be achieved via production of an undifferentiated cell mass called the blastema. These are processes demanding dedifferentiation and transdifferentiation, therefore providing a practical demonstration of adult stem cell plasticity.

Traditionally, tissue-resident adult stem cells were believed only able to differentiate into progeny within tissue lineage boundaries. Plasticity implies that stem cells can transdifferentiate into mature cell types outside their original lineage in response to microenvironmental cues. For example, haematopoietic stem cells (HSCs) may transdifferentiate into cardiomyocytes and blood vessels when transplanted into murine myocardium<sup>92</sup>.

These dramatic examples of restorative growth may be dependent on retained proliferative potential in a subset of adult cardiomyocytes kept in check under normal circumstances; the existence of such endogenous repair mechanisms suggests that cardiac repair might also be realised therapeutically in the clinical setting. Other mechanisms that could aid this process include:

- Overriding cell cycle checkpoints that constrain a reactive proliferation of ventricular myocytes
- Supplementing endogeneous cytoprotective mechanisms, or inhibiting cell death pathways
- Supplementing angiogenic mechanisms using growth factors or vessel-forming cells
- Provision of exogenous non-cardiac cells as surrogates or precursors for cardiac muscle.

Simultaneous to investigations of bone-marrow stem cell plasticity, searches for resident cardiac stem cells took place. Unlike the proposal that bone marrow serves as a reservoir for cells that can differentiate into myocardium when placed in the milieu of the injured heart, this hypothesis was in keeping with the basic tenet of developmental biology that stem cells reside in the tissue that they repair.

Contributors to the earlier landmark study by Orlic et al,<sup>92</sup> published a report in 2003 describing the isolation of adult cardiac stem cells from rat

myocardium. In the latter study, Beltrami et al. isolated a self-renewing clonogenic population of Lin<sup>-</sup>c-kit<sup>+</sup> cells from normal rat myocardium<sup>93</sup>. In vitro, these cells gave rise to cells with characteristics of myocytes, smooth muscle cells and endothelial cells. In vivo, implantation of  $1 \times 10^5$  of these cells into post-infarcted myocardium resulted in decreased infarct size, myocardial regeneration and preservation of cardiac function. The authors presented several lines of evidence to argue against cell fusion as a mechanism for myocardial regeneration. Finally, although they could not conclude whether these supposedly cardiac stem cells originally reached the myocardium through the circulation, they found no cells with similar characteristics in the bone marrow or peripheral circulation. A significant limitation of this study is that the techniques used to identify cardiac regeneration are the same as those used in the earlier study by Orlic et al.<sup>92</sup> which have been questioned as several other groups have been unable to reproduce the results<sup>94-96</sup>.

Oh et al.<sup>97</sup> also reported the existence of adult heart-derived cardiac progenitor cells in mice. Unlike Beltrami et al.<sup>93</sup>, these cells are Lin<sup>-</sup>c-kit-Sca-1<sup>+</sup>; in vitro, 5-azacytidine can induce cardiac differentiation of these cells. Moreover, in vivo, these cells can differentiate into myocytes when delivered into the infarct border-zone, with approximately half of these representing fusion products between native myocytes and the donor Lin<sup>-</sup>c-kit-Sca-1<sup>+</sup> stem cells.

Some of Beltrami's co-workers also reported the discovery of clusters of stem cells in the left ventricular outflow tract transitioning to cardiomyocytes, in patients undergoing aortic valve replacement for aortic stenosis<sup>98</sup>. These

cells were c-kit<sup>+</sup>Sca-1<sup>+</sup>, and some co-expressed cardiomyocyte markers GATA-4 and MEF2. They suggest that the presence of these cells strongly supports the existence of cardiac stem cells in humans. Messina et al,<sup>99</sup> performed studies in both mice and humans, isolating self-renewing clonogenic cells that grow as self-adherent clusters ('cardiospheres') in vitro and express the stem cell markers c-kit, CD-34 and Sca-1. These cells become beating cardiomyocytes when co-cultured with postnatal rat cardiomyocytes and can contribute to myocardium in vivo when injected into post-infarcted border-zone myocardium.

More recently, Laugwitz et al. described the isolation of cardiac progenitors from the heart of the postnatal rat, mouse and human which carry the genetic marker isl1; these cells can adopt a fully differentiated cardiomyocyte phenotype in vitro in the absence of cell fusion<sup>100</sup>.

Putting this work in perspective, there is emerging evidence that resident cardiac stem cells may exist in murine and human organ models. It is possible that different groups of cardiac stem cells have been isolated by different researchers; for example Beltrami et al.'s are c-kit<sup>+</sup>, whereas those of Oh et al. are c-kit<sup>-</sup>. Well-conceived mechanistic studies are now needed to more fully understand the nature of resident cardiac stem cells.

### **1.5.5 Could resident cardiac stem cells be subject to therapeutic supplementation or manipulation?**

With early studies supporting the notion that adult cardiac regeneration occurs naturally, albeit at low levels, several key questions arise as to which cells might participate in myocardial regeneration? Is the concept of resident cardiac stem cells irrefutable? Or do non-cardiac stem cells contribute to myocardial regeneration?

Intriguingly, CSCs can be clonally expand from human myocardial biopsies. It has been reported that intramyocardial injection of these cells after AMI in mice promotes cardiomyocyte and vascular cell formation and leads to an improvement in systolic function.<sup>99</sup> If these findings can be reproduced, CSCs hold great promise for clinical applications.

### **1.5.6 Candidates for myocardial regeneration or augmentation**

Stem and progenitor cell populations are currently available from a variety of sources including the embryonic cell mass, bone marrow, umbilical cord blood and placenta. Conceptually, autologous cells are the most appealing for clinical practice so that risks of infection or rejection are minimised; xenograft models have also been performed to determine the fate and viability of cross-species cell transfer. Each cell type has its own advantages, disadvantages and practicality issues in specific clinical settings; studies comparing the regenerative capacity of distinct cell populations are scarce (see Tables 1.5.1 and 1.5.2). Controversy remains as to the “ideal” stem cell

to use, with science in this area frequently and unfortunately clouded by self-interest and financial imperatives.

### *Embryonic Stem Cells*

Embryonic stem (ES) cells are totipotent stem cells derived from the inner cell mass of blastocysts and hold the greatest potential in terms of the diversity and number of cell types that could be produced. Under specific culture conditions, ES cells differentiate into multicellular embryoid bodies containing differentiated cells from all three germ layers including cardiomyocytes. The latter have been shown to display electrical coupling with host cardiomyocytes following transplantation into normal myocardium.<sup>101</sup> However, unresolved ethical and legal issues, concerns about the tumorigenicity of the cells, and the need to use allogeneic cells for transplantation currently hamper their use in clinical studies. Eventually, nuclear transfer techniques and therapeutic cloning may provide a means for generating an unlimited supply of histocompatible ES cells for the treatment of cardiac disease.

### *Skeletal Myoblasts*

Skeletal myoblasts, or satellite cells, are undifferentiated progenitor cells that normally lie in a quiescent state under the basal membrane of mature skeletal muscular fibres. Myoblasts can be isolated from skeletal muscle biopsies and expanded in vitro. Myoblasts differentiate into myotubes and retain skeletal muscle properties when transplanted into an infarct scar<sup>102,103</sup>. Although myotubes may not couple with resident cardiomyocytes electromechanically, myoblasts were the first clinically-relevant cells used in

transplantation models and have been shown to augment systolic and diastolic performance in animal models of myocardial infarction and clinical practice.

### *Bone Marrow*

Many clinical investigators have chosen a pragmatic approach using unfractionated bone marrow cells (BMSCs)<sup>104,105</sup> which contains a mixture of stem- and progenitor-cell populations, including haematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs). Ease of harvest and lack of extensive requirement for ex vivo manipulation are additional advantages of using unselected BMSCs.

### *Endothelial Progenitor Cells*

EPCs were originally defined by expression of haematopoietic surface marker proteins CD133 and CD34 and the endothelial marker vascular endothelial growth factor receptor-2 (VEGFR-2) together with their ability to incorporate into sites of neovascularisation.<sup>106</sup> Increasing evidence suggests that culture-expanded EPCs also contain a CD14<sup>+</sup>/CD34<sup>-</sup> mononuclear cell population which mediates its angiogenic effects by releasing paracrine factors.<sup>107</sup>

### *Mesenchymal Stem Cells*

MSCs comprise a rare population of CD34<sup>-</sup> and CD133<sup>-</sup> cells in bone marrow stroma (10-fold less abundant than HSCs) and other mesenchymal tissues. MSC clones can be expanded in vitro and can readily differentiate into osteocytes, chondrocytes, and adipocytes. Differentiation to cardiomyocyte-

like cells has been observed under specific culture conditions and after injection into healthy or infarcted myocardium in animals.<sup>108</sup> When injected into infarct tissue, MSCs may enhance regional wall motion and prevent remodeling of the remote, non-infarcted myocardium.

#### *Umbilical cord blood-derived stem cells*

Umbilical cord blood (UCB) left behind in the umbilical cord and placenta after delivery provides a readily available, cheap and potentially autologous source of haematopoietic progenitor stem cells of various lineages<sup>109</sup> devoid of the ethical issues surrounding embryonic stem cells and provides a rich source of very primitive stem cells compared to bone marrow and peripheral blood.<sup>110</sup> Additional advantages of CB include relatively naive immune status and well-preserved telomere lengths.<sup>111</sup>

#### *CD133+ Cells*

The cell surface antigen CD133 is expressed on early HSCs and EPCs from bone marrow and cord blood and collaborate to promote vascularisation of ischaemic tissues.<sup>112</sup> CD133<sup>+</sup> cells can integrate into sites of neovascularisation and differentiate into mature endothelial cells. Because CD133 expression is lost on myelomonocytic cells, this marker provides an effective means to distinguish “true” CD133<sup>+</sup> EPCs from EPCs of myelomonocytic origin. Less than 1% of nucleated BMCs are CD133<sup>+</sup> and at present, only limited numbers of CD133<sup>+</sup> cells can be obtained for therapeutic purposes but improved results of ex vivo expansion are emerging.<sup>113</sup>

**Tables 1.5.1 and 1.5.2**

The following tables summarise the results of key investigative work examining the efficacy of stem cell transplantation by cell type and xenogeneic model, respectively.

**Table 1.5.1: Potential donor cells, advantages, limitations & practical issues**

Cell type	Cell source	Ex vivo expansion?	Capacity for transdifferentiation		Paracrine effects	Clinical data	
			Cardiomyocytes	Vessels		Safety	Efficacy
Skeletal myoblasts	Skeletal muscle biopsy	Yes	No	No	Uncertain	Uncertain	Yes
Unfractionated bone marrow cells	Bone marrow	No	Probably no	Probably yes	Yes	Yes	Yes
Endothelial progenitor cells	Bone marrow, peripheral blood	Yes	Uncertain	Yes	Yes	Yes	No
Mesenchymal stem cells	Bone marrow/other	Yes	Yes	Uncertain	Yes	n.d.	n.d.
	mesenchymal tissues						
Fetal hematopoietic stem cells	Cord blood	Yes	Probably yes	Unknown	Uncertain	n.d.	n.d.
Resident cardiac stem cells	Cardiac muscle biopsy?	Yes	Yes	Yes	Uncertain	n.d.	n.d.
Embryonic stem cells	Allogeneic cell cells	Yes	Yes	Yes	Uncertain	n.d.	n.d.

nd: not defined

**Table 1.5.2: Cellular xenotransplantation for cardiac repair**

Cell type	Animal model	Observation period	Immunosuppression	Results
<i>Somatic</i>				
mAT-1 <sup>114</sup>	Rat	Various	CY 5mg/kg/d	Survived, formed connection with host heart cells, angiogenesis
mAT-1 hFCs <sup>115</sup>	MI pig	1 month	CY 15mg/kg/bd	Grafts formed close association with host myocytes, angiogenesis
hFVs <sup>116</sup>	MI rat	14-65 days	CY 12.5mg/kg/d	Survived in infarct, +ve staining for a-actin
mHL-1 <sup>117</sup>	Pig	4-5 weeks	CY 15mg/kg/d	Engrafts in gap junctions and normal myocardium but not infarct
rFCs <sup>118</sup>	Mouse	2-8 weeks	CY 5mg/kg/bd+other	Prolonged survival of xenografts
<i>Adult stem cells</i>				
mBSCs <sup>119,120</sup>	MI rat	13 weeks	No	Grafts appeared in bone marrow and infarcted myocardium

hMSCs <sup>121</sup>	MI pig	6 weeks	CY 15mg/kg/bd	Functional improvement, +ve staining for $\alpha$ -MHC and cTn-I
hMSCs <sup>122</sup>	Mouse	1-8 weeks	Genetic manipulation	Graft +ve for $\beta$ -MHC, $\alpha$ -actinin, cTn-I, desmin and phospholamban
C2C12 <sup>123</sup>	MI rat	1-8 weeks	Genetic manipulation	Grafts +ve for $\beta$ -MHC, $\alpha$ -actinin, cTn-I, desmin and phospholamban
hMYBs <sup>124</sup>	Ischaemic pig	6 weeks-5 months	CY 5mg/kg/d	Grafts +ve for desmin
<i>Embryonic Stem Cells</i>				
mESCs <sup>125,126</sup>	MI rat	6-32 weeks	No	Functional improvement, angiogenesis, grafts +ve for $\alpha$ -MHC and cTn-I
mESCs <sup>127</sup>	MI rat	5 weeks	No	Functional improvement, angiogenesis, grafts +ve for MLC2v, Cx-43
Beating cells from mEBs <sup>128</sup>	MI rat	30 days	No	Grafts +ve for sarcomeric myosin
Cardiac-committed mES <sup>129</sup>	MI sheep	1 month	Subset received CY 500 mg/kg	Colonisation of peri-infarct zone, 15% absolute difference in LVEF between control and cell-treated groups
<i>Fetal stem cells</i>				
mHSCs CD133 <sup>+</sup> <sup>116</sup>	MI rat	1 month		Prevented scar thinning and LV systolic dilatation

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### 1.5.7 Cell delivery

#### *Transvascular approaches*

Transvascular strategies are especially suited for the treatment of recently infarcted and reperfused myocardium when chemoattractants and cell adhesion molecules are highly expressed. Selective intracoronary application delivers a maximum concentration of cells homogeneously to the site of injury during first passage. Unselected BMCs, circulating blood-derived progenitors cells, and MSCs have all been administered to patients with AMI and ischaemic cardiomyopathy via the intracoronary route.<sup>130-132</sup> In these studies, through the central lumen of an over-the-wire balloon catheter during transient balloon inflations to maximize contact time with the microcirculation of the territory supplied by the infarct-related artery.

Intravenous delivery of EPCs or MSCs has been shown to improve cardiac function after AMI. However, homing of cells to non-cardiac organs<sup>133</sup> could be considered to limit the clinical applicability of this approach. Indeed, in a recent study in post-AMI patients, significant myocardial homing of unselected BMCs was observed only after intracoronary stop-flow delivery but not after intravenous application.<sup>134</sup>

### *Direct ventricular injection*

Direct injection is the preferred route for cell delivery for patients in whom occluded coronary arteries preclude transvascular cell delivery (for example patients with chronic myocardial ischaemia) or when cell homing signals are expressed at low levels in the heart (scar tissue). However, injection of cells into ischaemic or scarred myocardium could create islands of cells with limited blood supply leading to poor cell survival<sup>135</sup> or the development of arrhythmogenic foci. Direct injection is especially suited for large cells, such as MSCs or myoblasts, which may otherwise cause microembolisation after intracoronary delivery. Cell delivery by direct injection may be technically challenging in patients with AMI, particularly if cells are to be injected into the border zone of the infarct. The safety of such an approach is questionable given the fragility of recently necrotic tissue.

Alternatively, cells can be directly injected into endocardial aspect of the left ventricular wall using an injection needle catheter advanced across the aortic valve.<sup>136</sup> Electromechanical mapping of the endocardial surface can then be used to identify viable, ischaemic, and scarred myocardium before cell injections.

Transepicardial cell injection has been performed as an adjunct to coronary artery bypass grafting.<sup>137</sup> Such an approach permits direct visualization of the myocardium and a targeted application of cells to scarred areas and/or the border zone of an infarct scar but its invasiveness limits its use as a stand-alone

therapy. The contribution of cell transplantation performed simultaneous to surgical revascularisation of ischaemic myocardium has been investigated in pre-clinical<sup>138,139</sup> and clinical settings<sup>140,141</sup>, but claims regarding tangible benefits using readily-available functional outcome markers have been harder to ascertain. More frequently, single-digit percentage improvements in ejection fraction have been reported<sup>142</sup>. The use of a needle catheter system placed via the coronary venous system has been described to deliver BMSCs through the coronary veins into normal porcine myocardium.<sup>143</sup> The same approach has been used in a pilot trial in patients with ischaemic cardiomyopathy to deliver myoblasts to areas of nonviable myocardium.<sup>144</sup>

### **1.5.8 Mobilization of stem and progenitor cells**

Rather than cell transplantation, recruitment and mobilisation of circulating and extracardiac stem cells for the purposes of cardiac repair was conceived from attempts to increase EPC numbers in a model of hind limb ischaemia.<sup>107</sup> Stem cell mobilization with stem cell factor (SCF) and/or granulocyte colony-stimulating factor (G-CSF) has since been tested in animal models of AMI and in pilot studies in patients with AMI and chronic myocardial ischemia.<sup>145</sup> So far results have been mixed: improvements in cardiac function after AMI in mice<sup>146</sup> have been countered by findings to the contrary in non-human primate<sup>147</sup> and incidence of in-stent restenosis when combined with intracoronary stenting in humans.<sup>148</sup> Growth factors may also exert beneficial pleiotropic effects

independent of stem cells by enhancing macrophage infiltration and matrix metalloproteinase activation and suppression of cardiomyocyte apoptosis

### **1.5.9 Transplantation Kinetics & Mechanisms of Action**

Given that a small fraction of cardiomyocytes may be able to re-enter the cell cycle and regenerate through recruitment of resident and circulating stem cells, experimental studies have been undertaken to further investigate the effect of stem and progenitor cell transfer into the heart. These studies have demonstrated significant, albeit often small, improvements in tissue perfusion and contractile performance of the ischaemically-injured heart.

The mechanisms that might bring about such beneficial functional changes are the subject of ongoing debate. Following the dissemination of the early results of principally animal-based research, cell transplantation or “cellular cardiomyoplasty” has been explored clinically in a series small, mostly uncontrolled trials in patients unsuitable for existing therapeutic options such as revascularisation, transplantation or ventricular assist devices<sup>149</sup>. Preliminary efficacy data indicate that stem cells may enhance myocardial perfusion and/or contractile performance in patients with acute myocardial infarction, advanced coronary artery disease and chronic heart failure.

Some researchers favour engraftment by fusion<sup>150,151</sup> or transdifferentiation<sup>152</sup> into cardiomyocyte or vascular cell lineages as likely explanations for these beneficial effects. Others have proposed that transient cell retention and release of paracrine mediators<sup>127,153</sup> may be sufficient to effect functional improvement or bring about other advantageous changes by releasing angiogenic ligands to protect host cardiomyocytes from apoptotic cell death<sup>154</sup>, inducing proliferation of endogenous cardiomyocytes, modulating local immunomodulation thus escaping detection and removal and may recruit resident cardiac stem cells. Today it remains controversial as to how mechanistically stem cell therapy might improve perfusion and contractile performance of the injured heart. Pre-clinical animal studies have provided some suggestions as to how this might occur but to date significantly-powered clinical trials have proved frustrating in terms of displaying tangible functional improvements using conventional functional outcome measures<sup>155</sup>.

#### *Early haematopoietic stem cell work*

In attempt to track the locational and phenotypic fate of cells following transplantation, Jackson et al. lethally irradiated mice and then reconstituted their bone marrow with lacZ-labelled HSCs<sup>156</sup>. Ten weeks later, cardiac injury was induced by occluding the left anterior descending (LAD) coronary artery for 60 minutes. After 2–4 weeks, they found 0.02% of cardiomyocytes and 3.3% of endothelial cells in the peri-infarct region were b-gal<sup>+</sup>. This study lent support to the notion that bone-marrow-derived HSCs might contribute towards the

regeneration of myocardium, albeit in this study only slight functional improvements accompanied this and it was unclear to what extent cell fusion as opposed to true regeneration was responsible for the b-gal<sup>+</sup> cell populations.

In 2001, Orlic et al. took this further in an attempt to investigate the possibility that transplanted cells underwent differentiation rather than relying on innate bone-marrow-derived mechanisms for repair after myocardial injury<sup>92</sup>. They directly transplanted  $1 \times 10^5$  Lin<sup>-</sup>c-kit<sup>+</sup> (HSC-enriched) bone marrow cells into the border-zone myocardium of mice several hours after LAD ligation. Surprisingly, they found new donor-cell-derived myocardium comprising 68% of the infarct 9 days after transplantation. New myocytes, endothelial cells and smooth muscle cells, thought to be of donor origin, were detected. Moreover, ventricular function and haemodynamics were significantly improved in cell-treated animals compared with negative controls. This study drew enormous attention from both the scientific and clinical communities and set the stage for early human trials of bone marrow transplantation into the infarcted heart.

As experimental models emerged supporting the hypothesis that bone-marrow-derived cells can transdifferentiate into cardiomyocytes, a clinical study by Deb et al.<sup>157</sup> in human bone marrow transplantation provided further evidence. They found that, in the hearts of female recipients of gender-mismatched bone marrow transplants,  $0.23 \pm 0.06\%$  of the cardiomyocytes were Y chromosome positive. Thus it appeared that a small percentage of cardiomyocytes were derived from the donor bone marrow.

The concept that stem cells possess plasticity to permit differentiation into different cell types depending on their local environment challenges a long-held dogma of developmental biology: that tissue-specific stem cells derive from the tissue that they repair. Transdifferentiation was met with a great deal of scepticism in stem cell biology circles, and several alternative theories were put forward to explain the unexpected scientific findings.

A series of experiments by Wagers et al, critically examined the hypothesis that HSCs can give rise to non-haematopoietic cells in vivo<sup>158</sup>. Using a model in which bone marrow of lethally irradiated mice was reconstituted with a single GFP<sup>+</sup> (green fluorescent protein-positive) HSC, they found little contribution of HSCs to non-haematopoietic tissue. In their analysis of cardiac tissue in these animals, not a single donor HSC-derived cardiomyocyte was detected. In a parallel series of experiments, they used a parabiotic mouse model to look for chimerism in non-haematopoietic tissues. GFP-transgenic/wild-type parabionts developed substantial haematopoietic chimerism over the study period, but chimerism was not noted in non-haematopoietic tissue.

In 2002, further evidence emerged in support of the concept of fusion emerged as an alternative explanation for transdifferentiation events. Both Terada et al,<sup>159</sup> and Ying et al,<sup>160</sup> found that in co-culture experiments with bone marrow cells and embryonic stem cells or neural cells and embryonic stem cells respectively,

tetraploid hybrids could be detected that adopt embryonic stem cell characteristics. Using a Cre-lox recombination system to detect cell fusion events in lethally irradiated mice transplanted with labelled bone marrow cells, Alvarez-Dolado et al, identified fusion of bone marrow cells with Purkinje cells, hepatocytes and cardiomyocytes<sup>161</sup>. Camargo et al, also used a single HSC transplant model to demonstrate that HSC-derived hepatocytes in fact occur as a result of cell fusion<sup>162</sup>. Interestingly, Oh et al, found that Sca1+ cardiac progenitor cells delivered intravenously 6 h after cardiac ischaemia/reperfusion differentiate into myocytes within the infarct border-zone, with approximately half of these cells representing fusion products between native myocytes and the infused stem cells<sup>97</sup>. In this model, they were unable to determine whether fusion preceded differentiation or vice versa.

Re-examining the data on myocardial regeneration with HSC-enriched bone marrow and contemplating the human solid organ transplant experience, it is clear that only a minority of cells persist in the long-term following myocardial delivery.

In response to Orlic et al's study of 2001, which appeared to demonstrate that local implantation of HSC-enriched bone marrow into recently infarcted myocardium resulted in myocardial regeneration, several groups attempted to reproduce and extend these unexpected findings. The controversy surrounding Orlic's work was not really the identification of some of the  $1.5 \times 10^5$  Lin<sup>-</sup>c-kit<sup>+</sup> bone marrow cells injected into the border-zone myocardium, but the purported

identification of extensive regeneration of myocardium at the 9-day post-infarction study point.

Balsam's lab performed a series of experiments to examine the ability of c-kit-enriched bone marrow, Lin<sup>-</sup>c-kit<sup>+</sup> bone marrow, and purified HSCs to regenerate myocardium after infarction<sup>94</sup>. After inducing myocardial ischaemia by LAD ligation in mice, donor bone marrow cells were implanted into the border-zone myocardium. Histological analysis using confocal microscopy was performed at 10 and 30 days after infarction. Ten days after infarction, large number of cells were found in the areas of injection, but that by 30 days, the number of cells was much fewer.

Thus 'engraftment' into the myocardium was not stable, but rather was transient. Moreover, the donor cells identified were small and round with a haematopoietic phenotype. By co-staining with a variety of markers, many of the donor cells in the c-kit-enriched donor group stained with the pan-haematopoietic marker CD45; in addition, nearly all of the donor cells in the Lin<sup>-</sup>c-kit<sup>+</sup> and HSC donor groups were CD45<sup>+</sup> and stained with the myeloid (neutrophil) marker Gr-1. Balsam concluded that, when bone marrow progenitors and HSCs are implanted into ischaemic myocardium, they do not transdifferentiate into cardiomyocytes; but instead adopt traditional haematopoietic fates, differentiating primarily into neutrophils.

One hypothesis to explain this functional benefit in the absence of myocardial regeneration is that cell treatment may result in increased angiogenesis through local release of growth factors.

Nygren et al,<sup>95</sup> published similar results for a series of experiments studying the fate of implanted bone marrow into ischaemic myocardium in mice. Using labelled whole bone marrow and Lin<sup>-</sup>c-kit<sup>+</sup> cells as donor cells, they found that cells implanted into ischaemic myocardium after LAD ligation or cryoinjury maintained haematopoietic characteristics. Using both a 9-day and 28-day study point, they found transient engraftment of CD45<sup>+</sup> cells at the sites of injection; that is, many more donor cells were noted at the 9-day study point when compared with at 28 days. Twenty-eight days after infarction, the majority of engrafted GFP<sup>+</sup> cells within the heart were also CD45<sup>+</sup>. Rare GFP<sup>+</sup> cells in the myocardium stained with cardiomyocyte markers (0.75% of all GFP<sup>+</sup> cells analysed), but these were all lacZ<sup>+</sup> as well. This provided conclusive evidence that bone-marrow-derived cardiomyocytes were products of cell fusion rather than transdifferentiation.

