

**DEVELOPMENT OF METHODS FOR THE  
TISSUE ENGINEERING OF CARDIAC VALVES  
USING MESENCHYMAL STEM CELLS**

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# Summary

Cardiac valve replacement is the second most common heart operation in the Western World. Valve replacements currently available are poorly adapted for use in young patients who typically require multiple re-operations as they grow, resulting in increased morbidity and mortality. Tissue-engineering a living heart valve replacement would be an ideal substitute as it would not require drug therapies or re-operation. Bone marrow-derived mesenchymal stem cells have been shown to be capable of differentiating into a variety of lineages and may be advantageous as a cell source for tissue engineering applications.

Attempts were made to isolate human and porcine mesenchymal stem cells (MSC) from the respective bone marrows using gradient-centrifugation to separate the cells and complete Dulbecco's Modified Eagle's Medium (DMEM) to culture the cells. The cellular material from one bone marrow was transferred to one 25cm<sup>2</sup> tissue culture flask. Cell differentiation was attempted by supplementing complete culture medium with growth factors and biochemicals. The phenotype of the human MSC (hMSC) was then examined by FACS analysis. A previously developed decellularised porcine aortic valve matrix was biochemically characterised to ensure that the major matrix components had not been removed by the decellularisation procedure. Furthermore the biocompatibility of the decellularised tissue compared to fresh tissue was assessed by subcutaneous implantation into mice (n=4). hMSC were then seeded onto the decellularised porcine aortic valve matrices *in vitro* and the ability of the cells to migrate into the tissue compared to smooth muscle cells in a static culture system was assessed.

Putative porcine MSC (pMSC) and hMSC were successfully isolated from their respective bone marrows. Putative pMSC were found to have a cell doubling time (CDT) of 106 hours and hMSC had a CDT of 151 hours. A failure to successfully culture pMSC was found to be due to a change in the supplier of DMEM used. Attempts to differentiate porcine MSC (pMSC) produced adipogenic cells but failed to produce osteogenic, chondrogenic, neurogenic, myogenic or smooth muscle lineage cells. hMSC were successfully differentiated into cells of the adipogenic, myogenic and neurogenic lineage. However, attempts to clone the cells were unsuccessful. FACS analysis of hMSC indicated that the cells were CD45<sup>-</sup>, CD13<sup>+</sup>, D7FIB<sup>+</sup>, CD105<sup>+</sup>, CD10<sup>+/-</sup>, LNGFR<sup>+/-</sup>, CD55<sup>+</sup>, BMP<sup>-</sup> and AP<sup>+/-</sup>. Analysis of hydroxyproline, sulphated proteoglycan and DNA content of the decellularised porcine valve tissue indicated no change in collagen or GAG content and removal of cellular DNA. Implanted decellularised tissues were accepted by the mice in comparison to fresh tissue and appeared to be undergoing regeneration. Furthermore, the cell infiltrate into the matrices was favourable, being low in T-cells but more macrophages and endothelial cells. Seeding the decellularised matrix with hMSC showed that the cells migrated into the tissue to up to 2% of the cell density found in native valve tissue compared to 0% of the smooth muscle cells.

Failure to clone hMSC meant that differentiation could have been a result of multiple cell precursors being present in the bone marrow rather than a stem cell population. Biochemical and biocompatibility analyses of the decellularised porcine valve matrix showed that the tissue was unaltered by the procedure and biocompatible. hMSC were also observed to migrate into the tissue under static seeding conditions.

In conclusion, mesenchymal stem cells represent a promising cell source for tissue engineering a living aortic heart valve. However, more research is required to further characterise the cells and optimise their growth and differentiation. The decellularised porcine valve matrix developed by shows potential as a matrix for tissue engineering an aortic heart valve.

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## List of abbreviations

$\alpha$ -gal:	Gal $\alpha$ 1-3Gal epitope
$\alpha$ SMA:	$\alpha$ -smooth muscle actin
AP:	Alkaline phosphatase
APC:	Antigen presenting cell
AS:	Adult stem cell
BIO:	Biotin
BMP:	Bone morphogenetic protein
BSA:	Bovine serum albumin
BSE:	Bovine spongiform encephalopathy
CD:	Cluster of differentiation
CDT:	Cell doubling time
Col II:	Collagen II
DABCO:	1,4-diazobicyclo-(2, 2, 2)-octane
DMEM:	Dulbecco's modified Eagle's medium
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DNase:	Deoxyribonuclease
DSHB:	Developmental Studies Hybridoma Bank
ECACC:	European Collection of cell cultures
EDTA:	Ethylenediaminetetraacetic acid
ES:	Embryonic stem cell
FACS:	Fluorescence activated cell sorting
FCS:	Foetal calf serum
FGF-2:	Fibroblast growth factor-2

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GAGs:	Glycosaminoglycans
GAM:	Goat anti-mouse
HBSS:	Hank's balanced salt solution
HCMs:	Heavy chain myosin slow
HEPES:	N-(2-hydroxyethyl)piperazine-N'-(2-ethanulfonic acid)
HLA:	Human leukocyte associated antigen
hMSC:	Human mesenchymal stem cell
HRP:	Horseradish peroxidase
hSMC:	Human smooth muscle cell
IFN $\gamma$ :	Interferon gamma
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
ITSS:	Insulin-transferrin sodium selenite
LGI:	Leeds General Infirmary
LNGFR:	Low-affinity nerve growth factor receptor
MHC I:	Major histocompatibility complex type I
MHC II:	Major histocompatibility complex type II
MLR:	Mixed lymphocyte reaction
MSC:	Mesenchymal stem cell
NBS:	National Blood Service
NHS:	National Health Service
NSE:	Neurone specific enolase
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PDGF:	Platelet derived growth factor
PE:	Phycoerythrin

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PERVs:	Porcine endogenous retroviruses
PGA:	Polyglycolic acid
PLA:	Polylactic acid
pMSC:	Porcine mesenchymal stem cell
RNase:	Ribonuclease
rt-PCR:	Reverse-transcriptase polymerase chain reaction
SDS:	Sodium dodecyl sulphate
SEM:	Scanning electron microscopy
TBS:	Tris buffered saline
TBSS:	Tyrode's balanced salt solution
TGF $\beta$ :	Transforming growth factor beta



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# **CHAPTER ONE:**

## *Introduction*

# CHAPTER 1:

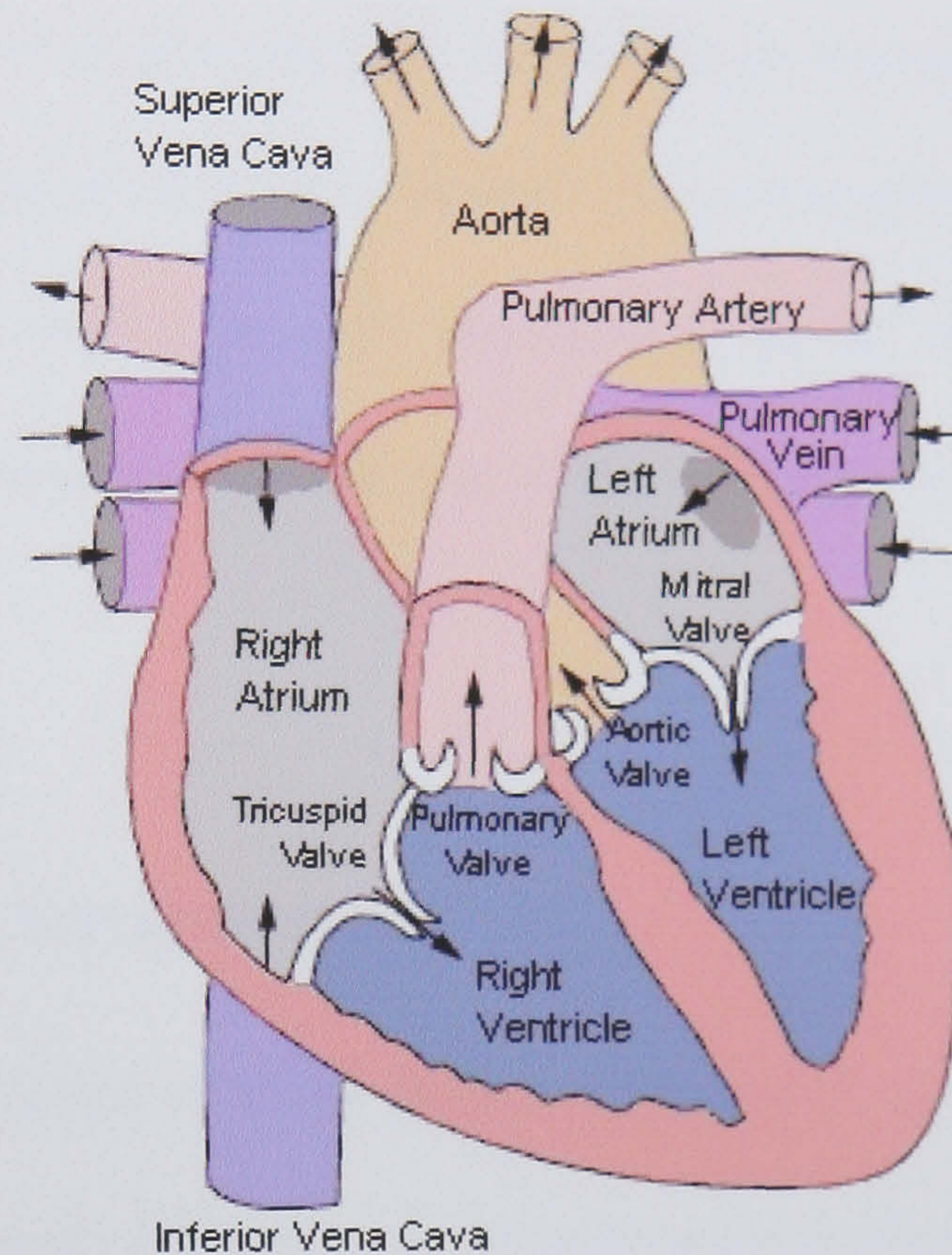
## *Introduction*

**C**ardiovascular disease is the leading cause of death in the Western World, with an estimated 62 million sufferers in the US and an additional 40,000 children suffering from congenital heart defects [Perry & Roth, 2003]. Some 20,000 people are thought to die in the United States alone as a result of heart valve dysfunction [Shinoka *et al.*, 1997]. If caught in the earliest stages therapies can be implemented to help alleviate the problems, however, the most severe cases will ultimately require some form of transplant, be it a whole heart, or just an individual valve. The availability of these replacements is restricted and unpredictable, leading to long waiting lists of patients requiring transplants, many of whom die before a replacement becomes available. Mitral valves are most frequently repaired rather than replaced, and the current techniques give favourable results. For aortic heart valves, mechanical and bioprosthetic alternatives are available, but these are not adequate for all patients, since they are unable to grow and repair, often requiring drugs to maintain their longevity. Thus, an alternative is required to overcome these problems, particularly for paediatric patients who currently require multiple reoperations as they grow.

### **1.1. *The Human Heart***

The human heart beats over 100,000 times a day, circulating a volume of approximately 5 litres of blood through, what is estimated to be nearly 100,000 miles of blood vessels in the average adult. In order to do this effectively, the pressure of the blood as it travels through the heart, particularly as it leaves through the aorta, is considerable. To

facilitate efficient blood flow in the correct direction there are four valves that prevent blood regurgitation between the chambers and major vessels of the heart during the resting phase of the cardiac cycle. The tricuspid valve separates the right atrium and ventricle; the mitral valve separates the left atrium and ventricle; the pulmonary valve separates the pulmonary artery from the right ventricle and the aortic valve separates the aorta from the left ventricle. An explanatory diagram can be seen in Figure 1.1.



**Figure 1.1. A diagram of the human heart**

[[http://www.pediheart.org/kidzone/The\\_heart/Anatomy/anatomy.html](http://www.pediheart.org/kidzone/The_heart/Anatomy/anatomy.html)].

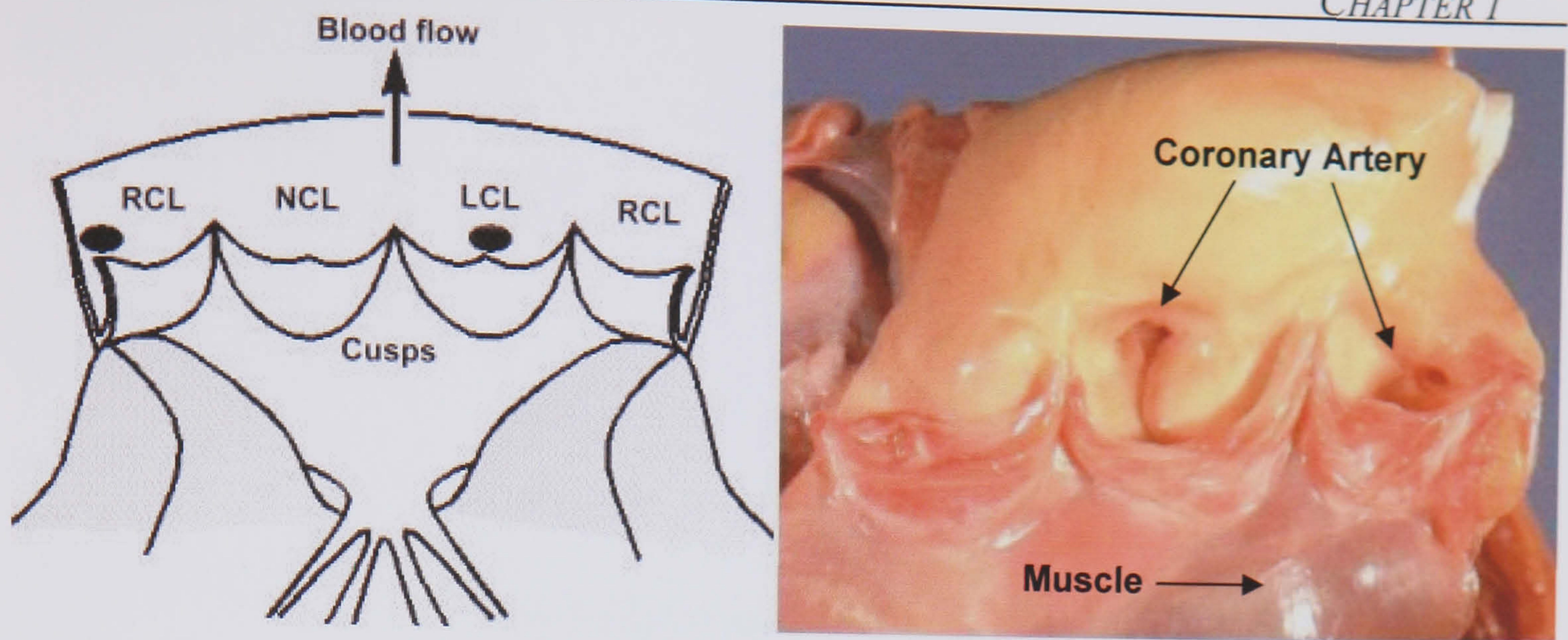
After coronary artery bypass grafting, valve replacement is the second most common major heart operation in the western world. Any one of the four valves in the heart may require repair (valvotomy) or, more commonly, replacement as a result of congenital problems or age and disease related degeneration of the valve tissue [Carabello & Crawford, 1997]. This results in progressive calcification, which can bring about stenosis, or valve failure, which induces valvular prolapse and blood regurgitation. This leads to a range of symptoms depending on the severity of malfunction, including shortness of breath, chest pain, tachycardia, palpitations, atrial fibrillation and orthopnea [Merck Manual of Medical Information (online), 2003]. Symptoms can also manifest in

other forms such as strokes, as thromboembolisms that originated in the heart valve become dislodged and move through the body's blood vessels before they become lodged elsewhere. In certain cases, such as endocarditis, death can result in a matter of days if surgical intervention is not rapidly implemented.

The most critical and most prone to failure of the four valves is the aortic valve. It is here that blood pressure is highest and the largest forces are applied to the tissues. This makes the valve very difficult to repair and adds to the complexity of creating a suitable replacement. Prior to a description of attempts to tissue engineer an aortic valve replacement, it is first important to understand the structure and function of the native tissue.

## **1.2. *Aortic Valve Structure***

The aortic valve is composed of three leaflets (or cusps), which can be identified as being the left-coronary, right-coronary, or non-coronary leaflet (Figure 1.2.). They are distinguished by the presence of coronary arteries just above the leaflets on the right and left coronary leaflets, which supply the heart tissue itself with blood. The non-coronary leaflet (NCL) has no such artery and the right (RCL) and left coronary leaflets (LCL) can be distinguished by the attachment of muscle near to the base of the right-coronary leaflet whilst there is none on the left (Figure 1.2.) [Sutton *et al.*, 1995].



**Figure 1.2. Schematic diagram identifying the individual leaflets of the aortic valve.** NCL: non-coronary leaflet; LCL: left coronary leaflet; RCL: right coronary leaflet. The ovals represent the coronary arteries. Muscle is found at the base of the RCL, making it distinct from the LCL

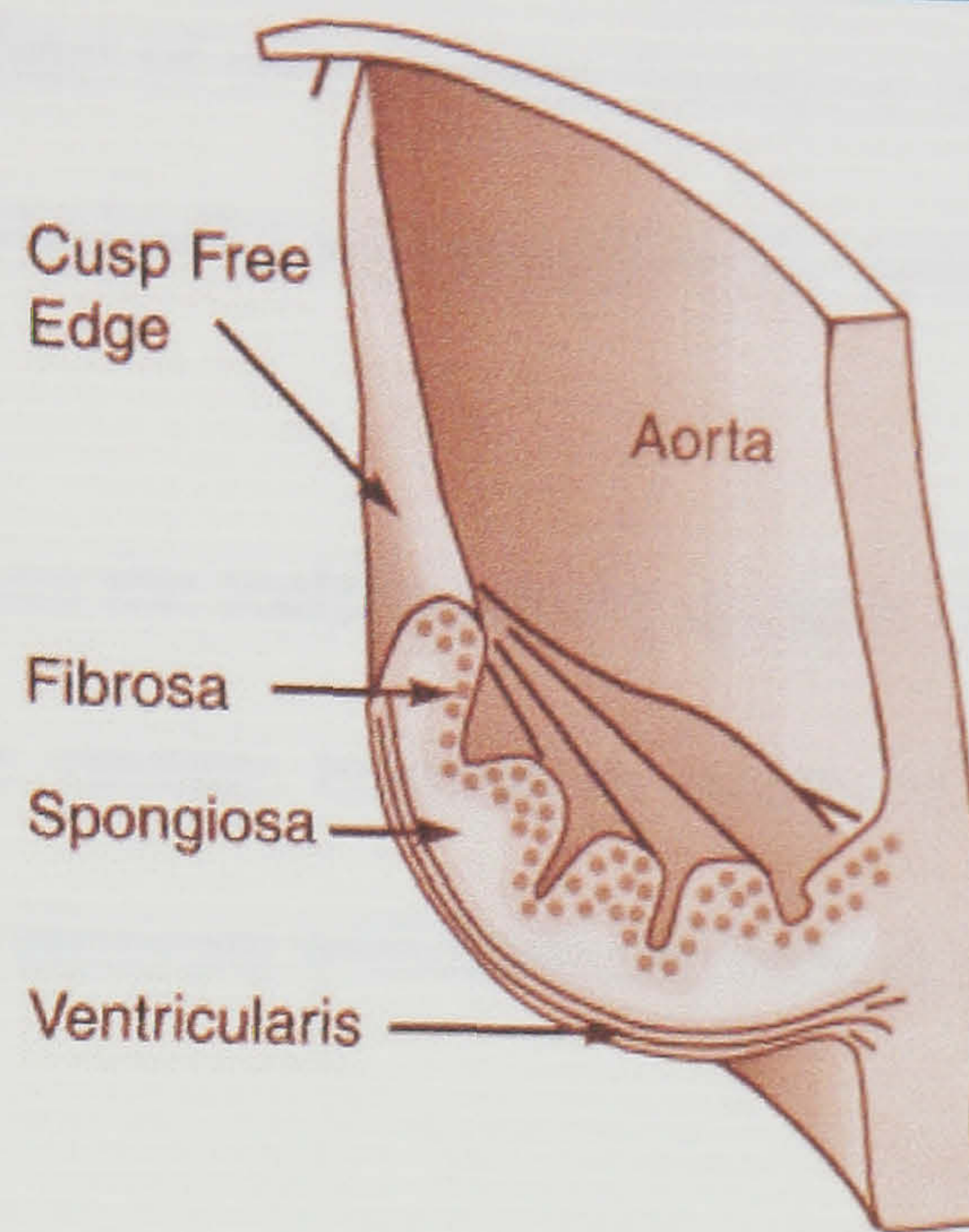
[<http://medlib.med.utah.edu/WebPath/CVHTML/CVIDX.html>].

When the valve is open, the leaflets bend outwards to let the blood flow through and upon closing each leaflet meets its neighbour along a junction extending from the periphery to the centre of the valve orifice to fully occlude the aorta and prevent blood regurgitation [Sutton *et al.*, 1995].

### 1.2.1. Tissue Layers

There are three well-defined tissue layers within each leaflet (Figure 1.3.) [Schoen, 1999]:

1. The *ventricularis* faces the inflow surface and is predominantly collagenous with radially aligned elastin fibres;
2. The centrally located *spongiosa* is composed of loosely arranged collagen and has an abundance of glycosaminoglycans (GAGs); and
3. The *fibrosa* faces the outflow surface and is composed predominantly of circumferentially aligned, macroscopically crimped, and densely packed collagen fibres; largely arranged parallel to the leaflet's free edge.



**Figure 1.3. The three-layered structure of the valve leaflet [Vesely, 1998].**

Within the leaflet layers, the structural elements are arranged in a non-random orientation, leading to properties that are highly anisotropic [Schoen, 1999]. Several structural features enable leaflets to be a) extremely soft and pliable when unloaded, b) virtually inextensible when back-pressure is applied, and c) to have a much higher tissue compliance in the radial direction than in the circumferential direction. These include i) gross corrugations of the fibrosa, which produce a visible surface rippling in systole but disappear during diastole; ii) an abundance of collagen cords in the fibrosa which radiate primarily from the commissures and confer strength; and iii) crimping of the collagen fibres. During closure, the crimping expands in the radial direction, permitting an initial increase in size with minimal stress. The stiffening induced by fully extended collagen crimps and taut collagen cords prevents excessive sagging of the leaflet centres when the valve is closed and thereby prevents leaflet prolapse [Schoen, 1999]. The elastin of the ventricularis extends when the leaflets stretch to enlarge the coaption area but recoils to make the leaflet smaller when the valve is open. The function of the spongiosa is poorly understood, but it is likely that shear stresses and flexion-fatigue caused by the differential movements of the layers and the shock of valve closure are both dissipated by the ability of the outer layers to glide upon this intervening spongy

layer, thus relieving the effect of compression forces on the fibrosa, and tensile forces on the ventricularis of flexing leaflet segments [Davila, 1989; Schoen, 1999].

Therefore, the collagen gives the leaflets tensile strength, the elastin allows for flexion and deformation, and the spongy connective tissue of the spongiosa permits the necessary movement of all elements within the leaflet body [Davila, 1989].

### 1.2.2. Aortic Valve Cells

Two cell types are present within the leaflets: an outer layer of endothelial cells and a layer of deep interstitial cells [Schoen, 1999]. Morphologically, heart valve interstitial cells have characteristics of both fibroblasts and smooth muscle cells [Messier *et al.*, 1994]. It is not clear, however, whether these cells represent a single cell type (myofibroblast) or whether there are two populations of cells within the valve [Lester *et al.*, 1988; Mulholland & Gotlieb, 1996; Roy *et al.*, 2000]. Amongst others Taylor *et al.* (2000), however, have found that two distinct populations of cells exist within the interstitium of porcine valve leaflets, one characterised by prominent stress fibres and the other by prominent synthetic and secretory organelles. Of the cells they examined, some had abundant Golgi complexes and rough endoplasmic reticulum with few filaments or microtubules, whilst others showed dense bundles of microfilaments throughout the cytoplasm with less rough endoplasmic reticulum and Golgi complexes present in the body of the cell. The bundles of microfilaments were most prominent along the periphery and extended into the long cell processes. In contrast, Rabkin *et al.* (2002), described the cells singularly as being myofibroblasts, but went on to demonstrate that the cells showed a varied protein expression that changed when a construct consisting of cells grown on a synthetic scaffold *in vitro* was implanted *in vivo*. To complicate matters further, Lester *et al.* (1988) have described electron dense



structures occurring along the microfilament bundles. Other features of interstitial cells include the presence of functional gap junctions between the cells. Gap junctions have been identified by electron microscopy of valve tissue [Lester *et al.*, 1988; Messier *et al.*, 1994; Mulholland & Gotlieb, 1996], suggesting that cells in the valve interstitium might be in communication with each other.

The outer surfaces of the valve leaflets also possess an endothelial cell lining. This cell layer plays a vital role in preventing platelet adhesion and subsequent embolus formation. It is also responsible for responding to and regulating the secretion and molecular transport of growth factors, cytokines and hormones in order to regulate perfusion and permeability in the microcirculation [Bachetti & Morbidelli, 2000].

### **1.2.3. Other Cellular Features of Aortic Valves**

Motor nerve terminals have been found in close juxtaposition to the valvular interstitial cells, mostly towards the base of the leaflets, although their relevance is currently unknown [Mulholland & Gotlieb, 1996]. In a study of the vasculature of the leaflets, Weind *et al.* (2000) found that of 15 porcine aortic valves examined, nine valves had vasculature in all three leaflets and two valves were completely avascular. Vessels were found predominantly in the basal third of the leaflets and extended in from the commissures almost to the level of the free edge. Thus it is possible that a normally functioning aortic valve receives oxygen from the oxygen-rich blood passing across its surfaces, but at the base diffusion distances exceed metabolic requirements and a compensatory microcirculation develops [Weind *et al.*, 2000].

#### 1.2.4. Aortic Valve Function

It is thought that no flow or pressure gradient across the valve is required to open the valve initially and purposeful contraction of the valve leaflets has been postulated [Messier *et al.*, 1994]. Higashidate *et al.* (1995) believed that the aortic valve opens before the onset of aortic blood flow because of the increase in the restoring force that tends to make a valve orifice triangular, owing to the increase in the distances between the commissures without forward flow. The reason for this can be deduced from the fact that the left ventricular pressure elevation induces aortic root expansion, subsequent commissure separation, and stretching of the free edges of the valve leaflet [Higashidate *et al.*, 1995]. Therefore it is believed that the valve has an intrinsic potential to open.