Finally, Murry et al, used a genetic reporter system to demonstrate that local implantation of HSC-enriched bone marrow into recently infarcted myocardium does not result in myocardial regeneration<sup>96</sup>. Mice were studied 1 to 4 weeks after infarction and implantation of Lin<sup>-</sup>c-kit<sup>+</sup> cells into the peri-infarct zone; only small round donor-derived cells were noted, and none of these co-stained with cardiomyocyte markers. Murry's group also studied the ability of circulating

bone-marrow-derived cells to regenerate myocardium after infarction using GFP+ bone marrow chimaeric mice. The hearts were studied at 1 week to 2 months after LAD ligation, and they found that only rare GFP-positive cardiomyocytes (1 to 3 cells/100,000 cardiomyocytes = 0.001%). This study however was not designed to answer whether fusion or transdifferentiation resulted in this low level of cardiomyocyte repopulation.

These three complementary studies raised many questions about the reproducibility and validity of the original bone marrow reports. Combined, they consistently demonstrate that local implantation of HSC-enriched bone marrow into ischaemic myocardium does not result in myocardial regeneration. Very low-level myocardial regeneration from bone marrow may occur after infarction, with or without peripheral stem cell mobilization, and this is probably a consequence of cell fusion. These mechanistic studies are of particular importance and suggest that, although such cell therapy may be of functional benefit to patients, the mechanism for improvement is not myocardial regeneration.

## **1.5.10 Potential cardiovascular applications of stem cell therapy**

### **Non-ischaemic cardiac disease**

#### *Adult*

Considering that functional benefits of cell transplantation have also been observed in animals with dilated cardiomyopathy<sup>163,164</sup> future trials may want to explore the role of cell therapy in patients with non-ischaemic heart failure.

Anecdotal reports of recovery of cardiac function following cardiotoxic chemotherapy followed by bone marrow or cord blood transplantation would also lend credence to the possibility of stem cell repopulation.

#### *Congenital heart disease*

- Cell-based myocardial therapy

Modulation of gene upregulation in response to increased workload

Stimulation of resident cardiac stem cells

- Tissue engineering of valves or conduits

As mentioned earlier, it is becoming increasingly recognised that congenital heart disease may occur either as a result of deficiencies or alterations in developmental pathways leading to suboptimal phenotypes with regards cardiomyocyte number and extracellular composition. Often these patients must be palliated with a less than physiological arrangement, for example with a systemic morphological right ventricle.

It takes only a small mind shift from an emphasis on regeneration in the adult to augmentation of the cardiovascular situation in the pediatric field. Opportunities for antenatal intervention are limited since by the time a fetal echocardiogram has been performed with a sufficient degree of certainty the practicalities of any intervention other than practical, flow-directed ones have missed the boat and almost certainly, “rescue” of the secondary heart field would be speculative.

With this in mind, the favourable modulation of natural history may be possible by supplementation with autologous or allogeneic stem cells with the intention of repopulating the heart with new cardiomyocytes or augmenting cardiac growth and function in the face of increased functional demands.

## **2.0 Differences in extra-cellular matrix and myocyte homeostasis between the neonatal right ventricle in Hypoplastic Left Heart Syndrome and Truncus arteriosus**

### **2.1 Abstract**

#### **Objectives**

The right ventricle in hypoplastic left heart syndrome (HLHS) works at systemic pressure and large volume loading before and after 1st stage palliation. There is a paucity of information regarding the intrinsic characteristics of the right ventricle in HLHS. We studied extra-cellular matrix composition, myocyte homeostasis and gene expression in right ventricular biopsies obtained from patients with HLHS undergoing neonatal 1st stage palliation and from patients undergoing neonatal truncus arteriosus repair.

#### **Methods**

Tissue was evaluated using histological and real time PCR techniques using the truncus group used as a comparative group. Mean difference in outcomes between the HLHS and truncus groups were estimated using linear regression models in unadjusted and age-adjusted analyses.

## **Results**

Markers of cell proliferation, apoptosis and fibronectin were significantly higher in the right ventricular myocardium of patients with hypoplastic left heart syndrome compared to truncus arteriosus. Type I collagen content and NKX2.5 expression were significantly lower in HLHS than the truncus group.

## **Conclusion**

The neonatal right ventricle in HLHS demonstrates a number of intrinsic differences compared to the right ventricle in truncus arteriosus including relative immaturity of the extracellular matrix, inappropriately low transcription factor expression and increased myocyte apoptosis.

## 2.2 Introduction

The right ventricle (RV) in hypoplastic left heart syndrome (HLHS) works at systemic pressure and increased volume loading before and after stage 1 palliation. There is growing evidence that the right ventricle of palliated HLHS patients has reduced function compared to other univentricular hearts with similar parallel circulations<sup>165-167</sup>. Despite innovations in surgical techniques and intensive care<sup>3,4,168</sup>, HLHS patients experience a more precarious course and persistent risk of early death than other palliated complex cardiac anomalies.

Little is known regarding the intrinsic structure of the right ventricular myocardium in HLHS. Analysis of archived HLHS specimens has identified reduced cardiac extracellular matrix collagen content that might contribute to myocyte slippage and suboptimal ventricular function<sup>169</sup>. Changes in myocyte homeostasis characterised by increased apoptosis and progressive net myocyte loss are known to contribute to ventricular dysfunction in both ischemic and non-ischaemic heart disease<sup>170,171</sup>. Differing myocardial composition and homeostasis in HLHS might affect the right ventricle's ability to adapt to the obligatory volume loading, increased wall stress and changes in coronary perfusion that accompany stage I palliation.

The modification of the originally described Norwood procedure to include a restrictive RV to PA conduit has also allowed access to right ventricular biopsies. The right ventricle in neonates with truncus arteriosus works under similar workloads with parallel circulations and large volume loading. Neonatal

repair of truncus arteriosus is the only procedure that provides access to RV samples both of similar age *and* where the loading conditions of the RV are similar to the un-palliated HLHS. The purpose of this cross-sectional comparative study was to ascertain whether the intrinsic properties of the right ventricular myocardium differ between patients with HLHS and truncus arteriosus (TA) in order to improve understanding of the response of the right ventricle to surgery and increased load.

## 2.3 Methods

### *Patient data*

Right ventricular myocardial biopsies were obtained from infants undergoing stage I Norwood procedure for typical HLHS (n=14) or neonatal repair of truncus arteriosus (n=7). Diagnosis of hypoplastic left heart syndrome was made by two-dimensional echocardiography and required underdevelopment of the left heart with significant hypoplasia of the left ventricle including atresia, stenosis or hypoplasia of the aortic or mitral valves, hypoplasia of the ascending aorta and aortic arch with duct-dependent systemic circulation and retrograde flow in the aortic arch. Patients with unbalanced atrioventricular septal defect or univentricular hearts with subaortic stenosis requiring Damus-Kaye-Stansel connection and shunt palliation in the neonatal period were excluded. Six patients with truncus arteriosus were of type 1 variant and one type 4. Median age at operation for HLHS and TA patients was 3.0 days (range, 2 to 8) and 14.0 days (range, 5 to 44), respectively. All patients were born after 38 weeks of gestation. One patient in the HLHS group had gut malrotation which required later correction. Two truncus arteriosus patients had 22q deletion and another had Di George syndrome. The study was approved by the Royal Children's Hospital Ethics in Human Research Committee and parents gave informed consent.

**Table 2.1: Patient demographics**

	HLHS (n=14)	Truncus (n=7)	<i>p</i> value
Male gender (n)	10	3	NA
Age at operation (days)	3.0 (2 to 8)	14.0 (5 to 44)	<0.001
Weight (kg)	3.3 (0.55)	2.75 (0.43)	0.09
BSA (m <sup>2</sup> )	0.20 (0.02)	0.18 (0.02)	0.28

BSA: Body surface area

Age, weight and BSA data presented as mean (SD) or median (range)

p-values based on *t*-test for weight and BSA on Mann-Whitney test for age at operation.

### *Operative details*

All stage I reconstruction procedures for HLHS were performed using right ventricle to pulmonary artery conduits as the source of pulmonary blood flow. Norwood operations were conducted using continuous cardiopulmonary bypass at a systemic temperature of 25°C. A 3.5mm diameter polytetrafluoroethylene graft was anastomosed end-to-side to the innominate artery and a cannula inserted at this level initially to maintain systemic perfusion. During arch repair the innominate artery was clamped and flow maintained through this cannula to provide antegrade regional perfusion at 30% of minimum predicted flow guided by inline and right upper limb arterial pressures. Coronary and lower body systemic perfusion during this phase was maintained via separate 2mm and 4mm diameter olive-tipped cannulae snared within the ascending and descending aorta, respectively to permit arch reconstruction with a beating heart. Repair of truncus arteriosus was carried out in the first month of life in keeping with the centre's routine practice, using aortobicaval cardiopulmonary bypass at 32°C. Intermittent antegrade cold blood cardioplegia was used in both groups; after giving half of the induction dose of cardioplegia at 32°C, the remainder together with further maintenance doses at 20 minute intervals were given at 24°C. Thus the myocardium in both groups was unloaded and arrested at equivalent temperatures. For a diagrammatic summary of the series of operations performed for HLHS and truncus arteriosus, please see Figures S2.1 and S2.2, respectively in the supplementary data section.

Transmural cylindrical cores of tissue were obtained from equivalent regions of the right ventricular free wall selected for ventriculotomy, below the pulmonary valve and adjacent to the left anterior descending artery. Samples were assigned a code to blind their origin and snap frozen in liquid nitrogen to optimally preserve immunogenicity until analysis.

### *Immunohistochemistry*

5 $\mu$ m sections from HLHS and TA tissue were mounted side-by-side on silane slides and fixed with 4% paraformaldehyde. Sections underwent treatment for antigen retrieval and were then incubated in a blocking solution to reduce non-specific binding. For each investigational target, a further 10 sections were prepared from the mesocardial layer of each sample. In all cases sections were double-stained with antibodies of different species origin using the second stain as an internal control to verify adequate preparation and staining. If the accompanying target did not stain correctly the run was repeated.

Primary antibodies were: ACAM/N-cadherin,  $\alpha$ -sarcomeric actin, connexin-43 (all Sigma-Aldrich, Castle Hill, Australia), caspase-3 active (R&D Systems, Minneapolis, USA), collagen types I and III (Southern Biotech, Birmingham, USA), desmin, fibronectin (both DakoCytomation, Denmark), heavy chain cardiac myosin (Abcam, Cambridge, UK), NKX2.5 (Santa Cruz Biotech, Santa Cruz, USA), phosphorylated histone H3 (Upstate, Charlottesville, USA) and

human troponin T (Chemicon, Temecula, USA). Secondary antibodies conjugated to the fluorophores Alexa Fluor 488 or 594 (Becton Dickinson) were used. Co-localisation of cardiac heavy chain myosin or troponin T with other markers of interest was used to verify their myocytic disposition.

#### *DNA-strand breaks in myocytes*

Myocyte apoptosis was also investigated by labelling nuclear DNA strand breaks with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) and counterstaining nuclei with 4',6-diamidino-2-phenylindole using a proprietary kit (Roche Diagnostics, Castle Hill, Australia). Tissue was labelled for TUNEL and myosin or desmin to delineate cell type. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlington, USA). The total number of cardiomyocyte nuclei in ten random high-power fields was identified, and the percentage of these containing TUNEL-labelled DNA breaks calculated.

#### *Image capture and analysis*

Slides were evaluated using fluorescent inverted (Olympus IX70, Olympus Corp., Tokyo, Japan) and laser scanning confocal microscopy (Leica TCS SP2 SE, Leica Microsystems GmbH, Wetzlar, Germany) with proprietary software (Spot version 3.4.2, Diagnostic Instruments Inc, Sterling Heights, USA) without gamma correction. Background levels of autofluorescence on unstained sections and those incubated with secondary antibodies only were used as controls. Images of the microscope field were acquired using a monochromatic camera

and proprietary software (Spot version 3.4.2, Diagnostic Instruments Inc, Sterling Heights, USA) without gamma correction. Once established for each target of investigation, microscope settings were retained throughout the series. Ten random fields were recorded from each section for later analysis. The microscope operator was blinded to the origin of the sections. Monochromatic image content was evaluated using an automated counting program and pre-specified morphometric criteria (ImagePro Plus, Media Cybernetics, Silverspring, USA). Mean intensity per microscope field was also recorded as in some circumstances this measurement better represented tissue staining. To avoid overestimation, areas immediately adjacent to blood vessels were excluded from assessment. False colours were later assigned to aid recognition upon merging images of the multiply-stained sections.

#### *Real-time Polymerase Chain Reaction*

Total RNA was extracted from samples of right ventricular myocardium from both patient groups using RNeasy (Qiagen) and reverse transcribed into cDNA. One  $\mu\text{g}$  of RNA sample was reverse transcribed using MMLV Reverse transcriptase (Promega Corporation) and cDNA stored at  $-20^{\circ}\text{C}$ . Intron-spanning primer sequences were selected for  $\beta_2$  microglobulin and NKX2.5; primer sequences are available in the Data Supplement. Real-time polymerase chain reaction (PCR) assays were carried out in triplicate using SYBR Green Master Mix (Sigma Aldrich) and a real-time PCR thermal cycler/fluorescence detection system (Rotor-Gene 3000, Rotor Gene Pty, Australia).  $25\mu\text{L}$  PCR

reactions included 12.5 $\mu$ L of SYBR Green 2x Master Mix (Sigma Aldrich) and 1.0 $\mu$ L of cDNA. Melting curve analysis was performed after the final PCR cycle to check for the presence of non-specific PCR products and primer dimers. We analysed relative differences in  $2^{-\Delta\Delta CT}$  values after normalising data using  $\beta_2$  microglobulin as a housekeeping gene.

## **2.4 Data analysis**

Mean scores of intensity and count derived from histological data were calculated for each patient. These mean scores were used as the observations in our analyses to circumvent issues with analysing correlated data<sup>172</sup>. The distribution of the outcome variables was examined using normal probability plots. Means and standard deviations are presented for the outcomes separately for each group. Linear regression was used to compare the mean outcome between the HLHS and truncus groups in unadjusted analyses and analyses that were adjusted for age as a linear effect. Partial residual plots did not suggest that the effect of age was non-linear for any of the 14 outcomes. There was little overlap in age between the comparison groups and the appropriateness of using linear regression to adjust for age relies on the relationship between outcome and age being the same in each comparison group. In order to assess this, scatterplots were drawn. Because some of the outcomes were not normally distributed and the sample size was small, bias corrected accelerated (BCA) bootstrap confidence intervals<sup>173</sup> were constructed

to validate the assumptions underlying the confidence intervals from the linear regressions. The non-parametric bootstrap algorithm was used to construct 2000 bootstrap datasets for each analysis. Where the bootstrap intervals were similar to those from the main analyses the latter results are presented. Where they were considered to be different the bootstrap intervals are presented. All data were analysed using Stata 9.0 (StataCorp, College Station, Texas, USA).

2.5 Results

Table 2.2 shows the results of image analysis for each of the variables, displaying mean count, mean intensity or percentage TUNEL positive nuclei per random microscope field.

**Table 2.2:** Results of immunofluorescent and histochemical analysis

Marker		Unadjusted		Adjusted			<i>p</i>
		HLHS	Truncus	Mean	Mean	95% confidence	
		Mean (SD)	Mean (SD)	difference	difference	intervals	
Caspase-3	C	65.8 (24.4)	34.8 (19.3)	30.9	19.0	-11.9 to 49.9	0.007
	I	36.3 (6.8)	23.4 (3.1)	12.9	11.8	3.59 to 20.0	
TUNEL	%	1.43 (0.36)	0.67 (0.19)	0.76	0.70	0.26 to 1.14	0.004
PH3	C	103.3 (33.6)	77.8 (26.5)	25.4	46.6	12.8 to 95.4	0.03
	I	39.3 (13.1)	30.7 (7.3)	8.7	6.2	-10.0 to 22.3	
Collagen I	C	165.1 (21.2)	218.7 (15.5)	-53.7	-45.5	-76.8 to -27.8	
	I	40.7 (3.0)	40.8 (4.4)	-0.1	-2.3	-7.0 to 2.4	
Collagen III	C	41.3 (15.0)	70.8 (32.6)	-29.4	-19.3	-60.0 to 49.2	
	I	31.6 (3.4)	34.4 (4.9)	-2.8	0.7	-4.3 to 5.6	
Fibronectin	C	417.8 (58.6)	303.5 (120.5)	114.3	159.5	-12.0 to 314.3	<0.001
	I	50.8 (3.2)	35.3 (6.6)	15.56	17.2	10.9 to 23.5	
NKX2.5	C	118.2 (14.9)	204.7 (13.8)	-86.5	-62.5	-60.1 to 186.2	0.02
	I	26.8 (4.3)	35.2 (4.7)	-9.4	-7.9	-14.1 to -1.7	

I Microscope field's mean intensity

C Automated count for discrete/nuclear activity

PH3 Phosphohistone H3

\* Where bootstrap method used,  $p$  value not shown

95% confidence intervals not containing zero imply  $p < 0.05$

### *Cell replication and apoptosis*

Cell replication, as indicated by the presence of phosphohistone-H3, was significantly higher in the HLHS group compared to TA (mean count 103.3 vs. 77.8; age-adjusted  $p=0.03$ ) as depicted in Figures 2.1a, 2.1b, 2.1c. To investigate myocyte apoptosis in HLHS, separate heart sections were stained for caspase-3 active or TUNEL. Apoptosis was assessed by staining for caspase-3 active, a key apoptotic mediator and member of a multigene family of cysteine proteases that cleave key cellular proteins, leading to the apoptotic demise of cells. Mean intensity of caspase-3 active staining in the HLHS group was significantly higher compared to TA (mean intensity 36.3 vs. 23.4; age-adjusted  $p=0.007$ , Figure 2.2). The frequency of myocyte apoptosis in the truncus arteriosus group as demonstrated by TUNEL staining was 0.67% of cardiac nuclei. In contrast, the frequency of myocyte apoptosis in HLHS tissue was 1.43%, twice as high as in TA (age-adjusted  $p=0.004$ ) (Figures 2.3a, 2.3b). These data demonstrate that myocyte apoptosis is higher in HLHS than in TA.

### *Extracellular matrix*

Type I collagen content was significantly lower in HLHS compared to TA, (mean count 165.1 vs. 218.7; BCA 95% confidence intervals -76.82 to -27.77, figures 2.1a, 2.1b, 2.4). This was accompanied by increased fibronectin content in HLHS (mean intensity 50.8 vs. 35.3; age-adjusted  $p<0.001$ ) as shown in figure 2.5. There were no significant differences in type III collagen nor laminin.

### *Cardiac transcription factors*

Semi-quantitative indirect immunofluorescent staining for the cellular expression of NKX2.5 was significantly lower in HLHS compared to TA (mean intensity 26.8 vs. 35.2; age-adjusted  $p=0.02$ ) as shown in Figure 2.6a. Real time PCR showed that NKX2.5 expression was significantly lower in HLHS compared to TA (mean relative concentration 0.62 vs. 0.98; age-adjusted  $p=0.04$ ). Figures 2.6b and 2.6c.

### *Cardiac proteins*

There were no statistically significant differences in the relative content of cardiac (heavy-chain myosin, troponin T), intermediate (desmin), transmembrane proteins (ACAM) nor connexin-43.

## 2.6 Discussion

Outcomes for staged surgical reconstruction for HLHS have improved<sup>174,175</sup>, however patients continue to experience impaired systemic cardiac output, RV dysfunction and tricuspid regurgitation, leading to increased risks of morbidity and mortality. Whilst there is a paucity of knowledge concerning the myocardial composition of the right ventricle in HLHS, imaging studies have demonstrated impaired right ventricular function in patients with HLHS compared to other single-ventricle physiologies<sup>166,167,176</sup>. In this study, we have shown the composition of the right ventricular myocardium in HLHS to differ significantly compared to truncus arteriosus despite similar loading conditions even when mitigating for age.

### *Cardiac development and transcription factors*

During fetal development, myocardial growth is co-ordinated by transcription factors including NKX2.5 which orchestrate developmental gene expression, cardiac-lineage determination and the formation of the multi-chambered heart<sup>177</sup>. Myocardial NKX2.5 expression normally undergoes a temporal decline from embryogenesis onwards, remaining at low levels post-natally where it assists in maintaining cardiac phenotype and facilitates responses to changing functional demands such as pressure overload<sup>178,179</sup>.

Knock-out murine models have highlighted the importance of NKX2.5 in normal structural development<sup>180</sup>, however similar mutations are relatively uncommon in clinically-pertinent structural phenotypes<sup>7</sup> which are likely to originate from interactions between multiple genetic and environmental influences. In this study, we found NKX2.5 expression to be significantly lower in the right ventricular myocardium of HLHS patients compared to those with TA, even when adjusting for age and despite similar right ventricular loading conditions. These differences are contrary to predicted chronological changes where higher levels of NKX2.5 would be expected in the younger HLHS group. It is therefore plausible that NKX2.5 expression either makes a positive contribution to a dynamic ventricular phenotype including adaptation to loading conditions, or that it is widely indicative of a chain of appropriate adaptive responses<sup>181</sup>.

### *Cardiomyocyte homeostasis*

The transition from late intra-uterine to neonatal life is normally accompanied by an increase in myocardial mass achieved principally by cellular hypertrophy with simultaneous cardiomyocyte cycling and renewal to aid adaptation to changes in hemodynamic workload<sup>182</sup>. Increased cell replication and apoptosis in the HLHS group, as manifest by increased expression of phosphohistone-H3 and caspase-3 active together with TUNEL staining may reflect attempts at adaptation in response to increased loading conditions simultaneous to ongoing myocyte loss. These findings, when considered with the reduced expression of NKX2.5 observed in the HLHS group suggests a different myocardial phenotype

compared to the truncus arteriosus group. As alluded to above, reduced levels of NKX2.5 may diminish recruitment and proliferation of new, and maintenance of existing cardiomyocytes or inhibit rescue from apoptosis, a phenomenon previously observed in the development of the cardiac conduction system of NKX2.5 knock-out mice<sup>46</sup>.

### *Extracellular matrix*

The extracellular matrix provides structural support to the myocardial interstitium, maintaining myocyte alignment, minimising slippage and permitting interdigitation throughout the cardiac cycle. The heart's principal extracellular matrix components are collagen types I and III and fibronectin. Typically extracellular matrix in the late embryonic period exhibits a shift in the fibronectin:collagen ratio in favour of collagen which serves to increase tissue robustness<sup>183</sup>, a phenomenon which also usually occurs in right ventricular hypertrophy induced by pressure overload<sup>184</sup>. In this work, type I collagen content was found to be significantly lower in the mesocardium of HLHS compared to TA and accompanied by increased expression of fibronectin in HLHS. The reduced content of type I collagen and increased fibronectin in the HLHS group may represent an inability to make such a switch or relative immaturity and may lead to greater ventricular compliance.

### *Implications*

The findings of this novel work support the notion that the neonatal systemic right ventricle in HLHS may be less able to adapt to increased loading conditions than the one in truncus arteriosus. In patients undergoing surgical reconstruction for HLHS, suboptimal right ventricular geometric and myocardial characteristics are compounded by a 40% increase in the hydraulic cost of work associated with the loss of a biventricular heart<sup>185</sup>. This may contribute to the more precarious course of these infants and supports the rationale for pharmacological or mechanical off-loading following stage I procedures.

### *Limitations*

In view of the limited quantities of tissue available, we elected to use immunofluorescent histological techniques and real-time PCR to investigate several targets of interest. With immunofluorescence, quantification of the various markers does not necessarily infer activity or efficacy of the gene products themselves where applicable nor their possible post-translational modification. We did not attempt to identify and correlate pre-operative risk factors nor outcomes to histological findings between groups.

An important limitation in this study is the differing maturity of the right ventricular myocardium in the two patient groups. We chose neonatal truncus arteriosus patients as a group for comparison because the right ventricle in neonatal truncus patients also works under increased pressure and volume loading conditions that would be comparable to the right ventricle in hypoplastic

left heart syndrome. In a study of this type, it is ideal to have age-matched subjects. However, in our institution we do not perform any other surgery in the first week of life that involves the routine removal of right ventricular myocardium. Similarly it is not possible to obtain myocardium from fresh, structurally-normal neonatal hearts in Australia for ethical reasons and TA was used in preference to other neonates with right to left shunts who lack significant volume loading.

The precise intra-operative timing of the biopsies between the two groups differed slightly but significant structural changes are unlikely within this timeframe. All study patients were born at or after 38 weeks gestation so prematurity should not be a confounder. There were fewer truncus samples than HLHS. Differences in age between the two groups were unavoidable. Scatterplots of outcome against age did not indicate a similar relationship for the HLHS and truncus groups for caspase-3 intensity, NKX2.5 intensity and type I collagen count and the effectiveness of age adjustment may be questioned here. The scatterplots for PH3 count, TUNEL and fibronectin intensity, however, did suggest the use of linear regression for age adjustment was appropriate and, overall, the pattern of results indicates morphological trends not explained by expected age-related changes alone.

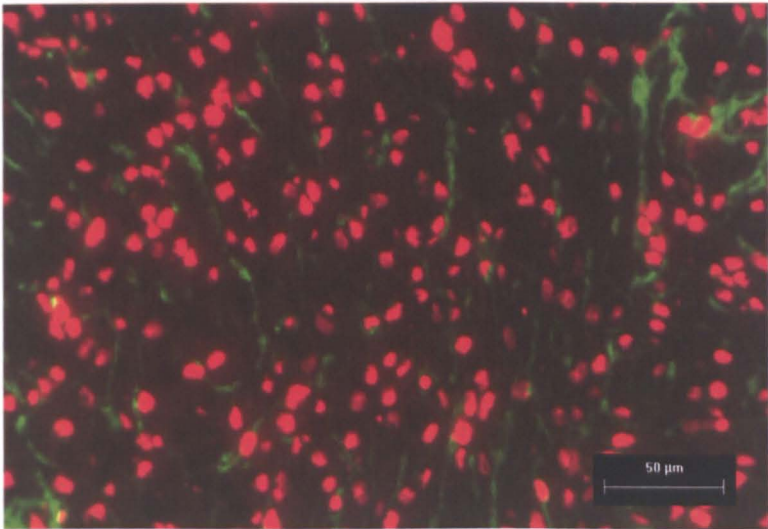
## *Conclusion*

This study has demonstrated a number of intrinsic differences between right ventricular myocardium in patients with HLHS and truncus arteriosus. Differing myocardial composition and myocyte homeostasis might affect the ability of the right ventricle to adapt to the obligatory volume loading, increased wall stress and changes in coronary perfusion that accompany stage I operations and resulting parallel circulation. Improved understanding of the composition of the right ventricle in hypoplastic left heart syndrome may help inform future treatment strategies in this group of patients.

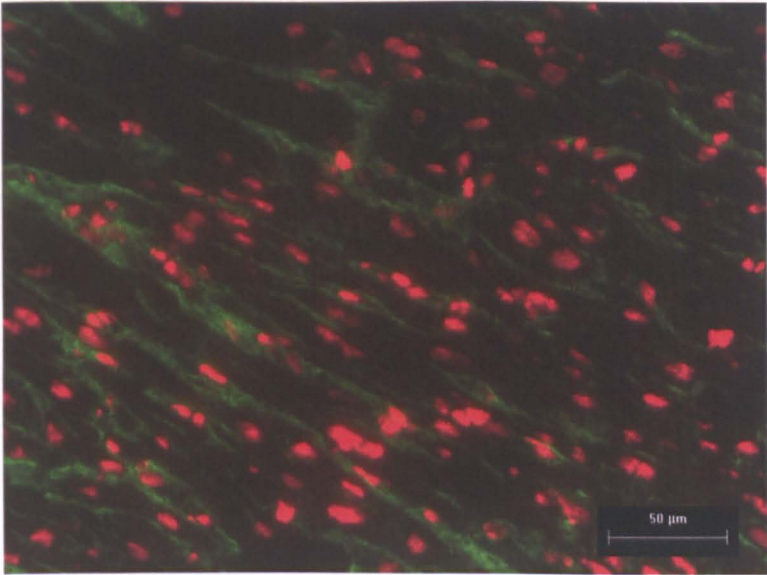
2.7      **Figures**

**Figure 2.1**    Representative photomicrographs of immunofluorescent staining for Phosphohistone H3 (red) and Type I Collagen (green); x400

**Figure 2.1a**    Hypoplastic left heart syndrome (HLHS)

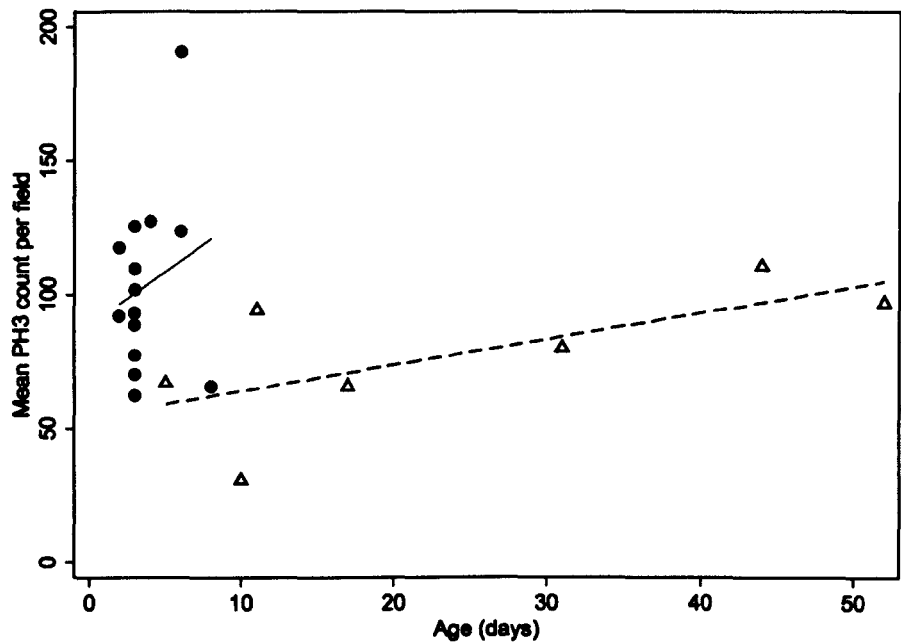


**Figure 2.1b**    Truncus arteriosus

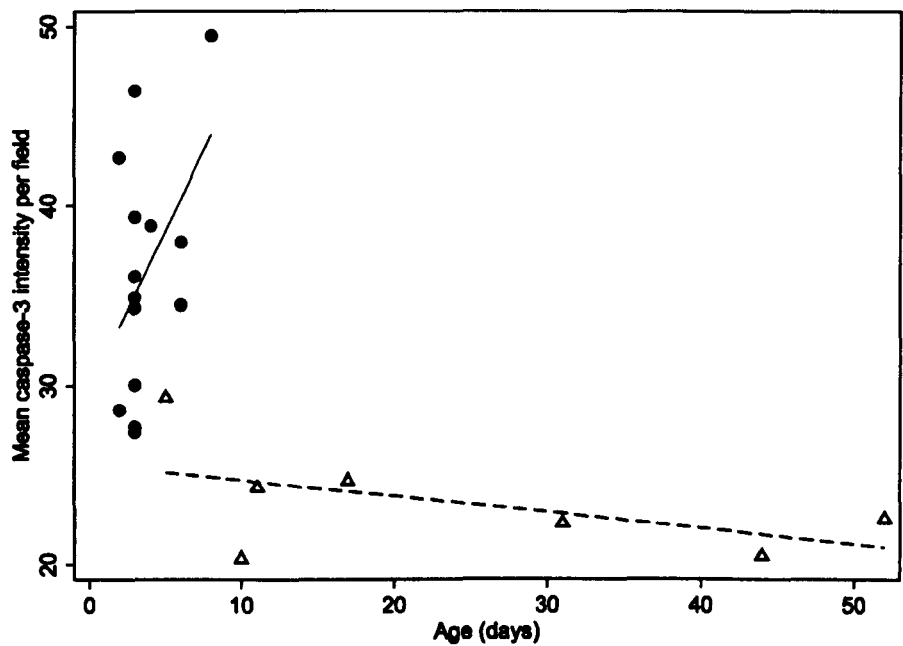


**Figure 2.1c** Scatterplot of phosphohistone H3 count against age in days.

HLHS outcomes are displayed as round dots and solid lines and  
TA with triangles and dashed lines, respectively



**Figure 2.2** Scatterplot of activated caspase-3 staining intensity in the right ventricle of patients with hypoplastic left heart syndrome and truncus arteriosus against age in days. HLHS outcomes are displayed as round dots and solid lines and TA with triangles and dashed lines, respectively.

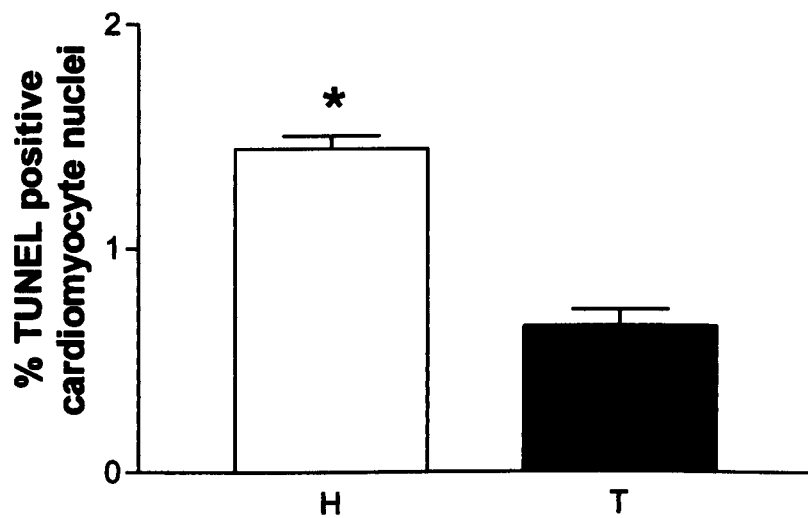


**Figure 2.3a** Percentage of TUNEL-positive cardiomyocyte nuclei in right ventricular myocardium in patients with hypoplastic left heart syndrome (H) and truncus arteriosus (T).

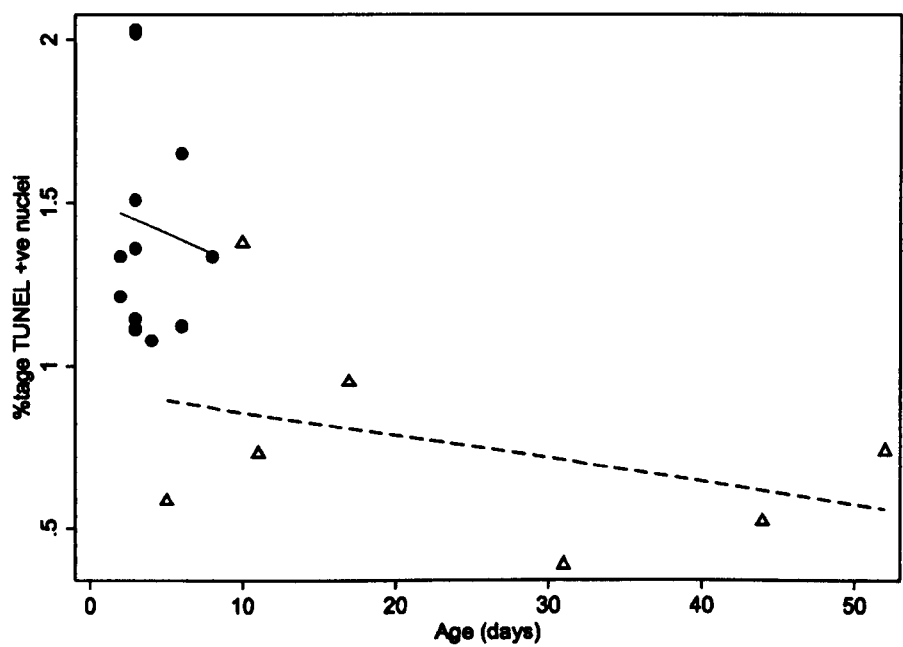
TUNEL positivity was significantly greater in HLHS than in TA.

Results are shown as mean  $\pm$  SEM.

\* significant difference where  $p < 0.05$

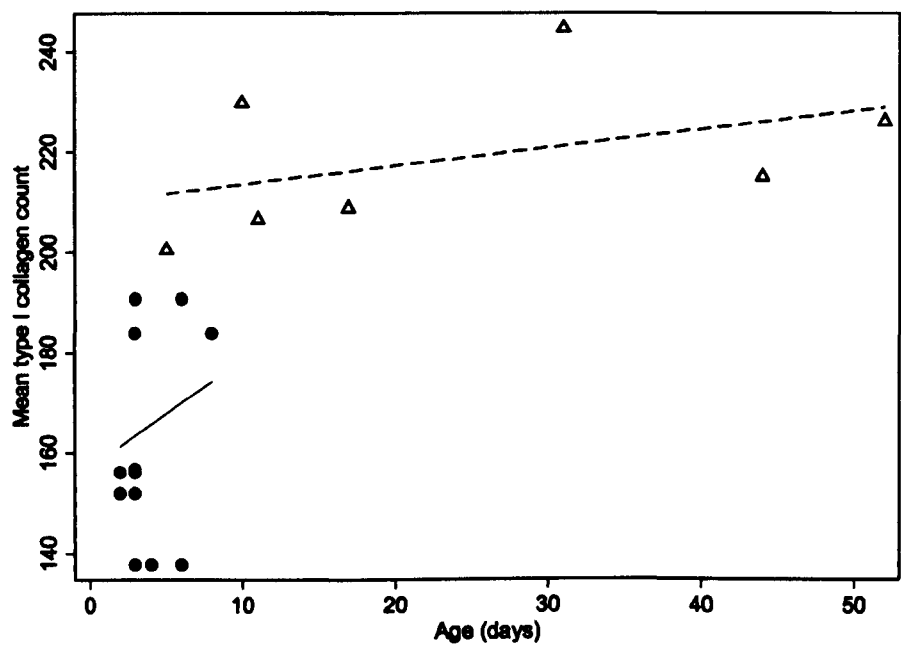


**Figure 2.3b** Scatterplot of percentage TUNEL-positive cardiomyocyte nuclei in the right ventricle of patients with hypoplastic left heart syndrome and truncus arteriosus against age in days. HLHS outcomes are displayed as round dots and solid lines and TA with triangles and dashed lines, respectively.



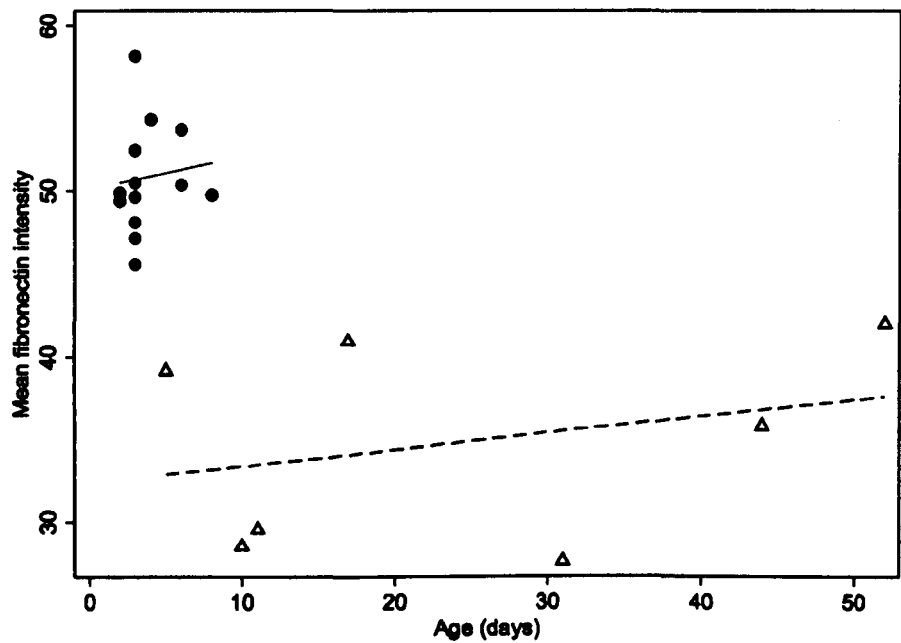
**Figure 2.4** Scatterplot of type 1 collagen count in the right ventricle of patients with hypoplastic left heart syndrome and truncus arteriosus against age in days.

HLHS outcomes are displayed as round dots and solid lines and TA with triangles and dashed lines, respectively.



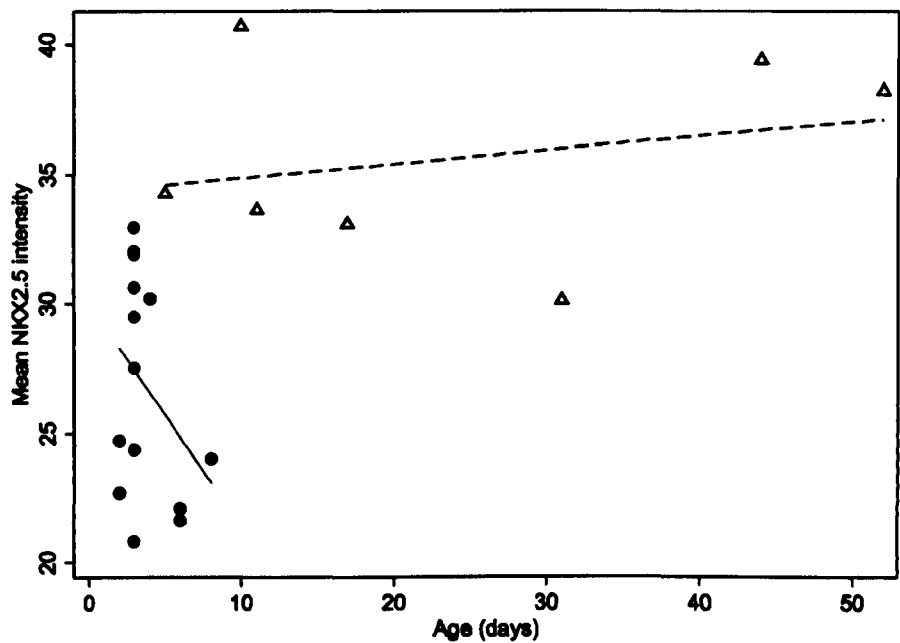
**Figure 2.5** Scatterplot of fibronectin staining intensity in the right ventricle of patients with hypoplastic left heart syndrome and truncus arteriosus against age in days.

HLHS outcomes are displayed as round dots and solid lines and TA with triangles and dashed lines, respectively.



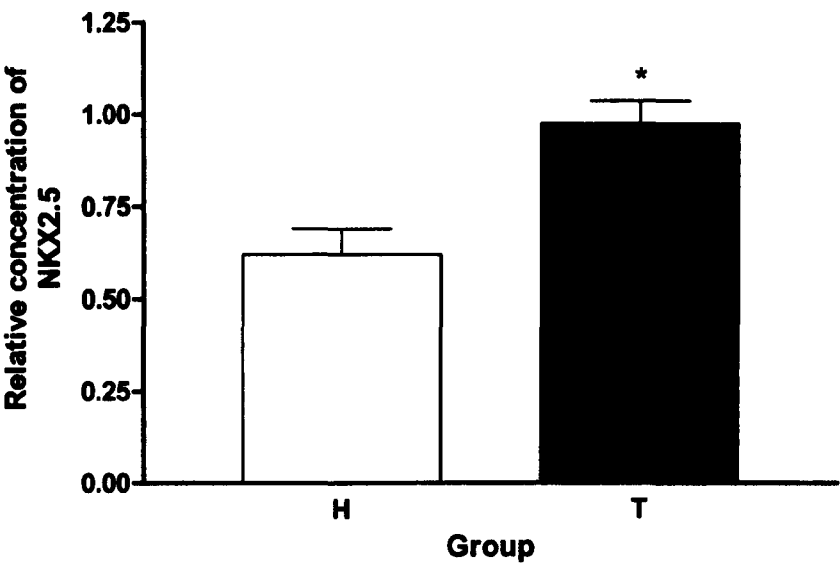
**Figure 2.6** Results of NKX2.5 studies

**Figure 2.6a** Scatterplot of NKX2.5 immunofluorescent staining intensity in the right ventricle of patients with hypoplastic left heart syndrome and truncus arteriosus against age in days. HLHS outcomes are displayed as round dots and solid lines and TA with triangles and dashed lines, respectively.

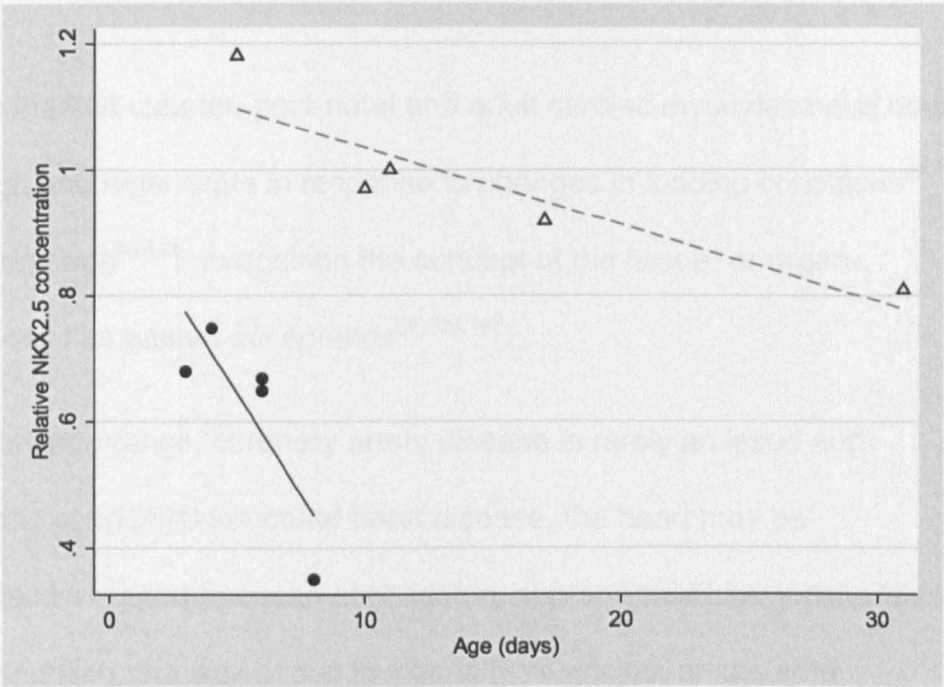


**Figure 2.6b** NKX2.5 expression as determined by real-time PCR

(Mean  $\pm$  SEM) by group, unadjusted for age. \*  $p=0.001$



**Figure 2.6c** NKX2.5 expression determined by real-time PCR against age, with linear regression lines (Truncus group boxes with dashed line, HLHS circles and solid line). Regression analysis shows significant inter-group differences for both unadjusted ( $p=0.001$ , Fig 2.6b) and age-adjusted analyses ( $p=0.04$ , Fig 2.6c)



### **3.0 Umbilical cord blood stem cells – a potential source of stem cells for ventricular augmentation in congenital heart disease?**

#### **3.1 Introduction**

As outlined in the first chapter, post-natal and adult cardiac myocytes have been shown to adapt and regenerate in response to changes in loading conditions<sup>98</sup> or ischaemic damage<sup>93,186</sup>. In addition the concept of the tissue- or organ-specific stem cell has gained acceptance<sup>99,100,187</sup>.

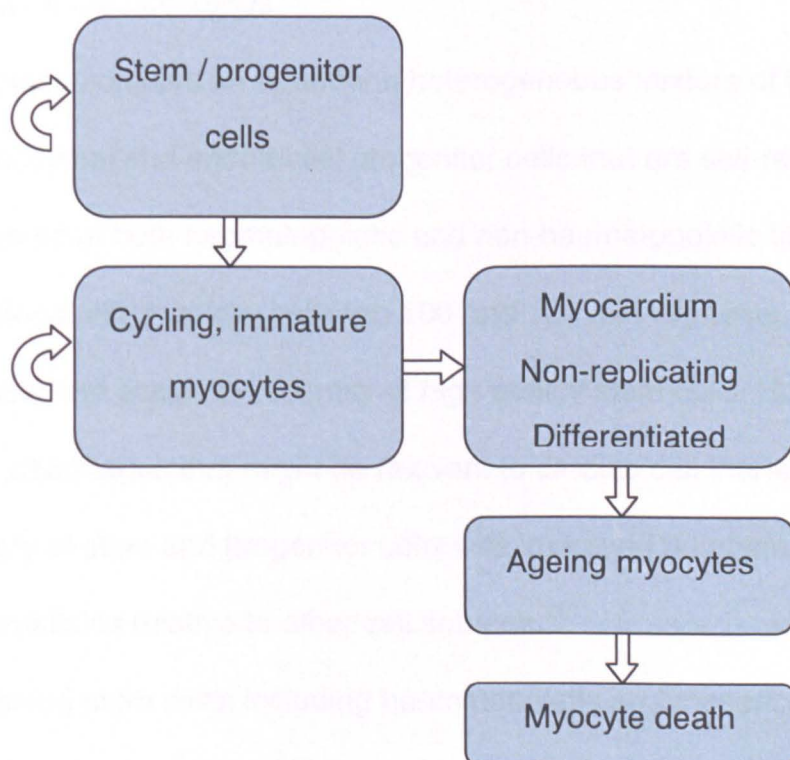
In the paediatric age range, coronary artery disease is rarely an issue and instead owing to congenital structural heart disease, the heart may be functionally disadvantaged because of obligatory supranormal haemodynamic loads due to shunting, the lack of two functionally ventricles, or acquired cardiomyopathies which directly impair myocardial function.

One avenue to approach the struggling heart could be to stimulate it by directly providing growth factors or indirectly via a paracrine effect, using a population of cells that could produce the same, irrespective of the still-disputed issues of whether such transplanted cells might integrate into the recipient via cell fusion transdifferentiation. A pharmacological precedent for such a process already exists. Clenbuterol, an anabolic steroid and  $\beta_2$  agonist, has been used to encourage an improvement in cardiac function firstly in animal models<sup>188-190</sup> and

then in human trials of heart failure combined with mechanical circulatory support<sup>191,192</sup>.

Exogenous stem cell transplantation into the heart might augment the adaptation of existing myocytes, increase cell cycling and recruit senescent cells into the functioning myocyte pool to promote a net increase in the functional myocyte mass and bring about an overall improvement in systolic and diastolic cardiac function.

**Figure 3.1** Myocardial homeostasis



### **3.1.2 Why use cord blood stem cells?**

Umbilical cord blood stem cells (CBSC) are readily available, free of ethical controversy and have an established track record as an alternative to bone marrow transplantation for stem cell reconstitution in the treatment of haematological disorders including genetic disease and haematological malignancies.<sup>193</sup> This has provided the foundations for many key stem cell biology research laboratory protocols and is remarkable for a resource once considered a redundant by-product of birth before its therapeutic potential was realised in the late 1980s.<sup>194</sup>

Cord blood contains an abundant, heterogeneous mixture of haematopoietic, mesenchymal and endothelial progenitor cells that are self-renewing, clonal precursors of both haematopoietic and non-haematopoietic tissues<sup>195</sup>. A typical cord blood unit, typically between 100 and 120 ml in volume, provides a workable and scaleable quantity of high quality stem cells. Human CBSC have many advantages that might be relevant to cardiac cell therapy: they are rich in a variety of stem and progenitor cells with improved numbers and proliferative characteristics relative to other cell sources.<sup>110</sup>

Cord blood stem cells, including haematopoietic and mesenchymal populations, can be expanded in vitro,<sup>109</sup> have the potential for enhanced self-renewal and angiogenic and myogenic differentiation<sup>196,197</sup> and can be stored for future use. Several studies have suggested that bone marrow- and cord blood-derived cells

can contribute to multiple non-haematopoietic tissues, including neurons<sup>198</sup>, hepatocytes, skeletal muscle and cardiomyocytes<sup>195,199,200</sup>. If and quite how this occurs, either through fusion events or transdifferentiation, remains contentious. It appears that there is reduced risk of rejection by the recipient's immune system with CB-derived stem cells.<sup>201</sup> Homing experiments have demonstrated that CBSCs can traffic through the intact human system to where they are required<sup>202</sup> and lineage-negative cell fractions enriched for the expression of CD34 are able to engraft in ovine and murine models over the longer term<sup>203</sup>.