Aortic heart valves are subjected to enormous stresses (both tensile and flexion) during over 100,000 cardiac cycles per day, and as a consequence of these forces, some internal damage must occur through cell shearing and general wear and tear of the tissues [Deck *et al.*, 1988]. Normal valve tissue has been shown to have a considerable metabolic activity [Weind *et al.*, 2000], and this most probably represents an active repair mechanism to maintain the structural integrity of the valve leaflets. The fact that vascularisation is found predominantly in the basal regions of the leaflets corresponds with the fact that this region endures the majority of the flexural stress imposed on the valve during the cardiac cycle, and therefore it will be the area that suffers the greatest structural damage [Weind *et al.*, 2000]. Deck *et al.* (1988) have shown a positive correlation between turnover rates of matrix components such as proteins and glycosaminoglycans (GAGs) and the amount of stress measured in distinct regions of rat aortic valves during the cardiac cycle. This lends support to the idea of ongoing repair within the tissue. The myofibroblastic cells within the interstitial layer in aortic valves are thought to play an important role in wound healing, and their presence

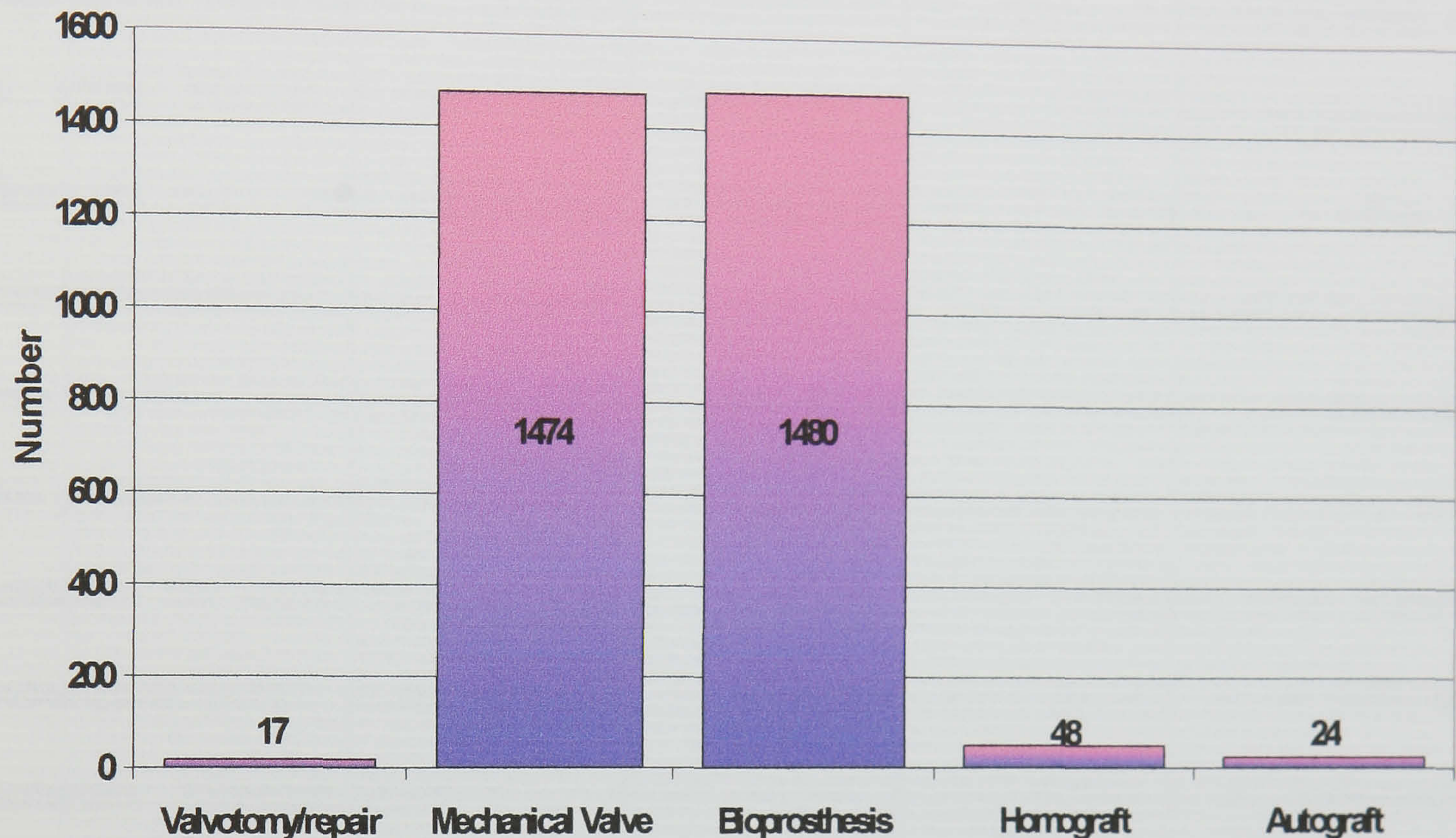
supports the concept of an active repair mechanism [Weind *et al.*, 2000]. The need for continual turnover within valve tissue could help explain the failure associated with glutaraldehyde-fixed bioprosthetic valves, which contain no viable cells [Weind *et al.*, 2000].

There remains considerable variability in the current understanding and characterisation of the heart valve leaflets. Of the information discussed here, there is much ambiguity in the reports written so far and these issues need to be addressed to fully understand the tissue and cell functions of the valves and maximise the potential benefits of tissue engineering replacement valves. For example, Weind *et al.* (2000) stated that vasculature although present in the majority of the leaflets they examined, was not ubiquitous in all the samples. This presents a potential problem to the success for all future tissue engineered valves because if vasculature is required and is not included during the engineering of the valves, all attempts will ultimately fail if the seeded cells die. However, if it is not required, much time could be saved in the production of the valves. It is also essential to assess the full importance of the motor nerve terminals found by Mulholland & Gotlieb (1996) as again there could be potential functional failure of any tissue engineered valve if they are not taken into account. Overall, further study and characterisation of aortic valve leaflets is essential.

### ***1.3. Currently Available Heart Valve Replacements***

Throughout the world, 95% of all valve replacements are performed for mitral and aortic valves, the vast majority of which are aortic valve replacements. There are approximately 225,000 heart valve procedures performed world-wide each year [St Jude Medical Inc., 2003], and replacement heart valves are estimated to be a \$259.1 billion industry [Gangireddy *et al.*, 2001]. This market is expected to continue to grow due to

the ageing population and the emergence of a need for the replacements in developing countries. Many different designs of prosthetic valves have been created since the 1950s and the replacement procedures all have a high success rate. The different types and numbers of single open valve replacement surgeries performed in the UK in 2000-2001 are shown in Figure 1.4.



**Figure 1.4.** The different types and numbers of single open valve replacement surgery performed in the UK in 2000-2001 without coronary artery bypass grafting (CABG) [Data included in the National Audit Cardiac Surgical Report 2000-2001: <http://www.scts.org/>].

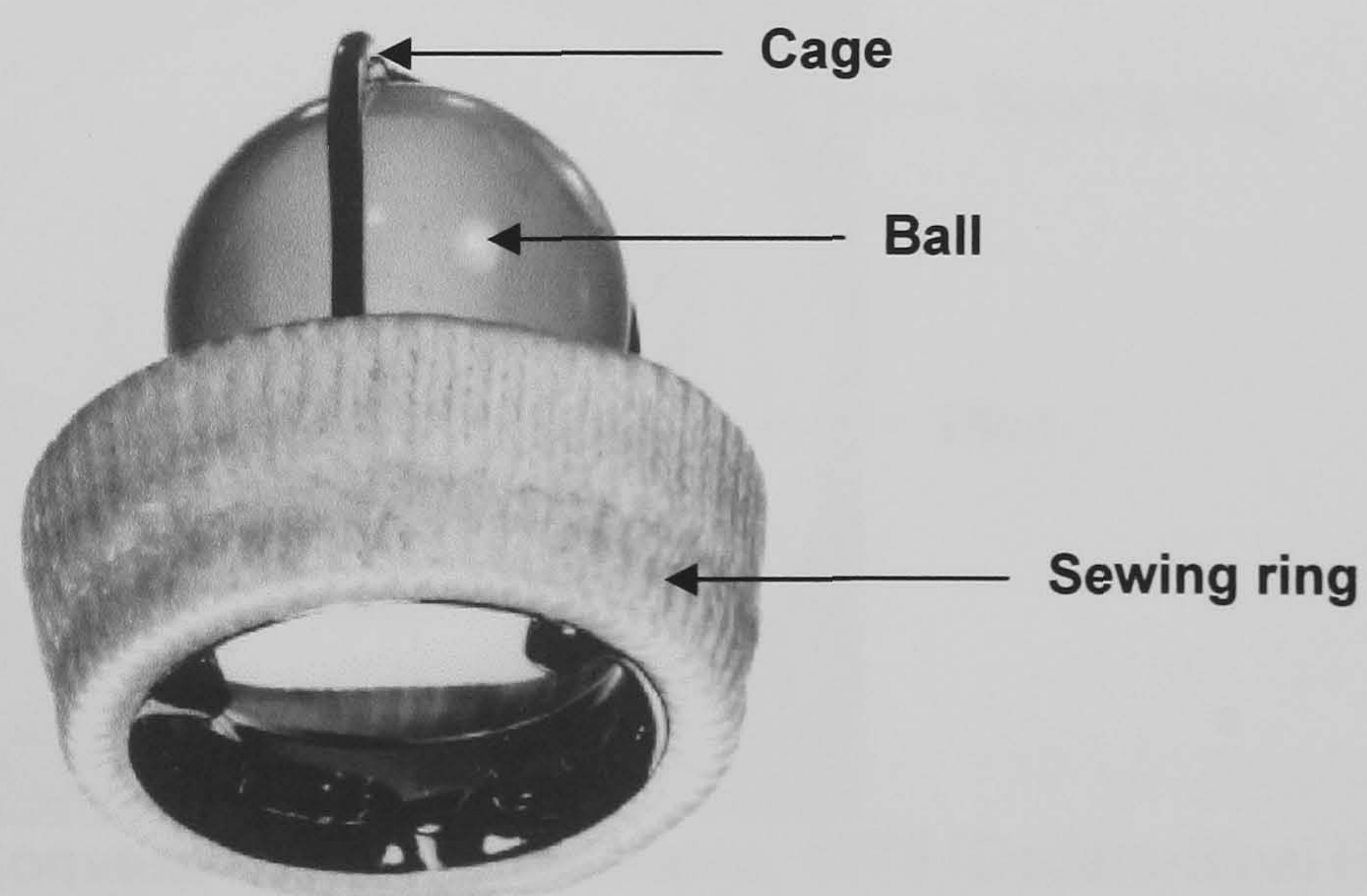
### 1.3.1. Mechanical Valves

#### 1.3.1.1. Ball Valves

The first artificial heart valve was implanted by Charles Hufnagel in 1952 to aid a poorly functioning natural heart valve [DeWall *et al.*, 2000]. This device consisted of a methacrylate ball contained in a methacrylate tube. The ball was situated at the proximal end of the tube during diastole, and three bulbous pouches that opened around the ball in its systolic position at the distal portion of the tube allowed blood to flow in one direction. The valve was sited in the descending thoracic aorta, secured by nylon rings

containing teeth on its inner surface to hold the aorta to the prosthesis. The valve was designed for use in patients with aortic insufficiency and was a major breakthrough for valvular disease.

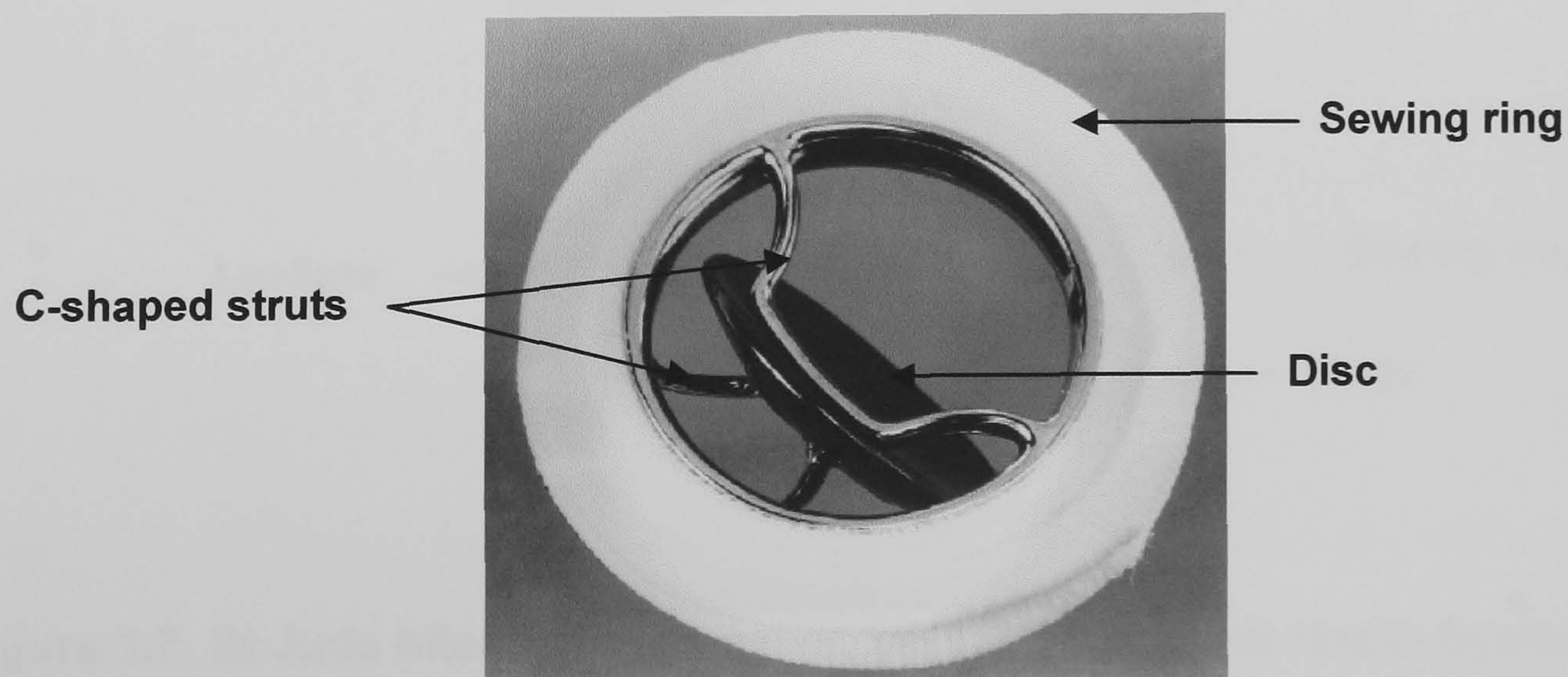
Modifications and improvements on this design brought the Starr-Edwards ball and cage valve, which became available in the early 1960s (Figure 1.5.) [Senthilnathan *et al.*, 1999]. This device consisted of a rubber ball in a cage in which the ball moved to close the valve under back-pressure. Although effective and durable as a valve, it altered the haemodynamics of the blood flowing over it because the ball had to be larger than the valve opening in order for it to effectively seal the valve when it was shut, and this partially obstructed blood flow, even when the valve was open. This resulted in a tendency for blood clotting; leading to thrombosis and ultimately heart failure. Subsequent models of this type aimed to reduce the size of the ball and use different materials. Although these initial valves functioned appropriately in the mechanical sense, they were totally different to the natural valve. Consequently, despite eliminating the original valve failure, they frequently induced additional problems. Therefore, despite these designs being some of the most durable designs developed, ball and cage valves are rarely implanted today.



**Figure 1.5. Starr-Edwards Ball and Cage valve, (se1000), 1960 [Cedars-Sinai Health System, 2003].**

### 1.3.1.2. Single Leaflet Disc Valves

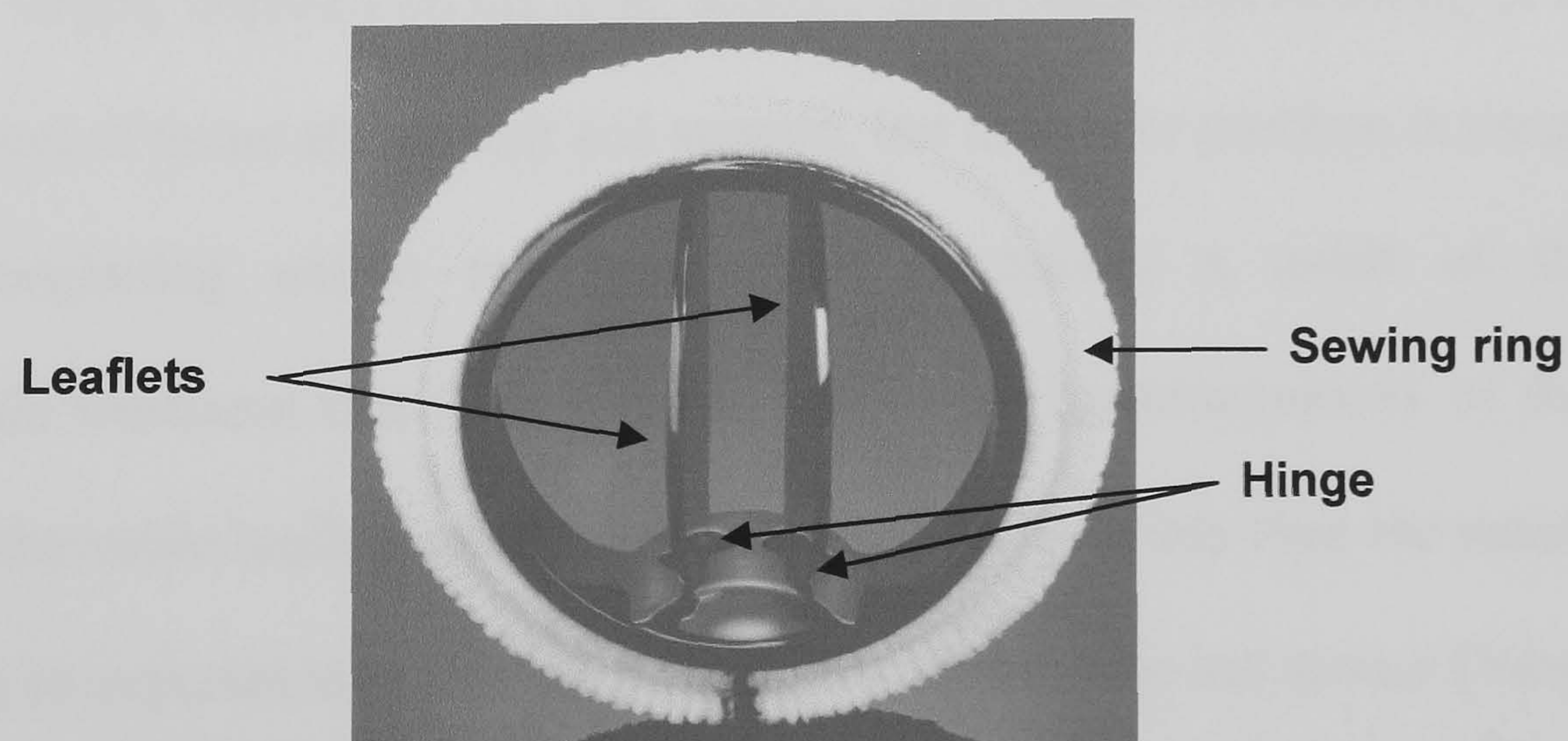
The next mechanical valves to be developed were the single leaflet disc valves. The most historically important single leaflet valve and initially one of the most successful, was the original Björk-Shiley valve (Figure 1.6.) of the late 1960s [Cedars-Sinai Health System, 2003]. The Björk-Shiley valve is a tilting disc valve with a single disc that is held in place by two small C-shaped metal inflow struts and an outflow strut. Tens of thousands have been implanted in the United States and across the world. The original model of the Björk-Shiley valve was an extremely reliable and durable valve. It had good haemodynamics and a low rate of thromboembolism, making it a very successful and durable design [Senthilnathan *et al.*, 1999; Cedars-Sinai Health System, 2003]. Unfortunately, attempts to improve the haemodynamics of the original Björk-Shiley valve by redesigning it led to disaster. Certain models of the Björk-Shiley valve developed strut fractures resulting in thromboemboli forming on the disc. This led to all models of the Björk-Shiley valve being removed from the United States valve market, although it is still available today in some countries [Cedars-Sinai Health System, 2003].



**Figure 1.6. Björk-Shiley Convexo-Concave UR valve, 1975 [Cedars-Sinai Health System, 2003].**

### 1.3.1.3. Bileaflet Heart Valves

Bileaflet heart valves were first developed in the late 1970s and one of the first and by far the most successful bileaflet valves has been the St. Jude Valve, shown in Figure 1.7, [Wang, 1989]. The leaflets and inner ring of the St. Jude Medical mechanical heart valve are made of graphite and coated with pyrolytic carbon. Pyrolytic carbon was chosen for the coating because of its hardness, strength, and durability. It is also resistant to platelet adhesion, which helps to minimise the risk of blood clot formation. The outer ring of the valve is a polyester cuff that is sewn into the remaining tissue of the heart. Since 1977, more than one million people have received St. Jude Medical mechanical heart valves [St. Jude Medical Inc., 2003]. It has excellent durability, good haemodynamics and is currently the most commonly implanted valve in the United States, with over 90% of valves being of this design [Senthilnathan *et al.*, 1999; Cedars-Sinai Health System, 2003]. Indeed it is regarded as the gold standard to which all other prosthetic valves are compared. The valve leaflets open easily, offer minimum resistance to flow, and when closed have insignificant regurgitation [Senthilnathan *et al.*, 1999].

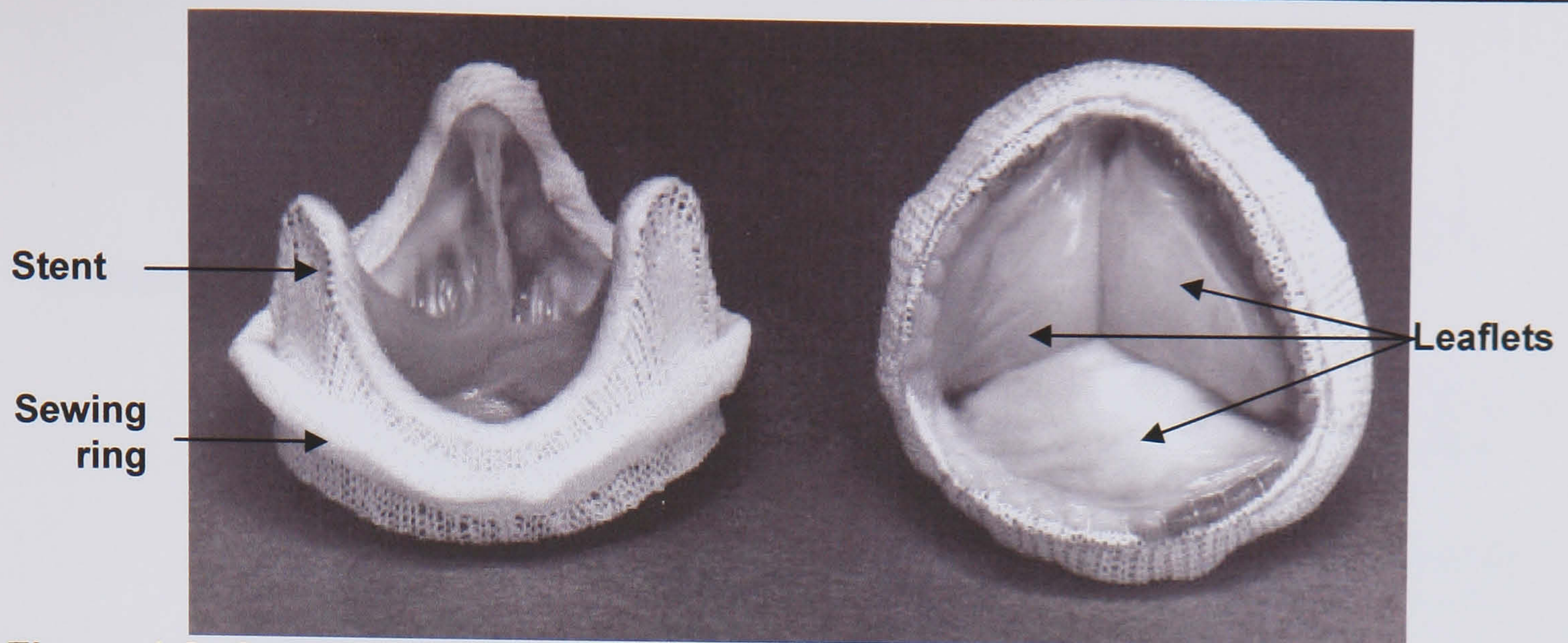


**Figure 1.7. St Jude bileaflet heart valve, 1977 [Cedars-Sinai Health System, 2003].**

### 1.3.2. Bioprosthetic Valves

The late 1960s saw the development of bioprosthetic valves in an attempt to overcome the haemodynamic problems of their mechanical counterparts. Bioprosthetic valves are usually hybrid structures consisting of either porcine cuspal tissue or bovine pericardial tissue, usually cross-linked with aldehydes, and a synthetic stent and sewing ring. Essentially, valve tissue is sewn onto a metal wire stent, which is bent to form three 'U' shaped prongs. A cloth (Dacron) sewing skirt is attached to the base of the wire stent and the stents are also covered with cloth. Non-stented valves are also available and are either completely or nearly completely composed of tissue and are sewn directly into the surgically prepared aortic valve sites [Schoen, 1999]. These implanted animal tissue valves are referred to as xenografts or heterografts. An example can be seen in Figure 1.8. Gluteraldehyde-fixed bioprosthetic valves were first introduced in 1969 in an attempt to reduce tissue immunogenicity and mechanical valve related thromboembolism and the subsequent risk of haemorrhage [Korossis *et al.*, 2000]. Such was the interest in these valves; about 60% of all valves inserted in the 1970s were of this type. Unfortunately tissue valves were subsequently found to have a high incidence of structural deterioration, [Senthilnathan *et al.*, 1999]. The function and success of these valves largely depends on the type, source, preservation and handling of the tissue and the method of tissue attachment and support, but the major problem is tissue failure; frequently beginning within ten years. This is mainly a result of the harsh gluteraldehyde treatment that is required to reduce the immunogenicity of the valves. This makes the valve leaflets much more rigid and less flexible than the natural valve, leading them to experience greater stresses and therefore wear out sooner [Vesely *et al.*, 1995]. Alternative fixative chemicals such as genipin, and altering other variables such as the fixation pressure on the valve leaflets have yet to provide leaflets with mechanical properties comparable to the natural leaflets [Sung *et al.*, 1999].





**Figure 1.8.** Carpentier-Edwards porcine aortic valve (CE2625), 1975 [Cedars-Sinai Health System, 2003].

### 1.3.3. Human Tissue Valves

Valvular tissue can be transplanted between persons and from one site to another in the same individual. Homografts are valves transplanted from another human (Figure 1.9.). A valve is removed from a human cadaveric donor and preserved in liquid nitrogen (cryopreserved) until needed. The valve must be thawed overnight prior to being transplanted into the recipient. This means that a surgeon must know in advance what size and type of valve he or she is going to use. Homograft valves tend to have good haemodynamics and good durability but are also technically harder to implant than standard bioprosthetic valves such as the Carpentier-Edwards valve [Cedars-Sinai Health System, 2003]. A major problem, as with all organ transplants, is that homograft availability is limited by donor availability.

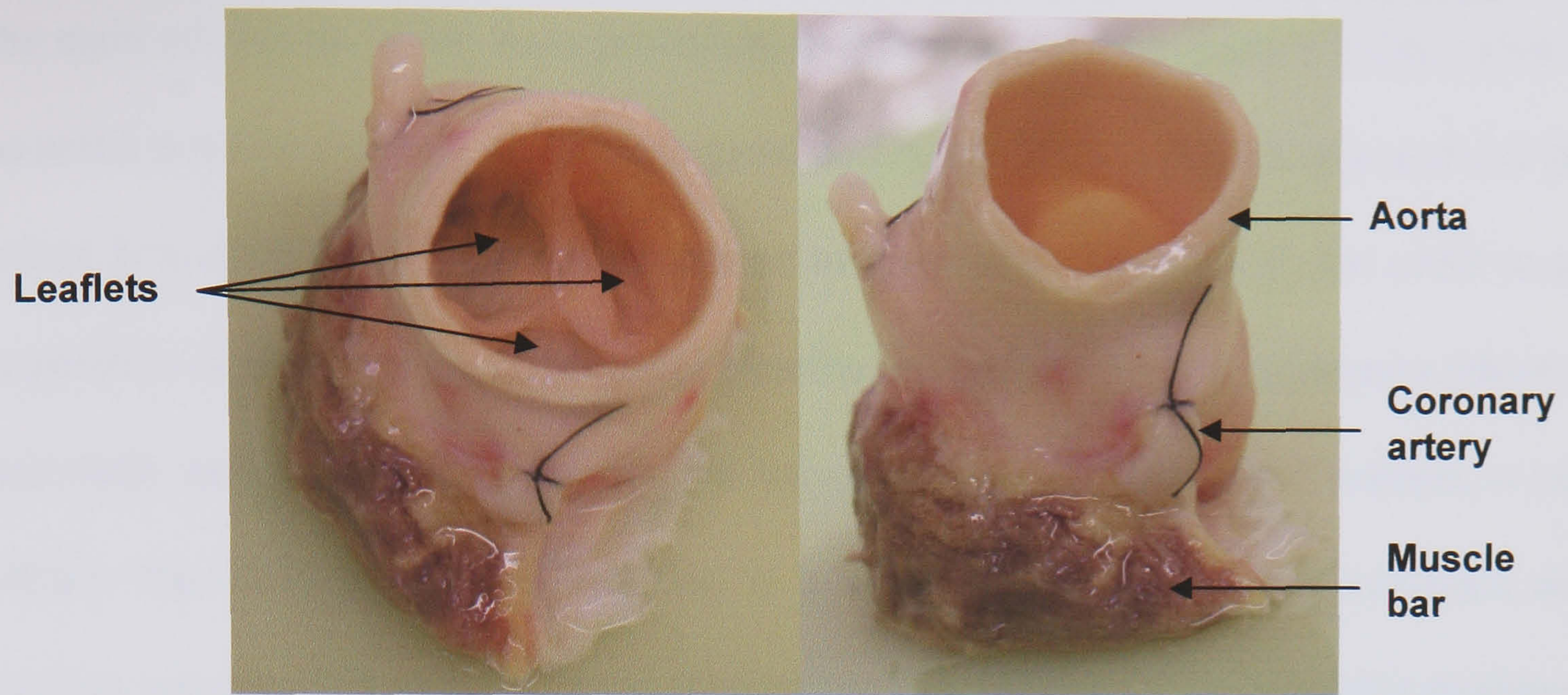


Figure 1.9. A human aortic homograft valve.