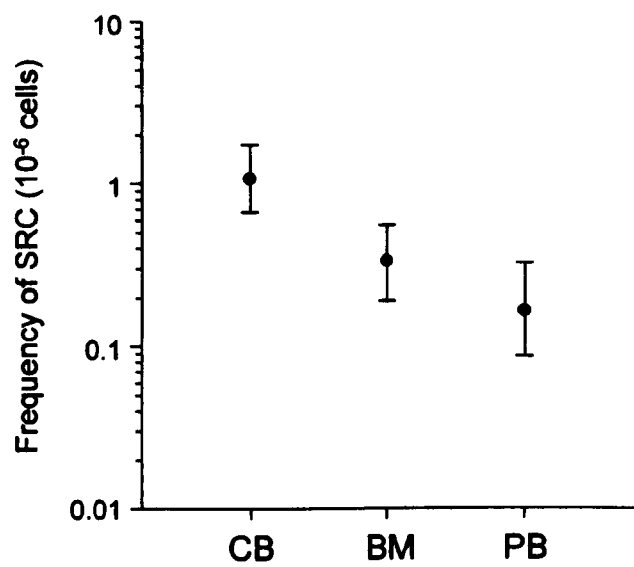
### *Haematopoietic stem cells*

Haematopoietic stem cells, which comprise a significant proportion of stem cells within cord blood,, are probably the best characterised tissue-specific stem cell. The traditional marker for immature HSC has been the CD34 antigen, a molecule with an important role in key migratory events including homing and adhesion. A long history of translational work in laboratory and clinical arenas has established some of the fundamental tenets of stem cell biology and at the same time advanced the treatment of haematological malignancies<sup>204-206</sup>. For example, differentiation, multipotentiality, and self-renewal were first demonstrated by Till and McCulloch who discovered that a subset of bone marrow cells could form macroscopic colonies upon transplantation into the spleens of lethally irradiated recipient animals<sup>207</sup>. The demand for greater mechanistic insights drove the development of technologies such as in vitro and

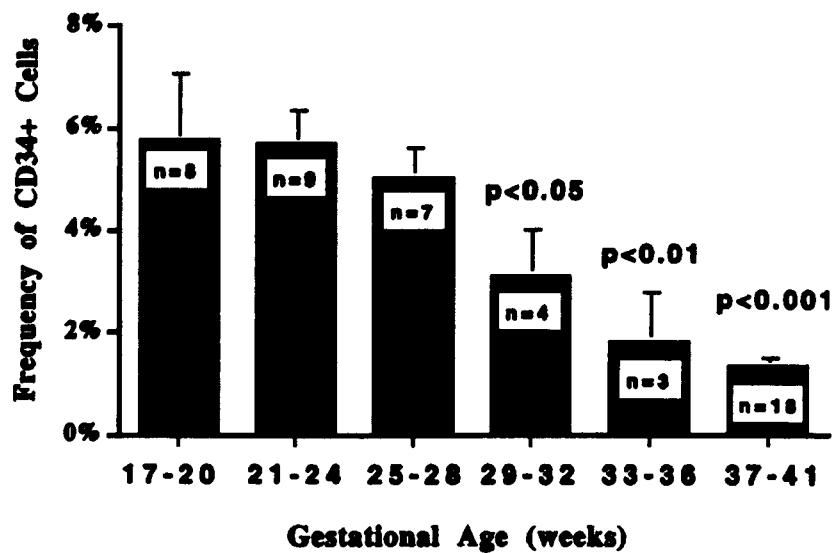
in vivo assays, fluorescence-activated cell sorting and monoclonal antibody technology, which are today taken for granted. HSCs were the first tissue-specific stem cells to be prospectively isolated<sup>208</sup> and today, heterologous bone marrow and cord blood remain the only stem cells in routine clinical use for the treatment of leukemias and autoimmune disorders<sup>209</sup>. The potential of utilising such stem cells, traditionally the preserve of haematologists, has stimulated several scientists, including those working in the cardiovascular field, to consider novel therapeutic applications for umbilical cord blood and bone marrow.

Bone marrow stem cells comprise a heterogeneous mixture of mesenchymal and haematopoietic stem cells that are relatively easy to collect, purify and store. For these reasons, bone marrow was selected for early pre-clinical<sup>92,94</sup> and clinical<sup>210</sup> work relating to ischaemic heart disease. Umbilical cord blood is arguably more attractive as a practical source of stem cells as it circumvents the ethic issues surrounding other cell types which is remarkable for a resource once considered a redundant by-product of birth before its therapeutic potential was realised in the late 1980s.<sup>194</sup> This. Cord blood contains HSC and MSC in relative abundance (see figures 3.2a and b) and a typical cord blood unit contains a workable and scaleable quantity of stem cells even at full term.

**Figure 3.1.2a**      Relative abundance of primitive human haematopoietic cells in cord blood (CB), bone marrow (BM) and mobilised peripheral circulating blood (PB), where the engrafting human cell was defined as a SCID-repopulating cell (SRC)<sup>110</sup>.



**Figure 3.1.2b**      Gestational age changes in circulating CD34<sup>+</sup>  
haematopoietic stem cells in fetal cord blood<sup>211</sup>

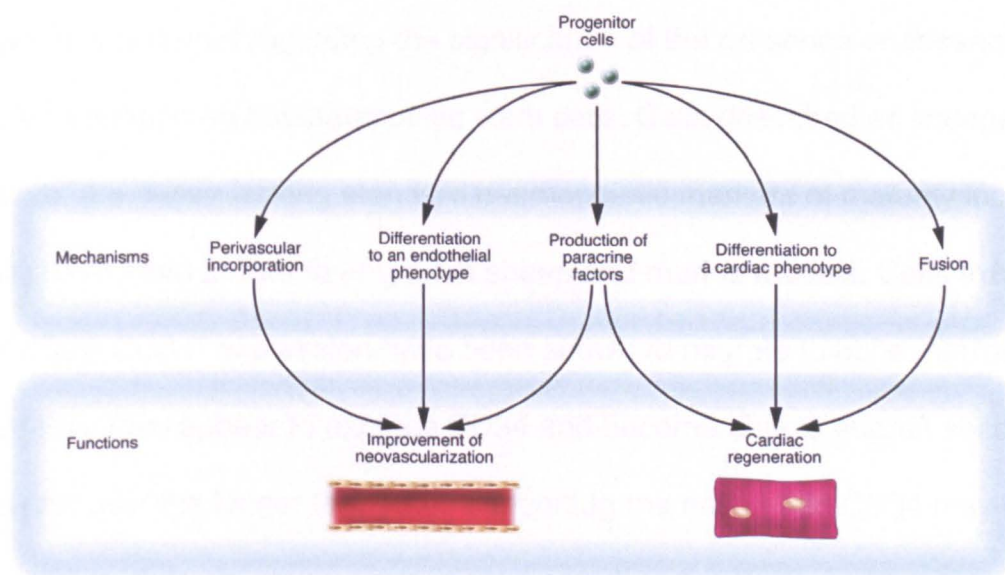


It is likely however that haematopoietic cells do what they are programmed to do and that expectations defying biological rules were not justified. Indeed several reports using HSCs in animal models of myocardial infarction demonstrated that transplanted cells maintain a haematopoietic phenotype<sup>212</sup>. Even in the absence of transdifferentiation improvements in cardiac function undeniably occur following ischaemic insults. CB-derived stem cells might improve resident stem cell function and therefore pump function in degenerative heart disease.

Other non-ischaemic forms of cardiac disease in different age groups have also benefited from cellular transplantation or cell-delivered gene therapy. Examples include the response to artificially-created dilated cardiomyopathies<sup>213</sup> or supraphysiological workload<sup>214</sup>; we have developed the latter principle in a novel, neonatal large animal model of right ventricular training:

The beneficial functional effects witnessed in these studies therefore probably results from the production of paracrine factors although the precise mechanisms remain poorly understood<sup>215</sup>.

**Figure 3.1.4** Proposed mechanisms for mediating improved cardiac function by exogeneous progenitor cells<sup>216</sup>



It is on this constantly changing background that we had to choose how best to characterise our cells. Taking a lead from existing cell therapy protocols used clinically for the treatment of haemato-oncological disease, we settled on CD34, CD33, CD38 and CD45. Criticism could be forthcoming for not including CD133 in this list, as CD133<sup>+</sup> populations are known to have the ability to develop into an endothelial lineage<sup>217</sup>. However the speculative advantages of “seeding” senescent, atherosclerotic capillary beds with such cells in a purported attempt to stimulate angiogenesis does not find conceptual favour in a non-ischaemic model. Taking a broader perspective, it is likely that a CD133<sup>+</sup> subpopulation will be included in a CD34<sup>+</sup> population anyway as their temporal changes in antigenic expression with developing maturity coincide<sup>218</sup>.

Debate has occurred regarding the significance of the presence or absence of the CD34 antigen on haematopoietic stem cells. Cells described as lineage-negative (i.e. those lacking standard haemopoietic markers of maturity including CD34) have been shown to engraft in sheep and murine models. Cells exhibiting low surface CD34<sup>+</sup> expression have been shown to migrate to bone marrow where they then appear to express CD34 and become able to engraft secondary recipients over the longer term, thus supporting the notion that CD34 may be an activation marker. However, such lineage-negative populations are extremely rare, require ex vivo expansion with liquid cultures and are at present clinically impractical.

We took a more practical pathway, committing to clinically-relevant quantities of material by using a lineage negative population depleted of mature haematopoietic cells, enriched for haematopoietic progenitors and also containing mesenchymal stem cells. From this we derived an easily handled, compact cell product that could be easily injected into the myocardium. From a cardiac perspective, CD34<sup>+</sup> cells have been shown to exhibit superior efficacy in preserving myocardial integrity and function after myocardial infarction compared to unselected circulating mononuclear cells<sup>219</sup> and in a dose-dependent manner<sup>220,221</sup>.

### **3.2 Cord blood stem cell isolation from normal pregnancies and children with Hypoplastic left heart syndrome**

As a prelude to xenotransplantation trials and also to build a bank of control data with which to compare cord blood stem cells obtained from pregnancies with congenital heart disease, I set out to obtain, characterise and store several normal human cord blood samples.

Prior to this, I undertook exploratory work looking at the feasibility of obtaining and using porcine or ovine cord blood in lieu of human blood. This proved logistically difficult and should I have pursued this further, this would have been hampered by a lack of cell surface antibody markers. Existing laboratory protocols and expertise made the use of human cells a logical choice and would also facilitate the detection of transplanted cells by exploiting their human characteristics detectable by PCR and antibody-based methodology.

Current understanding regarding the aetiology of clinically-recognisable and significant forms of congenital heart disease is characterised by a relative paucity of recognised single genetic defects. Many anomalies are thought to result from subtle mutations in regulators of heart development causing dysregulation in key transcription factor pathways in association with disturbed epigenetic phenomena such as hydraulic flow<sup>222</sup>. Therefore autologous stem cells with as yet undefined defective genotype may be suboptimal for use in

attempting to bring about favourable cardiac adaptations to increased haemodynamic load.

This report details the cell populations characterised in freshly-collected human cord blood samples. Analysed samples were selected from a pool of intact cord blood units, not rejects from cell therapy protocols nor cultured populations, which should be borne in mind when considering cell numbers for example. Cryopreserved cells were then subsequently used in a xenotransplantation neonatal model investigating the potential enhancement of right ventricular function in response to increased workload.

The aims of this work were several: to gain expertise in handling the materials, to establish a database of normal subject data, create a cryopreserved cells for later use in xenotransplantation experiments and also to compare the phenotype and enumeration and short-term culture potential of cord blood from normal subjects with that from children with HLHS.

### **3.2.1 Methods**

Informed consent was obtained from cord blood donors. Protocols were approved by the ethics review boards of the Royal Women's Hospital and the Murdoch Children's Research Institute, Melbourne, Australia.

Fresh human umbilical cord blood was obtained at delivery from consenting mothers with children with HLHS and processed within 12 hours of collection. Umbilical cord blood was diluted 1:2 with sterile phosphate buffered saline and mononuclear cells obtained by density centrifugation at 1,800rpm for 20 min (Ficoll-Paque Plus; Invitrogen) and ammonium chloride lysis. The mononuclear cell fraction was collected, counted using trypan blue exclusion and haemocytometry and  $1 \times 10^6$  cells put to one side for later use in the mononuclear cell culture plate (Figure 3.2.1).

A progenitor-enriched fraction was then obtained using immunomagnetic lineage depletion (StemSep<sup>®</sup>; StemCell Technologies). Cells were counted using trypan blue and manual haemocytometry. Representative samples of both unenriched and enriched cell suspensions were characterised using flow cytometry and three-colour combinations of monoclonal antibodies conjugated to fluorescein isothiocyanate, phycoerythrin or allophycocyanin (antibodies: human CD33, CD34, CD38, CD45) and using matched isotype-stained controls (all Becton Dickinson Biosciences, San Jose, CA, USA) to establish gates and 7-amino-actinomycin D (7-AAD) for assessment of cellular viability (Figure 3.2.2).

At least 50,000 cells were acquired with a LSR II® flow cytometer and analysed using FACSDiva software (both BD).

Growth potential of mononuclear and progenitor-enriched cells was also assessed. These were performed using commercially available culture medium and protocol (MethoCult H4434, StemCell Technologies, Vancouver, Canada). Mononuclear and progenitor-enriched cells were plated separately in duplicate and cultured for 14 days at 37°C in a humidified atmosphere with 5% carbon dioxide.  $5 \times 10^3$  cells were used in each well. Colonies were counted after this period according to their morphological characteristics by a single, experienced observer (N. Elwood) and results expressed as colonies per 1000 cells plated.

### **3.3 Results**

#### *Flow cytometric analysis of normal cord blood samples*

Flow cytometric analysis of standard lymphocyte gates of cord blood cell populations revealed a forward and side scatter characteristic of cord blood cells. The viability of these gated cells, as determined by 7-AAD was  $94\% \pm 2.5$ . Cells within the gate were then subphenotyped by CD34, CD45 and CD33/38 expression (Figure 3.3.1 A to D). Immunomagnetic negative selection resulted in a mean enrichment of CD34+/CD45+ cells of 28.0 fold (see Figure 3.3.2). The

results of enumeration and phenotyping of normal cord blood units are shown in table 3.3.1.

**Table 3.3.1** Summary of cell surface phenotyping studies on enriched cell populations (n= 50)

Surface markers		%	number
CD34+	Mean	<b>57.9</b>	2.67E+06
	SD	9.2	5.11E+05
	Median	<b>55.9</b>	2.84E+06
CD34-		<b>42.1</b>	2.02E+06
		9.2	7.90E+05
		<b>44.1</b>	1.76E+06
CD34+ CD45+		<b>59.2</b>	2.72E+06
		11.5	5.32E+05
		<b>57.4</b>	2.84E+06
CD34+ CD45-		<b>0.4</b>	1.94E+04
		0.4	1.75E+04
		<b>0.2</b>	1.13E+04
CD34+ CD33+ CD38+		<b>36.8</b>	1.77E+06
		19.0	1.08E+06
		<b>46.3</b>	1.80E+06
CD34+ CD33- CD38-		<b>28.4</b>	1.27E+06
		24.3	1.06E+06
		<b>16.9</b>	7.16E+05

Surface markers	%	number
CD34- CD45+	<b>37.8</b>	1.79E+06
	11.1	6.81E+05
	37.2	1.52E+06
CD34- CD45-	<b>6.7</b>	3.26E+05
	4.4	2.38E+05
	<b>6.2</b>	2.73E+05
CD45+	<b>91.8</b>	4.30E+06
	6.6	9.48E+05
	<b>92.5</b>	4.11E+06
CD45-	<b>8.2</b>	3.92E+05
	6.6	3.01E+05
	<b>7.6</b>	3.35E+05

*Comparison of normal and HLHS cord blood units*

50 normal cord blood units were collected and analysed, all within 4 hours of collection. Within the same time period, 8 units from HLHS births were collected. Using the data from the normal database, the flow cytometry results of the 8 HLHS samples were compared. For each of the markers of interest, frequency distributions were plotted of cell surface phenotypes, using 8 gestational-age matched controls for the 8 HLHS cord blood samples (Figure 3.3.3).

There were no significant differences between the two groups, either for number, cellular viability as assessed by 7-AAD or cell phenotypical classification (Tables 3.3.2 and 3.3.3).

**Table 3.3.2** Comparison of cell number and viability between normal and HLHS-derived cord blood units

	Normal	HLHS	P
	(n = 50)	(n = 8)	t test
<b>Number and viability</b>			
Number of mononuclear cells			
(x 10 <sup>8</sup> )	3.3 ± 1.7	3.4 ± 2.5	NS
Number of Lin <sup>+</sup> cells (x 10 <sup>6</sup> )	4.9 ± 1.3	4.7 ± 2.0	NS
Viability (%)	94 ± 2.5	94 ± 2.1	NS

**Table 3.3.3** Comparison of cell phenotype and number between normal and HLHS cord blood units

	Normal (n=50)	HLHS (n=8)	t test
CD34+ (%)	57.9 ± 9 (56)	57 ± 13 (58)	NS
absolute cell number x 10 <sup>6</sup>	2.7 ± 0.5 (2.8)	2.6 ± 1.6 (2.8)	NS
CD34- (%)	42.1 ± 9 (44)	43 ± 15 (42)	NS
absolute cell number x 10 <sup>6</sup>	2.0 ± 0.8 (1.7)	2.1 ± 1.3 (1.9)	NS
CD34+ / CD45+ (%)	59.2 ± 12 (57)	57 ± 16 (58)	NS
absolute cell number x 10 <sup>6</sup>	2.7 ± 0.5 (2.8)	2.6 ± 0.9 (2.8)	NS
CD34+ / CD45- (%)	0.4 ± 0.4 (0.2)	0.4 ± 0.8 (0.25)	NS
absolute cell number x10 <sup>4</sup>	1.9 ± 1.8 (1.1)	1.7 ± 1.8 (1.2)	NS
CD34+ / CD33+ / CD38+ (%)	36 ± 19 (46)	37 ± 21 (47)	NS
absolute cell number x 10 <sup>6</sup>	1.8 ± 1.1 (1.8)	1.7 ± 1.9 (1.8)	NS

	Normal	HLHS	t test
CD34+ / CD33- / CD38- (%)	28.4 ± 24 (17)	37 ± 27 (47)	NS
absolute cell number x 10 <sup>6</sup>	1.3 ± 1.0 (0.7)	1.2 ± 2.1 (0.7)	NS
CD34- / CD45+ (%)	37.8 ± 11 (37)	39 ± 14 (38)	NS
absolute cell number x 10 <sup>6</sup>	1.8 ± 0.7 (1.5)	1.8 ± 1.4 (1.6)	NS
CD34- / CD45- (%)	6.7 ± 4.4 (6.2)	6.3 ± 6.6 (4.8)	NS
absolute cell number x10 <sup>5</sup>	3.3 ± 2.4 (2.7)	2.9 ± 3.4 (2.2)	NS
CD45+ (%)	91.8 ± 6.6 (93)	94 ± 7.9 (94)	NS
absolute cell number x 10 <sup>6</sup>	4.3 ± 0.95 (4.1)	4.2 ± 1.1 (4.2)	NS
CD45- (%)	8.2 ± 6.6 (7.6)	6.1 ± 4.5 (6.7)	NS
absolute cell number x10 <sup>5</sup>	3.9 ± 3.0 (3.4)	2.7 ± 4.6 (2.5)	NS

All data expressed as mean ± SD (median)

% shown is percentage of lineage negative population

**Methocult colony forming assay culture**

There was no evidence of tumour formation, dysregulated growth or monoclonal expansions.

**Table 3.3. Colony enumeration**

Lin-	Normal	HLHS	Expected
	n=8	n=8	
CFU-E	13	24	9
	(0-28)	(0-49)	(1-48)
BFU-E*	0	0	104
			(1-310)
CFU-GM	128	88	115
	(0-284)	(0-212)	(1-303)
CFU-GEMM	72	58	25
	(0-127)	(0-199)	(1-59)

Mean number of colonies per 105 cells plated

Range derived from 2x standard deviations of mean

**Legend:**

- CFU-E** Colony-forming unit-erythroid, mature erythroid progenitors that require erythropoietin (EPO) for differentiation.
- BFU-E** Burst-forming unit-erythroid, more immature progenitors than CFU-E. Their low number in this context was to be expected as the immunomagnetic lineage depletion protocol should have depleted such cells.
- CFU-GM** Colony-forming unit-granulocyte, macrophage. Composed of  $\geq 20$  granulocytes (CFU-G), macrophages (CFU-M) or cells of both lineages (CFU-GM).
- CFU-GEMM** Multi-potential progenitor that produces a colony containing erythroblasts and cells of at least two other recognizable lineages.

Colony assays were unfortunately hampered by a small sample size and a wide range of “normal” expected values. In the limited data to hand, HLHS cord blood units appeared to have relatively fewer CFU-GMs and CFU-GEMMs compared to normal cord blood.

### **3.4 Discussion**

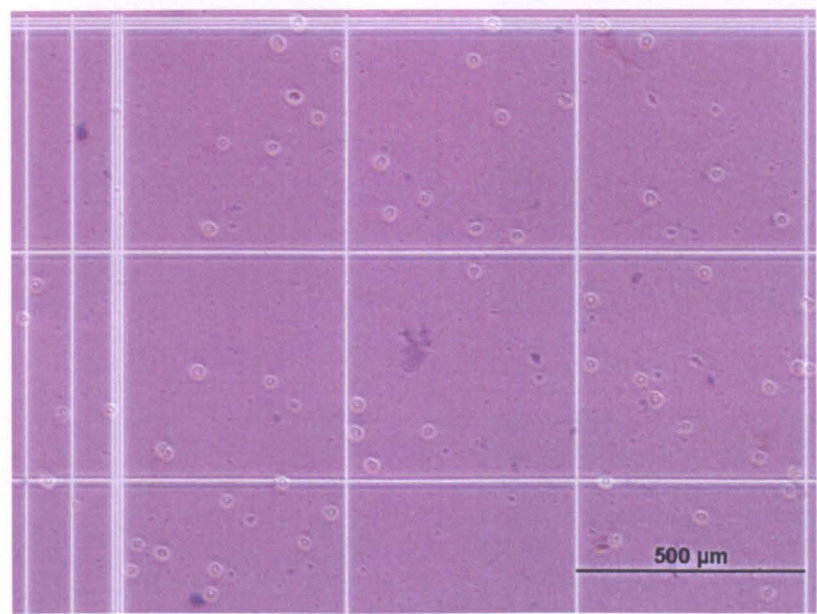
Both mononuclear cells and the lineage negative fraction of cord blood stem cells obtained from children with HLHS are similar in number and phenotypic enumeration to normal cord blood.

The results of short-term in vitro culture to investigate the relative growth potential of the two sample groups were inconclusive. Only 8 cord blood samples from HLHS children were processed to date during which time I was probably in the learning curve of the technique and might have been prone to operator error.

Umbilical cord blood proved easy to handle and with reproducible cell isolation under sterile conditions coupled with controlled freezing with cryoprotectants, umbilical cord blood warrants further examination as a cell therapy in congenital heart disease.