Autografts are valves taken from the same patient in which the valve is implanted. The most common autograft procedure is the Ross procedure developed by Mr. Donald Ross (Figure 1.10.), [Dohmen *et al.*, 2002a]. The abnormal aortic valve is removed and the patient's own pulmonary valve is transplanted to the aortic position to take its place. A homograft pulmonary valve is then used to replace the patient's pulmonary valve.

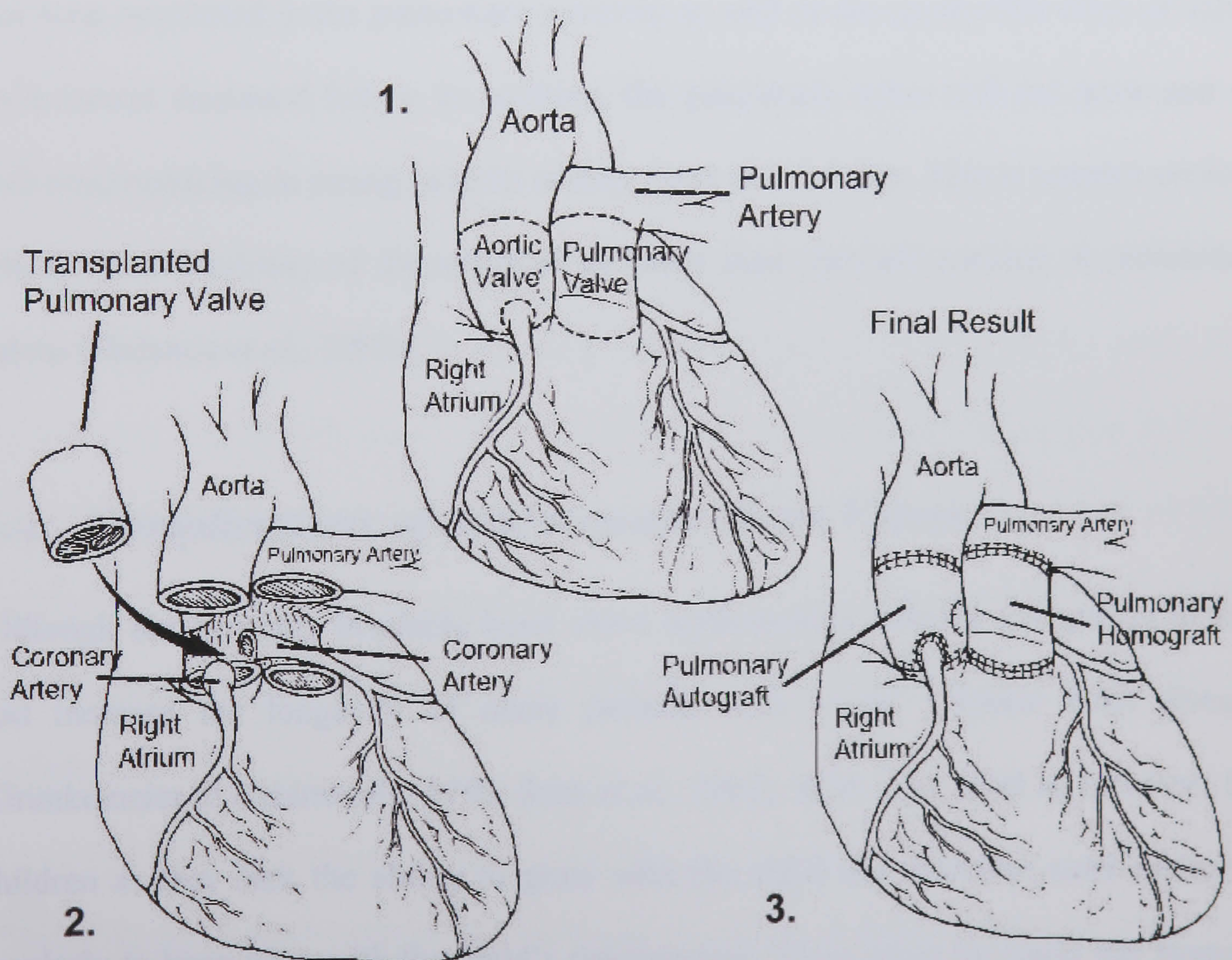


Figure 1.10. The Ross procedure [Cedars-Sinai Health System, 2003].

The main advantage of the Ross procedure is that the patient receives a living valve in the aortic position that will continue to grow and repair. This is particularly useful if the patient is a child as the valve can continue to grow and develop with the child so no reoperation is required. Other potential benefits are improved haemodynamics (there is essentially no pressure drop across the valve) and good durability [Dohmen *et al.*, 2002a]. The Ross procedure, however, is a very technically difficult procedure and involves considerable skill and time. The pulmonary valve must be carefully measured and sculpted to fit the aortic root and the replacement pulmonary homograft must similarly be shaped to fit the pulmonary root. There are many potential complications, the most common of which is aortic regurgitation [Dohmen *et al.*, 2002a]. Many patients have small amounts of aortic regurgitation but some have moderate or even severe amounts and require a second operation for valve replacement. Other problems include stenosis of a coronary artery, right-sided endocarditis (since a homograft valve has been implanted in the pulmonary position) as well as the usual difficulties of valve replacement discussed below. In addition, the pulmonary valve will not grow and so will need replacing in young patients as they grow and develop. Also it remains unclear whether the durability of the autograft is better than standard porcine or pericardial valves [Dohmen *et al.*, 2002a].

#### **1.4. *Complications of Replacement Heart Valves***

Although the currently available heart valve replacements enhance the quality of life and increase the longevity of many patients with severe valvular heart disease [Grunkemeier & Rahimtoola, 1990; Starr *et al.*, 2002], there is no ideal replacement for children as they lack the ability to grow with the child and therefore need replacing regularly to keep pace with the child's development. Even when the child has stopped growing, the valuable lifetime of the valves is frequently less than ten years before they

need to be replaced. Also, there may well be prosthetic valve related complications. Replacement associated problems necessitate re-operation or cause death within ten years postoperatively in at least 50-60% of patients undergoing heart valve replacement surgery [Schoen *et al.*, 1992, 1999; Korossis *et al.*, 2000]. The most common replacement related conditions include [Grunkemeier & Rahimtoola, 1990; Schoen, 1999; Korossis *et al.*, 2000]:

- i. Thromboembolism, thrombosis and anticoagulation related haemorrhage;
- ii. Non-structural dysfunction, including dehiscence (the separation of the prosthetic valve suture line from the heart, leading to para-valvular leakage, seen mainly in the immediate postoperative period), peri-valvular leak, disproportion (occurring when the prosthetic valve is not fitted well, leading to deficient blood flow), haemolysis, tissue overgrowth, and other extrinsic interactions of host tissues with the prosthesis;
- iii. Structural failure (degeneration of the prosthesis biomaterials); and
- iv. Prosthetic valve endocarditis.

Prosthetic valve endocarditis is particularly problematic in this situation as the bacteria responsible readily colonise the prosthetic valve and form a biofilm. This occurs when the bacteria irreversibly attach to the prosthesis and surround themselves with an extracellular polymeric substance that protects them from the effects of the host immune system and antimicrobial therapeutic agents administered to combat the infection [Donlan & Costerton, 2002]. Consequently the infection becomes very difficult to eradicate.

Clinical studies have indicated that the composite rates of valve related complications are similar for both mechanical and bioprosthetic valve replacements [Korossis, *et al.*,

2000; Starr *et al.*, 2002]. The frequency and nature of specific valve related conditions, however, can vary with the type of prosthesis, model, implantation site and patient characteristics. The main disadvantage of mechanical prosthetic valves is the substantial risk of systemic thromboemboli and local thrombotic occlusion [Grunkemeier & Rahimtoola, 1990; Schoen *et al.*, 1992], which requires chronic anticoagulation therapy as a preventative measure. The rate of thrombosis per patient per year for the St. Jude valve, for example, is approximately 5% [Wang, 1989]. Therefore in 20 years, a patient with one of these valves is essentially guaranteed to have some form of thromboembolic event. Chronic oral anticoagulation itself carries a risk of haemorrhage with a frequency of approximately 4% per patient per year, particularly retroperitoneal, gastrointestinal or cerebral, 15-25% of these events are fatal [Schoen *et al.*, 1992]. However, mechanical valves do offer patients extended durability, which limits the mortality and morbidity risks associated with reoperation for replacement of degenerated bioprostheses [Fradet *et al.*, 1995]. In contrast, since tissue valves maintain a low rate of thromboembolism without anticoagulation, their recipients are generally not anticoagulated and therefore have a superior quality of life. The main disadvantage with tissue valves, however, is structural failure due to progressive tissue deterioration (via both calcification and non-calcification mechanisms) [Butany & Leask, 2001]. Prosthetic valve endocarditis and non-structural dysfunction affect both tissue and mechanical valves almost equally. Unexpectedly, perhaps, noise can also be a problem with mechanical valves as they can be heard to make a ticking sound as they shut during the heart cycle, some valves being louder than others [Blome-Eberwein *et al.*, 1996].

Moreover, the cost of treating valve complications is not inconsequential. The average costs in the US of treating an episode of care for valve related complications in 1996

ranged from \$8,699 for the medical management of structural dysfunction to \$84,750 for the lifetime management of an embolism [Caro *et al.*, 1996].

### 1.4.1. Choice of Replacement Aortic Valve

Aortic valve replacement is performed primarily for degenerative valve disease in increasingly older patients [Birkmeyer *et al.*, 2000]. In elderly patients, mechanical valves may be less favourable because risks of anticoagulation-related bleeding increase with age. In addition, tissue valve failure, which typically occurs 10 to 15 years after valve implantation, may be less of a concern for older patients who have lower valve failure rates and shorter life expectancies [Grunkemeier & Rahimtoola, 1990; Birkmeyer *et al.*, 2000]. Therefore the optimal valve prosthesis depends on patient age, reflecting the time-dependent nature of trade-offs between mechanical and tissue valves. Consequently, although mechanical valves are associated with greater life-expectancy in younger patients, tissue valves confer greater benefit for the majority of patients undergoing valve replacement who are 60 years of age and older [Birkmeyer *et al.*, 2000].

The use of prosthetic devices for aortic valve replacement in infants and children with congenital heart valve defects is associated with numerous short-term and long-term complications and, in most cases, is considered palliative [Vitale *et al.*, 1999]. In short, tissue valves gradually deteriorate over time and mechanical valves, require anticoagulation therapy, which is especially undesirable in children [Turrentine *et al.*, 2001]. Of particular importance, neither is capable of growth and development and as such, multiple reoperations are frequently required as the child ages. Consequently, the Ross procedure has become the primary choice for aortic valve replacement in paediatric patients because of the growth potential, optimal haemodynamic

performance, and lack of the need for anticoagulation [Laudito *et al.*, 2001]. However, this also has drawbacks as concerns persist due to potential dilatation of the autograft, the incidence of reoperation for autograft dysfunction and the residual or increasing autograft insufficiency. It is for these reasons that an alternative must be sought that possesses the capability to adaptively grow and remodel without compromising other heart valves or necessitating further reoperation or drug therapies.

### ***1.5. Ten Commandments for the Prosthetic Valve***

An alternative heart valve prosthesis is therefore required that is suitable for all patients; particularly children. Harken (1989) defined ten commandments for the prosthetic valve:

1. It must not propagate emboli.
2. It must be chemically inert and not damage blood elements.
3. It must offer no resistance to physiological flows.
4. It must close promptly (less than 0.05 seconds).
5. It must remain closed during the appropriate phase of the cardiac cycle.
6. It must have lasting physical and geometric features.
7. It must be inserted in a physiological site (generally the normal anatomical site).
8. It must be capable of permanent fixation.
9. It must not annoy the patient.
10. It must be technically practical to insert.

No mechanical or bioprosthetic valve to date has completely fulfilled all of these objectives. To achieve these standards, the valve must resemble the natural living valve as closely as possible and so the most promising route is through the tissue engineering of a living aortic heart valve.

## 1.6. *Tissue Engineering*

Tissue engineering is a rapidly developing field that combines biological and engineering principles in an attempt to create replacement organs or structures from cells and biological or synthetic scaffolds to restore, maintain, or improve tissue function. Although the technology is still in its infancy, the potential of this field is vast, and the research is driven by the intrinsic problems of current techniques for repair and replacement of tissues, such as limited donor availability and poorly compatible materials. In the United States alone, approximately 7.5 million patients require artificial biological implants each year [Cassell *et al.*, 2002].

There are multiple challenges for the tissue engineering approach to aortic valve replacement. The physical and biological properties of the natural tissue must be recapitulated, and any synthetic materials used must be tolerated by the body and adequately degraded as the new tissue develops and remodels. The basic principle involves seeding cells onto a matrix scaffold. However, there are a number of ways of creating a suitable matrix scaffold and many potential cell sources.

There are four key processes occurring during the *in vitro* or *in vivo* phases of tissue formation and maturation [Rabkin & Schoen, 2002]:

- (1) Cell proliferation and differentiation;
- (2) Extracellular matrix production;
- (3) Degradation of the scaffold; and
- (4) Remodelling and potentially growth of the tissue.

Once combined in the correct manner, it has been proposed that a dynamic and interactive cell phenotype and extracellular matrix could be produced which would



ultimately develop a microarchitecture comparable to a native valve [Rabkin *et al.*, 2002]. This should then be able to grow, develop, repair and remodel itself just as the native valve tissue.

There has been considerable interest in the tissue engineering approach for designing cardiac valve substitutes, typically using a synthetic scaffold construct. [Bader *et al.*, 1998; Hoerstrup *et al.*, 1999; O'Brien *et al.*, 1999; 2002; Sodian *et al.*, 2000a; Elkins *et al.*, 2001a; Jockenhoevel *et al.*, 2001; Kim *et al.*, 2001; Cebotari *et al.*, 2002; Rabkin *et al.*, 2002; Rothenburger *et al.*, 2002; Shinoka, 2002; Taylor *et al.*, 2002; Perry & Roth, 2003].

### **1.6.1. Polymeric Scaffolds**

There are a number of different biodegradable materials with minimal immunogenicity currently being investigated. Scaffolds must possess sufficient structural integrity to temporarily withstand functional loading *in vitro* and *in vivo*. These scaffolds are usually designed to gradually degrade over time and be replaced with new host tissue and cells. Consequently, it must be ensured that materials used are non-immunogenic, non-toxic and are adequately reabsorbed by the body.

#### **1.6.1.1. Fibrin gel scaffold**

Fibrin gel is derived from blood and can be prepared in an autologous manner to negate immunogenicity problems. It can be polymerised and moulded into a valve structure to create a completely autologous and biodegradable scaffold. Histological investigations of cell seeding onto fibrin scaffolds by Jockenhoevel *et al.* (2001) have demonstrated good tissue development with viable fibroblasts surrounded by collagen bundles. However, fibroblasts surrounded by collagen bundles are a long way from a tri-laminar

tissue structure capable of withstanding the forces generated in the aortic valve. Moreover, a scaffold made from fibrin gel would lack the necessary mechanical strength and when placed into the aortic position would almost certainly disintegrate long before any cells could lay down a new extracellular matrix.

#### 1.6.1.2. Collagen matrix

Synthetic type I collagen has been used by Rothenburger *et al.* (2001; 2002) and Taylor *et al.* (2002) to construct a biodegradable, biocompatible sponge-like matrix which was reseeded *in vitro*. Taylor *et al.* (2002) showed that seeded valve interstitial cells developed a molecular phenotype similar to those of skeletal, cardiac, and smooth muscle cells, which may be valuable functional qualities for a tissue-engineered valve. However, although collagen is a fundamental part of the heart valve structure, without the damping effects of the glycosaminoglycans, and the elastic effects of the elastin, any scaffold made from collagen alone would be likely to deteriorate and degenerate very quickly as a result of the *in vivo* forces affecting the heart valve.

#### 1.6.1.3. Other Polymeric Scaffolds

Zünd *et al.* (1997) have combined polyglycolic acid (PGA) with polylactic acid (PLA) in a sandwiched copolymer for their scaffold material and seeded it with both fibroblasts and endothelial cells from an ovine source. They reported that after 14 days the construct was histologically similar to a native valve. However, no mention was made of any mechanical testing, which is an essential part of any analysis of a tissue engineered aortic heart valve construct.

Comparable studies have also been carried out by Shinoka *et al.* (1996; 1997; 1998) using a PGA/polyglactin sandwich scaffold implanted into a lamb model. They too

reported the generation of a 'proper' matrix, and in their case the scaffold was completely degraded after 8 weeks. However, they reported problems with polymer stiffness when it was first implanted, which meant that cells were subjected to greater forces and the leaflets were more prone to structural problems.

Sodian *et al.* (2000b) produced a scaffold fabricated from porous polyhydroxyalkanoate and seeded it with autologous cells prior to implantation in the pulmonary position in a lamb model for up to 17 weeks. They indicated that cell numbers increased and capillaries formed in the new scaffold, which was gradually overtaken by new host tissue as the seeded cells produced their own matrix. However, they reported insufficient degradation of the scaffold which meant that the cells were not able to remodel the scaffold as readily as expected.

Rabkin *et al.* (2002) have shown the potential of a PGA polymer by successfully creating an *in vitro* valve construct seeded with endothelial cells. However, whilst this appeared successful on gross morphological evaluation, immunohistochemical analysis showed that despite apparent proteoglycan accumulation and the presence of an appropriate three-layered architecture, the absence of elastin fibres and weak staining for collagen indicated limitations in the construct as these are the main strength and flexibility-giving qualities required for a suitable valve replacement.

Problems with PGA polymers have been noted by other researchers, however, as they may give toxic by-products upon degradation, and may be poorly reabsorbed by the body [Ye *et al.*, 2000]. Research into the use of chemical polymers and synthetic materials to produce tissue-engineered heart valves is in its infancy and both the

mechanical stability and potential complications of implanting the polymeric compounds used in scaffolds are currently unknown.

Each of these polymers has advantages and disadvantages. However, on the whole, they are severely limited when compared with the native tissue. Any synthetic material used must be designed to be as similar to the native tri-laminar structure as possible if it is to withstand the forces *in vivo* while new extracellular matrix is laid down and remodelled by the seeded cells. Concentrating simply on creating the correct polymer stiffness is not sufficient. The forces applied to the synthetic material must be adequately dissipated within the leaflet, and ideally the construct should have elastic properties to give a greater flexibility whilst maintaining polymer strength in order that the leaflet can effectively occlude the valve during backpressure whilst maintaining a minimal profile when the valve is open. These are all properties of the native valve tissue that have been honed through evolution to give the most effective design, and consequently it is the xenogeneic or allogeneic equivalent which represents the most promising route to creating a viable aortic heart valve. However, as with homografts, the allogeneic scaffold is beset with donor shortage problems.

### **1.6.2. Xenogeneic scaffolds**

Developing a heart valve tissue substitute from a naturally occurring decellularised biological matrix is an alternative strategy that would provide a ready-made scaffold, which already possesses optimal mechanical and biochemical qualities and characteristics. There is also an immediate distinct advantage in that there should be minimal host response to a naturally occurring extracellular matrix [Badylak, 2002]. Methods for producing acellular matrices typically use a combination of enzymes and detergents to remove the cells from a xenogeneic valve [Bader *et al.*, 1998; Booth *et al.*..

2002]. This approach could offer an unlimited source of valve matrices, which could then be reseeded with autologous cells from the valve recipient, to give a non-immunogenic product. The difficulty with this technique, however, lies in the decellularisation process: all of the cells must be completely removed, whilst not damaging the tissue matrix so that mechanical and structural properties are maintained as much as possible. Remaining cell fragments could readily act as nucleation points for calcification to occur, resulting in rapid stiffening and irreparable damage to any implanted tissues [Dohmen *et al.*, 2002b]. Any chemicals used in this process must be completely removed to ensure biocompatibility with cells and host tissue upon implantation. Also, questions remain as to the suitability of unfixed animal sources for human implantation due to the risks of transmitting animal viruses and prions, which are difficult to screen for and adequately remove if detected.

One important attempt at making a valve using a xenogeneic scaffold was the SYNERGRAFT™ valve produced by Cryolife Inc. This valve was made from decellularised porcine valve tissue stitched into a valve shape. The decellularisation process was reported to give a completely acellular matrix and biomechanical testing showed results comparable to those of a native human aortic valve. Despite favourable findings when the valves were implanted in an ovine model [O'Brien *et al.*, 1999], implantation of the valves into four paediatric patients resulted in catastrophic failure leading to the deaths of three patients and rapid replacement of the valve in the remaining recipient [Simon *et al.*, 2003]. It was reported that the collagen matrix induced a severe inflammatory response, which subsequently led to early development of a thick fibrous sheath both on the inside and the outside of the graft. Consequently, due to the sheep model failing to predict the failure of the valves in humans, it has been recommended that the implantation of these valves be stopped pending the development

of appropriate test models. It is possible that the failure of this valve was due to a lack of cells. Had the construct been reseeded *in vitro* prior to implantation, the remodelling carried out by the cells may have strengthened the construct and also remodelled any damaged areas of the matrix, thereby reducing the immune response by the host.

A patented method has been developed at the University of Leeds for the decellularisation of aortic valve leaflets using the detergent sodium dodecyl sulphate (SDS). Histological analysis showed that the matrix components remained intact (Booth *et al.*, 2002), whilst mechanical testing demonstrated that the SDS treatment produced valves with equal strength, competence under physiological pressures (120mmHg) and greater extensibility than untreated fresh valves (Korossis *et al.*, 2002).

### 1.6.3. Cell Source

In any tissue engineering strategy to produce a living aortic heart valve an appropriate source of suitable cells has to be established that have similar properties to the cells of the native tissue. It may be that multiple cell sources are required, i.e. endothelial and smooth muscle cells, in order that an endothelial lining is established to prevent thrombus formation, whilst smooth muscle cells can penetrate and remodel the tissue. Using MSC could be potentially better than terminally differentiated cells as they have the potential to differentiate into multiple cell phenotypes, potentially resulting in a cell lineage far closer to that of the native valve cells. Also, MSC could, in theory, be propagated indefinitely in culture, enabling vast numbers of cells to be grown *in vitro* without the loss of cell function seen in terminally differentiated cells [Hayflick, 1965]. The use of autologous cells would give the advantage of a functional tissue that would be an identical human leukocyte associated (HLA) antigen match, and therefore non-

immunogenic. In contrast, allogeneic cells would necessitate immunosuppressive therapy to prevent rejection, which has a high risk of morbidity and mortality [Adler & Turka, 2002].

It is thought that once a tissue-engineered product is implanted, host cells would gradually infiltrate and migrate through it over many weeks or months, thereby further remodelling the tissue and replacing the seeded cells. Therefore it could be argued that seeding cells prior to implantation is not strictly necessary. However, due to the high pressures and forces exerted on to heart valve tissue *in situ*, a lack of cells to undertake the ongoing repair that is required in order to maintain a native heart valve may well result in the tissue becoming irreparably damaged before any cells can reach the valve leaflets by migrating in from the surrounding tissues.

### **1.7. A Brief History of Stem Cells**

Mammalian tissues were first described to be composed of cells in the 19<sup>th</sup> century. This led Virchow and Schwann to the claim that cells originate exclusively from other cells (“*omnis cellula a cellula*”), [Wulf *et al.*, 2001]. Analysis of the phylogeny of haematopoiesis in the bone marrow by morphological observation led to the concept of tissue stem cells being the basis for tissue regeneration at the beginning of the 20<sup>th</sup> century. Pappenheim deduced the existence of an undifferentiated stem cell that gave rise to the plethora of blood cells via an intermediate state of progenitor cell [Wulf *et al.*, 2001]. In the 1950s, several groups corroborated the existence of the haematopoietic stem cell in the bone marrow by showing haematopoietic recovery from transplanted bone marrow after irradiation damage [Wulf *et al.*, 2001]. Till and McCulloch (1961) later traced haematopoietic repopulation capacity to clonogenic cells establishing spleen colony forming units and in 1976 experiments by Freidenstein *et al.* first brought

attention to a second population of stem cells within the bone marrow, mesenchymal stem cells. These findings led to the concept of tissue regeneration from a small population of resident tissue stem cells being widely accepted, and was extended to other tissues, such as gut and skin [Potten & Loeffler, 1990].

The principle of directed cellular proliferation was also used in the understanding of the early stages of embryogenesis, and led to the discovery of morphogenesis via germ layers in the early embryo [Wulf *et al.*, 2001]. Emerging technologies led to the functional isolation of stem cells with the capacity to differentiate into all tissues of the adult organism from pre-implantation embryos of mice in 1981 [Evans & Kaufman, 1981]. Thomson *et al.* followed this in 1998 by the isolation of the human equivalent. These cells were termed embryonic stem (ES) cells.

The successful cloning of a mammal by implanting the nucleus of an adult tissue cell into an enucleated fertilised ovum in 1997 [Wilmut *et al.*, 1997] established that the nuclei of at least some adult cells were capable of being reprogrammed. This spurred many groups to return to examining the differentiation capacity of adult tissue stem cells, and the credence of earlier postulations of the existence of adult tissue stem cells took on a new importance, which led to a vast increase in research in this area. It is now thought that adult stem cells may reside in all organs having a connective tissue component where they function as a source of cells for wound healing, tissue repair and regeneration [Calcutt *et al.*, 1993; Young *et al.*, 1995; Mackenzie & Flake, 2001a]. These include (in order of discovery) haematopoietic stem cells [Till and McCulloch, 1961], keratinocyte stem cells [Green *et al.*, 1979], gastrointestinal stem cells [Potten *et al.*, 1997; Potten & Booth, 2000], neural stem cells [McKay, 1997; Prockop, 1997; Solter & Gearhart 1999; Temple, 1999; Kennea & Memhet, 2002; Peterson, 2002],



hepatic stem cells [Alison, 1998; Strain & Crosby, 2000], muscle stem cells [Miller *et al.*, 2001; Asakura *et al.*, 2001; Zammit & Beauchamp, 2001; Goldring *et al.*, 2002; Wada *et al.*, 2002], amniotic fluid stem cells [Prusa, & Hengstschläger, 2002], cardiac stem cells [Hughes, 2002.], lung epithelial stem cells [Otto, 2002], pancreatic stem cells [Bonner-Weir & Sharma, 2002], and prostatic stem cells [Foster *et al.*, 2002].

## **1.8. *Adult versus Embryonic Stem Cells***

Both adult stem (AS) cells and embryonic stem (ES) cells have advantages and disadvantages in their potential use in tissue engineering. A comparison of ES and AS cells is shown in Table 1.1. At first, it seemed as though ES cells had the most potential as they have the capacity to differentiate into every cell in the human body once the correct conditions are established (totipotent), whereas it was originally thought that AS cells had some constraints on their developmental capacity (multipotent). However, AS cells continue to show a potential overlap in their functions and it is possible that in the future their capacity for differentiation will be shown to be equal to or almost equal to that of ES cells [Grompe, 2002]. In favour of AS cells, immunological problems can be overcome by extracting AS cells from the intended recipient of the final tissue engineered product, whereas ES cells will express the histocompatibility antigens of the parents and thus will be allogeneic. Without HLA matching, genetic manipulation or immunosuppression of the recipient they would be rejected. Hence there are considerable advantages of utilising AS cells, which can be extracted at any point through the life of the person.