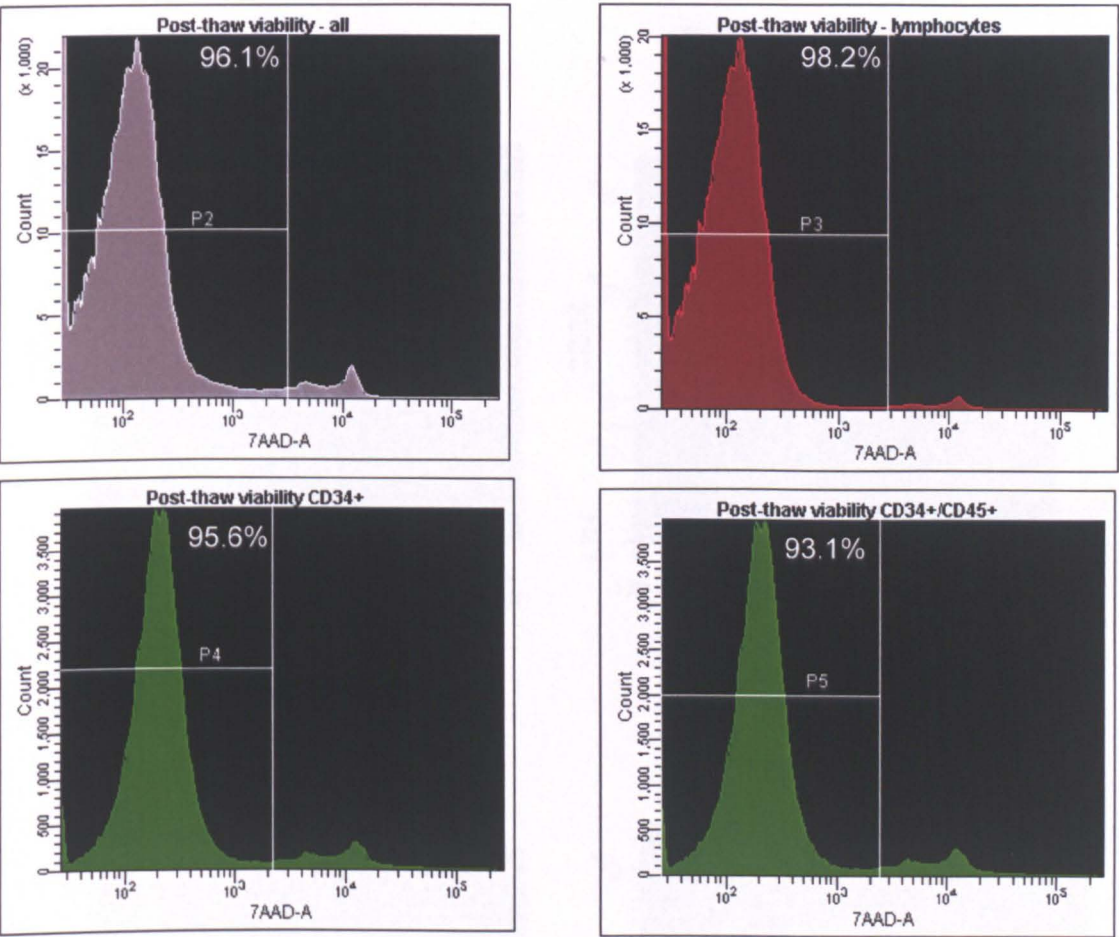
3.5    Figures

**Figure 3.2.1** Example of mononuclear cell count using haemocytometer

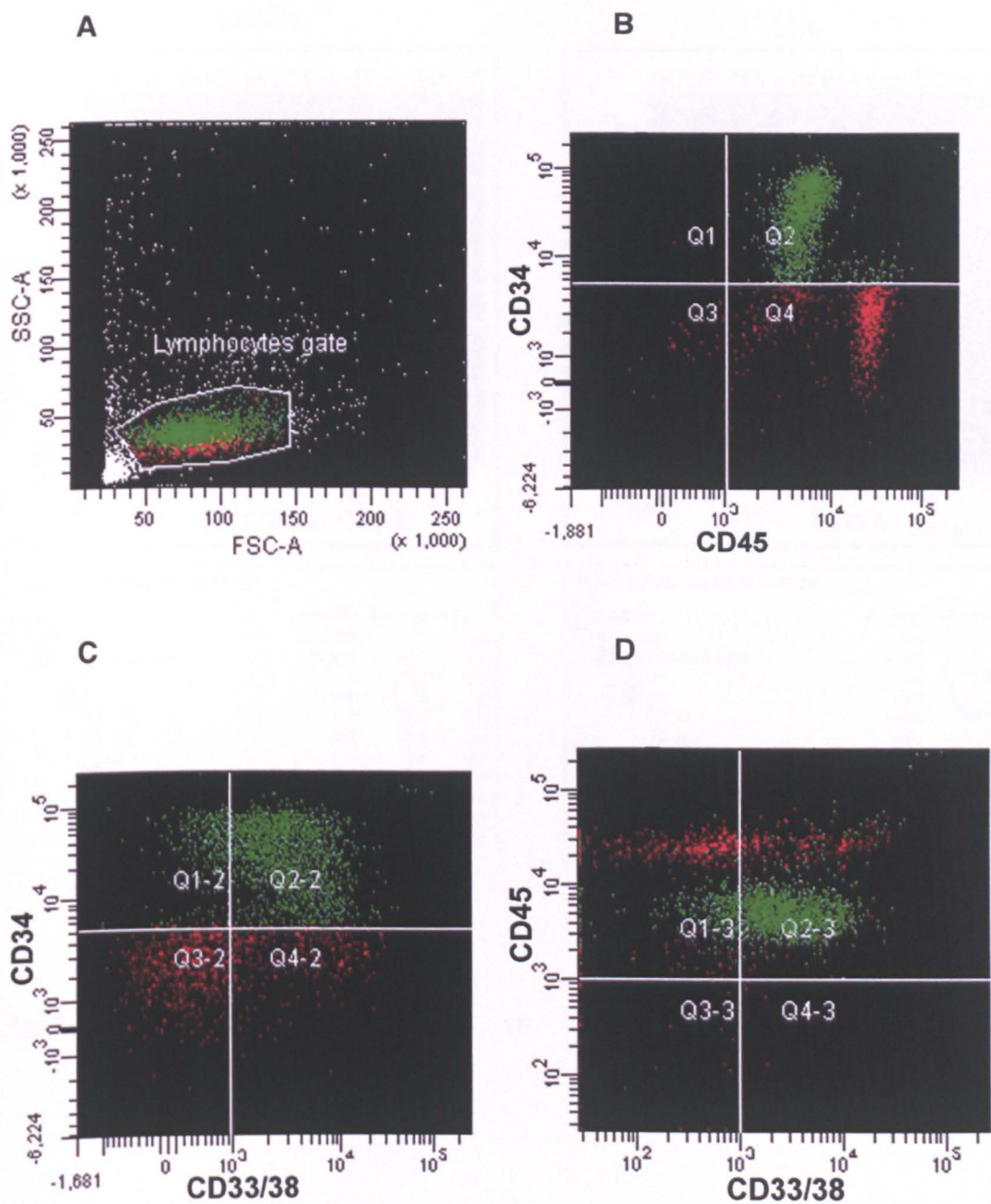


**Figure 3.2.2** Example of cellular viability of freshly-collected cord blood

mononuclear cells processed within 4 hours of collection using  
7-AAD

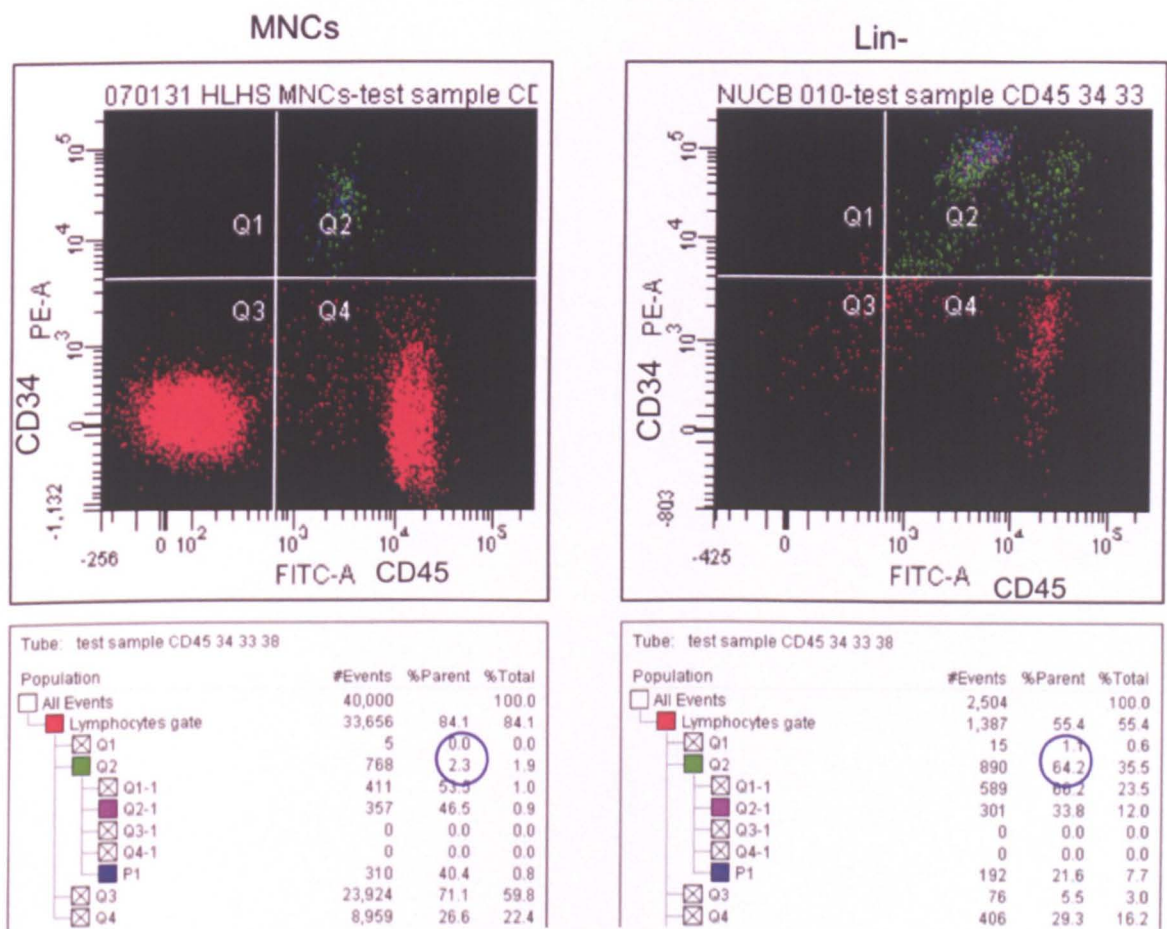


**Figure 3.3.1** Representative flow cytometric analysis of enriched CB populations showing forward versus sideward scatter plot and gating (A). The proportion of gated CD34/CD45 cells is shown in (B), CD34/CD33 and CD38 cells in (C) and CD45/CD33 and CD38 cells in (D).

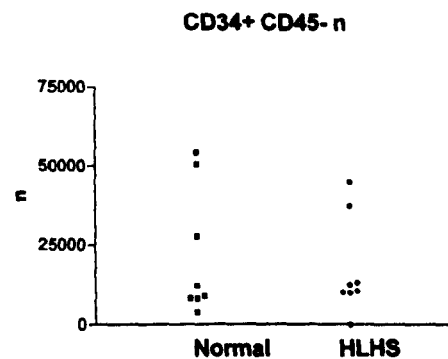
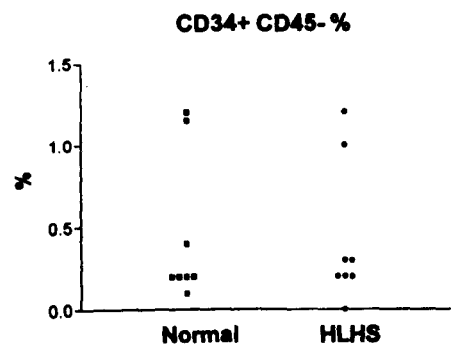
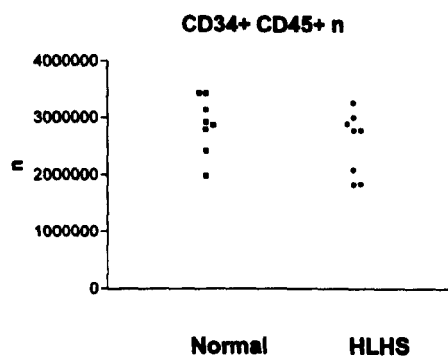
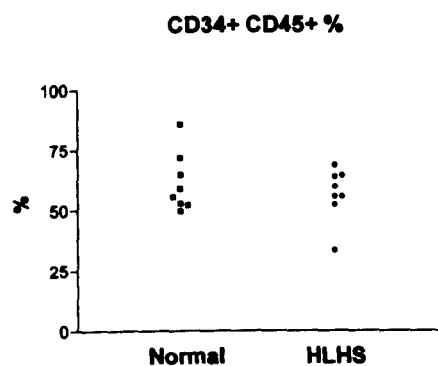
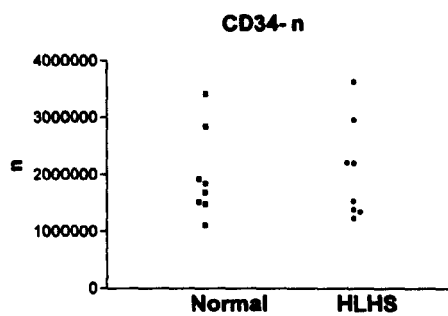
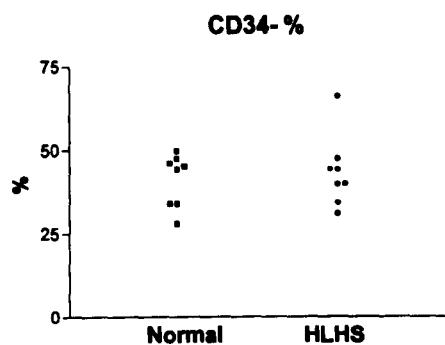
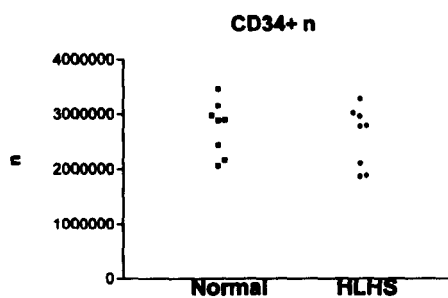
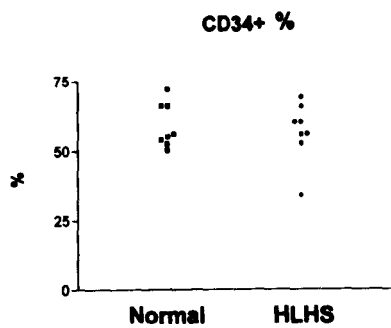


**Figure 3.3.2** Example flow cytometric analysis showing effects of

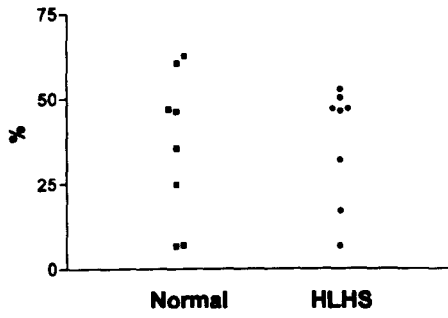
immunomagnetic enrichment: % CD34+/CD45+ content in mononuclear cells (MNC) compared to a sample enriched for primitive haematopoietic cells (Lin-)



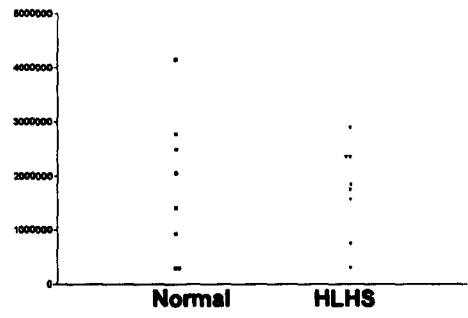
**Figure 3.3.3** Frequency distribution of cell surface phenotypes, using 8 gestational-age matched controls and 8 HLHS cord blood samples (see following pages)



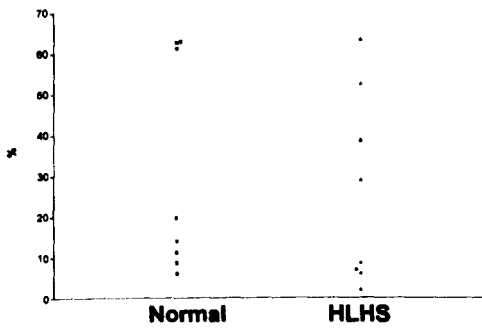
**CD34+ CD33+ CD38+ %**



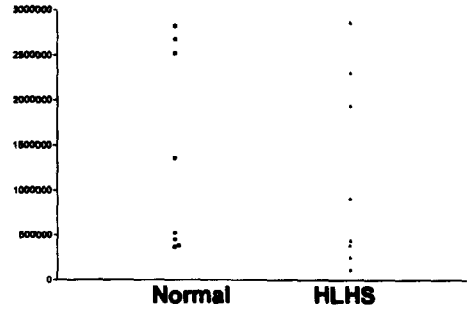
**CD34+ CD33+ CD38+ n**



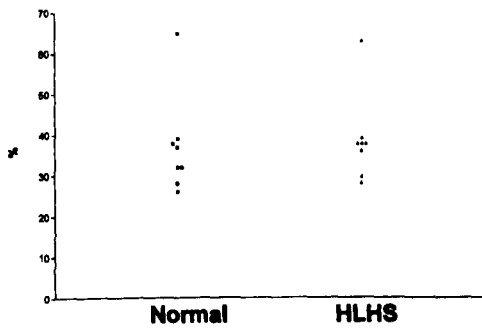
**CD34+ CD33- CD38- %**



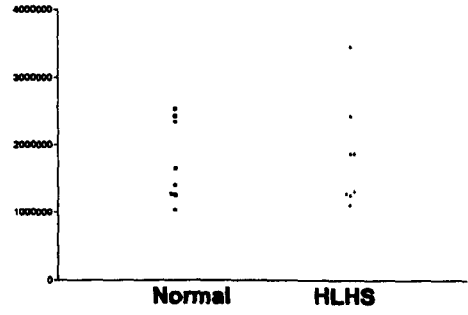
**CD34+ CD33- CD38- n**



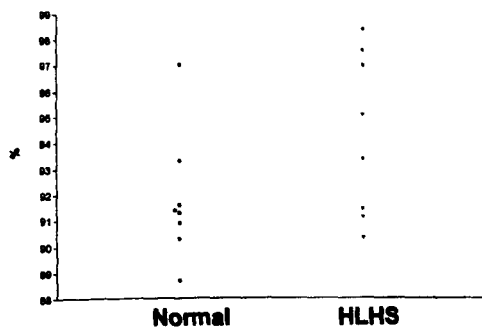
**CD34- CD45+ %**



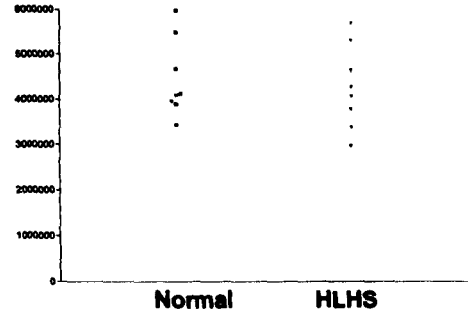
**CD34- CD45+ n**



**CD45+ %**

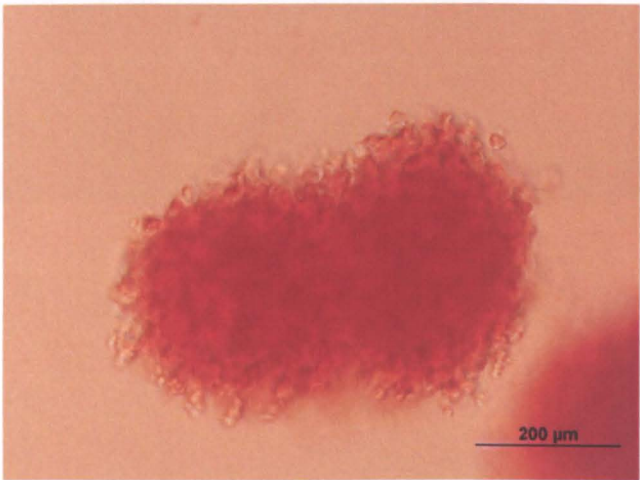


**CD45+ n**

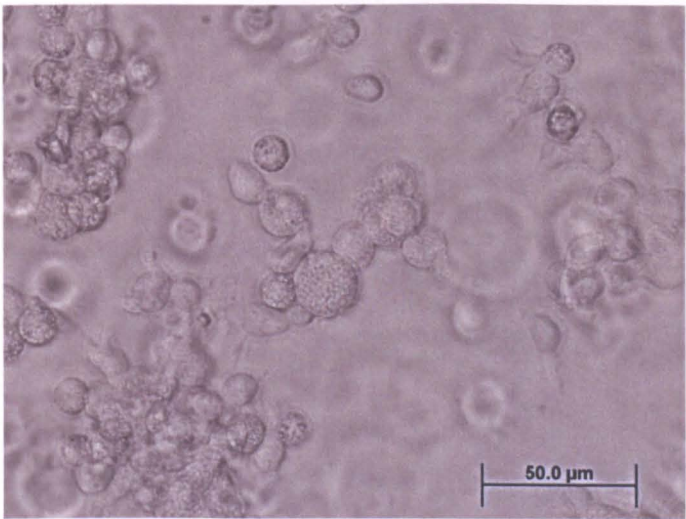


**Figure 3.2.**      **Colony photos**

**Figure 3.2.a**      Erythroid colony



**Figure 3.2.b,c**      Mixed colonies at 2 weeks



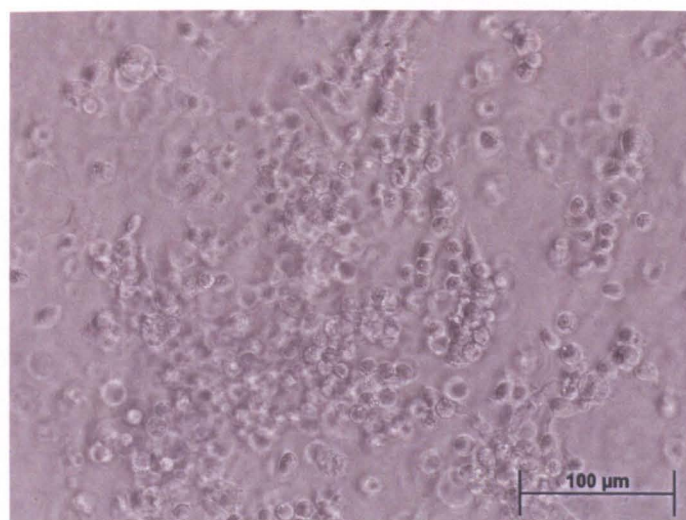
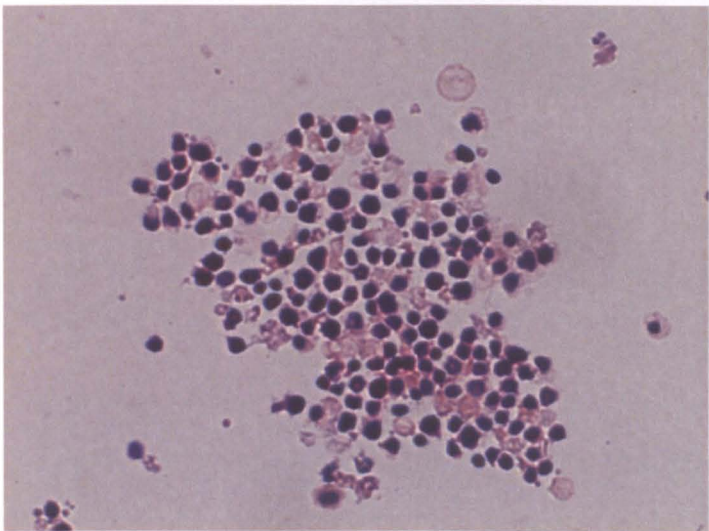
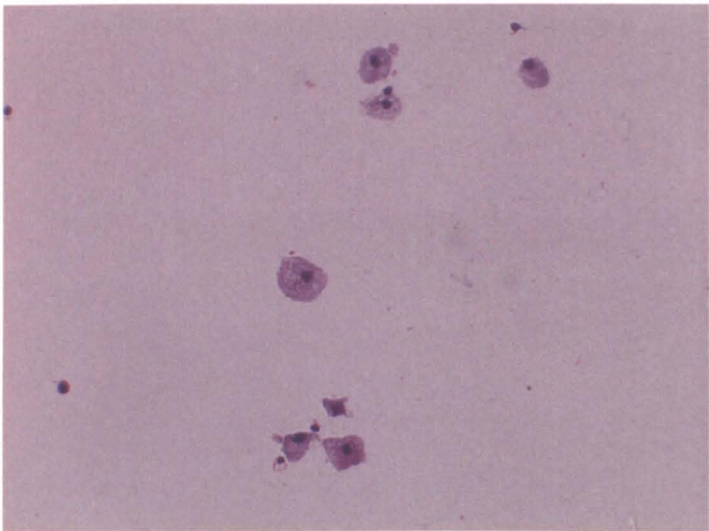


Figure 3.2.d,e (x40)



**Figure 3.2.** Example of CFU-GEMM: Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (x10)



## **4.0 Transplanted human cord blood stem cells improve cardiac function in a neonatal ovine model of right ventricular training**

### **4.1 Abstract**

Non-ischaemic right ventricular dysfunction and cardiac failure is a source of considerable morbidity in children with congenital heart disease. Despite work in adult ischaemic heart disease, cell transplantation has not previously been studied in the paediatric setting where enhancing ventricular function in response to supraphysiological workloads might be beneficial.

We transplanted human cord blood stem cells into an immunosuppressed neonatal sheep model of right ventricular training and took load-independent functional measurements at baseline and again after one month.

Transplanted human cells were detected in the myocardium, spleen, kidney and bone marrow up to 6 weeks after transplantation. The cells identified expressed the haematopoietic marker CD45 and in the bone marrow and spleen, also the mature B cell marker CD23. We did not identify any differentiation into cardiomyocytes, myocytes nor other mesenchyme-derived tissues. Significant

functional improvements were seen in the group receiving human cord blood stem cells compared to placebo.

Our data demonstrate that lineage negative-enriched cord blood stem cells engraft and augment right ventricular function. The cells adopt haematopoietic fates in both myocardium as well as the bone marrow and spleen.

## 4.2 Introduction

Increased right ventricular workload is an important feature in several types of congenital heart disease, particularly where the right ventricle is the sole functioning ventricle or where it acts as the systemic ventricle in lieu of the left. Despite improvements in treatment, early and late right ventricular dysfunction and cardiac failure due to pressure or volume overload is a source of persistent morbidity in children with congenital heart disease contributing to reduced survival<sup>175,223-225</sup>.

Cell transplantation has been shown to have beneficial effects on left ventricular function in animal<sup>116,226</sup> and human<sup>210,227-229</sup> studies of ischaemic heart disease. It has been hypothesised that haematopoietic stem cells (HSC) or mesenchymal stem cells (MSC) transplanted into ischaemic myocardium may contribute to recovery by differentiation into *de novo* cardiomyocytes<sup>230</sup>, reseeded damaged microvasculature, angioblast-mediated vasculogenesis or encouragement of endogenous reparative mechanisms by as yet incompletely-defined paracrine mechanisms<sup>153</sup>. Little is known about the potential of similar techniques to facilitate recovery in non-ischaemic heart disease or to augment adaptation of the systemic ventricle in younger age groups experiencing supraphysiological cardiac workloads due to congenital heart disease. It is likely that similar molecular mechanisms are at work in both embryonic and post-natal

cardiogenesis as well as regeneration of adult myocardium following ischaemic insults. Much as the recruitment or mimicry of regenerative processes in the adult heart offers therapeutic potential<sup>231</sup>, enhancing adaptive responses to augment cardiac function in response to increased workload or non-ischaemic insults in the young heart may involve similar processes which could be similarly open to augmentation.

Umbilical cord blood is readily-available and is a rich source of heterogeneous stem cell populations (CBSC) that are self-renewing, clonal precursors of both haematopoietic and non-haematopoietic tissues<sup>195</sup>. CBSCs have been well characterized through their clinical use in the treatment of haematological malignancies<sup>204-206</sup>. Ex vivo work has documented the ability of such cells to proliferate in culture and differentiate into a variety of cell types of differing lineages<sup>195,200</sup>. Homing experiments have demonstrated that CBSCs can traffic through the intact human system to where they are required. Furthermore, lineage-negative cell fractions enriched for the expression of CD34 are able to engraft in ovine and murine models over the longer term. It may be in fact that an earlier stem cell type, rather than the committed haematopoietic stem cell, can be harvested from CB and BM to account for the increasingly wide range of cell types that can be developed from these cell sources.

Transplantation of human CBSC into an immunosuppressed large animal model allows the detection of engraftment and evaluation of cell fate<sup>232</sup>. We used the immunosuppressed neonatal lamb model because of previous work in human

haemopoiesis, xenotransplantation and procedural experience within our group. In this model, long-term multi-lineage human chimerism has been established<sup>203</sup>. As human and sheep DNA and proteins differ significantly, human-specific markers can be used for unequivocal detection and characterisation of cells by independent yet complementary methodologies.

We isolated and purified human umbilical cord stem cells and set out to test the hypothesis that human umbilical cord blood stem cells can enhance myocardial performance after transplantation into a neonatal ovine model of right ventricular training. Our results document retention of human cells in the pressure-overloaded neonatal heart, expressing haematopoietic markers in association with improvements in systolic and diastolic function.

## **4.3 Methods**

### **4.3.1 Cord blood stem cell isolation**

Fresh umbilical cord blood was obtained for research purposes from consenting normal human donors shortly after delivery of healthy children >38 weeks gestation, with approval from the RCH Ethics in Human Research Committee. Cord blood stem / progenitor cells (CBSCs) were isolated and enriched using density centrifugation (Ficoll-Paque Plus; Invitrogen) and immunomagnetic lineage negative selection (StemSep; StemCell Technologies)<sup>233</sup>. Cells were suspended in a mixture of Iscove's modified Dulbecco's medium (IMDM; Gibcove) and fetal bovine serum containing 5% dimethylsulfoxide and stored in vapour phase above liquid nitrogen.

When required, cells were thawed and re-suspended in 500  $\mu$ l of bovine serum albumin and dextran-40. Thawed CBSCs had an immediate mean viability of  $92.6 \pm 2.6\%$  as assessed by staining with 7-amino-actinomycin and flow cytometry. Flow cytometry was used to characterise cell populations (LSR II, antibodies: human CD33, CD34, CD38, CD45) and matched isotype-stained controls (all BD Biosciences, San Jose, CA, USA). The reconstituted final cell or placebo preparation into the right ventricular myocardium was transplanted by transepical injection using a 30-gauge needle. Placebo medium consisted of a 1:1 ratio of bovine serum albumin and dextran-40.

#### **4.3.2 Animals**

Pregnant Border Leicester sheep of confirmed gestational age were housed in the large animal facility at a University of Melbourne. Lambs were born naturally and housed with their respective ewes. All lambs received 5 mg/kg/day oral cyclosporin solution (Neoral®, Novartis Pharmaceuticals Pty Ltd, Australia), achieving mean whole blood trough cyclosporin concentrations of 168.7 µg/l.

All experimental protocols were approved by the Animal ethics committees of the Murdoch Children's Research Institute and the University of Melbourne and conducted in accordance with the "Australian code of practice for the care and use of animals for scientific purposes" (National Health and Medical Research Council, Australian Government, 7th edition, 2004).

#### **4.3.3 Invasive haemodynamics and pulmonary artery banding**

Neonatal sheep (mean age  $10.0 \pm 3.1$  days; mean body weight  $6.7 \pm 1.5$  kg) were anaesthetised and ventilated using a standardised protocol comprising 2% inhalational isoflurane supplemented by buprenorphine, fentanyl and intercostal bupivacaine nerve blocks. Central venous and arterial catheters were placed, then 100 U/kg of unfractionated porcine heparin and atracurium administered.