	<b>EMBRYONIC STEM CELLS</b>	<b>ADULT STEM CELLS</b>
<b>ADVANTAGES</b>	Can be propagated indefinitely. Pluripotent differentiation capacity. Absence of any potential effects of age.	Autologous derivation. Respond to developmental signals in the injury environment and differentiate. Easy to obtain.
<b>DISADVANTAGES</b>	Need to be grown on mouse feeder cells. Can give rise to teratocarcinomas. Epigenetic instability. Allogeneic derivation.	Not pluripotent. May be donor-age-associated changes in proliferative and differentiative capacity. Current culture conditions do not maintain multipotency.

**Table 1.1. Comparing the advantages and disadvantages of ES and AS cells.**

The main drawback to the use of ES cells, however, remains an ethical one. With the currently available technology, extraction of ES cells for research purposes necessitates the death of the embryo from which they are harvested. This ethically contentious practice has promoted widespread public concern [Doerflinger, 1999]. Furthermore, confusion propagated by unhelpful publicity in the media in relation to human cloning, the use of human organs for research and a poor understanding of the concepts and potential of this kind of research have led to many countries implementing legislation to block or greatly limit the majority of ES cell research [Pedersen, 1999]. This has left a much clearer path open for research into AS cells, and has led to a huge surge in interest in this area.

### **1.9. *Definition of a Stem Cell***

To date, there is no universally accepted definition of a stem cell. Since there are no cell markers specific for stem cells, they are typically defined by virtue of their functional attributes. However, in order to assess whether a cell is a stem cell or not requires manipulation of the cell, which in turn changes its properties. So in reality, assessments have to be based on a population of cells clonally derived from a single cell that can be used in multiple experiments. When Till & McCulloch first identified haematopoietic

stem cells in 1961, they defined stem cells simply as clonogenic cells capable of both self-renewal and multilineage differentiation. Potten & Loeffler (1990) offered the definition as undifferentiated cells capable of:

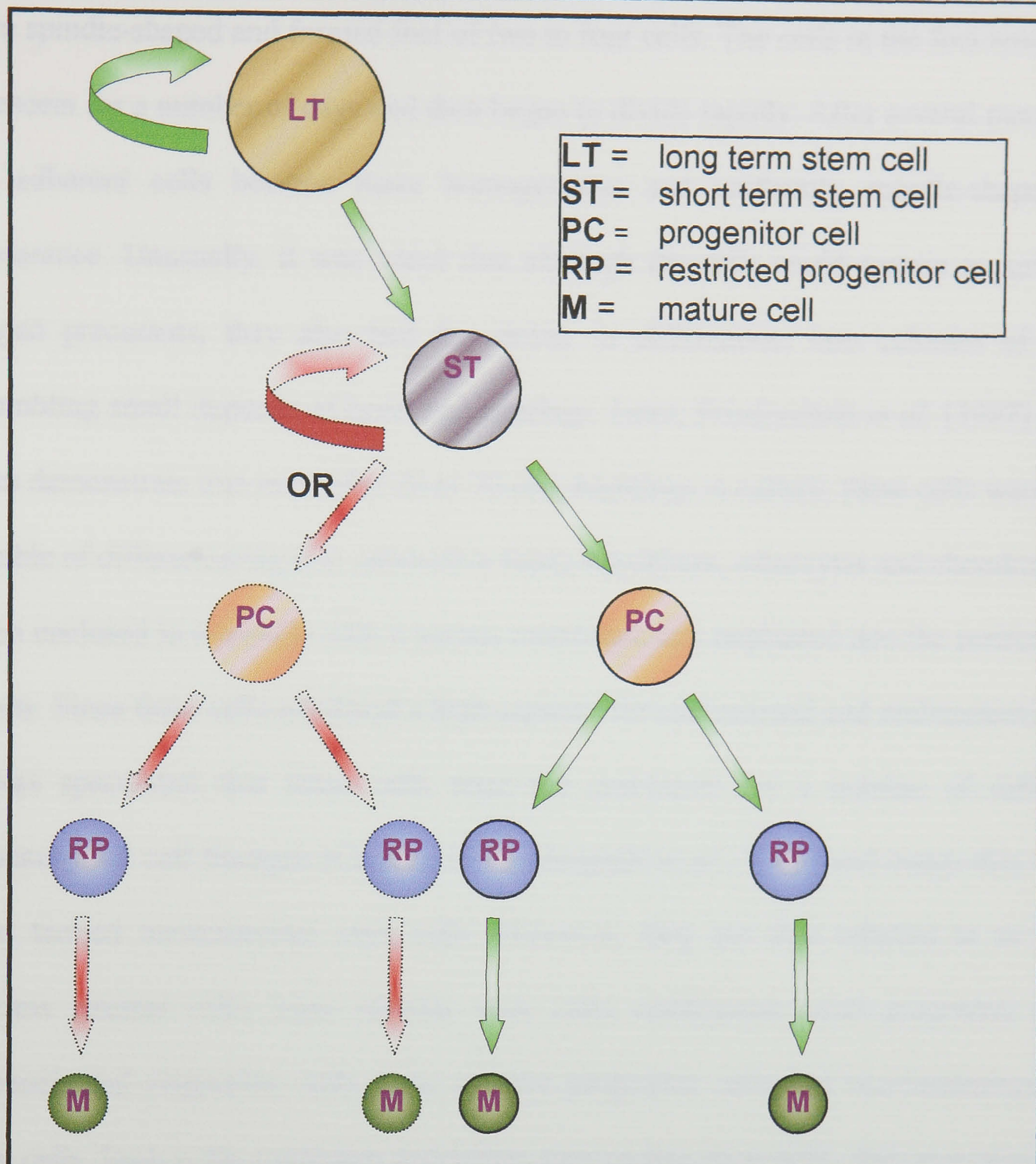
- a) Proliferation;
- b) Self-maintenance;
- c) The production of a large number of differentiated functional progeny;
- d) Regenerating tissue after injury; and
- e) A flexibility in the use of these options.

A more recent definition from Verfaillie [2002] states the minimal criteria for a stem cell to be:

1. The ability to self renew;
2. The ability for a single cell to differentiate into cells of the tissue of origin and into at least one cell type different from the tissue of origin; and
3. The ability to functionally differentiate *in vivo* into cells of the tissue of origin and at least one cell type of a tissue other than the tissue of origin.

These definitions are further complicated by the nature of stem cells as it seems that stem cells can be divided into a long-term subset, capable of indefinite self-renewal, as well as a short-term subset that self-renews for a defined interval [Weissman, 2000].

These stem cells then give rise to non-self renewing progenitor cells, which in turn give rise to more restricted progeny that become terminally differentiated fully functional mature cells (Figure 1.11). Therefore stem cell division is widely thought to be an asymmetrical process, which enables a constant number of self-renewing stem cells to be maintained whilst generating progenitor cells to undergo a differentiation process. This offers an explanation for the typically heterogeneous nature of freshly isolated adult stem cells, since they are likely to be a mixture of self-renewing cells, and other cells at different stages of the differentiation process.



**Figure 1.11. Stem Cell Lineage Progression.** Green arrows represent standard lineage progression. Short-term stem cell self-renews and produces progenitor cell for limited period before producing progenitor cells only (Red arrows).

### 1.10. Mesenchymal Stem Cells

The discovery of a population of adult stem cells in the bone marrow was made by Friedenstein *et al.* In 1976, Friedenstein *et al.* placed samples of murine whole bone marrow in plastic culture dishes and, after several hours, changed the culture medium, thereby pouring off the cells that had not adhered. In doing this, most of the haematopoietic stem cells and their progeny and any other non-adherent, non-haematopoietic cells were discarded. They reported that the small numbers of adherent cells remaining were heterogeneous in appearance, but that most tightly adherent cells

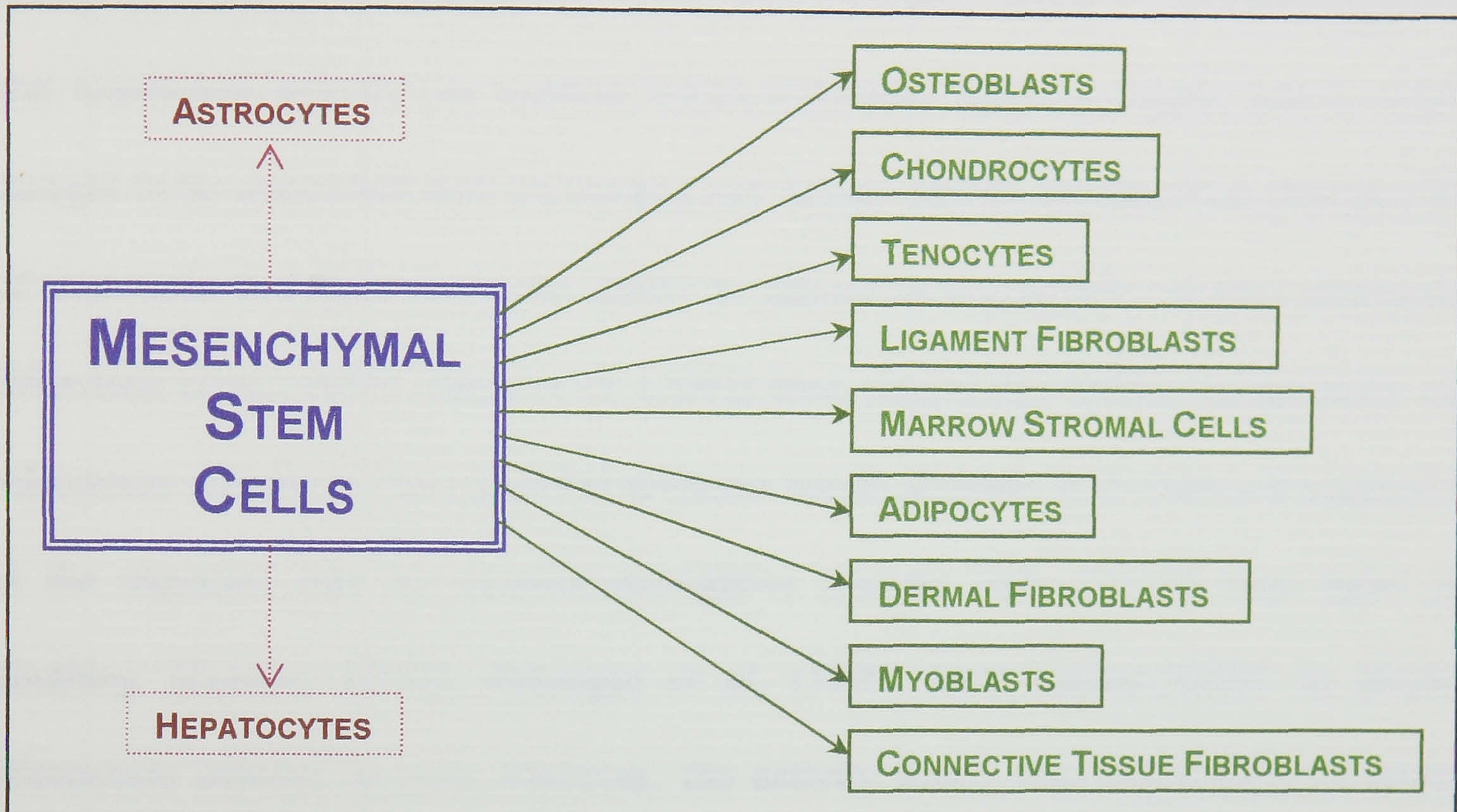
were spindle-shaped and formed foci of two to four cells. The cells in the foci remained quiescent for a number of days and then began to divide rapidly. After several passages, the adherent cells became more homogeneous and uniformly spindle-shaped in appearance. Unusually, it was noted that although the cells could remain as spindle-shaped precursors, they also had the ability to differentiate into colonies of cells resembling small deposits of bone and cartilage. Later, Friedenstein *et al.* (1987) went on to demonstrate that even after 20 or 30 cell doublings in culture, these cells were still capable of differentiating into connective tissue fibroblasts, osteocytes and chondrocytes when enclosed in a capsule with a porous membrane and implanted into the peritoneum of rats. Since these cells exhibited a high capacity for self-renewal and multipotentiality, it was speculated that these cells were the precursors for a number of different mesenchymal cell lineages [Caplan, 1994; Minguell *et al.*, 2000] and hence they have been termed mesenchymal stem cells. However, they are also referred to as bone marrow stromal cells, bone marrow stem cells, multipotent adult progenitor cells, mesenchymal progenitor cells, bone marrow progenitor cells and non-haematopoietic stem cells. Such is the confusion and debate surrounding these cells, they may also have been labelled with other names such as circulating endothelial progenitor cells [Hill *et al.*, 2003], which upon close examination bear more than a striking resemblance to cells that others have labelled MSC.

More recent advances in understanding of the differentiation processes and characterisation of mesenchymal phenotypes have led to the acceptance that MSC are precursors for a range of connective tissue-related cell types such as osteocytes, chondrocytes, adipocytes, myocytes, tenocytes, myofibroblasts, cardiomyocytes, endothelium and haematopoietic-supporting stromal cells (Figure 1.12.) [Caplan, 1991; Pereira *et al.*, 1995; Wakitani *et al.*, 1995; Cassiede *et al.*, 1996; Prockop, 1997; Ferrari,

*et al.*, 1998; Johnstone *et al.*, 1998; Nevo *et al.*, 1998; Makino *et al.*, 1999; Pittenger *et al.*, 1999; Deans & Moseley, 2000; Emura *et al.*, 2000; Minguell *et al.*, 2000; Pittenger *et al.*, 2000; Fukuda, 2002; Hakuno *et al.*, 2002; Reyes *et al.*, 2002]. Clonogenic studies of rat MSC have shown that multiple differentiation fates can be induced in a population of cells derived from a single cell [Young *et al.*, 2001]. This has been repeated in human MSC (hMSC) using a variety of methods such as ring-cloning [Prockop, 1997; Pittenger *et al.*, 1999] and retro-viral marking strategies [Reyes *et al.*, 2001; Jiang *et al.*, 2002; Schwartz *et al.*, 2002]. Although both of these techniques have their limitations (more than one cell may reside within a ring, and retroviral marking may cause unknown effects on cell DNA), success in each case further demonstrated the multipotent stem cell nature of MSC and strongly opposes the possibility of multiple progenitor cells for different cell lineages being present in the bone marrow. This extensive differentiation capacity, however, is thought to depend on factors such as the harvesting procedure, the frequency of MSC in the bone marrow and the condition and age of the donor [Caplan, 1994; Digorolamo *et al.*, 1999; Mendes *et al.*, 2002].

There have been increasing numbers of reports that there is some functional overlap between different stem cell lineages [Poulsom *et al.*, 2002], and mesenchymal stem cells have been reported to be capable of differentiating into non-mesenchymal cell types including astrocytes [Bjorklund & Svendsen, 1999; Woodbury *et al.*, 2000; Kabos *et al.*, 2002; Mezey *et al.*, 2003], and hepatocytes [Prockop, 1997; Avital *et al.*, 2001; Poulsom *et al.*, 2002; Theise & Krause, 2002a]. They have also been investigated for use in tissue engineering nephrons [Ito *et al.*, 2001]. Meanwhile, haematopoietic cells have been shown capable of differentiating into hepatic oval cells [Petersen *et al.*, 1999], neural stem cells into haematopoietic precursors [Bjornson *et al.*, 1999] and dermal stem cells have been differentiated into neurogenic, myogenic and adipogenic

cells [Toma *et al.*, 2001]. Indeed, such is the interest in MSC, a growing number of patents have been filed relating to the isolation, identification and differentiation of these cells for tissue engineering and other applications [Caplan, 1996; Caplan & Haynesworth, 1996; 2000; Pittenger, 1998; Marshak & Holecek, 1999; Fernández &



Minguell, 2001].

**Figure 1.12. Mesenchymal stem cell phenotypes.** Solid green arrows represent cells of mesenchymal lineage; dashed red arrows represent functional overlap with other adult stem cell lineages.

### 1.10.1. Characteristics of MSC

The term, mesenchyme, is derived from the Greek meaning ‘middle’ (meso) ‘infusion’ and refers to the ability of mesenchymatous cells to migrate and spread in early embryonic development between the ectodermal and endodermal layers [Caplan, 1991]. This characteristic migratory, space-filling ability is the key element of all wound repair in adult organisms involving mesenchymal cells in skin (dermis), bone (periosteum), or muscle (perimysium).

Human MSC (hMSC) cultures have been shown to have a population doubling time of 33 hours [Conget *et al.*, 1999]. However, there are conflicting reports over the longevity of hMSC, with some reports stating that the cells become senescent after only 8 or 9 passages, whilst others claim to have passaged hMSC in excess of 50 times without any change in viability or differentiation potential [Grompe, 2002]. It has been suggested that telomerase activity (an enzyme which maintains telomere length, and is widely thought to be associated with preventing cell aging) may be an important characteristic of stem cells that gives them the ability to self-renew indefinitely without senescence [Morrison *et al.*, 1997]. Banfi *et al.* (2002) have shown that hMSC do not show any telomerase activity *in vitro* and that telomere length shortens with each cell replication at the expected rate for telomerase-negative somatic cells (50-100 base pairs per doubling [Zakian, 1997]). Pittenger *et al.* (1999) have shown hMSC to display telomerase activity *in vitro*, however, the activity found was comparable to normal human foreskin fibroblasts, which have been consistently described by a number of investigators to be telomerase-negative [Kim *et al.*, 1994; Bodnar *et al.*, 1998]. Lack of telomerase activity does contradict the principle that true stem cells should be able to replicate indefinitely and suggests that hMSC life span would be no greater than that of other terminally differentiated somatic cells. Of course *in vivo* conditions may well promote telomerase activity and it may well be that current *in vitro* culture conditions do not provide an appropriate environment or stimulatory conditions for telomerase activity. Simonsen *et al.* (2002) have addressed this issue by demonstrating that it is possible to successfully transfect hMSC with a retroviral vector containing the catalytic subunit of the human telomerase gene, whilst maintaining full cell proliferation and differentiation functions.



The antigenic phenotype of hMSC has shown them to possess features of several cell lineages including mesenchymal, endothelial, epithelial and muscle cells [Galmiche *et al.*, 1993; Conget *et al.*, 1999; Pittenger *et al.*, 1999]. Isolated hMSC have been reported to constitute a single, phenotypically distinct population and show the following cluster of differentiation (CD) markers: CD44<sup>+</sup>, CD71<sup>+</sup>, CD90<sup>+</sup>, CD95L<sup>+</sup>, CD106<sup>+</sup>, CD120a<sup>+</sup>, and CD124<sup>+</sup> [Fontes & Thomson, 1999]. They have been shown to be CD34<sup>-</sup>, CD31<sup>-</sup> and CD133<sup>-</sup>, making them distinct from cells of the haematopoietic, endothelial and neurogenic stem cell lineages respectively. They have also been shown to cross-react with the monoclonal antibodies SH2 (anti-CD105/endoglin) and SH3 [Zvaifler *et al.*, 2000; Jones *et al.*, 2002; Lodie *et al.*, 2002].

Despite the fact that the multipotentiality of MSC has been recognised for several decades, there are surprisingly large gaps in the information about the cells themselves [Prockop, 1997]. MSC remain a very rare cell population within the bone marrow [van den Bos *et al.*, 1997; Nevo *et al.*, 1998], and it is estimated that less than 1 in 100,000 bone marrow cells are MSC [Caplan, 1994]. The cells, isolated by their adherence to plastic, were initially shown to be heterogeneous and have proved difficult to clone. A number of groups claim to have produced monoclonal antibodies specific for hMSC which could overcome this discrepancy [Simmons & Torok-Storb, 1991; Barry *et al.*, 1999; Caplan & Haynesworth, 2000], however almost as soon as they were produced, the monoclonal antibodies were found to cross-react with other non-MSCL lineages.

Cultured hMSC have been shown to synthesise an extracellular matrix that includes interstitial type I collagen, fibronectin, and the type IV collagen and laminin of basement membranes [Caplan, 1991; Clark & Keating, 1995]. A small fraction of the cultured cells have been reported to synthesise factor VIII-associated antigen, which is

characteristic of endothelial cells. The cells have also been shown to secrete cytokines, including interleukin-7 (IL-7), IL-8, IL-11 and stem cell factor (c-kit ligand) [Prockop, 1997].

### 1.11. *Defining Differentiation*

Differentiation can be defined as a qualitative change in the cellular phenotype that is the consequence of the onset of synthesis of new gene products and may be recognised by a change in the morphology of the cell or by the appearance of changes in enzyme activity or protein composition [Potten & Loeffler, 1990]. The specific route of transition from one lineage to another is a point of contention. Two groups [Terada *et al.*, 2002; Ying *et al.*, 2002] have claimed that there is a low incidence of cell fusion between murine adult stem cells (MSC and neural stem cells respectively) and host cells along side the differentiation of the majority of cells *in vivo*. In both these cases, donor cells were identified using Y-chromosome markers, and fusion was found but at a very low frequency. It was suggested that these fused cells could have the potential to become a dominant population should they gain a growth or survival advantage over their parental cells by supplementing deficient genes. However, in these experiments, it happened at a very low frequency *in vitro* (1 in  $10^5$  to  $10^6$  cells), and might only occur *in vivo* in circumstances of extensive selection pressures in tissues that 'tolerate' tetraploidy, such as muscle and liver [Vefaille, 2002].

There is also the concept of de-differentiation and re-differentiation (or nuclear reprogramming). De-differentiation has been used to describe the loss of the differentiated properties of a tissue when it is grown in culture [Freshney, 2000]. This mechanism is thought to underlie the ability of amphibians and fish to regenerate limbs spontaneously and can complicate the identification of a stem cell. Both chondrocytes

and adipocytes are known to de-differentiate in two-dimensional culture, losing the properties that normally define them as that cell phenotype, e.g. chondrocytes stop producing collagen II [Ma *et al.*, 2003]. Chondrocytes can be re-differentiated by placing the cells in a three-dimensional culture system, and adipocytes with the correct chemical stimulation. It is also thought that there may be a developmental link between adipocytes and osteocytes [Bennet *et al.*, 1991] and that culturing them under the correct conditions can cause the de-differentiation followed by re-differentiation into the other cell phenotype, or back into the original cell phenotype. This may be the situation behind the claims of adult stem cells being isolated from adipose tissue [Zuk *et al.*, 2001, 2002; De Ugarte *et al.*, 2003], and unknown similar relationships could be behind other reports of MSC differentiation. In other cases, in response to trauma or injury, mature cells may lose their differentiated properties and re-enter the cell division cycle. In this case, once the necessary repairs are made, cell division stops and differentiation is re-induced [Freshney, 2000]. Some investigators use the term “transdifferentiation” in describing the change in phenotype from one cell to another, typically referring to mature cells [Freshney, 2000].

It is for these reasons and ambiguities that there is currently no definitive proof that true pluripotent stem cells exist *in vivo* during post-natal life. However, if de- and re-differentiation is the mechanism involved, the potential benefits and uses of cells capable of this process remain unchanged. Whatever the mechanism, it will be vitally important to test whether *in vitro* culture and manipulation will not cause uncontrollable proliferation and/or differentiation similar to the teratomas seen when ES cells are transplanted *in vivo*.

### 1.11.1. Differentiation by Biochemical Supplementation *In Vitro*

From the earliest days of modern man, bone has been recognised to have the powerful capacity for self-repair [Caplan, 1991]. A variety of factors combine in a complex multicellular, multi-step response in which reparative cells are specifically attracted to the repair site. These cells then aggregate, multiply, bridge the bone gap, and differentiate into osteoblasts.

The culture conditions required for differentiating MSC, however, have been found to be somewhat species-dependent and influenced by poorly defined variables, such as the level of foetal calf serum used. Osteoblasts are thought to be highly orientated cells that secrete bone from the side of the cell opposite that which is in contact with the capillary, whereas chondrocytes develop away from vasculature [Caplan, 1991]. Therefore, basal nutrients, cell density, spatial organisation, mechanical forces, growth factors and cytokines have a profound influence on MSC differentiation [Pittenger *et al.*, 2000]. Chondrocytes have been shown to develop easily when MSC are plated under very compact, high-density conditions of approximately  $5 \times 10^6$  chick embryonic stage-24 limb mesenchymal cells per  $9.6 \text{ cm}^2$  dish [Caplan, 1991]. Densities below approximately  $2 \times 10^6$  cells per  $9.6 \text{ cm}^2$  dish have been shown to stimulate the generation of osteocytes [Caplan, 1991]. Using chemical methods, MSC from mouse, rat, rabbit and human sources have been reported to readily differentiate into colonies of osteoblasts under the influence of dexamethasone,  $\beta$ -glycerophosphate and ascorbate in the presence of 10% (v/v) foetal bovine serum (FBS). Chondrocyte differentiation has been induced by gently centrifuging isolated mesenchymal cells to form a pelleted-micromass and culturing the cells without serum and with transforming growth factor- $\beta 3$  (TGF $\beta 3$ ). Treatment with 1-methyl-3-isobutylxanthine, dexamethasone, insulin and indomethacin has been shown to induce differentiation into adipocytes [Friedenstein *et al.*, 1976; Piersma *et al.*, 1985; Friedenstein *et al.*, 1987; Caplan, 1991; Clark &

Keating, 1995; Wakitani *et al.*, 1995; Prockop, 1997; Bruder *et al.*, 1998; Pittenger *et al.*, 2000]. In response to 5-azacytidine and amphotericin B or amphotericin B alone, murine MSC have been reported to differentiate into myoblasts that fuse into rhythmically beating myotubes [Wakitani *et al.*, 1995; Makino *et al.*, 1999; Emura *et al.*, 2000]. Treatment with  $\beta$ -mercaptoethanol has been shown to differentiate hMSC into cells of the neurogenic lineage [Woodbury *et al.*, 2000]. The hMSC cultured in each of the differentiation conditions may also produce autocrine and paracrine factors that are essential for lineage progression [Pittenger *et al.*, 1999]. It is thought, however, that the osteogenic pathway is the 'default' lineage that these cells can progress through, possibly because of an intrinsic commitment or the *in vitro* culture conditions representing a microenvironment favouring osteogenesis [Muraglia *et al.*, 2000; Banfi *et al.*, 2002].

### **1.11.2. Differentiation by Co-culture**

The capabilities of MSC to differentiate as a result of the conditions created in a co-culture system have also been investigated. Emura *et al.* (2000) were able to differentiate hMSC into myofibroblasts by culturing the cells on a feeder layer of human colon carcinoma cells. Condorelli *et al.* (2001) have shown the potential for human endothelial cells and neural stem cells to differentiate into beating cardiomyocytes in differing degrees when co-cultured on a feeder layer of neonatal rat cardiomyocytes. This further demonstrates the plasticity of adult stem cells and also adds to the issue questioning the nature of the processes that induce cells to alter their phenotype.

### **1.11.3. Differentiation by Mechanical Stimulation**

Altman *et al.* (2002) have shown that it is possible for hMSC to be differentiated by mechanical stimuli *in vitro* without biochemical factors being present. In their experiments, MSC were stimulated to differentiate into a ligament cell lineage. They demonstrated a change in expression of collagens I and III and fibronectin, alignment of the cells and a changed morphology. After 14 days, mRNA levels were seen to approach those of native ligament cells. To date, this is the only work published examining the effects of mechanical stimulation on hMSC. However, the potential benefits of enhanced matrix production and cell alignment in response to mechanical stimulation have been shown on other cell types such as fibroblasts and aortic endothelial cells [Streppeti *et al.*, 1993; Banes *et al.*, 1995; Sato *et al.*, 1995; Eastwood *et al.*, 1998; Hishikawa & Lüscher, 1997; Sirois *et al.*, 1998; Breen, 2000; Grinell, 2000]. In a study on cardiac fibroblasts by Butt & Bishop (1997), it was found that mechanical load enhanced procollagen gene transcription in the presence of serum growth factors. More specifically, cardiac fibroblast procollagen synthesis in response to specific fibrotic cytokines such as TGF $\beta$ 1 and IGF-1 was enhanced when the cells were simultaneously subjected to mechanical load. These studies demonstrate the importance of not only biochemical, but also mechanical stimulation on both cell differentiation and cell signalling processes and serves to highlight the significance of culturing tissue-engineered products in appropriately designed bioreactors in order to accurately simulate the natural environment of the tissue prior to implantation.