A left thoracotomy was performed and cardiac catheterisation was performed via the right ventricular outflow tract. The patency of the ductal arteriosus was excluded, the inferior vena cava was dissected out immediately above the diaphragm and the circumference of the pulmonary artery measured 10mm distal to the pulmonary valve.

We studied the functional effect and fate of human CBSC or inert carrier medium in 16 immunosuppressed neonatal sheep in whom the main pulmonary artery was banded; two other groups (n=6 each) received either carrier medium or cord blood stem cells without pulmonary banding to establish the effect of cell transplantation on cardiac developmental changes in the neonatal period.

Haemodynamic indices were measured at baseline and again four weeks after initial surgery in placebo-treated (n=6), cell-treated (n=6), PAB and placebo treated (n=8) and PAB and cell treated animals (n=8) with a dual-micromanometer pressure transducer and conductance catheter used in conjunction with a pressure signal amplifier (Millar Instruments, Houston, Texas, USA) and signal conditioner (CD Leycom, Zoetermeer, The Netherlands).

Cardiac output was measured simultaneously using a non-occlusive perivascular ultrasonic flow probe (Transonics) placed around the pulmonary artery just distal to the pulmonary valve. Data were recorded at 1kHz during steady state and transient periods of inferior vena caval occlusion and stored for off-line analysis using customised software (Spike2, Cambridge Electronic Design, UK).

Parallel conductance of the right ventricle was evaluated by using injections of hypertonic saline solution into the right atrium; slope factor<sup>121</sup> was determined using cardiac output to allow for correction of volume measurements at post-hoc analysis.

All animals underwent baseline haemodynamic studies followed by PAB, placebo or human cord blood stem cell injection, according to treatment allocation. Where applicable, a non-absorbable 3 mm-wide Dacron® band was placed around the pulmonary artery, empirically reducing the cross-sectional area of the native vessel by 30%. After 30 minutes, a series of 5 to 6 injections of human CBSCs in 500µl (mean cell number  $4.7 \times 10^6$ ) or an identical volume of non-human serum albumin and dextran-40 medium as a placebo, were made into the right ventricular wall, as demarcated by the left anterior descending artery, atrioventricular junction and posterior descending artery. Measuring devices were removed, the chest closed and animals weaned from the ventilator and extubated. All lambs received a four day course of prophylactic antibiotics post-operatively.

#### **4.3.4 Tissue processing**

Sheep were killed after repeat cardiac catheterisation one month following placebo or cell transplantation. Heart, liver, spleen, kidney and bone marrow samples were collected and analysed for the presence of human cells by DNA

isolation and immunohistochemistry. Tissue was fixed in 4% paraformaldehyde for 4 hrs, stored overnight in 30% sucrose with 0.1 M phosphate-buffered saline, pH 7.2, embedded in OCT and then stored at  $-80^{\circ}\text{C}$ . Additional samples from each tissue were frozen and stored in liquid nitrogen for subsequent total cellular DNA extraction.

#### **4.3.5 DNA extraction and PCR analysis**

Total cellular DNA was extracted (DNeasy, Qiagen) and analysed by PCR for human-specific  $\beta$ -2 microglobulin. Specific primers for human  $\beta$ -2 microglobulin were selected based on the published human sequence (upstream primer, 5'-GTGTCTGGGTTTCATCAATC-3'; downstream primer, 5'-GGCAGGCATACTCATGTTTT-3') that was shown to selectively amplify human, not ovine DNA. All samples were also amplified to detect the  $\beta$ -actin gene (upstream primer, 5'-CGGGACCTGACTGACTAC-3'; downstream primer, 5'-GAAGGAAGGCTGGAAGAG-3') as a control for presence of amplifiable DNA. Amplification conditions included initial denaturation at  $95^{\circ}\text{C}$  for 9 min followed by 45 cycles of denaturation at  $95^{\circ}\text{C}$  for 30s, annealing at  $55^{\circ}\text{C}$  for 30s, extension  $72^{\circ}\text{C}$  for 15s, followed by a final extension at  $72^{\circ}\text{C}$  for 5 min. PCR products were resolved by electrophoresis through 2% agarose gels containing ethidium bromide. The sensitivity of the assay was assessed by human/sheep dilution studies which established standard curves demonstrating a sensitivity of detection of 1 ng human DNA in 1,000 ng ovine DNA.

#### **4.3.6 Immunohistochemistry**

Ovine heart, lung, liver, spleen, kidney, bone marrow and peripheral blood were screened for the presence of human cells by PCR. Tissues positive by PCR were then analysed by immunohistochemistry to investigate cell fate. Tissues negative by PCR and from control group sheep were used as negative controls. Cells isolated from the buffy coat of ovine peripheral blood was also analysed using flow cytometry for human haematopoietic and stem cell markers.

Staining was performed for human nuclear antigen, human CD23, CD34, CD45, human sarcomeric smooth muscle actin, SERCA-2, connexin-43, human myosin heavy chain, sarcomeric smooth muscle actin, N-cadherin, vimentin, cytokeratin-18, Nkx2.5, and von Willebrand Factor. Serial cutting sections 10  $\mu\text{m}$  in thickness were taken from each of the OCT-embedded tissues and placed on Superfrost Plus slides (Menzel-Gläser). To enhance antigen retrieval, slides were heated at 90°C for 10 minutes in 10 mmol/L citrate buffer (pH 6.0) and washed in phosphate buffered saline. Sections were then blocked in non-immune serum from the species in which the primary antibody was raised, followed by 14 hours incubation with primary antibodies. After incubation slides were rinsed with PBS and secondary detection performed using appropriate fluorescent antibodies (Jackson ImmunoResearch) or a chromagen-based biotinylated antibody kit (Vectastain ABC kit; Vector Laboratories).

#### **4.3.7 In situ hybridization**

Antigen retrieval was performed as described above. A fluorescein-conjugated human ALU probe (Innogenex, San Ramon, California) was applied and hybridization was done at 80°C for 5 min, followed by overnight incubation at 37°C. Slides were developed using an in situ hybridization kit for fluorescein-labelled probes (Innogenex) with DAB used as a substrate to develop the stain. For cardiac sections, in situ hybridization was done before staining with other primary antibodies as required.

#### **4.3.8 Statistical analysis**

Between-group differences were examined using analysis of variance testing. P values of less than 0.05 were considered significant. All P values are two-sided.

## 4.4 Results

### 4.4.1 Cell isolation and purification

CBSCs were isolated from freshly-collected human umbilical cord blood and stored in liquid nitrogen until required. Mononuclear cell yields per cord blood unit before and after immunomagnetic lineage depletion were  $3.3 \times 10^8 \pm 1.6 \times 10^8$  and  $4.7 \times 10^6 \pm 1.0 \times 10^6$ , respectively. Cellular viability of the final cell product following thawing was  $93.6\% \pm 2.3$  (94%). There were no significant differences in viability between samples used for group 2 or 4 ( $93.6 \pm 2.5\%$  vs.  $93.7 \pm 2.1\%$ ). Greater than 90% of lineage negative cells were CD45<sup>+</sup> and 6 to 8% ( $3.5 \times 10^5$ ) CD45<sup>-</sup>. An average of 58% of all cells were CD34<sup>+</sup>; of which half were CD33<sup>+</sup>/CD38<sup>+</sup> committed progenitor cells and half CD33<sup>-</sup>/CD38<sup>-</sup> primitive haematopoietic stem cells<sup>234</sup>. Of the CD34<sup>-</sup> cells, the majority were CD45<sup>+</sup> and therefore haematopoietic in lineage. There were no statistically significant differences in number, viability nor phenotype between cells injected into the two groups of sheep receiving stem cell treatment in the xenotransplantation model.

### 4.4.2 Transplanted CBSCs distribute to cardiac and non-cardiac tissues

We assessed the *in vivo* effects of CBSC transplantation by injecting  $4.7 \times 10^6$  hCBSC into the right ventricle of week-old neonatal sheep 30 mins after pulmonary artery banding (PAB) (Figure 4.1). We chose this age as this is a common time for the surgical intervention for the most therapeutically-

challenging forms of congenital heart disease. 28 sheep were studied, all of whom were immunosuppressed.

One month after *in vivo* injection we screened tissues by PCR for human-specific B<sub>2</sub>-microglobulin DNA to investigate the presence and distribution of viable human cells. All 14 transplanted sheep had detectable human cells in the right ventricle at one month. These cells were located either in isolation or small clusters of 3 to 5 cells throughout the pressure-loaded tissue. Transplanted cells were also detected in a variable fashion in non-cardiac tissues including lung, liver, bone marrow, spleen and kidney (Figure 4.2a, 4.2b; Table 4.1). Peripheral blood samples were negative for human-specific DNA sequences or haemotopoietic markers by PCR and flow cytometry, respectively. These results support a model of haematogenous distribution.

Following epicardial injection, some of the heterogeneous populations of CBSCs remained local to the site of injections with a proportion distributed systemically, migrating across endothelial barriers, to take up residence within organ-specific microenvironments and achieve survival at one month. The pattern of distribution in extra-cardiac tissues might be indicative of the distributive effects of the bloodstream once cells had passed back into the left side of the heart or the relationship between the pre-determined fate of transplanted cells and the various host tissues providing specific and favourable microenvironments. Transplanted xenogeneic cells may have also temporarily integrated into other tissues but lacked the ability to survive. We did not attempt a detailed

quantification of the number of human cells surviving or proliferating one month after transplantation. The total number of human cells within the right ventricle was estimated in a subset of animals. 0.07% of all nuclei examined in the right ventricular free wall were of human origin at 30 days. On first impression this frequency of donor cells may be considered disappointing, but this must be considered in light of the near-exponential somatic growth that occurs in the neonatal phase of large animal models and also by the results of quantitative xenogeneic studies of haematopoietic cell turnover in chimeric bone marrow that have demonstrated significant expansion in the total number of donor cells following transplantation despite detection of similarly low frequencies of donor cells<sup>235</sup>.

To confirm the presence of human cells in PCR-positive tissues, we stained sections with an antibody specific for human nuclear antigen, a human-specific nuclear component (Figures 4.3a-d) and performed *in situ* hybridisation studies using a human-specific *Arthrobacter luteus* (*Alu*) gene probe (Figures 4.4a-d). Human cells were found in injected hearts in a scattered pattern as rare events in the right ventricular free wall. We also identified cells positive for human nuclear antigen, principally in the perivascular regions, in the kidney, bone marrow and spleen, all tissues with haematopoietic or lymphopoietic roles (Figures 4.3b-d). Negative controls, consisting of tissues from transplanted sheep that were negative by PCR and tissues from normal sheep, were uniformly negative and confirmed the human specificity of the staining methods.

#### ***4.4.3 Transplanted CBSCs express CD45 in cardiac tissue***

We evaluated the fate of transplanted cells in both cardiac and non-cardiac tissues using a combination of approaches. Double- and triple-staining immunofluorescent or immunohistochemical antibody techniques were used incorporating an antibody against human nuclear antigen or a human *Alu* probe combined with a panel both human-specific and non-human-specific differentiation markers to investigate the fate of the transplanted human cells. Cardiac tissue was stained with antibodies against myofibrillar components human cardiac myosin, skeletal myosin and human troponin, smooth muscle actin, smooth endoplasmic reticulum ATPase-2 (SERCA-2), connexin-43 and Nkx2.5, as well as von Willebrand Factor (vWF), a marker for endothelial cells. Human cells located in the myocardium were found to be CD45 positive suggesting that these cells were from a haematopoietic fraction of the enriched cord blood (Figure 4.5). We found no evidence to support transdifferentiation of transplanted human cells in the sheep myocardium. Specifically, human cells did not co-localise with contractile filaments nor exhibit positivity for mesenchymal markers.

#### ***4.4.4 CD45-positive or CD23-positive transplanted CBSCs identified in non-cardiac tissue***

We then further characterised human cells identified in sections of kidney, bone marrow and spleen. Human cells in the kidney stained positively for CD45, demonstrating a haematopoietic fate one month after injection (Figure 4.6).

Staining of these human cells with antibodies against CD23, a marker of B-cell progenitors and bone marrow stromal cells, endothelial and mesenchymal markers was consistently negative. One month after transplantation, several clusters of human cells strongly expressing CD45 and human nuclear antigen or human CD23 were present in the marrow and spleen (Figures 4.7a, 4.7b, 4.8a, 4.8b). Human cells in sheep bone marrow or spleen were positive for either CD45 or CD23, simultaneous co-expression of these markers was not seen. This suggests that least two distinct populations of human cells had taken up residence or matured in haematopoietic and lymphopoietic tissues within the host animal. Although human cells were identified by PCR in the liver and lung tissue of four transplanted animals, they could not be located on sections by our immunohistochemical and immunofluorescent methods. Finally, there was no evidence of uncontrolled growth or inflammatory infiltrate in any of the examined sections

#### *4.4.5 Intramyocardial CBSC injection enhances right ventricular systolic function in the presence of increased afterload*

We studied the functional effect and fate of human CBSC or inert carrier medium on right ventricular function in 16 immunosuppressed neonatal sheep in whom the main pulmonary artery was banded with either CBSC or carrier medium injection; two other groups (n=6 each) received either carrier medium or cord blood stem cells without pulmonary banding to establish the effect of cell transplantation on cardiac developmental changes in the neonatal period.

Load-independent functional indices were measured at baseline and again four weeks later. Baseline variables of right ventricular function, pressures and volumes did not differ significantly between the four groups (Table S2, supplementary data).

One month after initial functional assessment, right ventricular performance was found to be slightly reduced in the control group, consistent with expected post-natal changes that include decreased beta-adrenoceptor expression.

Transplantation of CBSCs without pulmonary artery banding had no significant effects on haemodynamic variables and load-independent indices of systolic and diastolic function.

One month following pulmonary artery banding and placebo injection, we observed improvements in load-independent indices of systolic function in the animals. Intramyocardial injection of CBSC with pulmonary artery banding was accompanied by improvements in systolic function greater than that of pulmonary artery banding alone (Figures 4.9a, 4.9b, 4.9c).

Load-independent indices of right ventricular systolic function were significantly greater in group 4 receiving stem cells and pulmonary artery banding group compared with group 3 who received pulmonary artery banding and placebo; in groups 4 and 3 respectively, end systolic elastance ( $E_{es}$ ) increased by a mean of  $1.4 \pm 0.2$  mmHg/ml compared to  $0.9 \pm 0.1$  mmHg/ml  $P < 0.001$ ; the slope of preload recruitable stroke work ( $M_w$ ) increased by  $21.1 \pm 2.9$  mmHg compared to  $15.8 \pm 2.5$  mmHg,  $P < 0.05$ ; the slope of the relation between  $dP/dt_{MAX}$  and

end diastolic volume ( $[dP/dt_{\max}]_{\text{edv}}$ )  $17.3 \pm 2.2$  mmHg/s/ml compared to  $11.6 \pm 1.6$  mmHg/s/ml,  $P=0.06$ ).

#### ***4.4.6 CBSC transplantation improves diastolic function***

At four weeks, diastolic parameters tended to be improved, albeit non-significantly, in animals receiving PAB and CBSC injection compared to PAB and placebo injection. These two groups both had increases in right ventricular mass as expected in response to pulmonary artery banding.  $\tau$ , the time constant of relaxation, increased by  $8.7 \pm 5.7$  ms in the group receiving CBSC compared to  $13.2 \pm 6.2$  ms in the group receiving placebo ( $P>0.05$ ). These observations of improved relaxation and reduced myocardial stiffness may indicate that CBSC encourage a more beneficial and less restrictive growth response to increased afterload characterised by relatively reduced myocardial interstitial oedema and consequently less fibrosis, a chain of events recognised clinically<sup>236,237</sup>.

## 4.5 Discussion

We have shown here that transplanted human cells can be detected in the myocardium, spleen, kidney and bone marrow up to 6 weeks after transplantation. Human cells identified in the heart and kidney adopted a haematopoietic fate expressing CD45; in the bone marrow and spleen cells also expressed the mature B cell marker CD23. We did not identify any differentiation into cardiomyocytes, myocytes nor other mesenchyme-derived tissues.

Our data demonstrates that lineage negative-enriched cord blood stem cells adopt haematopoietic cell fates in the myocardium where their presence augments right ventricular function in the presence of increased afterload. Following intramyocardial injection into the neonatal heart, viable transplanted cells are distributed systemically and take up residence in natural haematopoietic niches such as bone marrow and spleen and also migrate to distant tissues.

The novel findings of this work demonstrate that intramyocardial injection of human cord blood stem cells enhances right ventricular function following pressure overload beyond functional responses to pulmonary artery banding alone. Trends in diastolic performance, although not significant, indicate that a more desirable adaptation was stimulated with improved relaxation indices compared to the banding alone. Interstitial oedema developing in the myocardium following acute pulmonary artery banding has been shown to

evolve into fibrotic tissue resulting in a stiffer, physiologically more restrictive ventricle.<sup>236,237</sup>

These results show that CBSCs can be transplanted and are capable of integrating in multiple tissues, requiring migration across endothelial barriers and integration into host tissue microenvironments. This supports a model of haematogenous distribution, with subsequent selective survival in specific and appropriate tissue niches. These findings suggest myocardial retention of a small number of cells, followed by trafficking of other cells to tissues either along ontologically pre-determined positions or by virtue of haematogenous distribution. This may be a function of the ability of specific microenvironments to support the engraftment and differentiation of CBSCs; alternatively the lack of engraftment in some tissues may be due to heterogeneity of the transplanted cell population with respect to differentiation potential, replicative capacity or longevity. A third possibility is that the xenogeneic microenvironment can support the viability and differentiation of human CBSCs, but not their self-replication. Although insufficient homing of cells, among other factors, could account for the lack of proper transdifferentiation, our study suggests a biological effect on right ventricular function and adaptation to increased loading conditions. Potential mechanisms for this beneficial effect include paracrine-mediated effects by transplanted cells.

We have not quantitatively assessed expansion of the cells transplanted in this experiment. Minimal expansion of donor cells in this xenogeneic model would not be unexpected. However, the apparent low frequency of donor cells indicated by immunohistochemistry here may be misleading. This data should be interpreted with caution as cell division or proliferation of transplanted cells after intramyocardial injection may occur in the early time period. However, over time, most of the locally-delivered cells in each group examined either die or migrate elsewhere. In this neonatal sheep model, rapid growth of the sheep occurred in the first month after transplantation (typically 75% increase in bodyweight).

Experiments in the sheep model of human haematopoiesis in which human cells have been quantitatively assessed in chimeric bone marrow have shown that even when low frequencies of donor cells can be detected, there is tremendous interval expansion in the total number of donor cells since transplantation. While 0.07% represents only low-level engraftment, this does represent a significant number of cells, for example a year-old lamb is estimated to contain 1.5 kg of bone marrow with 1 mg being equivalent to one million cells. In comparison, it has previously been shown with 67 000 human cord blood CD34<sup>+</sup> cells that 2% to 3% chimerism was typical<sup>238</sup>.

Although we found no evidence of myocardial transformation in cell-treated hearts, we hypothesized that cell treatment could still have positive effects on cardiac geometry, mass acquisition and function after pulmonary artery banding.

Our study had several limitations. Questions about responsible cell type, optimum dose, and timing of cell transfer still remain. We transferred cells 30 mins after pulmonary artery banding. In many respects, the clinical utility of such an approach is needed, after all bone marrow transplantation has been performed using an unrarefied buffy coat fraction for many years and the precise cellular dynamics and transplantation kinetics are still being elucidated.

CD34<sup>+</sup> cells have been shown to exhibit superior efficacy in preserving myocardial integrity and function after myocardial infarction compared to unselected circulating mononuclear cells<sup>219</sup> and in a dose-dependent manner<sup>220,221</sup>. From a practical perspective, a mixture of mutually supportive heterogeneous cell populations is probably preferable to using a population far more purified and smaller in number. We therefore took a more practical approach, committing to clinically-relevant quantities of material by using a lineage negative population depleted of mature haematopoietic cells and enriched for haematopoietic progenitor cells. From this we derived an easily handled, compact cell product that could be easily injected into the myocardium.

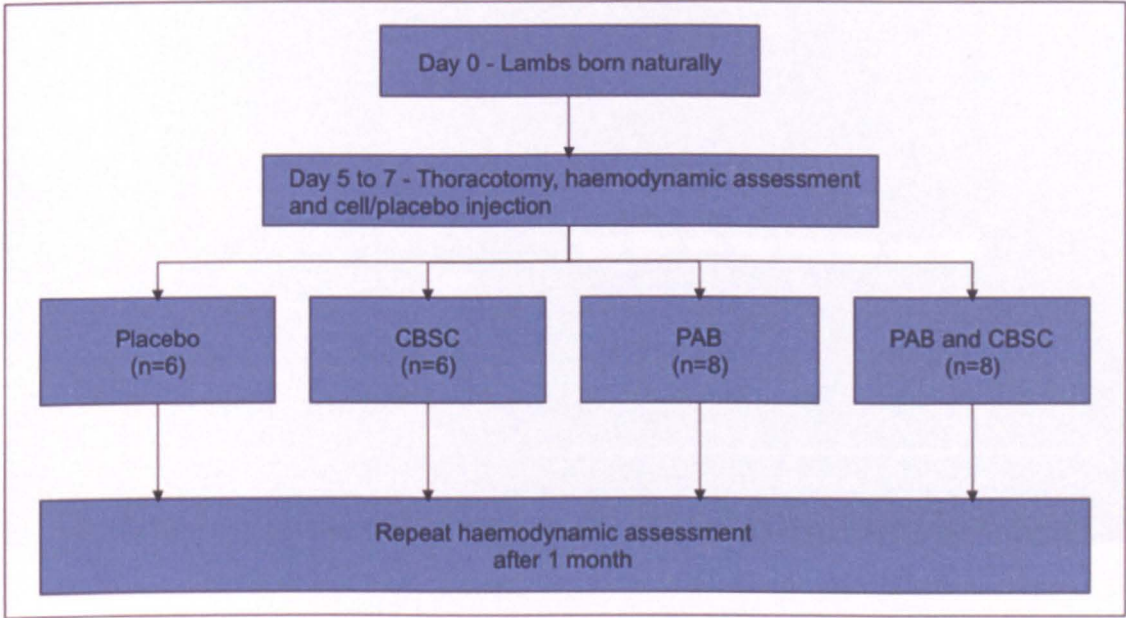
The predictable adoption of mature haematopoietic fates of transplanted CBSCs supports the potential of these cells in cardiac transplantation and tissue engineering applications. Further insight is required into the normal and disease-induced regulation of cell populations contained within CBSC, a greater insight into their transplant immunology and the development of strategies for diffuse or site-specific delivery for therapeutic applications.

#### **4.5.1 Conclusion**

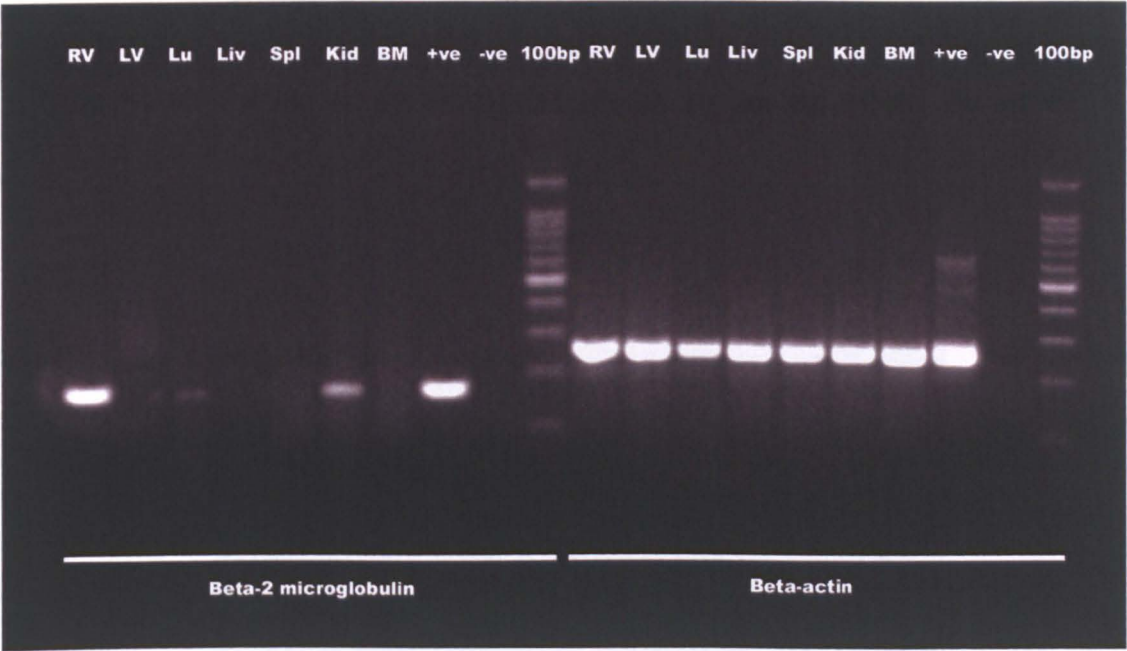
Cell transplantation with human CBSCs provides some short- to mid-term benefit in enhancing right ventricular function in response to increased workload. These findings suggest the potential therapeutic use of cord blood stem cells to augment myocardial function in paediatric and non-ischaemic heart disease.

4.6 Figures

Figure 4.1 Trial design



**Figure 4.2a** Screening of sheep tissues one month after transplantation, by DNA PCR using probes specific for human  $\beta$ -2 microglobulin sequences.  $\beta$ -actin lanes demonstrate presence of amplifiable DNA.