#### **1.11.4. Homing and Differentiation *In Vivo***

Both Almeida-Porada *et al.* (2001) and Liechty *et al.* (2000) investigated the fate of hMSC when they were introduced into foetal sheep early in gestation, before and after the expected development of immunological competence. Liechty *et al.* (2000) demonstrated that hMSC underwent site-specific differentiation into chondrocytes,

adipocytes, myocytes and cardiomyocytes, bone marrow stromal cells and thymic stroma. The cells were capable of migration across the peritoneal cavity and engrafted and persisted in multiple tissues for as long as 13 months after transplantation, even when cells were transplanted after the expected development of immunocompetence.

Devine *et al.* (2001) went on to investigate the intravenous infusion of baboon-derived autologous and allogeneic mesenchymal stem cells combined with haematopoietic stem cells in a lethally irradiated non-human primate model. They found that MSC were capable of homing to the bone marrow and persisted for over a year without significant toxicity. A control animal that was not irradiated, but still infused with MSC had cells present in its bone marrow up to 442 days post-infusion.

Mackenzie & Flake, co-authors of Liechty *et al.* (2000) speculated that the homing ability of the cells may be a response to tissue injury, further supporting the idea that MSC exist as part of a wound repair mechanism [Mackenzie & Flake, 2001a; 2001b]. Also, since the cells appeared to be capable of persisting in xenogeneic and allogeneic hosts, it may be that the cells possess unusual immunological properties.

### **1.11.5. Stem Cell Therapy**

To date, animal studies have shown the potential usefulness of lineage-committed mesenchymal stem cells in the site-directed repair of cartilage [Wakitani *et al.*, 1994; Grande *et al.*, 1995; Yoo *et al.*, 1998], skeletal muscle [Saito *et al.*, 1995], heart muscle [Wang *et al.*, 2000; Tomita *et al.*, 2002], bone [Kadiyala *et al.*, 1997], and tendon [Young *et al.*, 1998]. Also, uncommitted MSC have been used in the simultaneous site-directed repair of both cartilage and bone [Grande *et al.*, 1995]. In all cases, MSC were

simply injected into the site of interest, and then the tissue excised and examined after varying time periods.

### **1.12. Do hMSC Possess Immunomodulatory Properties?**

hMSC have been shown to constitutively express a number of molecules required for antigen specific interactions with T-cells including major histocompatibility class-I (MHC I), but not MHC class II molecules, unless induced by interferon gamma (IFN- $\gamma$ ), [McIntosh & Bartholomew, 2000; Le Blanc *et al.*, 2003]. They also constitutively express intracellular adhesion molecule-1 (ICAM-1), ICAM-2, vascular adhesion molecule-1 (VCAM-1), L-selectin, CD72, and lymphocyte function-associated antigen-3 (LFA-3) adhesion molecules [Pittenger *et al.*, 1999]. hMSC typically do not express the co-stimulatory molecules B7-1 (CD80) or B7-2 (CD86), although mRNA for B7-1 has been detected by reverse-transcriptase polymerase chain-reaction (rt-PCR) [McIntosh & Bartholomew, 2000].

Several groups have shown that hMSC are not stimulatory to alloreactive T-cells [McIntosh & Bartholomew, 2000; Le Blanc *et al.*, 2003; Tse *et al.*, 2003]. Culture of hMSC with resting allogeneic T-cells failed to elicit T-cell proliferation or the expression of T-cell activation molecule CD40-ligand. This was apparently not due to a lack of co-stimulatory molecule expression, since retroviral transduction of MSC with either B7-1 or B7-2 did not result in a significant response. It has also been shown that although the ability to present antigen in the absence of co-stimulatory molecules might result in the induction of T-cell tolerance, MSC appeared not to be tolerogenic, since T-cells cultured with allogeneic MSC or donor-matched irradiated peripheral blood mononuclear cells (PBMC) for seven days and rested for three days were able to be re-



stimulated with irradiated PBMC with secondary kinetics. This suggests that MSC prime T-cells without inducing proliferation.

In addition to this, some groups have carried out experiments to investigate the effect of MSC on mixed lymphocyte reactions (MLR). The MLR is used as an *in vitro* correlate of the transplantation reaction. The MLR involves mixing populations of lymphocytes from two allogeneic donors. One donor's cells are irradiated to prevent cell proliferation whilst maintaining metabolic activity and are termed the stimulators, and the other donor's cells are termed responders. The T-cells of the responder population react with the foreign MHC on the surface of stimulator antigen presenting cells (APCs) causing the T-cells to rapidly proliferate and this can be assayed by measuring tritiated thymidine incorporation into the DNA of the dividing cells. The level of T-cell proliferation is directly proportional to the degree of human leukocyte antigen (HLA) mismatch between the two cell sources: closely matched cells show a minimal T-cell proliferation where mismatched cells show a high T-cell proliferation. The MLR was previously used to type tissues prior to organ transplantation in order to try to minimise organ rejection. Experiments by Klyushnenkova *et al.* (1999) and Le Blanc *et al.* (2003) suggested that hMSC may suppress activated T-cells, since the addition of MSC to an MLR resulted in near complete suppression of T-cell proliferation whether the MSC were added to the MLR at the initiation culture or midway through a seven day culture period. Suppression was also shown to be independent of treatment with IFN- $\gamma$  and fas-ligand, and was not MHC restricted, since MSC from third party donors were as equally suppressive as autologous MSC matched to responder or stimulator cells in the MLR. Furthermore, this immunosuppressive effect appeared to be mediated at least in part by a soluble factor based on the result of MLR cultures undertaken in transwells [McIntosh & Bartholomew, 2000]. Interestingly, Le Blanc *et al.* [2003] showed that although

proliferation activity in the MLR was suppressed with the addition of between 10,000 and 40,000 MSC, the addition of just 10-1000 MSC led to a less consistent suppression or a marked lymphocyte proliferation, showing the effect to be largely dose dependent. However, another interpretation of these results suggest that competition for nutrients could cause the reduction in T-cell proliferation since when the MSC were added to the MLR, the volume of tissue culture medium was kept constant, failing to compensate for the increased consumption of nutrients by the extra cells. However, no other cell source was used as a control for the experiment therefore there are no means to verify whether lack of nutrients was the cause of these results. Moreover, the wide inconsistency of the findings when only 10-1000 cells were added suggests more research is required before any real conclusions can be made. More recent experiments by Bartholomew *et al.* (2002) showed that T-cell suppression was reduced when MSC were added midway through an MLR and could be partially reversed using interleukin-2 (IL-2), however again without adequate experimental controls. They also demonstrated that the intravenous introduction of MSC in baboons prolonged the survival time of both an MSC donor matched skin graft and a third party skin graft from approximately 7 days to approximately 11 days.

Despite the short fallings of these experiments, taken together, these data suggest that hMSC may possess immunosuppressive effects which may render them either “immunoprivileged” or perhaps immunosuppressive *in vivo*. These findings may explain the lack of rejection of xenogeneic, allogeneic, or gene transduced MSC following their transplantation in large animal models [Devine *et al.*, 2001; Mackenzie & Flake, 2001a; 2001b; Liechty *et al.*, 2000]. This offers great potential benefits for tissue engineering purposes as MSC from any donor could be used to create the ideal off-the-shelf tissue-engineering product suitable for any recipient, whilst retaining the

ability to grow and develop. However, the same findings also highlight potentially catastrophic consequences: If MSC do cause suppression of T-cell proliferation, then should implanted cells become infected with a virus or become neoplastic once transplanted, the immune system will be unable to deal with the problem, as cell mediated immunity is paramount in host defence against virus infected cells and tumours. Therefore, any tissue-engineered product seeded with MSC might be unprotected and exempt from the body's immune surveillance systems.

### ***1.13. Alternative Sources of Mesenchymal Stem Cells***

It is possible that MSC may reside in tissues other than the bone marrow. Zuk *et al.* (2001, 2002), Gronthos *et al.* (2001), De Ugarte *et al.* (2003), Morizono *et al.* (2003) and Winter *et al.* (2003), for example, claim to have isolated hMSC from adipose tissues. If this source of MSC was found to be reliable, it would be a far more useful source of cells both for research and future applications as the cells could be readily isolated from waste material from liposuction procedures which are increasingly common. This would give a far superior supply of cells than procurement from bone marrow biopsies which are both painful to the donor and do not provide a great deal of material to isolate cells from. It remains to be seen whether this source perhaps represents another pool of adult stem cells or if they are in fact MSC. If they are MSC, then it could be argued that all adult stem cells are in fact the same and have the same capabilities and activities but are merely found in different reservoirs throughout the body as a ready source for wound healing.

Other groups believe that hMSC can be found circulating through the blood [Zvaifler *et al.*, 2000; Blau *et al.*, 2001], although others have previously disagreed [Lazarus *et al.*, 1997]. Again this could represent a new source of adult stem cells, although it seems

more likely that they have been mobilised from their initial location of the bone marrow, perhaps by wound response factors, and are destined for a wound healing role in a specific damaged tissue. De Bari *et al.* (2001) and Jones *et al.* (2002) claim to have isolated hMSC from adult synovial membrane, where again they may provide wound healing response to the surface of the joint concerned and could be another form of adult stem cell. The presence of hMSC in full term cord blood is still a point of contention [Erices *et al.*, 2000; Labat, 2001; Romanov *et al.*, 2003]. hMSC have, however, been isolated in first trimester foetal blood, liver and bone marrow, suggesting that hMSC migrate during the first trimester of foetal life from the liver to the bone marrow [Campagnoli *et al.*, 2001].

#### **1.14. Mesenchymal Stem Cells and Neoplasia**

A number of researchers have noted similarities between adult stem cells and metaplastic cells. Both cell types have been shown capable of indefinite proliferation, form heterogeneous populations, have the capability to migrate and differentiate and can be tissue specific. This has led a number of people to draw on the possibility that the two may well be linked [Reya, *et al.*, 2001; Alison *et al.*, 2002; Pathak, 2002]. Since the main functional purpose of adult stem cells are as a source of cells for wound repair, it follows that pools of adult stem cells of different sources, in various locations throughout the body, would need to be maintained in some form throughout the life of an individual. Since these cells are immortal, their environment would have to be stringently controlled to prevent unnecessary proliferation beyond base line levels required for maintenance of the cell population while they are in a quiescent state. This environment and the rigorous controls placed on the cells could be affected by the ageing and/or health state of an individual, which could ultimately lead to relaxation of these strict controls. Under these conditions, the known proliferation and differentiation

capabilities of mesenchymal stem cells alone could enable a single pool of de-regulated cells to readily differentiate into the broad range of cell phenotypes found in neoplasias throughout the body. The recognition that different varieties of adult stem cells may display functional overlap in their diversity of differentiation capabilities could easily account for most, if not all, varieties of neoplasia. Needless to say, no proof of any link currently exists and further investigation is required to link the two, but it would seem to be an intriguing coincidence and possibility.

# *Aims and Objectives*

## *Aims*

To date, no valve has completely fulfilled all of the objectives of the ideal aortic heart valve replacement as set out by Harken in 1989. The aims of this study therefore were to develop methods for the isolation and characterisation of mesenchymal stem cells as a cell source for re-seeding a decellularised aortic heart valve matrix in order to create a living valve that is capable of growth, repair and regeneration. Furthermore, a previously developed decellularised porcine valve matrix was further characterised and its biocompatibility assessed prior to being reseeded with mesenchymal stem cells.

## *Specific objectives*

- To investigate the extraction and isolation of mesenchymal stem cells (MSC) from porcine and human bone marrow.
- To characterise porcine and human MSC by means of their differentiation capacity and phenotypic analysis.
- To further characterise a previously developed decellularised porcine aortic heart valve matrix.
- To assess the biocompatibility of the decellularised valve matrix in a murine model.
- To examine the potential of re-seeding a decellularised aortic heart valve matrix with human mesenchymal stem cells *in vitro*.

## **CHAPTER TWO:**

### ***General Materials & Methods***

# CHAPTER 2:

## *General Materials & Methods*

### **2.1. *Equipment & consumables***

#### **2.1.1. Balances**

A Mettler AE50 four-figure balance and a Sartorius BL50 two-figure balance were used.

#### **2.1.2. Centrifuges**

An MSE Mistral 3000i (Fisher Scientific) centrifuge was used for general centrifugation. High-speed centrifugation was carried out on samples contained in Sorvall 'Oakridge' polycarbonate (50 ml) centrifuge tubes using a Sorvall RC-5B refrigerated super-speed centrifuge.

#### **2.1.3. Class II safety cabinet**

Hazardous procedures, such as handling unscreened tissue, and procedures in which workers' protection was required were carried out in a Heraeus 85 Class II safety cabinet. All items entering the cabinet were sprayed thoroughly with a 70 % (v/v) ethanol (VWR International) solution as a surface disinfection step. Gloved hands of those using the cabinet were also sprayed with alcohol prior to entering the work area within the cabinet.

#### **2.1.4. Microtitre plate reader**

A Dynex MRX plate reader with preloaded software was used.



### **2.1.5. Fluorescence activated cell sorting (FACS)**

FACS analysis was carried out using a Beckman Coulter EPICS XL-MCL model.

### **2.1.6. Glassware**

Glassware (beakers, bottles (100 ml, 500 ml, 1000 ml), flasks and measuring cylinders) was cleaned by immersion in a 1 % (v/v) solution of a phosphate-free detergent (Neutracon<sup>®</sup>, Decon Laboratories Ltd) overnight. They were then rinsed thoroughly in tap water followed by three rinses in distilled water to remove all traces of detergent. The glassware was then dried or sterilised (160 °C for 2 hours, as required) by dry heat.

### **2.1.7. Incubators**

Tissue culture experiments were incubated in a humidified 5 % (v/v) CO<sub>2</sub> incubator (Sanyo Gallenkamp) maintained at 37 °C.

### **2.1.8. Microscopy**

Bright-field and phase contrast microscopy was carried out using a CK40 Olympus inverted microscope. Fluorescent microscopy was carried out on the same microscope using a CK40-RFA fluorescent vertical illuminator. Cells were photographed by attaching an Olympus OM-4Ti Camera using 400 and 800 speed Fuji colour film for bright field and fluorescence microscopy respectively and later an Olympus Camedia C4040 Zoom digital camera. Photograph negatives were digitally scanned for computer manipulation using a Minolta Dimage Image Scanner attached to a personal computer using Adobe Photoshop 5.5 software. Latterly, cells and tissues were viewed and photographed using an Olympus Inverted IX71 microscope and an Olympus BX51

microscope respectively with an Evolution MP Color Digital camera from MediaCybernetics attached and controlled through Image Pro Plus imaging software.

### **2.1.9. Sterile plasticware**

Flat-bottomed 96-well, 48-well, 24-well, 12-well and 6-well plates, and 25 cm<sup>2</sup> and 75cm<sup>2</sup> tissue culture flasks, all Nunc brand, were purchased from Fisher Scientific. The 'Stripette' disposable pipettes (2 ml, 5 ml, 10 ml and 25 ml) were supplied by Corning Costar. Bibby Sterilin bijou containers (7 ml), universal containers (25 ml) and specimen containers (60 ml, 150 ml and 250 ml) were purchased from SLS. Finnpiettes and pipette boys were purchased from Scientific Laboratory Supplies. Both filter and non-filter tips (2 µl, 20 µl, 200 µl and 1000 µl) were purchased from Star Labs.

### **2.1.10. Dissection**

A size 22 scalpel handle, dissecting scissors, rat-tooth forceps, and standard forceps were purchased from Fisher Scientific and sterilised with dry heat prior to use. Single use sterile scalpel blades were also purchased from Fisher Scientific.

### **2.1.11. Measurement of pH**

The pH of solutions was measured using a Jenway 3020 pH meter. The pH meter was calibrated using solutions of pH 4, 7 and 10 made from buffer tablets (VWR International) dissolved in deionised water. The pH of solutions was measured using temperature compensation. To adjust the pH of the solution, 1 M hydrochloric acid (HCl, VWR International) or 1 M sodium hydroxide (NaOH, VWR International) was added drop-wise with stirring.

### **2.1.12. Microtome and cryostat**

Paraffin sections were cut at 4  $\mu\text{m}$  on a Leica RM2125RTF microtome. Frozen sections were cut at 5  $\mu\text{m}$  on a Bright Instruments OTF/AS 88100588 cryostat.

## **2.2. *Sterilisation***

### **2.2.1. Dry heat sterilisation**

Items to be sterilised by dry heat were placed in an oven and held at a temperature of 160 °C for 1 hour.

### **2.2.2. Moist heat sterilisation**

Objects and solutions not suitable for dry heat sterilisation were sterilised by autoclaving at 121 °C, 15 pounds per square inch (psi) for 20 minutes.

### **2.2.3. Filter sterilisation**

Solutions not suitable for autoclaving were sterilised with single use 0.2  $\mu\text{m}$  filters purchased from Schleider and Schuell UK Ltd.

## **2.3. *General solutions & reagents***

### **2.3.1. Bovine serum albumin**

Bovine serum albumin (BSA) fraction V (tissue culture tested) was purchased from Sigma-Aldrich and stored at 4 °C.

### **2.3.2. Foetal calf serum**

Foetal calf serum (FCS) of New Zealand origin was supplied by Invitrogen. Complement within the FCS was inactivated by incubation at 56 °C for 60 minutes. The FCS was then aliquoted and stored at -20 °C until required.

### **2.3.3. Formalin solution**

A 40 % (v/v) solution of formaldehyde (VWR International) was diluted in phosphate buffered saline (PBS) to 4 % (v/v) and stored at room temperature.

### **2.3.4. L-Glutamine solution**

L-Glutamine was purchased from Sigma-Aldrich at a concentration of 200 mM. The solution was aliquoted and stored at -20 °C.

### **2.3.5. Hanks' balanced salt solution (HBSS)**

Both complete HBSS and calcium and magnesium free HBSS were purchased from Sigma-Aldrich and stored at room temperature until required. After opening, HBSS was stored at 4 °C.

### **2.3.6. N-(2-hydroxyethyl)piperazine-N'-(2-ethanulfonic acid) (HEPES)**

A 1 M solution of HEPES was supplied by Sigma-Aldrich and was stored at room temperature until required. Once open, the HEPES was stored at 4 °C.

### **2.3.7. Penicillin/streptomycin solution**

A solution of 100,000 Units.ml<sup>-1</sup> penicillin and 100,000 µg.ml<sup>-1</sup> streptomycin was purchased from Sigma-Aldrich and was aliquoted and stored at -20 °C.

### **2.3.8. Transport medium**

Transport medium consisted of 100 ml complete HBSS supplemented with 1 ml penicillin/streptomycin solution, 10 KIU aprotonin (Tyrasol, Leeds General Infirmary Pharmacy), and 2 ml 1 M HEPES.

### **2.3.9. Phosphate buffered saline (PBS)**

Sterile PBS solution for tissue culture experiments was purchased from Sigma-Aldrich. PBS for general experiments was prepared from PBS tablets (BR14a OXOID). One tablet for each 100 ml of PBS required was dissolved in distilled water according to the manufacturer's instructions and autoclaved.

### **2.3.10. Culture medium**

Dulbecco's modified Eagles medium (DMEM) without L-glutamine and sodium pyruvate was purchased from Invitrogen up to February 2002 and after October 2002. The same DMEM was supplied by Sigma-Aldrich between February and December 2002. Between August and October 2002, human MSC cells were cultured in Mesencult with supplements for human mesenchymal stem cells. One porcine MSC sample (pBM37) was cultured in Mesencult with supplements for human mesenchymal stem cells during December 2002. All culture medium was stored at 4 °C and warmed to 37 °C prior to use.

### 2.3.11. Gas mixtures

Laboratory grade CO<sub>2</sub> was supplied by BOC Ltd.

## 2.4. *Tissue culture*

### 2.4.1. Complete medium and standard culture conditions

DMEM was supplemented with 1 ml penicillin/streptomycin solution and 1 ml L-glutamine solution per 100 ml, and 10 % (v/v) FCS. This was stored for up to 2 weeks at 4 °C. Cells were cultured in a humidified Sanyo incubator at 37 °C with 5 % CO<sub>2</sub> (v/v) in air.

### 2.4.2. Cell counting & cell viability

**Materials:** An improved Neubauer haemocytometer chamber was purchased from Fisher Scientific. Trypan blue solution (4 %w/v) was purchased from Sigma-Aldrich.

**Method:** A trypan blue dye exclusion test was used when counting cells to ensure that only viable cells were included. Trypan blue solution was added to a small aliquot of cell suspension at a ratio of 1:1. The plasma membrane of a viable cell did not permit entry of the dye substance; therefore viable cells and non-viable cells could be distinguished. The sum of the number viable cells visible by bright field microscopy within any five of the nine major squares on either side of the central divide of the haemocytometer were multiplied by a factor of 10<sup>3</sup> to calculate the number of cells in 1ml of suspension.

### 2.4.3. Calculating cell doubling time (CDT)

Once a confluent cell layer was obtained from the original cell isolation, 5×10<sup>5</sup> cells were transferred to a 75 cm<sup>2</sup> tissue culture flask. The cells were then cultured further

until a confluent monolayer had formed and the time to reach confluence noted. The cells were then counted using a haemocytometer as described in section 2.4.2. Typically,  $5 \times 10^5$  human mesenchymal stem cells formed a confluent monolayer in a 25 cm<sup>2</sup> tissue culture flask, and  $1.5 \times 10^6$  cells in a 75 cm<sup>2</sup> tissue culture flask. The cell doubling time (CDT) in that time period was then calculated using the following formula:

$$\text{CDT} = \frac{t}{\log_{10}(N/N_0) \times 3.33}$$

Where: t = time in hours for confluent monolayer to form after cells seeded  
 N = final number of cells in confluent monolayer  
 No = original number of seeded cells

#### 2.4.4. Passaging cells

**Materials:** Trypsin-ethylenediaminetetraacetic acid (1×; EDTA) solution was obtained from Sigma-Aldrich.

**Method:** Cell culture medium was aspirated and then discarded. The cell layer was then rinsed once with calcium and magnesium-free HBSS. Enough trypsin-EDTA was added to just cover the cell monolayer (0.5 ml for 25 cm<sup>2</sup> flasks, and 1 ml for 75 cm<sup>2</sup> flasks). The flask was then rocked to coat the surface with trypsin-EDTA prior to being incubated at 37 °C for 10 minutes. The flask was then firmly tapped to assist cell detachment from the surface of the tissue culture flask until all the cells were detached. Once detached, the cells were resuspended in complete medium and passaged at a 1:3 ratio.

#### 2.4.5. Cryopreservation of cells

**Materials:** Cryovials and a Nalgene freezing container were obtained from Fisher Scientific. Dimethylsulphoxide (DMSO) was obtained from Sigma-Aldrich. Cryo-

medium consisted of complete medium containing 20 % (v/v) foetal calf serum and 10 % (v/v) DMSO.

**Method:** The cells were trypsinised as described above and resuspended in chilled (4 °C) cryo-medium. Cells were then split at a ratio of 1:2 and 1:6 for 25 cm<sup>2</sup> and 75 cm<sup>2</sup> flasks respectively into pre-cooled cryovials to give 1ml volumes. The cryovials were then placed in a Nalgene freezing container, which was placed in a -80 °C freezer for a minimum of 4 hours in order to provide a cooling rate of -1 °C.min<sup>-1</sup>. The cryovials were then quickly transferred to liquid nitrogen storage.

#### **2.4.6. Resurrection of cryopreserved cell cultures**

**Method:** Cells were removed from liquid nitrogen storage and rapidly defrosted in a 37 °C water bath. Pre-warmed complete medium (10 ml) was then added drop-wise to the cells prior to centrifugation at 200 g for 10 minutes. The supernatant was then discarded and the pellet resuspended in 5 ml of complete medium. This was then transferred to a 25 cm<sup>3</sup> tissue culture flask and the cells incubated under standard conditions.

### **2.5. *Basic histological techniques***

#### **2.5.1. Tissue harvesting for paraffin embedding**

**Materials:** Ethanol, xylene and paraffin were obtained from VWR International.

**Methods:** Tissue samples were placed in 20 ml of formalin solution for one hour, before being placed in 20 ml of 70 % (v/v) ethanol until ready for further processing. Tissues were then dehydrated through graded alcohols and xylene. This involved first replacing the 70 % (v/v) ethanol with 20 ml 90 % (v/v) ethanol and leaving the samples overnight. The tissues were then placed in 20 ml absolute ethanol for one hour, before the ethanol was replaced for a second hour. This was then repeated with xylene and



liquid paraffin. Tissues were then embedded in liquid paraffin before it was cooled and solidified.

### **2.5.2. Tissue sectioning and slide preparation of paraffin-embedded tissues**

**Materials:** The hotplate was purchased from Raymond A Lamb (London, UK). Superfrost Plus slides were obtained from VWR International.

**Method:** Tissue sections were made by sectioning the paraffin block at a thickness of 4  $\mu\text{m}$  on a microtome. Sections were then melted onto Superfrost Plus slides by leaving the slides on a hotplate set at 56 °C for 10 minutes.

### **2.5.3. Dewaxing and rehydrating paraffin-embedded tissue sections**

**Materials:** Ethanol, xylene and paraffin were obtained from VWR International.

**Method:** This was carried out by sequentially immersing the cells in two lots of 100 ml of xylene for ten minutes each followed by three changes of 100 ml absolute ethanol for 1 minute each, followed by 100 ml 70 % (v/v) ethanol for 1 minute and finally rinsing the slides in running tap water.

### **2.5.4. Dehydration and mounting of tissue sections**

**Materials:** Ethanol, xylene, DPX mountant and 22 mm<sup>2</sup> coverslips were obtained from VWR International. Slide racks were purchased from Raymond A Lamb Ltd.

**Method:** Once sections had been stained, the slides were transferred to a metal slide rack. This was then sequentially immersed in 70 % (v/v) ethanol then three changes of ethanol for 1 minute at a time. The ethanol was then followed by two changes of xylene for 1 minute each. Slides were then individually removed from the xylene, a drop of

DPX mountant placed over the tissue section, and a coverslip carefully placed over the section.

### **2.5.5. Tissue harvesting for cryo-embedding**

**Materials:** Cryo-embed was purchased from VWR international.

**Methods:** Tissues requiring cryo-embedding were first lightly dried using tissue paper.

A drop of Cryo-embed was then placed on a cryostat chuck in the freezing chamber of the cryostat. The tissue section was then placed into the cryo-embed drop before more cryo-embed was added to cover the tissue. The sample was then left to freeze for 10 minutes. Frozen blocks were then stored at  $-40^{\circ}\text{C}$ .

### **2.5.6. Tissue sectioning and slide preparation of cryo-embedded tissues**

**Methods:** Tissues were sectioned at a thickness of  $5\ \mu\text{m}$  on a cryostat and the section transferred to Superfrost Plus slides and allowed to air dry. Prior to any histological or immunohistochemical staining, sections were rehydrated by immersing the slides in 5 ml of PBS for 5 minutes.

### **2.5.7. Haematoxylin & eosin Staining**

**Materials:** Modified Mayer's haematoxylin consisted of 3 g of haematoxylin (Raymond A Lamb) added to 20 ml absolute ethanol (VWR International) This was then mixed with 850 ml distilled water containing 0.3 g sodium iodate (Fisher Scientific), 1 g citric acid (VWR International), 50 g chloral hydrate (Fisher Scientific) and 50 g aluminium potassium sulphate (VWR International). Once mixed, 120 ml of glycerol (Sigma-Aldrich) was added and the solution filtered before use. Eosin was obtained from Raymond A Lamb.

**Method:** Paraffin sections were first dewaxed and rehydrated through graded alcohols. Sections were then dipped in 100 ml Modified Mayer's Haematoxylin for 1 minute, rinsed in tap water, and immersed in 100 ml eosin for 3 minutes. Sections were then dehydrated and mounted.