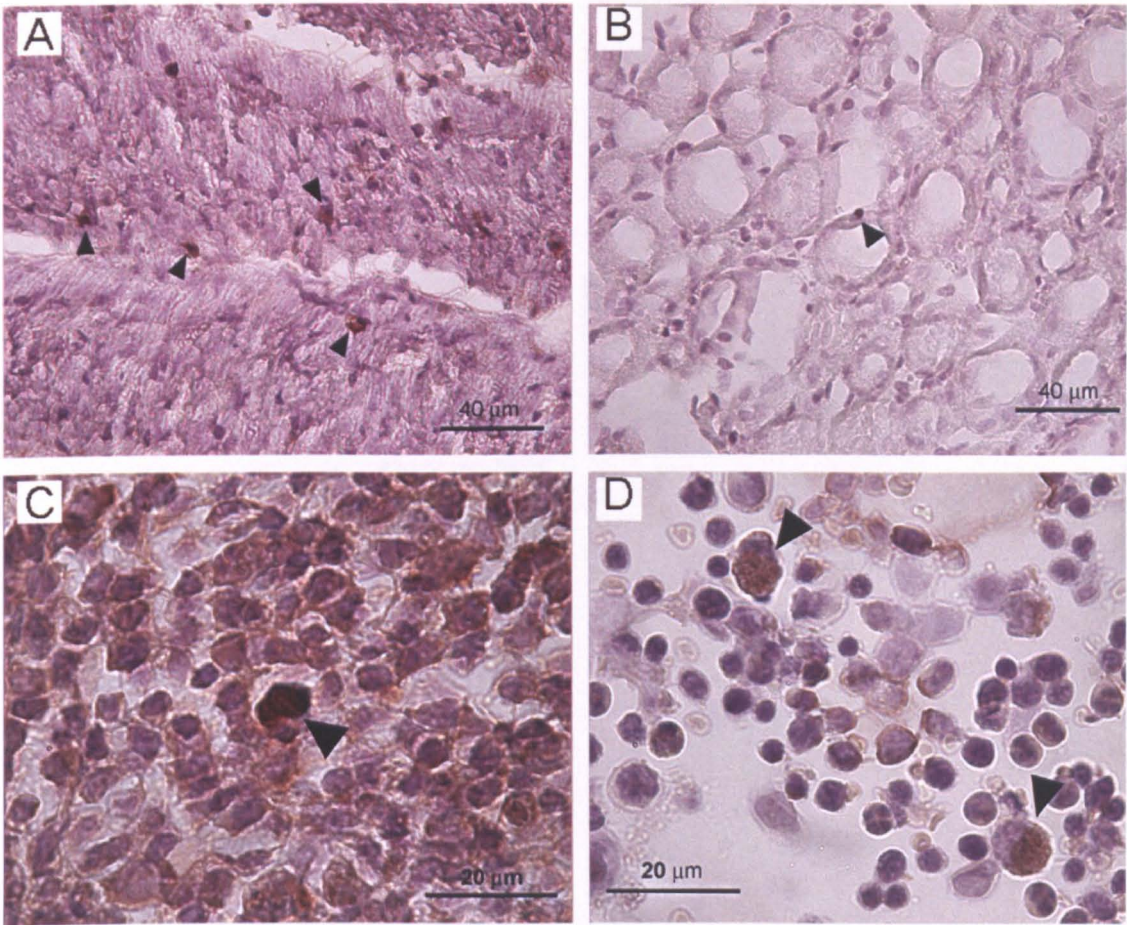
**Figure 4.2b** Serial dilutions of genomic human DNA in sheep DNA to establish sensitivity of DNA screening test with molecular size markers in right-hand lane (100 base-pair ladder)



**Table 4.1**      Frequency and distribution of human-sequence-positive tissues one month following injection, by DNA PCR using probes specific for human  $\beta$ -2 microglobulin sequences.

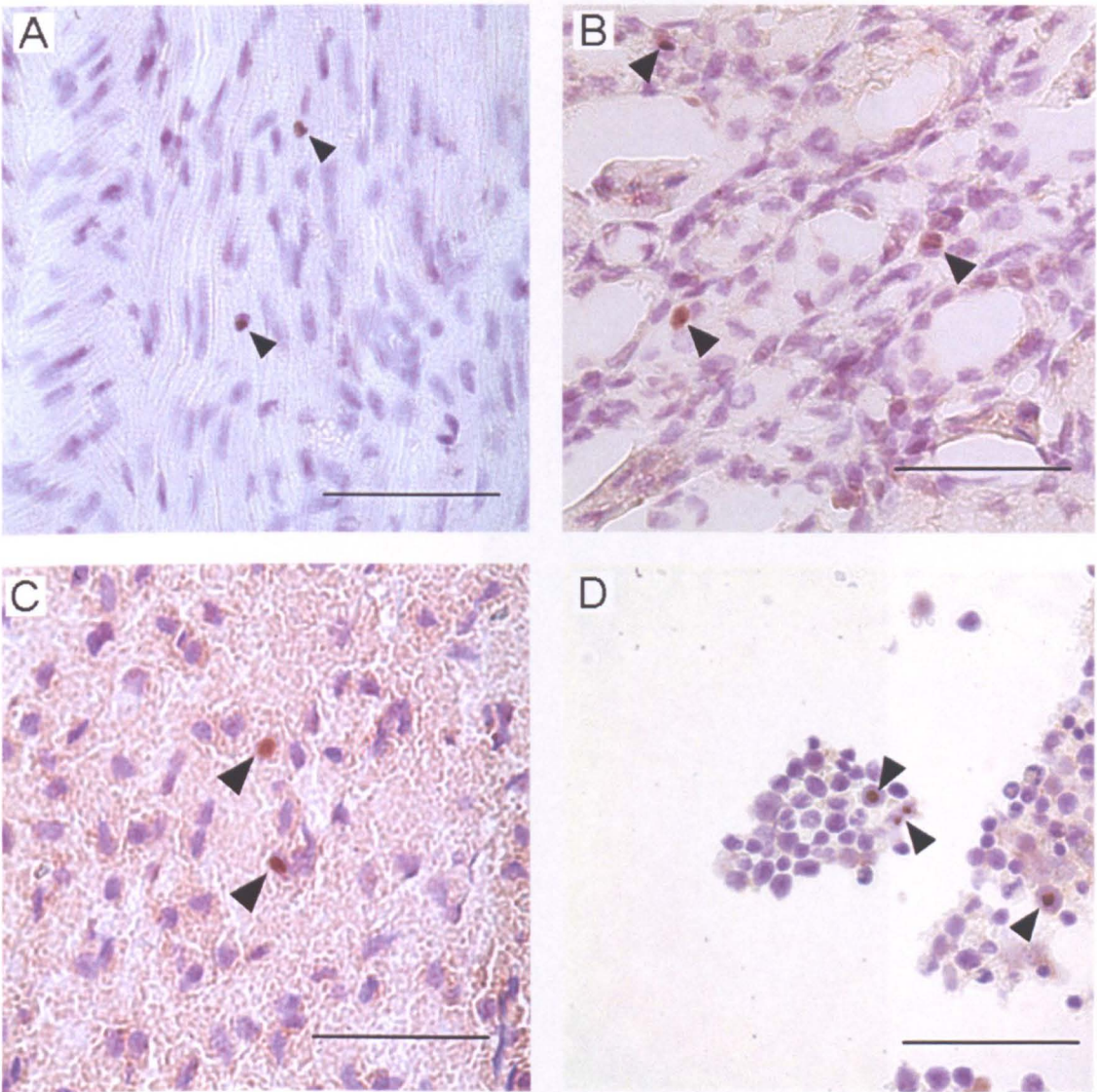
	CBSC injection	PA banding	All
	and		
	CBSC injection		
	(Group 2)	(Group 4)	
<i>n</i>	6	8	14
RV - Right ventricle	6/6	8/8	14/14
LV - Left ventricle	2/6	3/8	5/14
Lu - Lung	1/6	1/8	2/14
Liv - Liver	0/6	2/8	2/14
Spl - Spleen	0/6	2/8	2/14
Kid - Kidney	5/6	7/8	12/14
BM - Bone marrow	3/6	5/8	8/14

**Figure 4.3** Immunohistochemistry for Human Nuclear Antigen.



- A Right ventricle
- B Kidney
- C Spleen
- D Bone marrow

**Figure 4.4** Immunohistochemistry for human-specific *Alu* gene



- A Right ventricle
- B Kidney
- C Spleen
- D Bone marrow

**Figure 4.5** Right ventricle

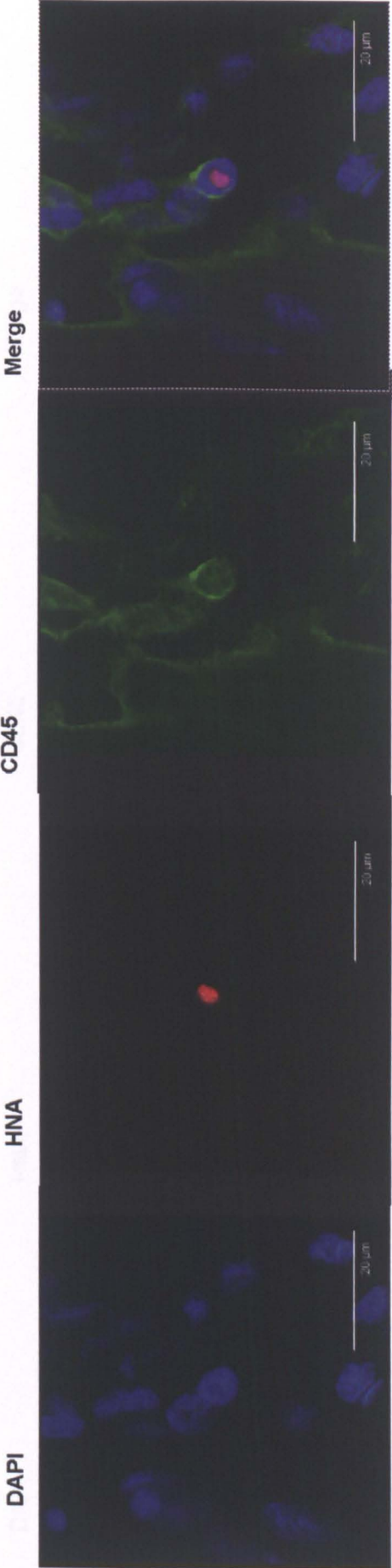
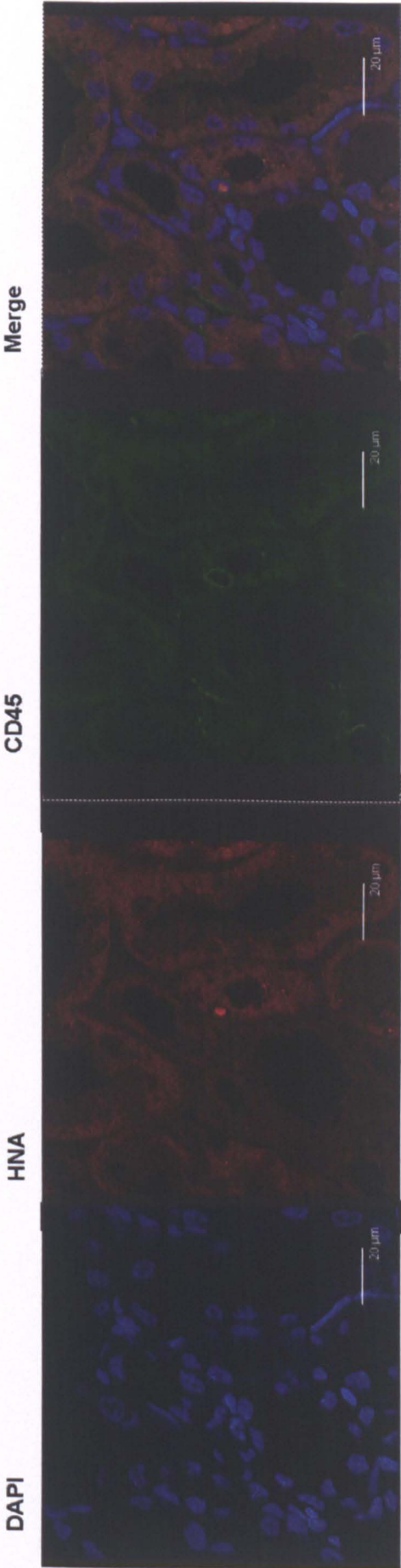


Figure 4.6 Kidney



**Figure 4.7** Spleen

**Figure 4.7a** CD45



**Figure 4.7b** CD23 positivity

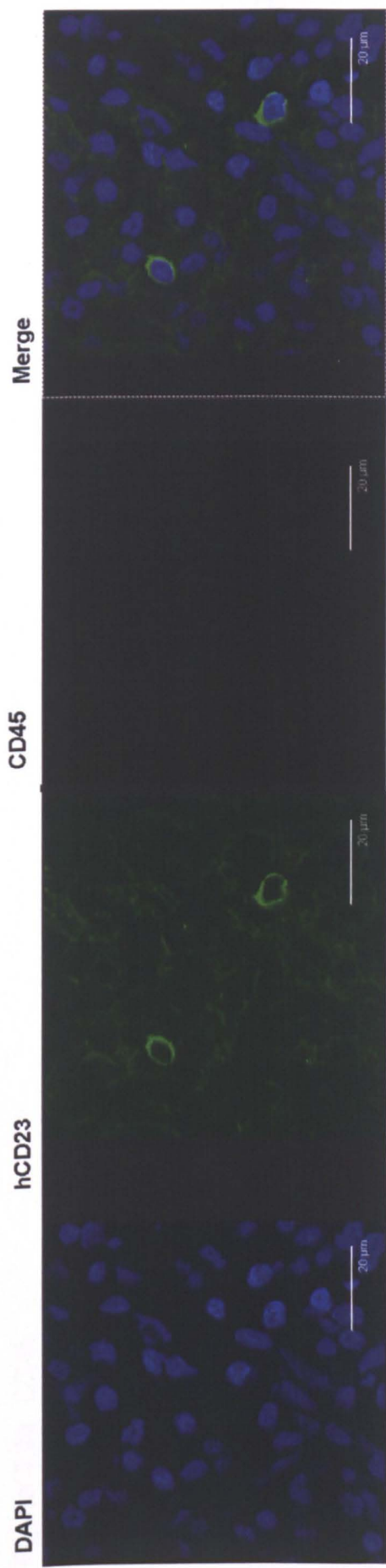


Figure 4.8 Bone marrow

Figure 4.8a CD45

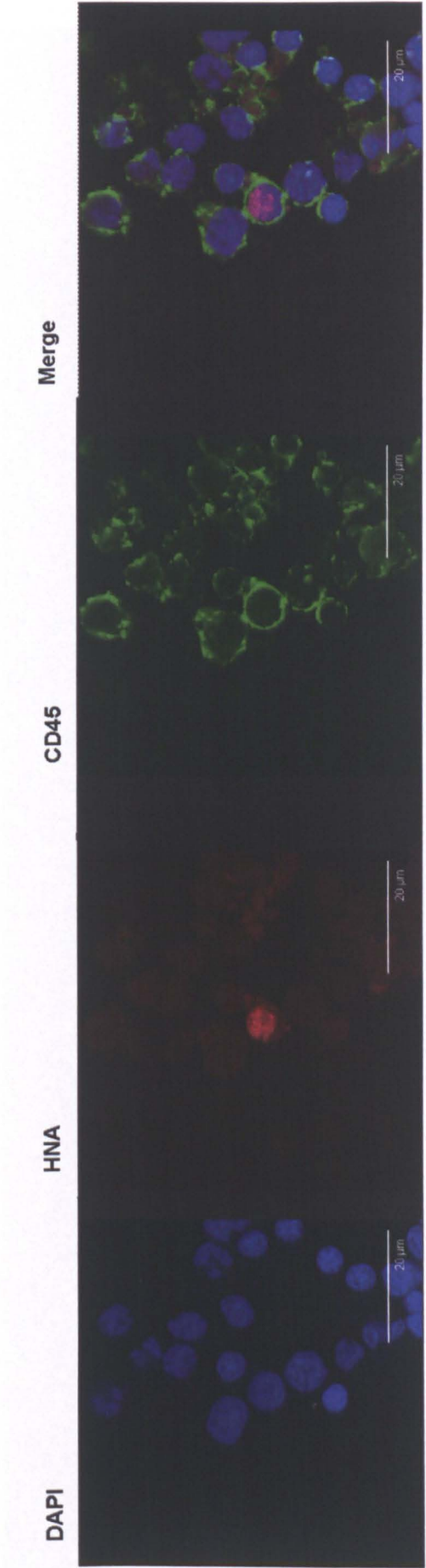


Figure 4.8b CD23 positivity

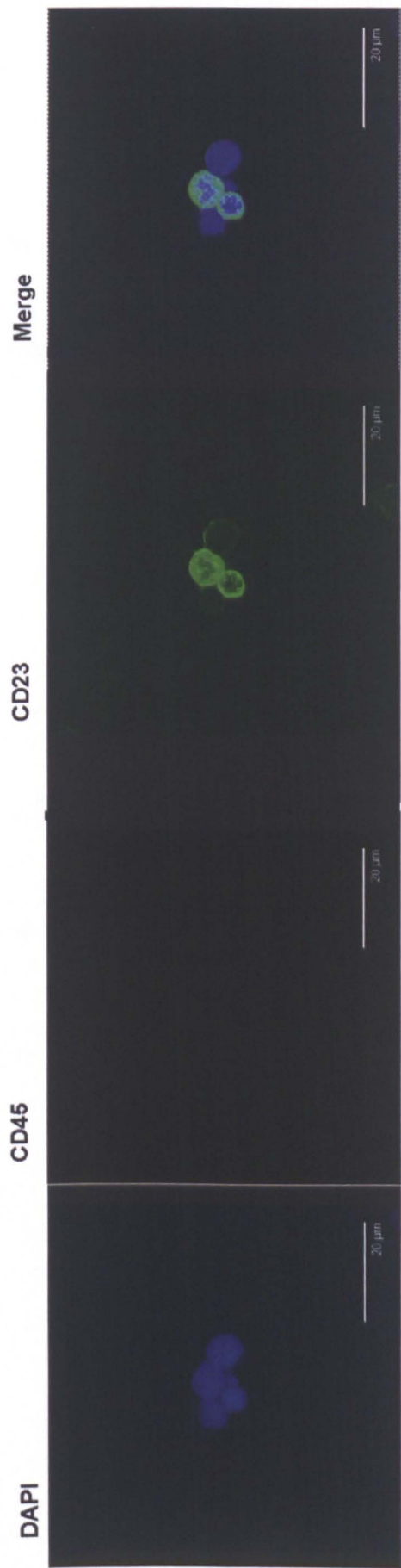
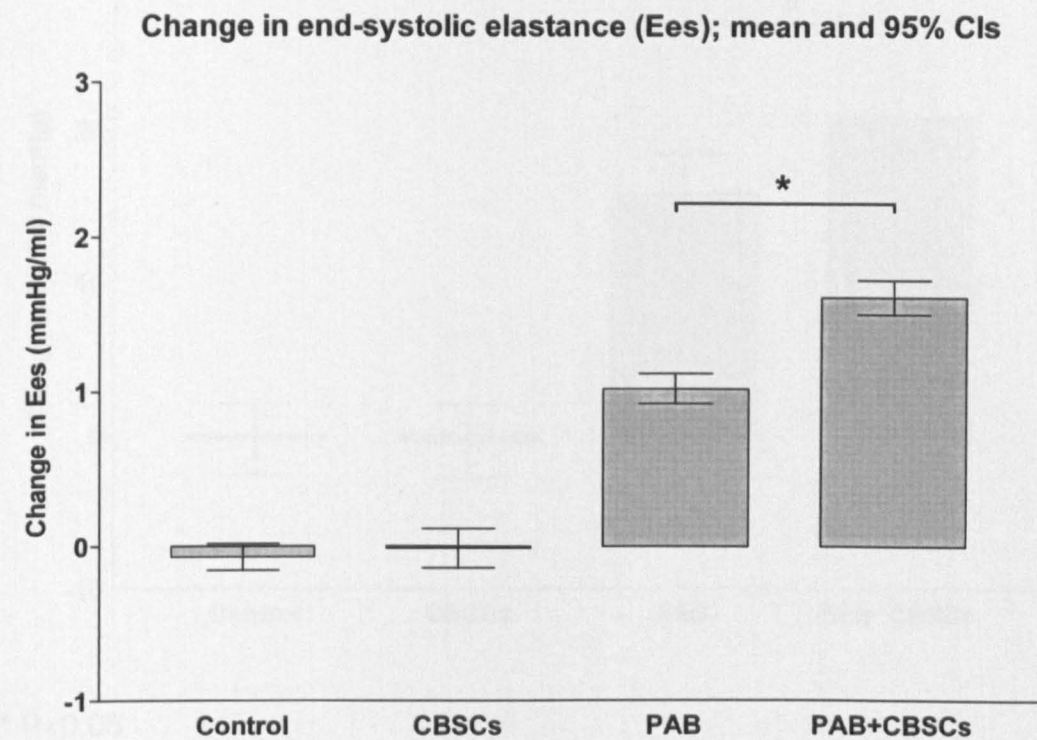


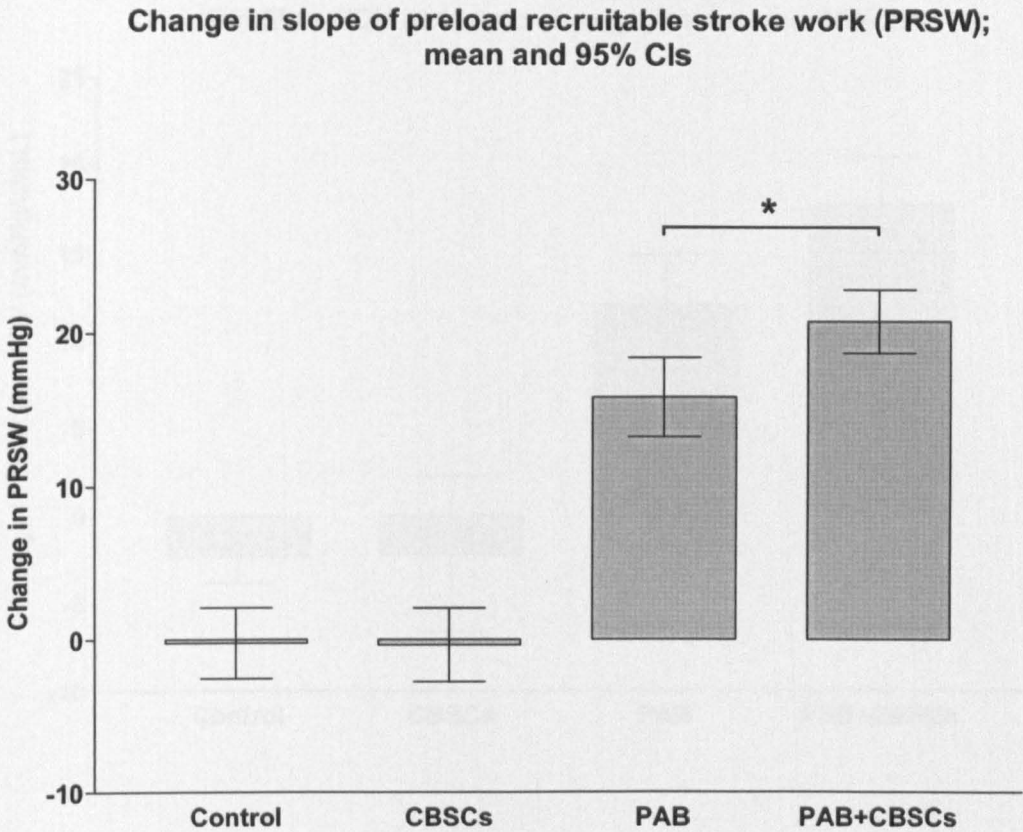
Figure 4.9

Figure 4.9a



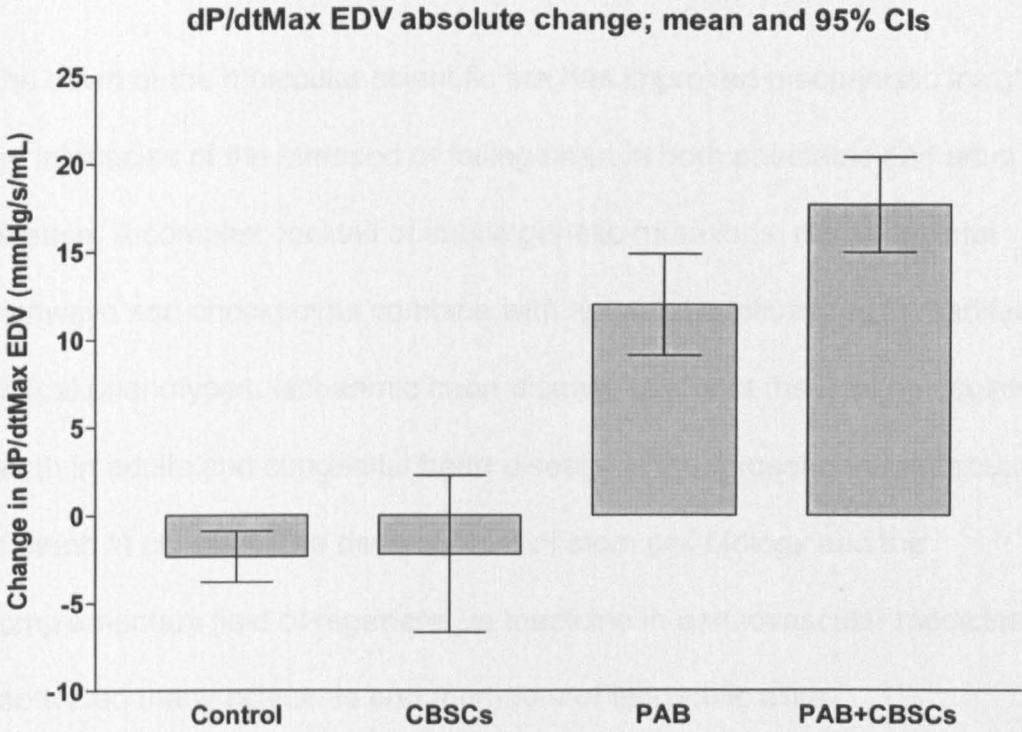
\*  $P < 0.001$

Figure 4.9b



\*  $P < 0.05$

Figure 4.9c



## 5.0 Conclusion

The dawn of the molecular scientific era has improved mechanistic insight into the intricacies of the stressed or failing heart in both paediatric and adult heart disease. A complex cocktail of subtle genetic mutations, transcriptional pathways and checkpoints combine with epigenetic influences to manifest as clinical phenotypes. Ischaemic heart disease is one of the leading causes of death in adults and congenital heart disease is the largest non-infectious cause of death in children. The development of stem cell biology and the complementary field of regenerative medicine in cardiovascular medicine has captivated many scientists and members of the public alike.

Attempts at myocardial augmentation or regeneration by harnessing or stimulating the heart's intrinsic regenerative capacity remains in their infancy. Functional improvements brought about by cell transplantation in acute animal models of ischaemic heart disease have not infrequently only translated into single digit improvements in left ventricular ejection fraction. Indeed, given the sheer global burden of ischaemic heart disease and the lack of evidence for differentiation or mechanical contribution of exogenous stem cells into myocardium, a cocktail of growth factors delivered locally into the myocardium, may prove more a viable approach than a tailor-made cell therapy.

At present, I believe the transplantation of stem cells or progenitor cells for purported paracrine effects remains justifiable within the context of a trial given that multiple factors are likely to be functioning synergistically. Paracrine factors, either eluted from transplanted cells or introduced directly in a concentrated and refined form, could promote a shift in cardiac homeostasis encouraging cell survival and replication. Where hereditary considerations may exist, for example in congenital heart disease, autologous cells might not be desirable as repopulating a damaged organ with the same genotype is conceptually flawed. Allogeneic cells that can be processed to a high level of quality control and stored ready for 'off the shelf' are one possibility. If it is proven that paracrine cell-derived factors are indeed responsible for improvements in cardiac function, then protein-based therapy might be more clinically and logistically viable than a cell-based therapy.

Whilst some would contend that there is no place for clinical trials on imperfectly understood science, I would disagree. In fact medical history is testament to the use of incompletely understood therapies being employed effectively, for example Edward Jenner and smallpox vaccination or bone marrow transplantation, in the latter the responsible cell type and kinetics took years to clarify. If we wait for every single nuance regarding a new treatment or drug to be clearly defined, we would be denying potentially life-saving treatments that

are already in wide-spread use. Such enthusiasm should be tempered by the potential pit-falls of accelerating too soon which could resemble the uncomfortable fanfare with which gene therapy arrived a decade ago and has since failed to deliver. Many questions remain regarding the optimal cell type, number, method of delivery and mode of action. The recent development of in vivo cell tracking technology has facilitated more meaningful studies looking at transplantation kinetics.

This experimental work has shown that the right ventricular myocardium in hypoplastic left heart syndrome may be at an inherent disadvantage structurally and in terms of mounting an appropriate genetic response to generate sufficient haemodynamic force to sustain the circulation as a whole.

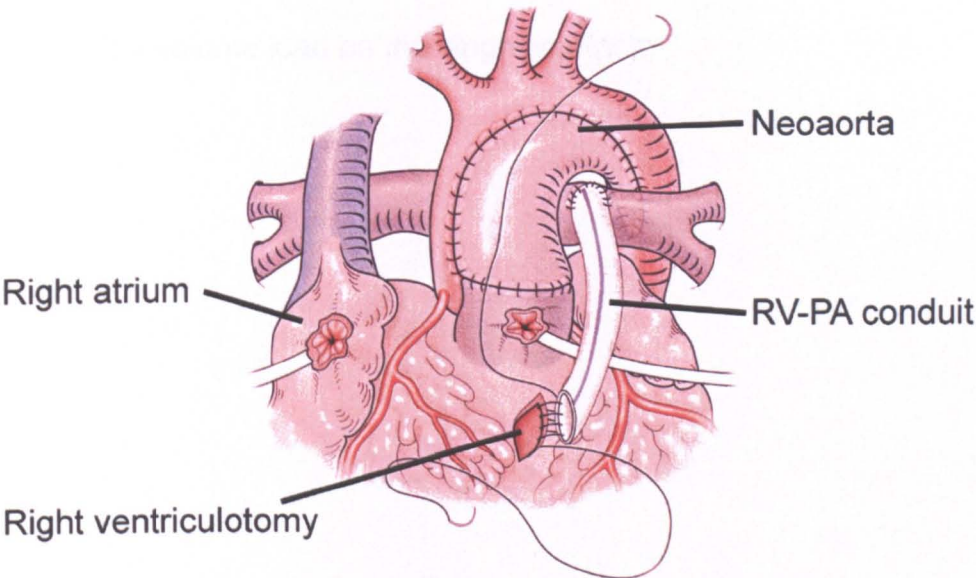
I have also demonstrated a novel proof of concept using human umbilical cord blood stem cells in a clinically-relevant neonatal large animal model of right ventricular training and cellular xenotransplantation. Cell therapy has the potential to assist myocardial adaptation to supraphysiological loads and deserves further investigation.

In the future, I would like to examine in closer detail the viral cardiomyopathies and also more genetically-subtle small animal models of congenital heart disease. In the former, I postulate that the homeostatic balance of the myocyte mass becomes disturbed secondary to a viral infection and the occurrence of molecular mimicry. It is conceivable that stem cell transplantation in this situation could stimulate the recovery of resident stem cell activity and myocyte cycling. The latter offer an improvement of traditional lethality models and I would like to attempt stem cell “rescue” of Id mice or similar knock-out strain prone to cardiac defects using haematopoietic stem cells from normal and HLHS pregnancies.

6.0                      **Supplementary data**

**Figure S2.1** Diagrams summarising the typical sequence of palliative surgery performed for HLHS

Stage 1                      **Modified Norwood Operation**



Usually performed in the first week of life and aims to create unobstructed outflow to the systemic circulation and well-balanced systemic and pulmonary circulations. The atrial septum is excised to allow unrestricted pulmonary venous return to the single morphological ventricle. The pulmonary artery is anastomosed to the native hypoplastic aorta which is typically reconstructed using a patch of homograft material. A shunt from the right ventricle to the pulmonary arteries provides pulmonary blood flow.

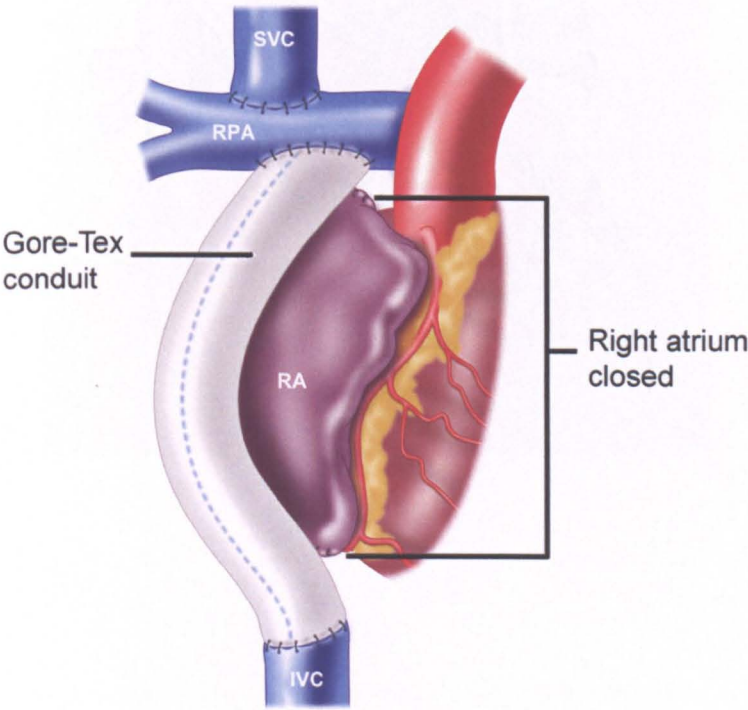
## **Stage 2      Bidirectional cavopulmonary shunt**

Generally performed on infants between 3 and 6 months of age.

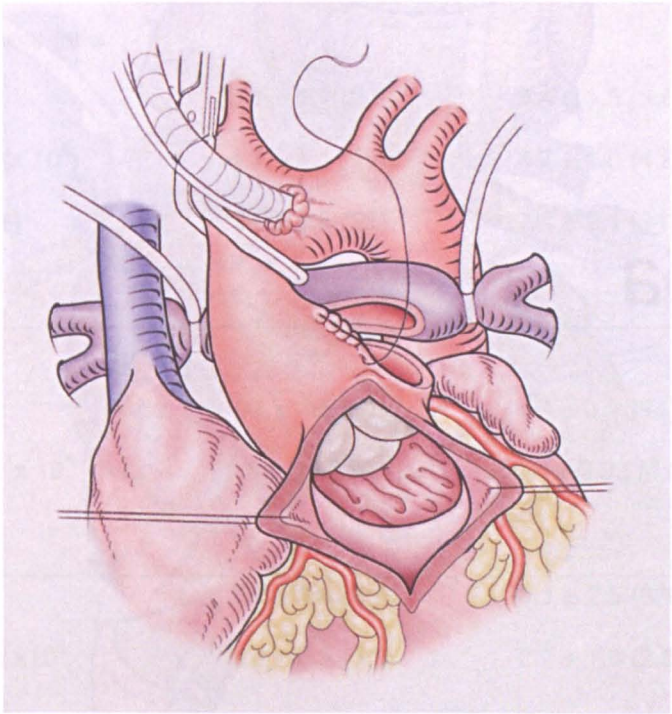
The superior vena cava is anastomosed to the ipsilateral pulmonary artery so that systemic venous return from the head and neck returns to the pulmonary circulation directly. This reduces the volume load on the single ventricle

Stage 3      Extra-cardiac Fontan

This completes the reconstructive surgery for HLHS and is performed around 5 years of age. A Gore-Tex tube is used to direct inferior caval blood to the pulmonary artery, allowing systemic venous blood to flow directly and passively to the pulmonary circulation. Using an external conduit means minimal suturing of the right atrium, a risk factor for late arrhythmias.



**Figure S2.2** Diagram showing the site of the right ventriculotomy made during the operative repair of truncus arteriosus. This ventriculotomy was the source of the biopsy material and more importantly, permits the establishment of right ventricle to pulmonary artery continuity



**Table S4.1** Stem cell characteristics

	<b>Group 2</b>	<b>Group 4</b>	<b>P</b>
	<b>CBSC Injection</b>	<b>PAB &amp; CBSC</b>	
	<b>(n = 6)</b>	<b>(n = 8)</b>	<b>t test</b>
<b>Number and viability</b>			
Number of mononuclear cells			
(x 10 <sup>8</sup> )	3.1 ± 1.7 (2.9)	3.4 ± 1.5 (3.6)	0.72
Number of Lin <sup>-</sup> cells (x 10 <sup>6</sup> )	4.7 ± 1.1 (4.4)	4.7 ± 1.0 (4.8)	0.88
Post-thaw viability (%)	94 ± 2.5 (95)	94 ± 2.1 (94)	0.93
<b>Surface markers</b>			
CD45+ (%)	92 ± 2.5 (91)	94 ± 3.1 (94)	0.11
absolute cell number x 10 <sup>6</sup>	4.5 ± 0.87 (4.1)	4.3 ± 0.93 (4.2)	0.72
CD45- (%)	7.7 ± 3.4 (8.5)	6.1 ± 2.5 (6.7)	0.32
absolute cell number x10 <sup>5</sup>	3.9 ± 2.5 (3.5)	2.7 ± 1.6 (2.5)	0.29
CD34+ (%)	59 ± 8 (56)	57 ± 11 (58)	0.63
absolute cell number x 10 <sup>6</sup>	2.8 ± 0.5 (2.9)	2.6 ± 0.6 (2.8)	0.53
CD34- (%)	41 ± 8 (45)	43 ± 11 (42)	0.63
absolute cell number x 10 <sup>6</sup>	2.0 ± 0.8 (1.8)	2.1 ± 0.9 (1.9)	0.83
CD34+ / CD45+ (%)	62 ± 12 (57)	57 ± 11 (58)	0.42
absolute cell number x 10 <sup>6</sup>	2.9 ± 0.5 (2.9)	2.6 ± 0.6 (2.8)	0.25

	<b>Group 2</b>	<b>Group 4</b>	<b>P</b>
	<b>CBSC Injection</b>	<b>PAB &amp; CBSC</b>	
	<b>(n = 6)</b>	<b>(n = 8)</b>	<b>t test</b>
CD34+ / CD33+ / CD38+ (%)	36 ± 22 (41)	37 ± 17 (47)	0.91
absolute cell number x 10 <sup>6</sup>	1.8 ± 1.3 (1.7)	1.7 ± 0.9 (1.8)	0.89
CD34+ / CD33- / CD38- (%)	31 ± 27 (17)	37 ± 17 (47)	0.70
absolute cell number x 10 <sup>6</sup>	1.4 ± 1.1 (0.9)	1.2 ± 1.1 (0.7)	0.68
CD34- / CD45+ (%)	37 ± 12 (34)	39 ± 11 (38)	0.80
absolute cell number x 10 <sup>6</sup>	1.7 ± 0.6 (1.5)	1.8 ± 0.8 (1.6)	0.80
CD34- / CD45- (%)	7.1 ± 3.1 (6.5)	6.3 ± 5.6 (4.8)	0.74
absolute cell number x10 <sup>5</sup>	3.7 ± 2.4 (3.1)	2.9 ± 2.4 (2.2)	0.54
All data expressed as mean ± SD (median)			

% shown is percentage of lineage negative population

## **6.1 PCR primers**

Human  $\beta$ -2 microglobulin

Forward/Sense: 5'-GTGTCTGGGTTTCATCAATC-3'

Reverse/Antisense: 5'-GGCAGGCATACTCATCTTTT-3'

$\beta$ -actin

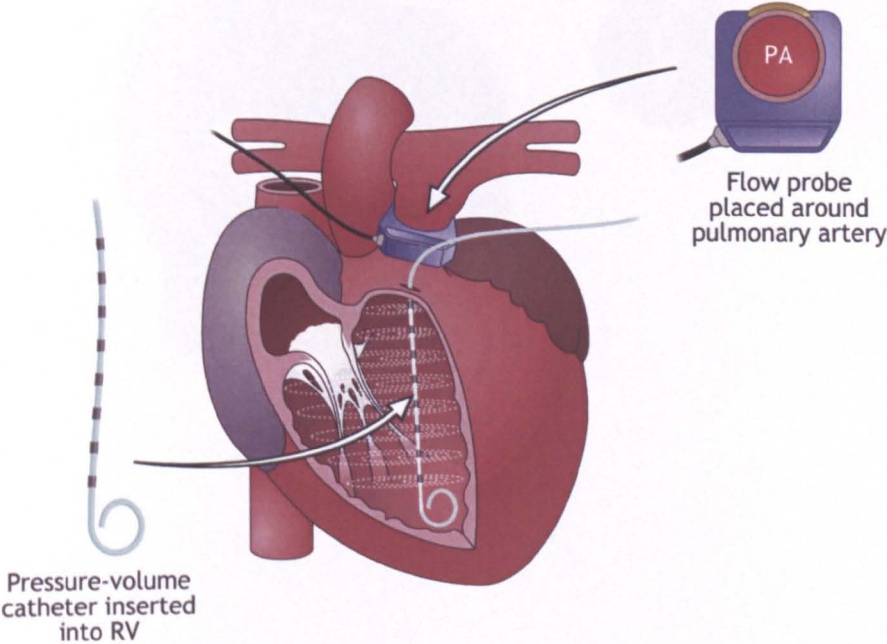
Forward/Sense: 5'-CGGGACCTGACTGACTAC-3'

Reverse/Antisense: 5'-GAAGGAAGGCTGGAAGAG-3'

6.2 Haemodynamic data acquisition and analysis

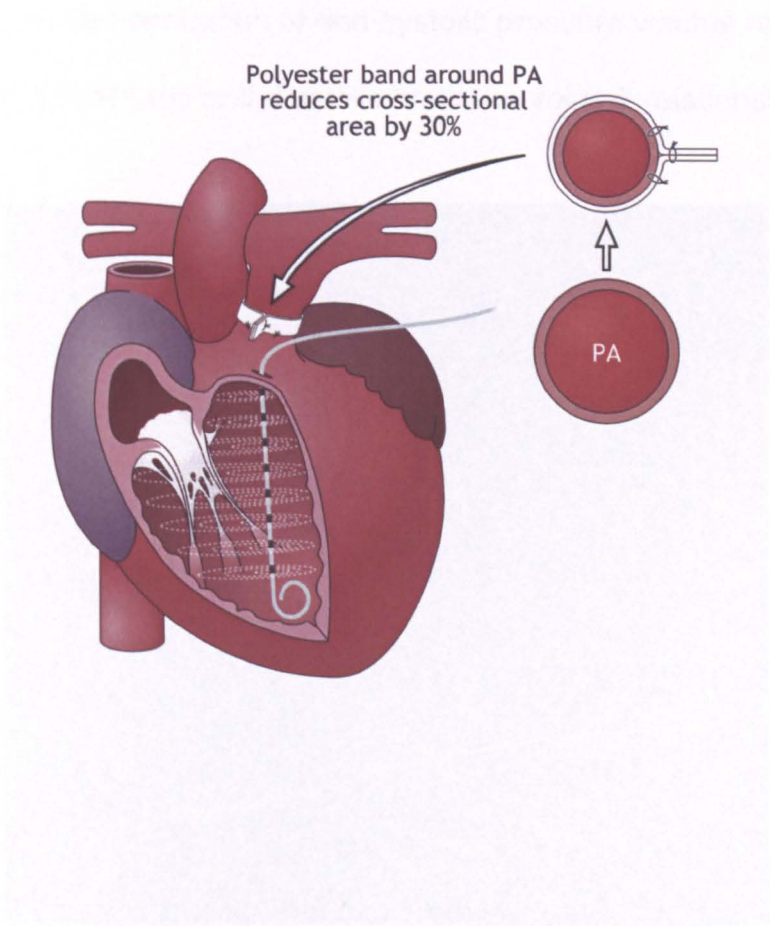
The pressure-volume catheter was used in conjunction with a pressure signal amplifier (Millar Instruments, Houston, Texas, USA) and signal conditioner (Leycom Sigma 5/DF, CardioDynamics, Zoetermeer, The Netherlands). A perivascular ultrasonic flow probe (Transonics, Ithaca, NY, USA) placed around the pulmonary artery just distal to the pulmonary valve was used to give simultaneous cardiac output (Figure S1).

Figure S4.1 Diagram of operative set up



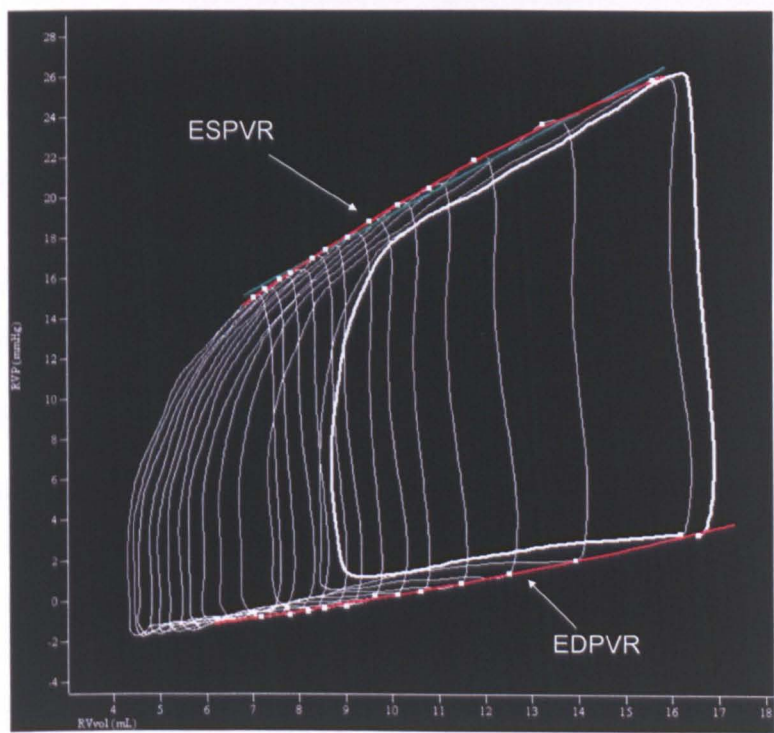
Right ventricular pressures and volumes, maximal slopes of systolic pressure increment ( $dP/dt_{\text{Max}}$ ) and diastolic decrement ( $dP/dt_{\text{Min}}$ ) and relaxation time constant ( $\tau$ ; exponential fit method) were calculated using customised software.

**Figure S4.2** Diagram of pulmonary artery banding, demonstrating reduction of cross-sectional area by 30%



Right ventricular systolic and diastolic functional indices were also obtained during transient occlusions of the inferior vena cava to reduce preload. Systolic contractile indices included the slope and volume-axis intercept of the linear end-systolic pressure volume relationship (End-systolic elastance); preload-recrutable stroke work (PRSW), the slope of the relationship between stroke work and end-diastolic volume; and the quadratically modelled end-diastolic pressure volume relationship ( $dP/dt_{\text{Max}}$ -EDV). Diastolic indices included the end-diastolic pressure volume relationship, tau and  $dP/dt_{\text{Min}}$ , as described above.

**Figure S4.3** Pressure-volume loops following occlusion of inferior vena cava with demonstration of end-systolic pressure volume relationship (ESPVR) and end-diastolic pressure-volume relationship



Parallel conductance of the right ventricle was evaluated by using injections of hypertonic saline solution into the right atrium; slope factor was determined using cardiac output to allow for correction of volume measurements at post-hoc analysis. Linear modelling was found to be adequate over the range of pressures experienced within the right ventricle. Intraclass correlation of quadratic versus linear modelling of ESPVR regression during preload reduction was assessed using 386 data sets by one-way analysis of variance. 0.989; [95% CI 0.979 to 0.999]

### **6.3            Immunohistochemistry & laser scanning                   confocal microscopy**

Slides were examined using an Olympus IX70 fluorescent inverted microscope (Olympus Corp., Tokyo, Japan) and a Leica TCS SP2 SE laser scanning confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Negative controls were used to calibrate laser power and detector gain settings and control for autofluorescence.

**Table S4.2** Summary table of Invasive haemodynamic measurements

Group		1	2	3	4
		Control	CBSC	PAB	PAB & CBSC
Cardiac output (l/min)	Baseline	1.6 ± 0.3	1.6 ± 0.5	1.6 ± 0.4	1.6 ± 0.2
	One month	2.3 ± 0.4	2.3 ± 0.3	2.3 ± 0.4	2.3±0.4
Cardiac index (l/min/kg)		0.24 ± 0.04	0.27 ± 0.07	0.26 ± 0.07	0.26 ± 0.06
		0.19 ± 0.04	0.2 ± 0.02	0.21 ± 0.04	0.22 ± 0.06
End diastolic volume (ml)		16.2 ± 5.2	15.0 ± 4.7	16.3 ± 4.1	17.2 ± 2.9
		32.1 ± 6.3	31.8 ± 4.9	27.9 ± 6.6	29.4 ± 7.0
End systolic volume (ml)		7.9 ± 2.3	7.7 ± 2.2	8.2 ± 1.1	8.1 ± 1.5
		16.2 ± 3.1	15.7 ± 2.0	18.6 ± 3.6	17.7 ± 4.5
Stroke volume (ml)		8.4 ± 1.0	8.5 ± 1.2	8.2 ± 1.3	8.4 ± 1.8
		15.9 ± 2.0	15.9 ± 2.7	14.6 ± 2.2	14.6 ± 2.0
Stroke work (mmHg.ml)		120.7 ± 16.5	126.6 ± 33.0	125.2 ± 33.5	128.2 ± 20.3
		257.1 ± 32.8	249.8 ± 26.9	445.8 ± 73.9	465.4 ± 90.6

<b>Group</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
	<b>Control</b>	<b>CBSC</b>	<b>PAB</b>	<b>PAB &amp; CBSC</b>
<b>End diastolic pressure</b> (mmHg)	5.5 ± 1.8	5.9 ± 4.5	5.9 ± 3.9	6.0 ± 2.4
	3.8 ± 2.6	3.1 ± 2.3	6.7 ± 2.7	6.5 ± 2.4
<b>End systolic pressure</b> (mmHg)	19.3 ± 1.9	19.6 ± 5.0	20.7 ± 2.5	20.3 ± 2.9
	14.9 ± 2.4	14.8 ± 2.1	41.7 ± 6.9	41.0 ± 6.8
<b>dPdtMax</b> (mmHg/s)	461.2 ± 61.8	471.3 ± 42.6	461.0 ± 53.0	460.0 ± 64.0
	412.8 ± 39.1	427.3 ± 33.1	632.3 ± 86.2	673.3 ± 97.5
<b>dPdtMin</b> (mmHg/s)	317.3 ± 33.9	322.7 ± 43.6	333.9 ± 56.3	337.9 ± 34.7
	271.2 ± 21.7	276.8 ± 27.8	585.3 ± 93.7	606.1 ± 93.8

Group	1	2	3	4
	Control	CBSC	PAB	PAB & CBSC
tau	36.1 ± 3.8	36.0 ± 7.9	35.2 ± 8.5	35.0 ± 4.7
(ms)	31.3 ± 2.8	30.6 ± 2.6	48.4 ± 3.9	43.7 ± 3.4
Diastolic stiffness constant	0.23 ± 0.06	0.23 ± 0.04	0.23 ± 0.05	0.24 ± 0.05
(k)	0.16 ± 0.03	0.16 ± 0.02	0.28 ± 0.05	0.25 ± 0.03
Ees slope	1.11 ± 0.07	1.11 ± 0.11	1.18 ± 0.11	1.09 ± 0.13
	1.04 ± 0.05	1.10 ± 0.11	2.20 ± 0.18	2.70 ± 0.16
PRSW slope	10.9 ± 0.8	11.3 ± 1.0	11.7 ± 1.4	11.3 ± 2.0
	10.6 ± 1.4	11.0 ± 1.6	27.5 ± 3.3	32.2 ± 3.8
dPdtmax-EDV slope	12.7 ± 4.2	12.5 ± 3.4	13.3 ± 2.6	11.9 ± 2.1
	10.3 ± 3.4	10.3 ± 3.1	25.3 ± 4.5	29.6 ± 4.3
All values mean ± SD				

**Table S4.3** Summary morphometric data

		Placebo	Cells	Band	Band & cells	P
		(n=6)	(n=6)	(n=8)	(n=8)	
Age (days)	Baseline	10.8 ± 3.0	9.0 ± 1.7	10.9 ± 2.8	10.6 ± 2.1	NS
	One month	43.8 ± 6.3	43.2 ± 3.7	43.6 ± 6.2	44.8 ± 6.0	NS
Body weight (kg)	Baseline	6.8 ± 0.7	6.0 ± 0.2	6.1 ± 0.9	7.2 ± 1.2	NS
	One month	10.9 ± 1.5	11.0 ± 0.9	11.1 ± 1.0	12.5 ± 2.4	NS
Total heart weight (g)		64.0 ± 8.4 (62.0)	64.2 ± 10.2 (62.0)	83.9 ± 16.6 (82.0)	90.0 ± 17.0 (84.5)*	<0.05
Heart to body weight index (g/kg)		6.1 ± 0.7 (6.0)	5.8 ± 0.6 (5.9)	6.5 ± 1.3 (5.9)	6.5 ± 0.8 (6.5)	NS
RV weight (g)		11.1 ± 1.4 (11.0)	11.3 ± 1.5 (11.0)	15.6 ± 3.1 (15.0)	18.1 ± 4.4 (18.0)*†	<0.05
RV to body weight index (g/kg)		1.1 ± 0.2 (1.1)	1.0 ± 0.1 (1.0)	1.4 ± 0.6 (1.3)	1.4 ± 0.3 (1.3)	NS

	Placebo (n=6)	Cells (n=6)	Band (n=8)	Band & cells (n=8)	P
RV thickness (mm)	4.5 ± 0.5 (4.5)	4.5 ± 0.8 (4.0)	5.9 ± 0.6 (6.0)*†	6.2 ± 0.9 (6.0)*	<0.05
LV thickness (mm)	9.2 ± 0.7 (9.3)	9.6 ± 0.8 (9.5)	8.9 ± 1.1 (9.0)	9.4 ± 0.7 (9.5)	NS
RV:LV thickness ratio	0.50 ± 0.08 (0.49)	0.47 ± 0.11 (0.46)	0.67 ± 0.05 (0.46)*†	0.66 ± 0.09 (0.68)*	<0.05

Mean ± SD (range)

One-way ANOVA or Kruskal Wallis with Tukey or Dunns post-testing, as appropriate

\* versus Placebo

† versus Cells

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