## **2.6. Immunohistochemical labelling**

### **2.6.1. Immunoperoxidase labelling of paraffin-embedded sections**

**Materials:** Citric acid buffer consisted of 10 mM citric acid (Sigma-Aldrich) adjusted to pH 6.0 using NaOH pellets (Sigma-Aldrich). A streptavidin/biotin blocking kit, rabbit serum and ready-to-use streptavidin horseradish peroxidase were purchased from Vector Laboratories Ltd. Tris-buffered saline (TBS buffer) consisted of 25 ml 2 M Tris (Sigma-Aldrich), adjusted to pH 7.6 using HCl (VWR International), 50 ml 3 M NaCl (VWR International) and 925 ml distilled water. TBS azide consisted of TBS buffer containing 0.01% (w/v) sodium azide (VWR International). TBS azide BSA consisted of TBS azide containing 10% (w/v) bovine serum albumin (Sigma-Aldrich). Sigma Fast 3,3'-diaminobenzidine tablets (1 ml) were purchased from Sigma-Aldrich. Antibodies were obtained from various suppliers and were diluted according to the accompanying data sheet in TBS azide BSA prior to use. Biotinylated secondary antibody was diluted to according to the manufacturer's instructions in TBS azide BSA containing 10 % (v/v) pig serum.

**Method:** Sections were first dewaxed and rehydrated prior to undergoing antigen retrieval and peroxide blocking if required. The slides were then placed into separate compartments of a slide box prior to covering the sections with 50 µl of streptavidin block for 10 minutes. The slides were then washed three times in TBS buffer and 50 µl of biotin block added to each section for a further 10 minutes. Again the sections were washed three times in TBS buffer and 50 µl of 20 % (v/v) rabbit serum (diluted in TBS

azide) added to each section for a further 5 minutes. This was then washed three times in TBS buffer and 50  $\mu$ l of the appropriate diluted antibody added to each section (see Table 2.1). This was then left for 1 hour at room temperature before being washed three times in TBS buffer and 50  $\mu$ l of secondary antibody was added to each section. After 30 minutes, the slides were washed three times in TBS buffer and 50  $\mu$ l of horseradish peroxidase streptavidin was added to each section for 45 minutes at 37 °C. This was then washed three times in TBS buffer. Sigma Fast 3,3'-diaminobenzidine tablets were dissolved in 1ml of distilled water according to the manufactures instructions and 50  $\mu$ l of the working solution was added to each section. After 10 minutes, the reaction was stopped by immersing the slides in running water. The sections were then counterstained by dipping the slides in haematoxylin for 5 seconds, prior to dehydrating the sections through graded alcohols as previously described. Sections were then mounted in DPX mountant prior to viewing.

### **2.6.2. Peroxide blocking**

**Materials:** Hydrogen peroxide (30 %; v/v) was purchased from Sigma-Aldrich.

**Method:** For paraffin sections, slides were placed in 3 % (v/v) H<sub>2</sub>O<sub>2</sub> in distilled water for 10 minutes at room temperature, prior to being washed for 3×2 minutes in distilled water. For frozen sections, slides were placed in 0.6 % (v/v) H<sub>2</sub>O<sub>2</sub> in methanol for 10 minutes at room temperature prior to being washed for 3×2 minutes in distilled water.

### **2.6.3. Antigen retrieval**

#### **2.6.3.1. Microwave treatment**

**Materials:** EDTA buffer was prepared using a solution of 1 mM EDTA (Sigma-Aldrich) adjusted to pH 8.0 using NaOH (Sigma-Aldrich). Citric acid buffer was prepared using 10 mM citric acid adjusted to pH 6.0 using NaOH pellets (Sigma-

Aldrich). The microwave was an 800W model from Sanyo. The Pyrex dish and clingfilm were purchased from Wilkinson's.

**Method:** Where indicated by the manufacturer of the antibody, microwave treatment was used to retrieve antigens on paraffin embedded tissue sections. The slides were placed in a Pyrex dish and covered in 400 ml of either EDTA or citric acid buffer accordingly. The dish was then covered with clingfilm and placed in a microwave, before being microwaved on full power for 10 minutes. The dish was then placed on ice and allowed to cool.

#### **2.6.3.2. Trypsin treatment**

**Materials:** Trypsin and  $\text{CaCl}_2$  were purchased from Sigma-Aldrich.

**Methods:** Sections were incubated at 37 °C in distilled water for 10 minutes. Water was then replaced with a solution containing 0.1 % (w/v) trypsin in 0.1 % (w/v)  $\text{CaCl}_2$  at pH 7.8. Sections were then rinsed in running tap water prior to further processing.

#### **2.6.3.3. Hyaluronidase treatment**

**Materials:** Buffered hyaluronidase was prepared by dissolving 10 mg of bovine testicular hyaluronidase Type IV (Sigma-Aldrich) in 10 ml PBS).

**Method:** Antigen retrieval was carried out by immersing each slide in 5 ml of buffered hyaluronidase enzyme for 3 hours at 37 °C.

#### **2.6.4. Immunofluorescent labelling of cultured cells**

**Materials:** Slide boxes were obtained from Fisher Scientific. Antibodies were obtained from various suppliers and were diluted according to the accompanying data sheet (see Table 2.1) in TBS azide BSA prior to use. Fluorochrome labelled goat anti-mouse secondary antibody was obtained from Molecular Probes Europe BV and was diluted

1/500 in TBS azide containing 10 % (v/v) pig serum (Vector Laboratories Ltd). Tween 20 (polyoxyethylene sorbiton monolaurate) was purchased from Sigma-Aldrich. DABCO/glycerol solution consisted of 9:1 solution of glycerol (VWR International): 2.5 % (w/v) 1,4-diazobicyclo-(2, 2, 2)-octane (DABCO, Sigma-Aldrich) in 0.1M NaHCO<sub>3</sub> (VWR International), adjusted to pH 9.0 using NaOH pellets (Sigma-Aldrich).

**Method:** Multi-spot slides were placed into a 4-well slide box. The cells were then washed three times in TBS buffer. Antibody was diluted according to the manufacturer's instructions in TBS-azide BSA and 50 µl was added to each section and left for 1 hour at room temperature. The slides were then washed three times in TBS buffer prior to fixation in a 50:50 solution of methanol and acetone for 2 minutes. Diluted fluorochrome-labelled anti-mouse secondary antibody (50 µl) was added to each section and the sections were incubated at room temperature for 30 minutes. The slides were then washed twice in PBS containing 0.25 % (v/v) Tween 20. The sections were then rinsed once with distilled water before being dried and stored in a dark box prior to viewing. Finally, the sections were mounted in DABCO/glycerol solution.

### 2.6.5. Antibodies used for immunohistochemistry

Where necessary, antibodies were diluted with TBS azide BSA as described in section 2.6.1. Few antibodies were available that were known to bind to porcine antigens, therefore, antibodies to human antigens were frequently purchased and their suitability for porcine tissue assessed using porcine positive control tissue.

ANTIGEN	TYPE	SUPPLIER	ISOTYPE	ANTIGEN RETRIEVAL	DILUTION	POSITIVE CONTROL
Ki67	Mouse anti-human	DAKO	IgG1	None	1:75	Porcine skin
Elastin	Mouse anti-bovine	Vector	IgG1	Trypsin	1:100	Porcine small intestine
Collagen I	Mouse anti-	Chemicon	IgG1	Trypsin	1:100	Porcine skin

Collagen II	human Mouse anti-human	Sigma	IgM	Hyaluronidase	1:1500	Bovine cartilage
Active caspase-3	Mouse anti-human	Vector	IgG1	None	1:25	Porcine tonsil
Tau	Mouse anti-bovine	Vector	IgG1	None	1:100	Porcine optic nerve
NSE	Mouse anti-human	Vector	IgG2a	Citrate microwave	1:50	Porcine optic nerve
$\alpha$ SMA	Mouse anti-human	Vector	IgG2a	None	1:50	Porcine bowel wall
HCMs	Mouse anti-rabbit	Vector	IgG1	Citrate microwave	1:20	Porcine heart muscle
Vimentin	Mouse anti-pig	Vector	IgG1	Citrate microwave	1:50	Porcine lymph node
Desmin	Mouse anti-pig	Vector	IgG1	Citrate microwave	1:50	Porcine heart muscle
X-gal	Baboon anti-pig	Chemicon	Poly-clonal	None	1:500	Porcine valve leaflet
Alkaline Phosphatase	Mouse anti-human	DSHB	Poly-clonal	None	1:10	Osteosarcoma cell line
Osteonectin	Mouse anti-human	Vector	IgG1	Citrate microwave	1:40	Osteosarcoma cell line
Osteopontin	Mouse anti-human	Vector	IgG1	Citrate microwave	1:25	Gall bladder
CD3	Rat anti-mouse	Caltag	IgG2a	Not suitable for paraffin	1:4	Murine spleen
CD4	Rat anti-mouse	Caltag	IgG2a	Not suitable for paraffin	1:4	Murine Spleen
F4/80	Rat anti-mouse	Caltag	IgG2b	Not suitable for paraffin	1:4	Murine Spleen
CD34	Rat anti-mouse	BD Pharmingen	IgG2a	Not suitable for paraffin	1:20	Murine Spleen

**Table 2.1. Details of the primary antibodies used during the study. Antigen retrieval only required when paraffin sections used.**

ANTIGEN	TYPE	SUPPLIER	DILUTION
2° Rabbit anti-mouse	Biotin-ylated	DAKO	1:200
2° Goat anti-mouse	FITC	Molecular Probes	1:500
2° Rabbit anti-rat	Biotin-ylated	DAKO	1:200

**Table 2.2. Details of the secondary antibodies used during the study.**

ANTIGEN	TYPE	SUPPLIER
Isotype control IgG1	Mouse control	DAKO
Isotype control IgG2a	Mouse control	DAKO
Isotype control IgG2a	Rat control	Caltag
Isotype control IgG2b	Rat control	Caltag

**Table 2.3. Details of the isotype control antibodies used during the study.**

## **2.7. Control cell lines and tissues**

A human osteosarcoma cell line (HOS38094) was obtained from The European Collection of Cell Cultures (ECACC), as a positive control for the alkaline phosphatase assay. Human smooth muscle cells obtained from saphenous vein explants were used as a negative control for the alkaline phosphatase analysis. All control cells were cultured in complete medium and under standard conditions as described in Section 2.4.1. Control tissues for immunohistochemistry are described in Table 2.1. Human aortic homograft valves were obtained from the National Blood Service for cell counting analysis and comparison with reseeded tissues.



### **2.7.1. Isolation of human smooth muscle cells from saphenous vein**

**Materials:** Saphenous vein samples were obtained with ethical approval (ethics number: 01/044) from Leeds General Infirmary from patients undergoing a coronary artery bypass.

**Methods:** Isolation of human smooth muscle cells from saphenous vein was carried out by Dr Helen Wilcox. The tissue sample was placed into a petri dish and rinsed twice with PBS. The vein was then cut longitudinally and the internal surface (intima) scraped with a scalpel in order to remove the endothelial cell layer. The outer adventitial layer was then carefully peeled away from the remainder of the tissue and discarded. The remaining tissue was then finely diced before being transferred to a 25 cm<sup>2</sup> tissue culture flask and the pieces spread out evenly on the base of the flask. Complete medium (1 ml) was then carefully added to the flask so as not to disturb the tissue pieces, and the tissue cultured under standard conditions. Culture medium was replaced every other day at this point, and the migration of cells out of the tissue samples monitored using an inverted microscope. Once cells could be observed to be migrating out from the majority of tissue pieces, the flask was flooded with 5ml of complete medium and re-incubated for 24 hours. The flask was then gently tapped in order to dislodge the tissue pieces, which were then poured out of the flask. This was then replaced with 5ml of complete medium and the cells cultured using standard conditions and techniques, as described in Section 2.4.1.

## ***2.8. Leaflet decellularisation and disinfection***

### **2.8.1. Leaflet decellularisation**

**Materials:** Aprotinin was purchased from the LGI pharmacy as Tyrasol. Sodium ethyldiaminotetraacetic acid, Tris, magnesium chloride, bovine serum albumin (BSA), RNase A and DNase I were purchased from Sigma-Aldrich. Hydrochloric acid was

obtained from VWR International. Sodium dodecyl-sulphate was obtained from Calbiochem. An orbital plate shaker (IKA KS130 basic) was purchased from Jencons. Wash buffer consisted of PBS containing 10 KIU aprotinin and 0.1 % sodium ethyldiaminotetraacetic acid (EDTA). Hypotonic buffer consisted of 10 mM Tris with 10 KIU aprotinin and 0.1 % EDTA adjusted to pH 8.0 using HCl. Reaction buffer was made by adding 10 mM magnesium chloride and 50  $\mu\text{g}\cdot\text{ml}^{-1}$  bovine serum albumin to 50 mM Tris and adjusted to pH 7.5 using hydrochloric acid.

**Method:** This technique was developed by Booth *et al.* (2002). Porcine hearts were obtained from a local abattoir and the aortic valve leaflets excised. Leaflets were then washed 3×30 minutes in wash buffer (5 ml per leaflet) on a plate shaker set at 300 rpm. Leaflets were then incubated for 16 hours in hypotonic buffer at 4 °C (5 ml per leaflet) before being incubated for 24 hours in hypotonic buffer containing 0.1 % (w/v) SDS (5 ml per leaflet) at room temperature on a plate shaker set at 300 rpm. Leaflets were again washed in wash buffer (5 ml per leaflet) for 3×30 minutes in PBS (5 ml per leaflet) prior to enzymatic digestion. RNase (1  $\text{U}\cdot\text{ml}^{-1}$ ) and DNase (50  $\text{U}\cdot\text{ml}^{-1}$ ) were added to 100 ml of reaction buffer and filter-sterilised and the leaflets incubated in 5 ml reaction solution per leaflet for 3 hours at 37 °C with gentle agitation on an orbital plate shaker. Leaflets were then washed for 3×30 minutes in wash buffer. At this stage, decellularised leaflets were either cryopreserved and stored in liquid nitrogen prior to further use, or formalin fixed for histological analysis.

### 2.8.2. Leaflet cryopreservation

**Materials:** Cryovials and a Nalgene freezing container were obtained from Fisher Scientific.

**Methods:** Once decellularised, leaflets were transferred to chilled (4 °C) cryo-medium. Two leaflets were then transferred to each cryovial in 1 ml of cryo-medium. The

cryovials were then placed in a Nalgene freezing container, which was placed in a  $-80^{\circ}\text{C}$  freezer for a minimum of 4 hours in order to provide a cooling rate of  $-1^{\circ}\text{C}\cdot\text{min}^{-1}$ . The cryovials were then quickly transferred to liquid nitrogen storage.

### 2.8.3. Thawing of cryopreserved leaflets

**Methods:** Leaflets were removed from liquid nitrogen storage and rapidly defrosted in a  $37^{\circ}\text{C}$  water bath. The leaflets were then transferred to 20 ml complete medium until required for further manipulation.

### 2.8.4. Tissue disinfection

**Materials:** Aprotinin and imipenem were purchased from the LGI pharmacy as Tyrasol and Primaxin respectively. Amphotericin B, vancomycin, gentamicin, polymixin B and PBS were purchased from Sigma-Aldrich.

**Method:** Decellularised leaflets placed in PBS containing  $25\ \mu\text{g}\cdot\text{ml}^{-1}$  amphotericin B,  $0.05\ \text{mg}\cdot\text{ml}^{-1}$  vancomycin,  $0.5\ \text{mg}\cdot\text{ml}^{-1}$  gentamicin,  $0.2\ \text{mg}\cdot\text{ml}^{-1}$  polymixin B,  $0.2\ \text{mg}\cdot\text{ml}^{-1}$  imipenem, and  $10\ \text{U}\cdot\text{ml}^{-1}$  aprotinin. Approximately 5 ml of solution was prepared per leaflet. Leaflets were then incubated on a plate shaker set at 300 rpm for 18-24 hr at  $4^{\circ}\text{C}$ .

## 2.9. Statistical analysis

### 2.9.1. Basic statistical terms

Mean  $(\bar{x}) = \frac{\sum x}{n}$

$\Sigma$  = Sum of  
 $x$  = individual values  
 $n$  = the number of individual values

Standard Deviation (S) =  $\sqrt{\frac{(\sum x)^2}{n-1}}$

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$$\text{Standard Error (SE)} = \frac{S}{\sqrt{n}}$$

### 2.9.2. 95 % Confidence limits

Confidence values, 95 %, were calculated for data with several replicates. The results were expressed as the mean  $\pm$  95 % confidence limits.

$$95 \% \text{ confidence limit} = \text{SE} \times \text{t-value}$$

Statistical tables were used to calculate the t-value where:

$$\alpha = \text{probability level (0.05)}$$

$$v = \text{degrees of freedom (n-1)}$$

### 2.9.3. Arcsine transformation of data

Where experimental data was expressed as percentages it was necessary to transform the data to arcsine values in order to generate 95 % confidence limits. Arcsine is also known as inverse sine. The percentage data (proportions) were converted to the angle whose sine was the given quantity. At the end of the analysis the value for 95 % confidence was back transformed to a percentage and plotted as normal.

## **CHAPTER THREE:**

### ***Isolation and Characterisation of Porcine Mesenchymal Stem Cells***

# CHAPTER 3:

## *Isolation and Characterisation of Porcine Mesenchymal Stem Cells*

### **3.1. Introduction**

In any tissue engineering strategy involving living cells, an appropriate source of cells has to be established that possess similar properties to the cells of the native tissue. The use of terminally differentiated cells such as fibroblasts and smooth muscle cells for tissue engineering an aortic heart valve is potentially beset with cell-age related problems as it is widely known that terminally differentiated cells become senescent after prolonged culturing *in vitro* [Hayflick, 1965]. For a tissue-engineered product in which cells would ultimately be derived from the recipient of the construct to circumvent rejection, it is likely that a relatively small number of cells isolated from a simple biopsy would have to be expanded to large numbers *in vitro* prior to reseeded and subsequent implantation of the construct. Since these cells would already have aged *in situ* within the patient, subsequent *in vitro* culture could yield cells possessing only a limited lifespan. This could lead to seeded cells becoming senescent before host cells were able to infiltrate the tissue engineered implant, leaving a period of time in which the implant would not have cells needed for repair and remodelling of the tissue. This could be particularly problematic for a heart valve since the tissue is continually subjected to strong shear forces and flexion fatigue *in vivo* [Davila, 1989; Schoen, 1999] and is consequently highly susceptible to damage. Also, whilst fibroblasts and smooth muscle cells may have properties similar to the native cells, differences will remain. Since these cells are terminally differentiated, their capacity to adapt to the new environment will be limited and they may have limited capacity for changing

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phenotype, therefore they would be potentially suboptimal until host cells infiltrate the new tissue.

Adult stem cells, such as mesenchymal stem cells, have two distinct advantages over terminally differentiated cells. One of the definitions of any stem cell is that they are thought to be immortal in their undifferentiated state [Till & McCulloch, 1961]. This immortality could enable large numbers of cells to be grown *in vitro* without loss of proliferation capacity or age-related deterioration of the cells which is seen when terminally differentiated cells are cultured for prolonged periods [Hayflick, 1965]. Although it has been found that hMSC do not possess any telomerase activity [Banfi *et al.*, 2002] this could be a result of inappropriate culture conditions. Improving the culture conditions could permit the cells to maintain the immortality that the cells should in theory possess *in vivo*, allowing large cell expansion without loss of function. Once sufficient cells are available, differentiation into the appropriate required lineage(s) could then either be induced biochemically or mechanically *in vitro* by culturing the cells in a bioreactor. This latter route could be advantageous as it has been shown that mechanical stimulation can differentiate MSC and can even enhance matrix production and cell orientation of some terminally differentiated cells [Streppeti *et al.*, 1993; Banes *et al.*, 1995; Sato *et al.*, 1995; Butt & Bishop (1997); Eastwood *et al.*, 1998; Hishikawa & Lüscher, 1997; Sirois *et al.*, 1998; Breen, 2000; Grinnell, 2000; Altman *et al.*, 2002]. In tissue engineering an aortic heart valve, it is likely that the environment created in a bioreactor and later *in situ* within the patient would provide the correct mechanical and biochemical stimuli necessary to differentiate the cells into a phenotype more closely related to the native heart valve cells than could otherwise be achieved.

In order to perform pre-clinical assessment of a tissue engineered living heart valve, a large animal model would be required. Since only inbred pigs were available at the start of the project, this was the model of choice. Isolation and identification of porcine mesenchymal stem cells would provide a source of cells ultimately capable of being used to create a valve that could be tested in a large animal model. This construct could then be implanted into pigs to assess the *in vivo* functionality of the construct, which would be a vital step in the development of a tissue-engineered product suitable for human implantation.

At the beginning of the project, no work had been published regarding porcine MSC. It was therefore necessary to derive methods for isolating and identifying pMSC from investigations on MSC from other sources. An increasing number of groups have shown that it is possible to isolate and differentiate both human and murine MSC into a variety of cell lineages, including osteogenic, adipogenic and chondrogenic lineages [Caplan, 1991; Pereira *et al.*, 1995; Wakitani *et al.*, 1995; Cassiede *et al.*, 1996; Prockop, 1997; Ferrari, *et al.*, 1998; Johnstone *et al.*, 1998; Nevo *et al.*, 1998; Makino *et al.*, 1999; Pittenger *et al.*, 1999; Deans & Moseley, 2000; Emura *et al.*, 2000; Minguell *et al.*, 2000; Pittenger *et al.*, 2000; Fukuda, 2002; Hakuno *et al.*, 2002; Reyes *et al.*, 2002]. Thus methods for isolating the MSC from both sources are widely documented and essentially follow the same principle of centrifuging whole bone marrow on a continuous gradient (usually Ficoll or Percoll) and culturing the adherent cells from the uppermost fraction under standard conditions. The first medium change is left until 4 or 5 days after isolation to allow the MSC to attach, and the non-adherent haematopoietic cells can be easily poured off and discarded. Since this method has been shown to be effective for isolating murine and human MSC, it was decided to attempt the same protocol using porcine bone marrow.



Once cells are isolated, it would first be necessary to try to identify the cells as being MSC prior to investigating their potential in tissue engineering an aortic heart valve. Currently there are no definitive markers available specific for MSC of any origin, and so their identification has to be based on virtue of their differentiation capacity. However, this requires manipulation of the cell, which in turn changes the cells' properties. So in practical terms, the differentiation capacity has to be assessed using populations of MSC derived from a single cell. In identifying cells in this way, it would be important to try to differentiate the cells into a number of different cell lineages in order to rule out the possibility of multiple cell lineage precursors being present in the bone marrow and to demonstrate the multipotential differentiation capacity of the cells. Once differentiation had been attempted, a range of markers would be used to identify the cells. The myogenic lineage would be identified using markers to  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), desmin and heavy chain myosin slow (HCMs). Measuring alkaline phosphatase activity would identify the osteogenic lineage. The adipogenic lineage would be identified by looking for the accumulation of Oil Red O stain in the lipid vesicles. The chondrogenic lineage would be identified using markers to collagen II, and the neurogenic lineage would be identified using markers to neurone specific enolase (NSE) and tau.

When this work was started, it was noted that no successful attempts to perform clonogenic analyses of the cells had been published in the literature and consequently it was decided to try to differentiate cells derived from a single bone marrow sample rather than a single cell, with the idea that clonogenic analyses could be performed at a later stage if evidence of a multipotent differentiation capacity could be found in these initial experiments.

## **3.2. *Aims & objectives***

The aims of this part of the study were to adapt methods developed for human and murine MSC to isolate and differentiate porcine mesenchymal stem cells into cells of the osteogenic, myogenic, chondrogenic, adipogenic, neurogenic and smooth muscle cell lineages. Success with at least three of these attempts would indicate the cells to be porcine mesenchymal stem cells and would enable investigation of their application in an animal model of a tissue engineered aortic heart valve.

## **3.3. *Materials & methods***

### **3.3.1. Isolation of MSC from porcine bone marrow**

**Materials:** Porcine shoulder bones were obtained from pigs recently slaughtered at a local abattoir. A Percoll gradient was prepared by centrifuging 22.05 ml Percoll, (Sigma-Aldrich), 2.45 ml 1.5 M NaCl, (Sigma-Aldrich) and 10.5 ml Tyrode's balanced salt solution, (Sigma-Aldrich) at 20,000 g for 15 minutes. Centrifuge tubes (50 ml) were obtained from Fisher Scientific.

**Method:** The bones were dissected and the bone marrow within each bone was scraped out into separate sterile centrifuge tubes containing 25 ml of complete medium, as described in Section 2.4.1. The tube was then inverted a number of times in order to disperse the bone marrow prior to being centrifuged at 600 g for 5 minutes. The supernatant and overlying fat layer were carefully removed leaving approximately 5 ml of medium to avoid aspirating the pellet. The pellet was then resuspended by repeated pipetting in the remaining 5 ml of medium, before the suspension was carefully layered onto a preformed Percoll gradient. The Percoll-marrow solution was centrifuged at 460 g for 15 minutes before 14 ml of the upper fraction was removed and added to 25 ml of complete medium. The solution was then centrifuged at 600 g for 5 minutes, the

supernatant removed, the pellet resuspended by repeat pipetting, and a further 20 ml of complete medium added. The tube was then centrifuged at 600 g for a further 5 minutes. The supernatant was then removed and the cells resuspended in 5 ml of complete medium. The cell suspension was then transferred to a 25 cm<sup>2</sup> tissue culture flask and the flask incubated under standard conditions as described in Section 2.4.1. Culture medium was first changed after five days and then every three to four days. If not immediately required, the cells were cultured this way until the first passage when they were cryopreserved as described in Section 2.4.5. Otherwise the cells were grown until required for a differentiation assay. Cells were counted as described in Section 2.4.2.

### **3.3.2. Porcine mesenchymal stem cell differentiation assay 1**

**Materials:** Osteogenic medium consisted of complete medium supplemented with  $2 \times 10^{-4}$  M ascorbic acid (Sigma-Aldrich),  $7 \times 10^{-3}$  M  $\beta$ -glycerophosphate (Sigma-Aldrich), and  $1 \times 10^{-8}$  M dexamethasone, (Sigma-Aldrich).

Chondrogenic medium consisted of complete medium supplemented with  $2 \times 10^{-4}$  M ascorbic acid and  $1 \text{ ng.ml}^{-1}$  TGF $\beta$ 1 (Product code T5050, Sigma-Aldrich).

Adipogenic medium consisted of complete medium containing only 1 % (v/v) FCS,  $1 \times 10^{-7}$  M dexamethasone, and  $1 \times 10^{-9}$  M insulin (Product code I5523, Sigma-Aldrich).

Plates (96-well) were obtained from Invitrogen. Multi-spot slides were obtained from C. A. Hendley Ltd. Square petri-dishes were obtained from Appleton and Woods. Methanol and acetone were purchased from VWR International.

Cells from sample number pBM2 were used as they were the first cells successfully isolated that did not succumb to contamination or other problems. Cells were used at passage 3 for the chondrogenic and adipogenic assays, and at passage 5 for the osteogenic assay.

**Method:** At the third passage,  $5 \times 10^5$  cells were transferred to each of four 75 cm<sup>2</sup> flasks containing 10 ml of the three different differentiation media and a complete medium control, as established by Muraglia *et al.* (2000). The medium was changed every three to four days and the cells passaged when confluent. This continued until the cells no longer divided. At each passage the cells were resuspended in a total volume of 3 ml. Aliquots of cells ( $1 \times 10^5$  cells) were removed to 12 wells of a 96-well plate, and  $2.5 \times 10^4$  cells were removed to 24 spots of multi-spot slides. Of the remaining cell suspension  $5 \times 10^5$  cells were then passaged into a fresh 75 cm<sup>2</sup> flask (Figure 3.1). The cells in the 96 well plate, when confluent (usually 1-2 days), were assayed for alkaline phosphatase (AP) to establish the activity of this enzyme which is only thought to be expressed by cells of the osteogenic lineage. Multi-spot slides were cultured for a minimum of three hours to ensure that the cells had attached and were not washed off the slides prior to the slides being flooded with the appropriate culture medium. The cells were then grown until confluent (usually 2 or 3 days) and fixed in a 50:50 methanol/acetone solution for 2 minutes prior to storage at  $-20^\circ\text{C}$  until they could be analysed for phenotypic markers.

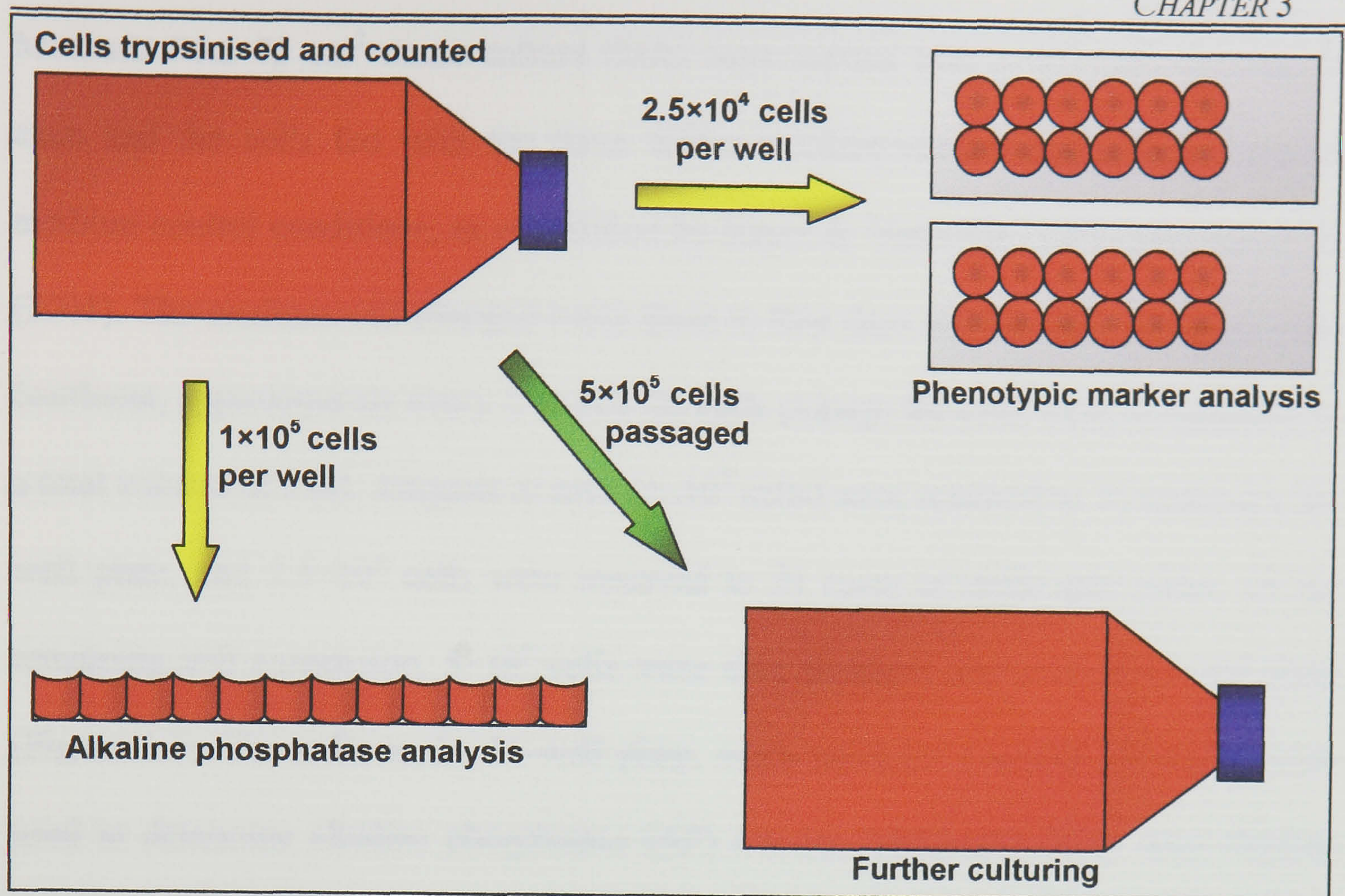


Figure 3.1. Diagram showing how the cells were divided at each passage.

### 3.3.3. Porcine mesenchymal stem cell differentiation assay 2

**Materials:** Osteogenic differentiation medium consisted of complete medium supplemented with  $50 \mu\text{M}$  ascorbate-2-phosphate (Sigma-Aldrich),  $10 \text{ mM}$   $\beta$ -glycerophosphate (Sigma-Aldrich), and  $0.1 \mu\text{M}$  dexamethasone, (Sigma-Aldrich).

Myogenic differentiation medium consisted of complete medium supplemented with 5% (v/v) horse serum (Invitrogen),  $0.1 \mu\text{M}$  dexamethasone and  $50 \mu\text{M}$  hydrocortisone (Sigma-Aldrich).

Smooth muscle cell differentiation medium consisted of serum-free DMEM supplemented with  $100 \text{ ng.ml}^{-1}$  platelet-derived growth factor (PDGF) [P8953, Sigma-Aldrich]. Plates (96-well) were obtained from Invitrogen. Multi-spot slides were obtained from C. A. Hendley Ltd. Square petri dishes were obtained from Appleton and Woods. Methanol and acetone were purchased from VWR International.

Cells from sample number hBM2 were used at passage 5 as they were the first cells successfully isolated that did not succumb to contamination or other problems.

**Method:** Four 75 cm<sup>2</sup> tissue culture flasks were seeded with  $5 \times 10^5$  cells (passage 3) each and the cells fed with the three different differentiation media and complete medium control conditions, as established by Reyes & Verfaillie (1999) and Zuk *et al.* (2001). The medium was changed every three to four days and the cells passaged when confluent, approximately every 2 weeks. At each passage the cells were resuspended in a total volume of 3 ml. Aliquots of cells ( $1 \times 10^5$  cells) were removed to 12 wells of a 96-well plate, and  $2.5 \times 10^4$  cells were removed to 24 spots of multi-spot slides. Of the remaining cell suspension,  $5 \times 10^5$  cells were then passaged into a fresh 75 cm<sup>2</sup> flask (Figure 4.1). The cells in the 96-well plate, when confluent (usually 1-2 days), were used to determine alkaline phosphatase (AP) activity. Multi-spot slides were flooded with the appropriate culture medium a minimum of 3 hours after cells were aliquoted to ensure that the cells had attached and were not washed off the slides. The cells were then grown to confluence (usually 2 or 3 days) and fixed in a 50:50 methanol/acetone solution for 2 minutes prior to storage at  $-20^{\circ}\text{C}$  until they could be analysed for phenotypic markers.

### 3.3.4. Porcine mesenchymal stem cell differentiation assay 3

**Materials:** Adipogenic medium consisted of 50 ml of adipogenic differentiation supplement (Stem Cell Technologies) added to 450 ml of human Mesencult medium (Stem Cell Technologies).

Myogenic medium consisted of complete medium supplemented with 10  $\mu\text{M}$  5-azacytidine (Sigma-Aldrich).

Neurogenic induction medium consisted of complete medium containing 20 % FCS (Invitrogen) supplemented with 1 mM  $\beta$ -mercaptoethanol (Sigma-Aldrich). Neurogenic differentiation medium consisted of serum-free DMEM supplemented with 10 mM  $\beta$ -mercaptoethanol (Sigma-Aldrich). Tyrode's balanced salt solution (TBSS) was

purchased from Sigma-Aldrich. Cells were grown on multi-chamber slides purchased from Bibby Sterilin. Methanol/acetone solution was made from 50:50 methanol:acetone which were both purchased from VWR International. Polypropylene centrifuge tubes (15 ml) were purchased from Fisher Scientific.

Cells isolated from sample number pBM9A were used at passage 3 for this differentiation attempt and were chosen randomly from the available cryopreserved cell stock.

**Method:** For the adipogenic, myogenic and neurogenic differentiation assays,  $5 \times 10^4$  cells (passage 3) were cultured in each well of four multi-chamber slides in complete medium for 24 hr to enable the cells to become fully attached.

For adipogenic differentiation, complete medium was then removed and replaced with adipogenic medium. Cells were then fed adipogenic differentiation medium twice weekly for up to four weeks with two slides fixed at 2 weeks, and two slides fixed at 4 weeks in formalin solution for 30 minutes. Slides were then stored at  $-20^\circ\text{C}$ . Lipid vesicles were visualised by staining the cells with Oil Red O.

The myogenic differentiation assay was derived from Wakitani *et al.* (1995). Briefly, complete medium was replaced with myogenic medium for 24 hr before this was replaced with complete medium again. Cells were then fed complete medium twice weekly for up to four weeks with two slides fixed at 2 weeks, and two slides fixed at 4 weeks in methanol/acetone for 2 minutes. Slides were then stored at  $-20^\circ\text{C}$  prior to phenotypic evaluation using immunofluorescence. Immunofluorescent staining as described in Section 2.6.4 using antibodies to  $\alpha$ -smooth muscle actin, myosin heavy chain-slow and desmin was used to show if differentiation had taken place.

The neurogenic differentiation assay was derived from Woodbury *et al.* (2000). Briefly, complete medium was first replaced with neurogenic induction medium for 24 hr. The cells were then briefly rinsed in TBSS, before this was replaced with neurogenic

differentiation medium. Slides were then fixed for 2 minutes in methanol/acetone at 2, 4, 6 and 8 hours after differentiation medium was introduced before being stored at  $-20^{\circ}\text{C}$  prior to phenotypic evaluation. Immunofluorescent staining using antibodies to tau and NSE was used to show if differentiation had taken place.

### 3.3.5. Alkaline phosphatase assay

**Materials:** Tyrode's balanced salt solution, ready to use alkaline phosphate colour substrate, *p*-nitrophenyl phosphate, and sodium hydroxide were obtained from Sigma-Aldrich.

**Method:** Culture medium was removed from cells grown to confluence in wells of a flat-bottomed 96-well plate and the cells rinsed in Tyrode's balanced salt solution. *p*-nitrophenyl phosphate (200  $\mu\text{l}$ ) was added to each well and the plate incubated at  $37^{\circ}\text{C}$  for 20 minutes. NaOH (50  $\mu\text{l}$ , 3 M) was then added to each well to stop the reaction. The supernatant (200  $\mu\text{l}$ ) was then removed to wells of a second flat-bottomed 96-well plate and the absorbance read in a microtitre plate reader at 405 nm.

### 3.3.6. Oil red O staining

**Materials:** Isopropanol was obtained from VWR International. Oil Red O Stock solution consisted of 300 mg Oil Red O (Sigma-Aldrich) added to 99 % (v/v) isopropanol. This was stable for up to 6 months. Working solution was made by mixing 3 parts oil red O stock solution with 2 parts distilled water and filtered using a number 5 filter purchased from Whatman International. This was stable for 1 hour.

**Methods:** Cells cultured for Oil Red O staining were fixed in formalin solution for 30min. Formalin solution was then removed and the slides rinsed with distilled water. Sufficient isopropanol (60 % v/v) was then added to cover each slide and let stand for 3 minutes. This was then replaced with oil red O working solution for 10 minutes. This

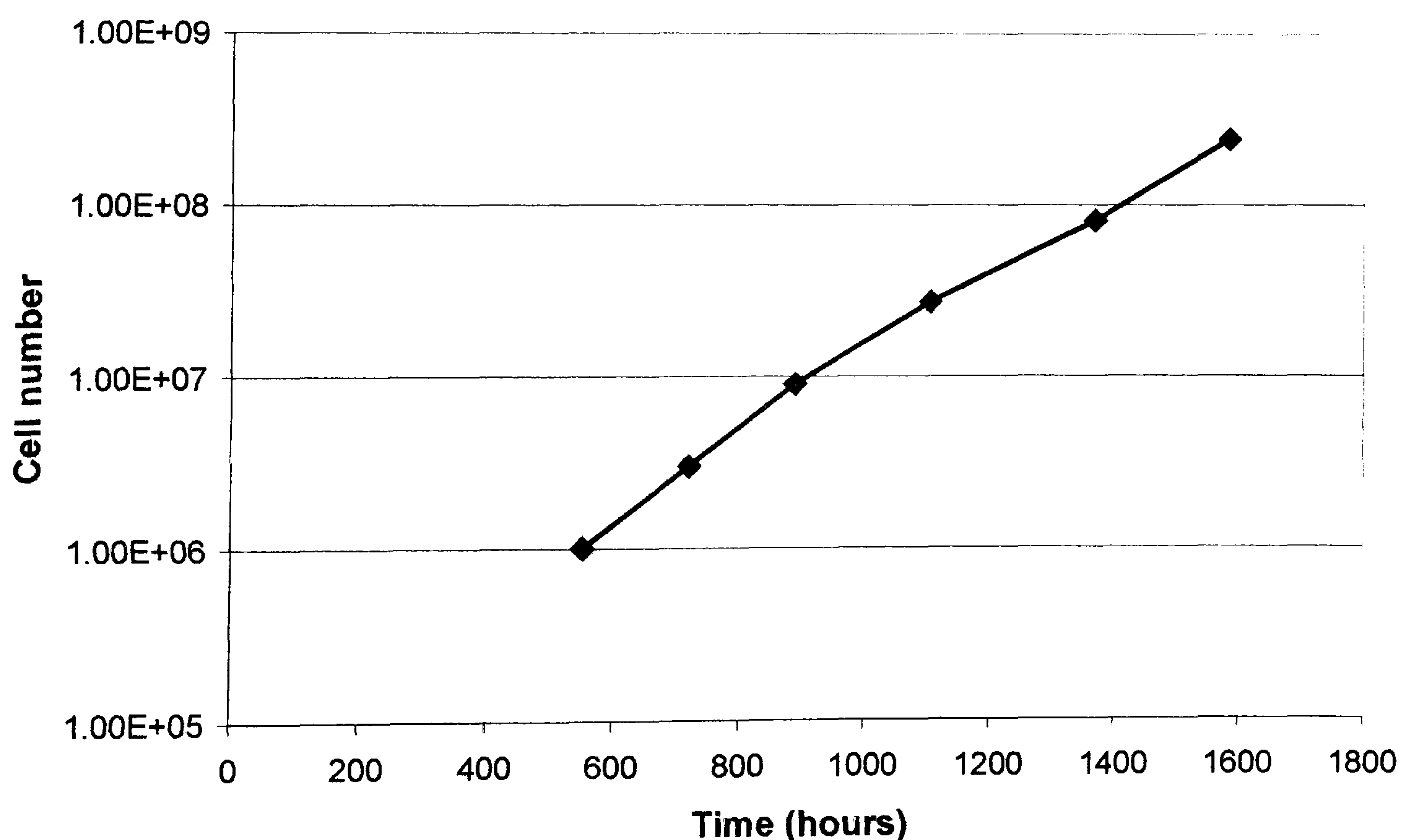


was then carefully removed and the slides rinsed again with distilled water. Sufficient haematoxylin was then added to cover each slide and let stand for 1 minute. This was then poured off and the slides carefully rinsed 5 times using distilled water. Cells were then viewed wet under phase contrast microscopy.

### 3.4. Results

#### 3.4.1. Cultured porcine mesenchymal stem cells

A growth curve was plotted using the first successfully isolated putative pMSC (pBM2, Figure 3.2) and the cell doubling time (CDT) was calculated as described in Section 2.4.3. Typically,  $1 \times 10^6$  porcine mesenchymal stem cells formed a confluent monolayer in a  $25 \text{ cm}^2$  tissue culture flask, and  $3 \times 10^6$  cells in a  $75 \text{ cm}^2$  tissue culture flask, which was when the cells were passaged. The CDT was calculated to be approximately 106 hours initially, although by the sixth passage, this had risen to approximately 136 hours.

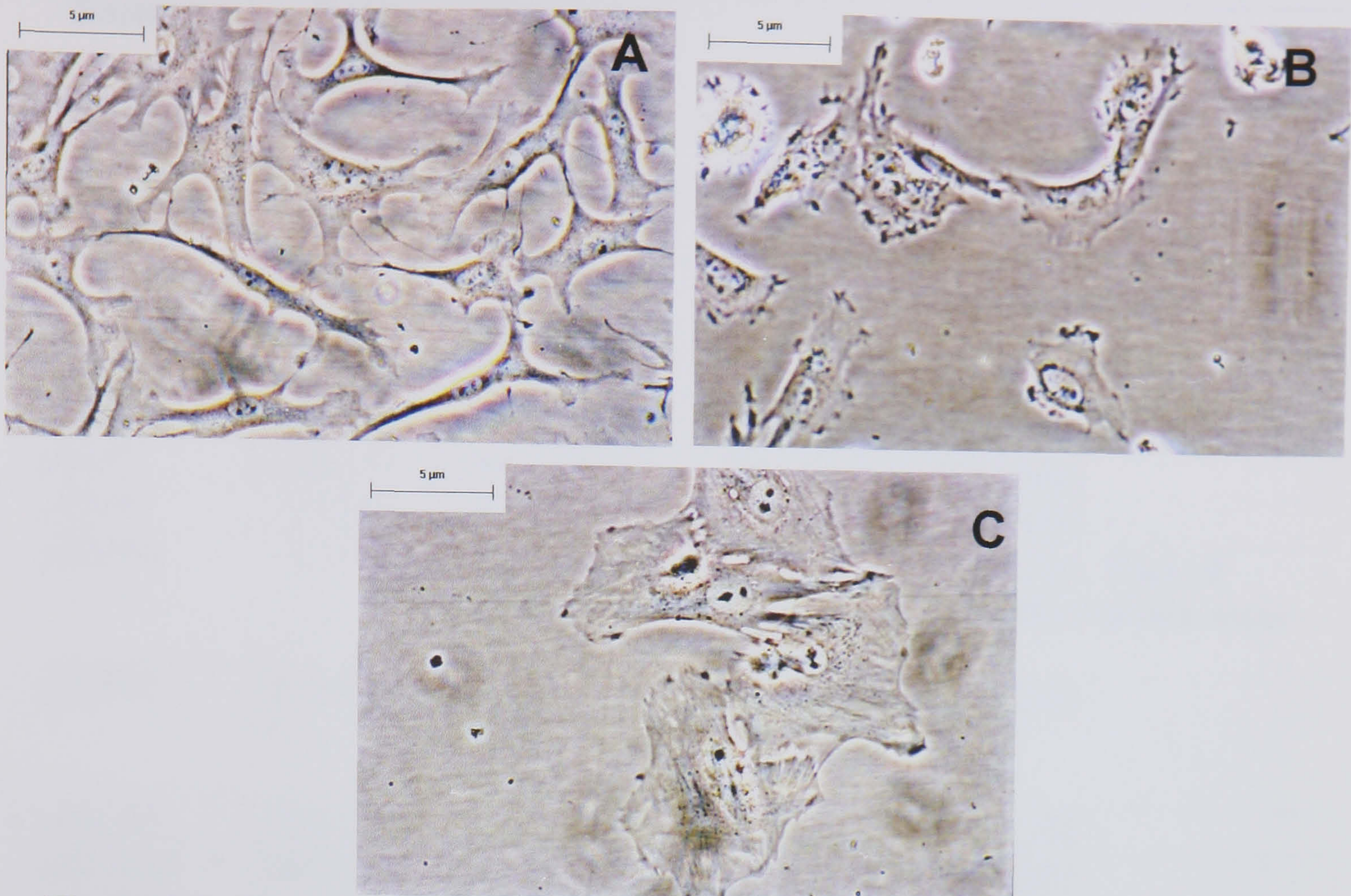


**Figure 3.2. Growth curve of porcine mesenchymal stem cells.** Each point on the line represents a cell passage. The original cell number could not be calculated due to difficulties in seeing the cells through other bone marrow debris.

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The apparent reduction in proliferation rate was matched by a change in cell morphology. Images of cells freshly isolated from the porcine bone marrow of a recently slaughtered pig are shown in Figure 3.3. The cells were seen to be fibroblastic in appearance. Observation of cell morphology at different passages showed that the cells could generally be categorised into three age-related morphologies based on their appearance:

1. Cells one week after isolation (passage 0) are shown in Figure 3.3a. Cells were relatively small, becoming confluent in approximately 2 weeks.
2. Cells at an intermediate stage (passage 4) after which their rate of proliferation had decreased slightly and the cells were beginning to take on a more flattened and spread morphology are shown in Figure 3.3b.
3. Quiescent cells (passage 6) are shown in Figure 3.3c. At this stage, cells were largely quiescent and divided very slowly, if at all. Cells became highly flattened and spread with prominent filaments visible in the cell cytoplasm, and typically took over 4 weeks to become confluent.



**Figure 3.3. Porcine mesenchymal stem cells at different ages.** A: Proliferating primary morphology (P0); B: Secondary intermediate morphology (P4); and C: Quiescent tertiary morphology (P6).

During the course of the investigation, a number of cell isolations were made and are listed in Table 3.1. There was a long period between February and November 2001 when cells could not be isolated due to a national outbreak of Foot & Mouth disease restricting access to the abattoir. The yield of cells was observed to vary from bone to bone, however it was impossible to accurately count the number of cells initially isolated because of the large amount of debris still present after the isolation procedure. Although seemingly healthy cells were isolated in the first few attempts, during February and November 2001, later attempts between January and December 2002 resulted in cells that rapidly ceased proliferation and broke away from the culture flask. Cryopreserved samples were retained as a cell bank for future studies and analysis.

SAMPLE	DATE	PASSAGES	NOTES
pBM2	12/02/01	P1 07/03/01 P2 14/03/01 P3 21/03/01 P4 30/03/01 P5 10/04/01 P6 19/04/01	Used in differentiation assay 1, chondrogenic and adipogenic at P3, osteogenic and control at P5. Used in differentiation assay 2 at P5. Samples cryopreserved at each passage.
pBM5	21/02/01	P1 12/03/01	Cryopreserved 12/03/01
pBM6	21/02/01	P1 12/03/01	Cryopreserved 12/03/01
pBM7	21/02/01	P1 14/03/01	Cryopreserved 14/03/01
pBM8	21/02/01	P1 19/03/01	Cryopreserved 19/03/01
pBM9A	15/11/01	P1 04/12/01 P2 10/12/01	Cryopreserved 10/12/01. Thawed 08/01/03, used in differentiation assay 3 at P3.
pBM9C	15/11/01	P1 26/11/01 P2 04/12/01 P3 20/12/01	Cryopreserved 20/12/01
pBM9D	15/11/01	P1 10/12/01	Cryopreserved 01/02/02
pBM10A	06/12/01	P1 20/12/01	Cryopreserved 20/12/01
pBM10B			
pBM11	11/01/02	-	Discarded 21/01/02, too few cells
pBM12	22/01/02	-	Discarded 01/03/02, cells died
pBM13	27/02/02	-	Discarded 18/03/02, too few cells
pBM14	04/03/02	-	Discarded 12/04/02, cells died
pBM15	04/03/02	-	Discarded 03/04/02, no cells
pBM16	11/03/02	-	Discarded 09/04/02, cells died
pBM17	20/03/02	-	Discarded 12/04/02, cells died
pBM18	09/04/02	-	Discarded 26/04/02, cells died
pBM19	17/04/02	-	Discarded 29/04/02, cells died
pBM20	23/04/02	-	Discarded 13/05/02, cells died
pBM21	02/05/02	-	Discarded 13/05/02, no cells
pBM22	21/05/02	-	Discarded 10/06/02, cells died
pBM23	28/05/02	-	Discarded 01/07/02, too few cells
pBM24A	24/06/02	-	Discarded 24/07/02, cells died
pBM24B			
pBM24C			
pBM24D	24/06/02	P1 12/07/02	Discarded, 12/08/02, cells died
pBM25	04/07/02	-	Discarded 24/07/02, cells died
pBM26	09/07/02	-	Discarded 26/07/02, cells died
pBM27	16/07/02	-	Discarded 09/09/02, cells died
pBM28	01/08/02	-	Discarded 09/09/02, cells died
pBM29	08/08/02	-	Discarded 09/09/02, cells died
pBM32	03/10/02	-	Discarded 10/12/02, cells died
pBM33A	18/10/02	-	Discarded 06/01/03, cells died
pBM33C			
pBM33B	18/10/02	P1 08/11/02	Discarded 06/01/03, cells died
pBM33D	18/10/02	P1 01/11/02	Discarded 06/01/03, cells died
pBM34	22/10/02	-	Discarded 01/11/02, no cells
pBM35	29/10/02	-	Discarded 06/01/03, cells died
pBM36A	04/11/02	P1 19/11/02	Discarded 06/01/03, cells died

pBM36B	04/11/02	P1 13/11/02 P2 22/11/02	Discarded 06/01/03, cells died
pBM36C pBM36D pBM36E	04/11/02	-	Discarded 06/01/03, cells died
pBM36F	04/11/02	P1 13/11/02 P2 25/11/02	Discarded 06/01/03, cells died
pBM37A	03/12/02	-	Discarded 06/01/03, cells died
pBM37B	03/12/02	P1 13/12/02	Discarded 06/01/03, cells died
pBM37C pBM37F	03/12/02	P1 13/12/02	Cryopreserved 13/12/02
pBM37D pBM37E	03/12/02	-	Discarded 20/12/02, too few cells
pBM38A	24/02/03	P1 10/03/03 P2 18/03/03	Cryopreserved 18/03/03
pBM38B	24/02/03	P1 14/03/03	Cryopreserved 14/03/03

**Table 3.1. Putative porcine mesenchymal stem cell isolations.** Each isolation attempt tried to isolate putative pMSC from four porcine shoulder bones. Unless otherwise stated, one sample number corresponds to all four bones. A number followed by a letter indicates individual bones.

When the success in isolating pMSC began to deteriorate, a number of factors in the procedure were investigated to try to establish why this was happening. This involved systematically checking individual components of both the solutions used to isolate the cells and also those used in the complete medium used to culture the cells. This was carried out by sequentially replacing each reagent with freshly bought reagents and attempting to isolate cells to assess whether there was an improvement. Despite this process revealing potential sources of problems, such as ageing reagents, no changes were observed in the success of isolating the cells. Consequently, the source of the reagents used was investigated. This revealed the problem to be a change of supplier of DMEM. Samples from pBM1 to pBM12 and pBM38 were isolated and cultured using DMEM supplied by Invitrogen, whereas samples from pBM13 to pBM36 were isolated and cultured in DMEM supplied by Sigma-Aldrich. Sample pBM37 was isolated and cultured in Mesencult, supplied by Stem Cell Technologies, with supplements designed specifically for hMSC. Invitrogen and Sigma-Aldrich DMEM were both reported to be

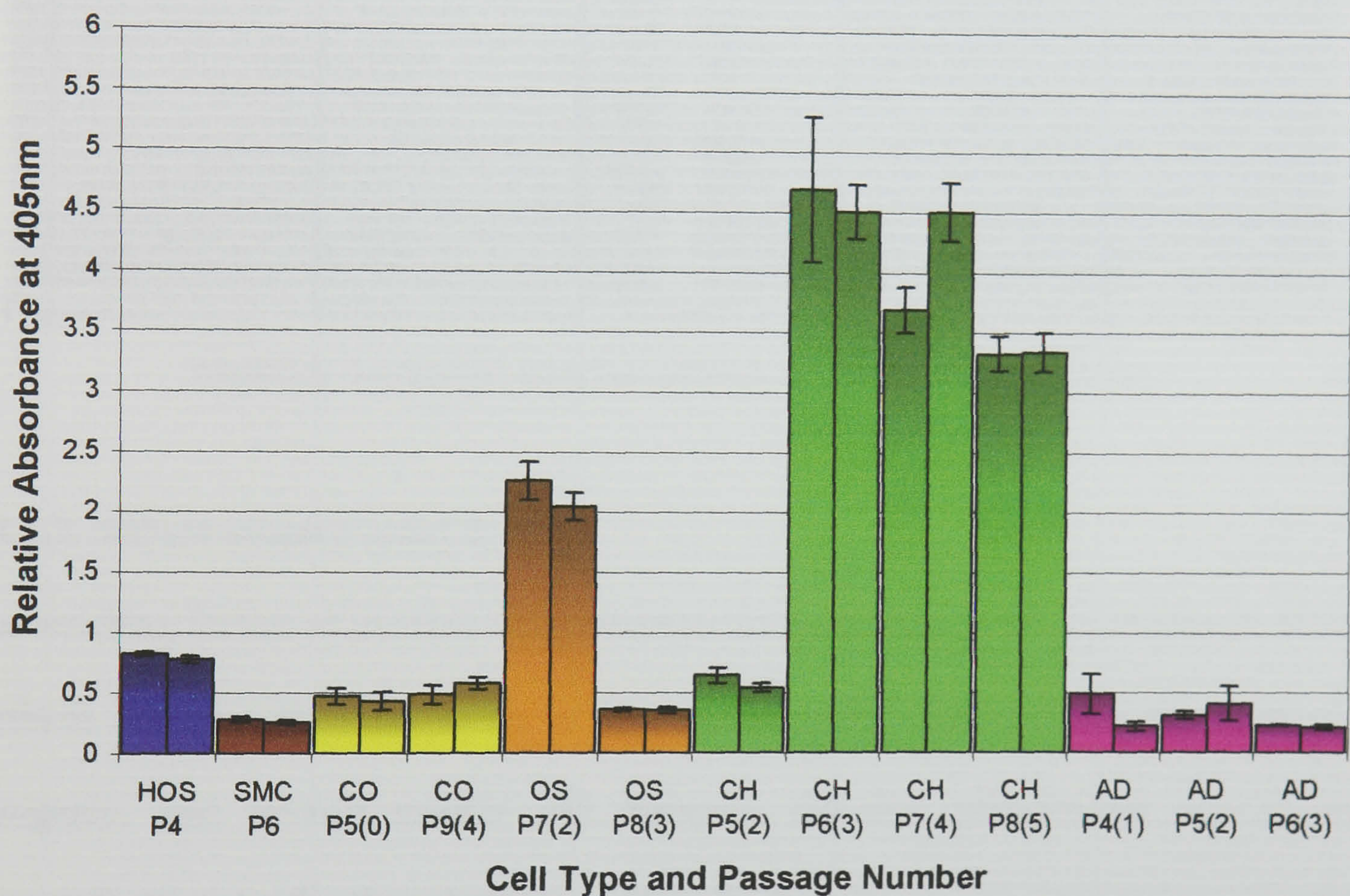
identical, however, a comparison of their specific ingredients revealed numerous small differences in components and concentrations which ultimately seemed to affect the isolation and growth of the cells. Human Mesencult medium was used to grow the cells to see what happened before it was realised that the DMEM itself was the problem. This also seemed to support the cells adequately, despite being aimed specifically at human MSC. However, this was a very expensive medium, which was why attempts at using Invitrogen DMEM were resumed once the source of DMEM was suspected as being the source of the problem.

### **3.4.2. Porcine mesenchymal stem cell differentiation**

#### **3.4.2.1. Differentiation assay 1**

Once putative MSC had been successfully isolated, it was decided to try to differentiate the cells into multiple phenotypes in order to show their multipotentiality. A review of the literature provided the conditions thought necessary to try to differentiate the pMSC initially into cells of the osteogenic, chondrogenic and adipogenic lineages. The differentiated cells were then identified by measuring alkaline phosphatase activity for the osteogenic lineage, immunohistochemical staining for collagen II for the chondrogenic lineage, and oil red O staining for the adipogenic lineage. The results of the alkaline phosphatase (AP) of the cells grown in each of the differentiation media are shown in Figure 3.4. A human osteosarcoma cell line was used as a positive control for AP activity and human smooth muscle cells as a negative control. The AP activity of cells grown in the osteogenic medium increased initially, and then decreased again at each subsequent passage. The cells grew very slowly at this point, and it was not possible to transfer further aliquots from the flask to the multi-spot slides as the cells became senescent and failed to attach to the slide surface after trypsinisation from the tissue culture flask. More intriguingly was the huge amount of AP activity demonstrated

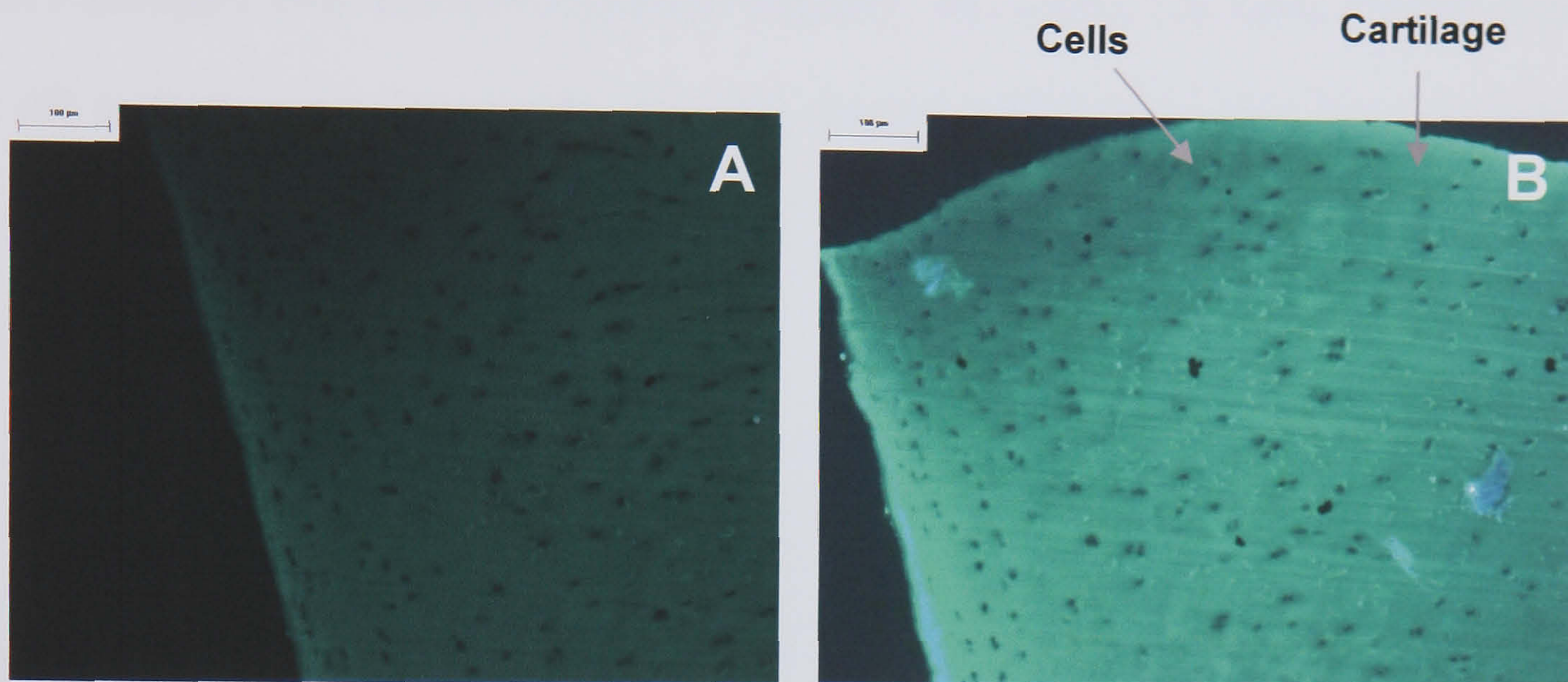
by the cells grown in the chondrogenic medium. These cells had a low activity initially, but cells from the third passage had an extremely high activity, producing an absorbance over four times greater than the osteosarcoma cells used as a positive control for the assay. This had dropped by the fifth passage in the differentiation medium, but was still significantly higher than the positive control. Meanwhile, the cells grown in adipogenic medium had a level of AP activity comparable to the negative control smooth muscle cells.



**Figure 3.4. Alkaline phosphatase supernatant absorbance of pMSC from differentiation assay 1.** HOS= human osteosarcoma cell line; SMC= human smooth muscle cell; CO= control undifferentiated pMSC; OS= pMSC grown in osteogenic differentiation medium; CH= pMSC grown in chondrogenic differentiation medium; AD= pMSC grown in adipogenic differentiation medium. Two bars for each sample represent repeat samples. P number refers to the passage number of the cells. Number in brackets represents the number of passages since differentiation medium was introduced. Results are expressed as mean ( $n=6$ )  $\pm$ 95 % confidence limits.

Attempts to differentiate the putative MSC into cells of the adipogenic and chondrogenic lineage both failed. The cells in adipogenic medium failed to produce

fatty vesicles that could be stained and visualised with Oil Red O. The cells in chondrogenic medium failed to produce collagen II, which is the principle marker for chondrogenic differentiation. Bovine cartilage was used as a positive control for the collagen II antibody (Figure 3.5).



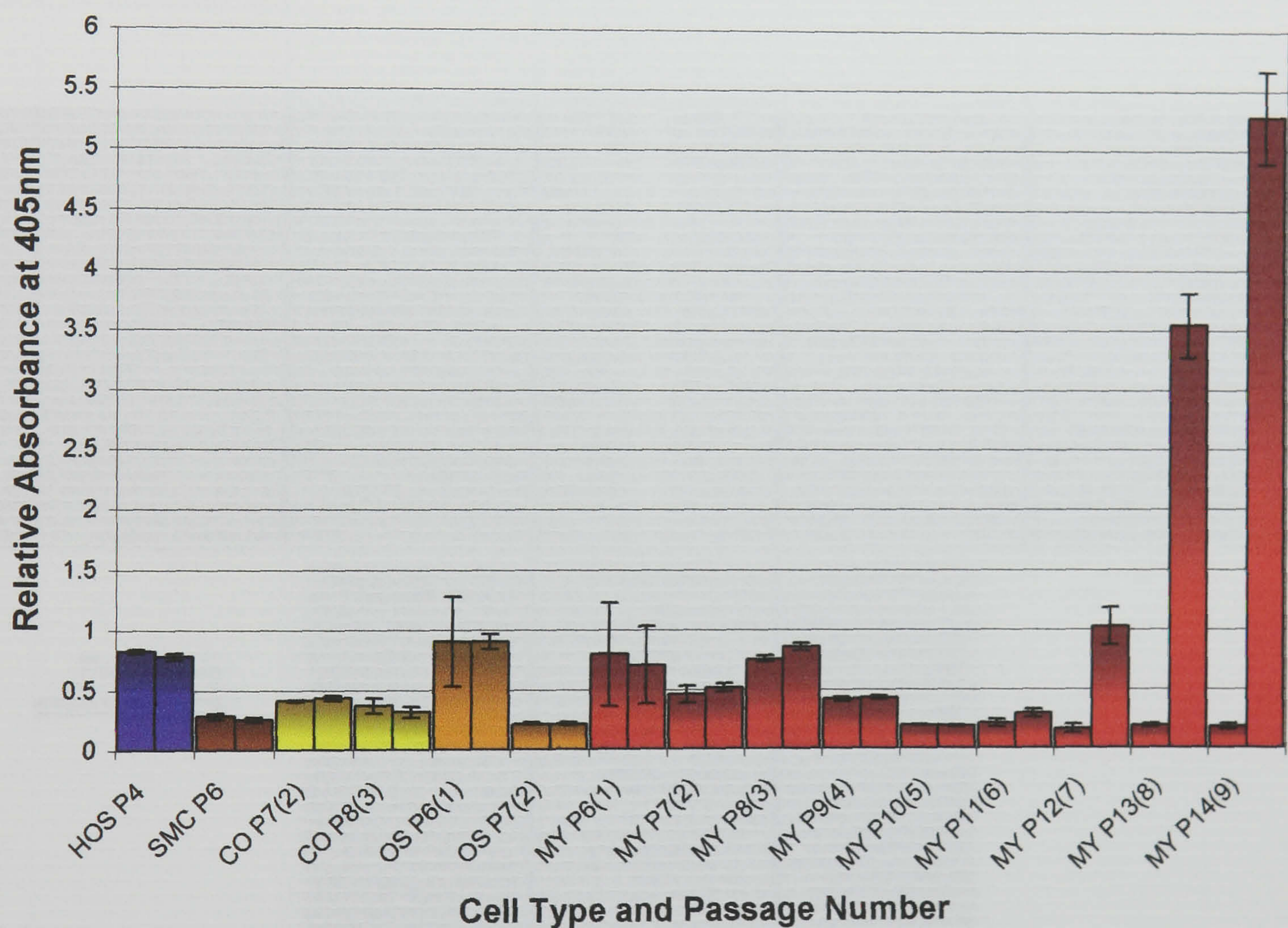
**Figure 3.5.** Anti-collagen II immunofluorescent staining of bovine cartilage. A: negative control; B: positive control. Bar represents 100 µm.

#### 3.4.2.2. Differentiation assay 2

The resulting failure of the first differentiation assay resulted in the development of new methods from the literature to try to differentiate pMSC into cells of the osteogenic, myogenic, and smooth muscle cell lineages. Alkaline phosphatase activity was measured as a mark of osteogenic differentiation, decreased immunohistochemical staining for  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), increased heavy chain myosin slow (HCMs) staining and increased desmin staining was measured as a marker for myogenic differentiation and  $\alpha$ SMA, HCMs, desmin and vimentin was measured as a marker for the smooth muscle cell lineage. The results of the alkaline phosphatase (AP) activity of the cells grown in each of the differentiation media is shown in Figure 3.6. A human osteosarcoma cell line was used as a positive control for AP activity and human smooth muscle cells as a negative control. The AP activity of cells grown in the osteogenic medium increased initially, and then decreased again at subsequent passage. The cells grew very slowly at this point, and it was not possible to transfer further aliquots from

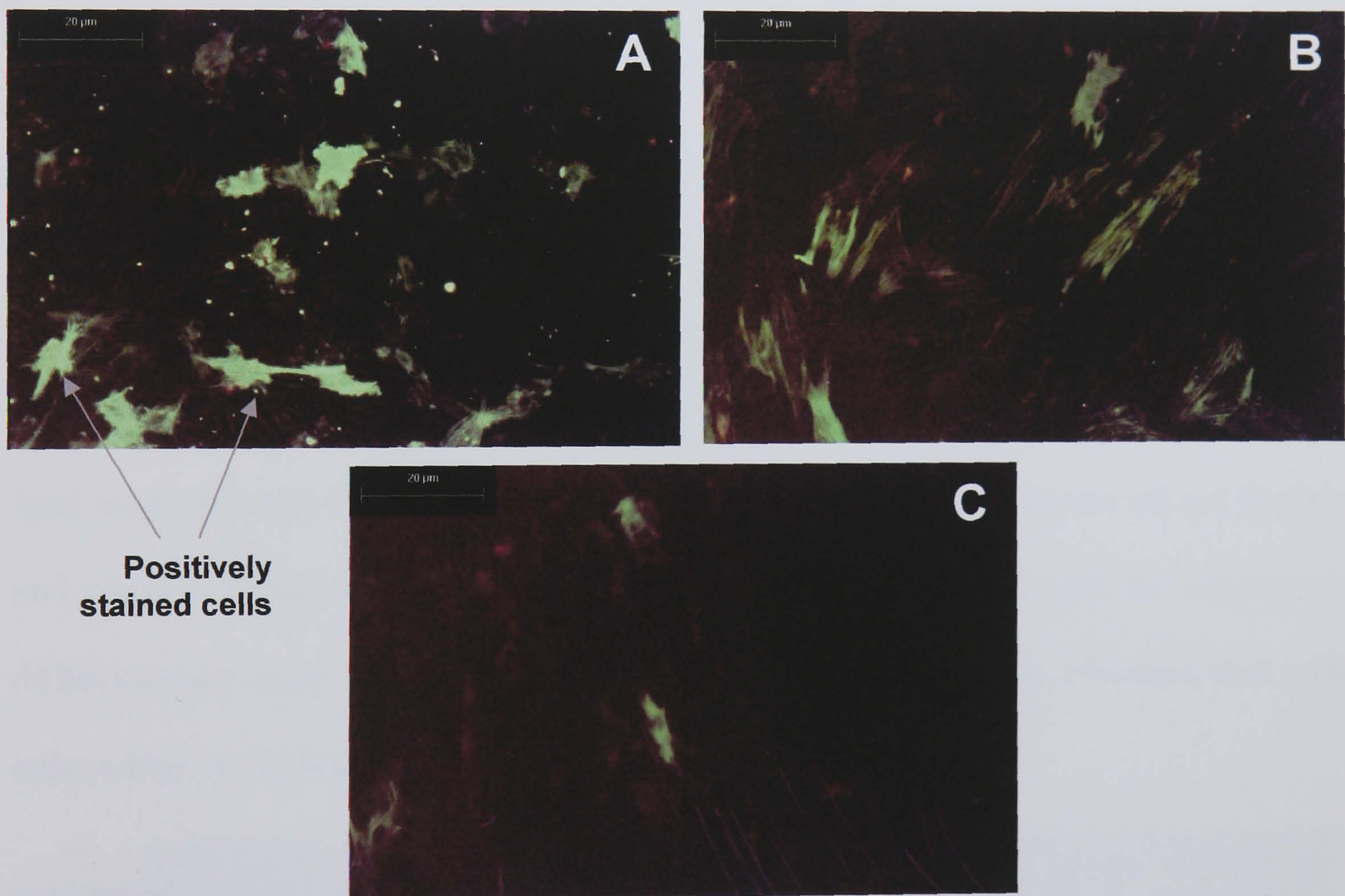


the flask to the multi-spot slides as the cells became senescent and failed to attach to the slide surface after trypsinisation from the tissue culture flask. More intriguingly was the huge amount of AP activity demonstrated by the cells grown in the myogenic medium. The activity of the AP seemed to fluctuate between the samples at each passage, and then 7 passages after the differentiation medium was added, one flask of cells showed an AP activity equivalent to the negative control, whilst the other flask showed an increasing activity up to 5 times greater to that of the cells grown in osteogenic differentiation media.



**Figure 3.6. Alkaline phosphatase supernatant absorbance of pMSC from differentiation assay 1.** HOS= human osteosarcoma cell line; SMC= human smooth muscle cell; CO= control undifferentiated pMSC; OS= pMSC grown in osteogenic differentiation medium; MY= pMSC grown in myogenic differentiation medium. Two bars for each sample represent repeat samples. The P number refers to the passage number of the cells. The number in brackets represents the number of passages since differentiation medium was introduced. Results are expressed as mean ( $n=6$ )  $\pm$  95% confidence limits.

Attempts to differentiate pMSC into other lineages were unsuccessful. Cells grown in myogenic medium failed to label with antibodies to  $\alpha$ -smooth muscle actin, slow heavy-chain myosin and desmin. However, they did show one change from freshly isolated cells, which were  $\alpha$ -smooth muscle actin positive (Figure 3.7). Porcine heart muscle was used as a positive control for the myogenic antibodies. Cells grown in smooth muscle cell medium changed morphology within a few days of the change in differentiation medium, however as there was no serum or equivalent substitute included in the growth medium, the cells became quiescent and died before any further phenotyping or analysis could be carried out.

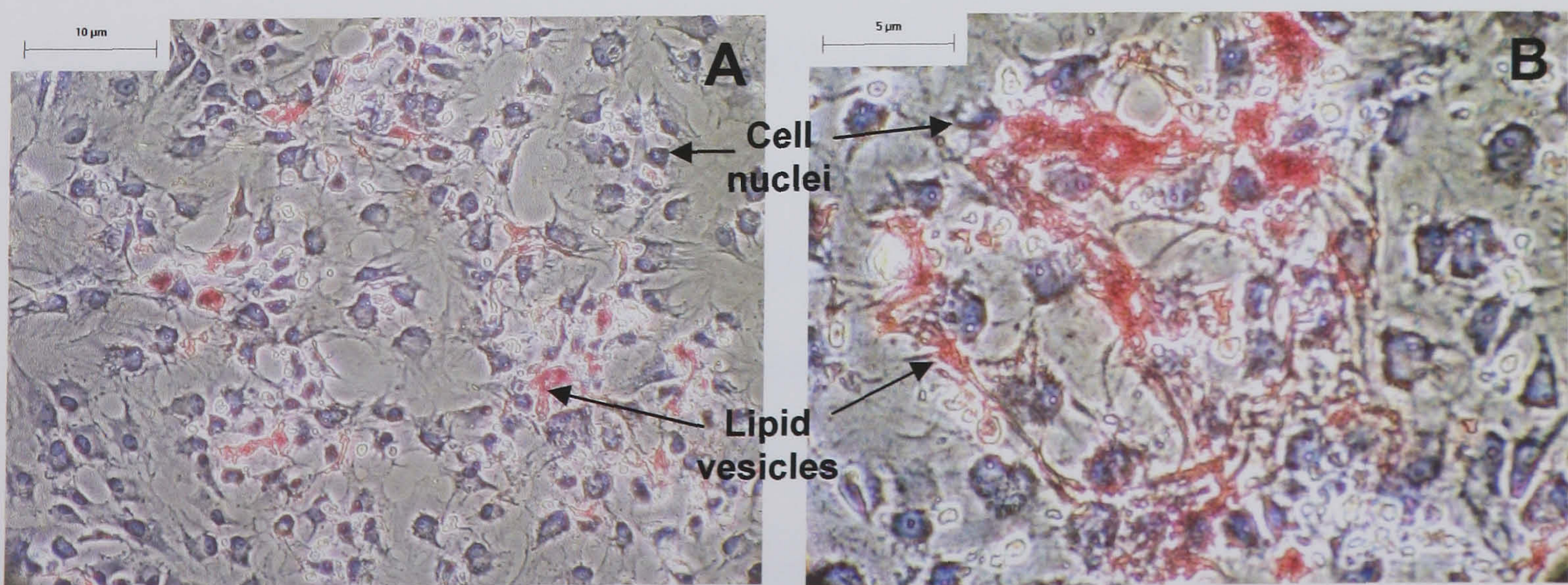


**Figure 3.7.**  $\alpha$ SMA immunofluorescent staining of pMSC grown in myogenic differentiation medium. A: control human smooth muscle cells (hSMC); B: control pMSC; C: pMSC grown in myogenic differentiation medium

### 3.4.2.3. Differentiation assay 3

The lack of success of the first two differentiation assays lead to the development of further methods to try to differentiate the putative pMSC into cells of the adipogenic,

myogenic and neurogenic lineages. Oil Red O stain was used to identify cells of the adipogenic lineage, decreased immunohistochemical staining for  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), increased heavy chain myosin slow (HCMs) and increased desmin indicated myogenic differentiation and immunohistochemical staining for tau and neurone specific enolase (NSE) was used to identify cells of the neurogenic lineage. Porcine heart muscle and porcine optic nerve were used as positive controls for myogenic and neurogenic differentiation attempts respectively. Attempts to differentiate pMSC into cells of the myogenic lineage were unsuccessful, as the cells were negative when stained with antibodies to  $\alpha$ -smooth muscle actin, slow heavy-chain myosin, and desmin although again this did represent one change in that freshly isolated pMSC stained positive for  $\alpha$ -smooth muscle actin. Attempts to differentiate pMSC into cells of the neurogenic lineage were also unsuccessful as the cells were negative when stained with fluorescent antibodies to tau and neurone-specific enolase. However, the results of the experiment to differentiate the cells into cells of the adipogenic lineage can be seen in Figure 4.8 below. Oil Red O is a lipid soluble dye; therefore the areas stained pink mark lipid deposits made by the cells. The lipid could only be seen using the oil red O stain and seemed diffuse across the cells, especially when compared with the adipogenic differentiation assay carried out on hMSC (see Figure 3.2). It was observed that only approximately 20 % of cells differentiated successfully.



**Figure 3.8. Differentiation of porcine mesenchymal stem cells into adipogenic lineage.** Oil red O staining of lipid vesicles. A:  $\times 10$  magnification; B:  $\times 40$  magnification.

### 3.5. Discussion

Since there was no literature available on pMSC at the start of this study, methods used for human cells were adopted. The isolation method developed for isolating pMSC yielded cells with a fibroblastic appearance. Some isolation methods examined had passed the bone marrow material through sequentially narrower hypodermic needles to break apart cell clumps prior to centrifugation [Friedenstein *et al.*, 1987]. However, this was not included in the final developed method as it proved difficult to pass porcine bone marrow through even the highest calibre needles. This was probably due to the fact that porcine bone marrow was observed to contain a lot of fat, which was very solid in texture.

Attempts at isolating putative pMSC met with mixed success over the course of the investigation. Although initially successful, cell yields became reduced and cells frequently died during the initial incubation period before a confluent monolayer could be obtained. After much thought, discussion and investigation, it was deduced that the unexpected change was due to a change in supplier of DMEM. The supplier had been changed from Invitrogen to Sigma-Aldrich during the course of isolating the cells during February 2002, essentially because of a change in the prices from the supplier. Although the suppliers each sold the DMEM as the same product, analysis of the exact contents of the media revealed numerous differences in concentrations of constituent components. A return to Invitrogen DMEM during December 2002 resulted in improved cell yields that survived to the point of cryopreservation.

The cells isolated appeared to become senescent with prolonged culturing, in a manner similar to terminally differentiated cells. The cell doubling time also slowed from 106

hours initially to 136 hours with older cells. This was in contrast to the notion that stem cells are immortal and compared well with reports by others which have shown that human MSC do not possess telomerase activity [Banfi *et al.*, 2002], which is thought to be an important enzyme involved in maintaining telomere length and consequently cell immortality. However, it may well be that the *in vitro* conditions used to grow the cells could not sustain the immortal capacity of the cells and instead exerted a selective environment that preferentially encouraged the cells to become terminally differentiated.

A number of attempts to differentiate the cells were made using biochemical supplements to complete medium and serum-free medium. The methods for the first differentiation assay were derived from Muraglia *et al.* (2000). Reyes & Verfaillie (1999) and Zuk *et al.* (2001) provided the conditions for the second differentiation assay and Stem Cell Technologies, Wakitani *et al.* (1995), Woodbury *et al.* (2000), and Jones *et al.* (2002) provided the conditions for the third differentiation assay. In differentiation assay 1, osteogenic differentiation medium consisted of complete medium supplemented with 200  $\mu$ M ascorbic acid, 7 mM  $\beta$ -glycerophosphate and 10 nM dexamethasone. Ascorbic acid is a free radical scavenger and can also act as a reducing agent. Furthermore, it functions as a co-factor in the hydroxylation of lysine and proline residues in collagen and is essential for its normal synthesis and secretion [Beresford & Owen, 1998]. Therefore its role in matrix production and consequently cell differentiation is clear.  $\beta$ -glycerophosphate is an organic phosphate donor which, in the presence of ascorbate, can enhance the formation of mineralised matrix *in vitro*, consequently its role in osteogenic differentiation is clear [Beresford & Owen, 1998]. Dexamethasone is a glucocorticoid steroid with numerous effects, including its role as a broad-spectrum transcription inducer. So here its role could be to switch on genes that

were previously inactive in order to induce differentiation of the cells. Although the effects on specific osteogenic-related genes are unknown, used in combination with a phosphate donor such as  $\beta$ -glycerophosphate, it is possible that osteogenic differentiation could be induced. The osteogenic differentiation medium in the second differentiation assay contained essentially the same components, but at different concentrations. Here, ascorbic acid was replaced by 50  $\mu$ M ascorbate-2-phosphate, which is a more stable form of ascorbic acid,  $\beta$ -glycerophosphate was increased in concentration from 7 to 10 mM, and dexamethasone was increased in concentration from 10 to 100 nM. This variation in concentrations producing apparently the same effects clearly demonstrates the unknown nature of what is really required to biochemically induce cell differentiation.

In differentiation assay 1, chondrogenic differentiation medium consisted of complete medium supplemented with 200  $\mu$ M ascorbic acid, along with 10 ng.ml<sup>-1</sup> transforming growth factor beta-1 (TGF $\beta$ 1). There are three forms of mammalian TGF $\beta$  and they can be found in a variety of developing and regenerating tissues and in pathological responses. They have a variety of functions including attracting inflammatory cells to a wound site, stimulating the release of cytokines and also regulating the extracellular matrix by stimulating fibroplasia and collagen deposition whilst inhibiting proteases [O’Kane & Ferguson, 1997]. It is likely that its function in promoting collagen production is its role in inducing chondrogenic differentiation here. The main problem here is that it is widely known that if chondrocytes are cultured in the 2-dimensional environment of a tissue culture flask, they rapidly de-differentiate and stop producing a collagen II extracellular matrix. Therefore, trying to induce the differentiation of MSC into chondrocytes in a tissue culture flask has this problem to overcome as well.

The adipogenic differentiation medium used in the first differentiation assay consisted of complete medium with the serum content reduced to 1 % (v/v) FCS and then supplemented with 100  $\mu$ M dexamethasone and 1 nM insulin. As previously discussed, dexamethasone is a glucocorticoid steroid which can act as a wide-ranging transcription inducer. Insulin plays a major part in the uptake of glucose by cells and stimulates glycogen synthesis. This in turn increases the synthesis of fatty acids and therefore lipids. Consequently, the combination of a transcription inducer with a molecule which controls lipid synthesis is likely to promote adipogenic differentiation. Also, the reduction in serum content from the standard 10 % to 1 % (v/v) in this differentiation medium could have mimicked starvation conditions for the cells, thereby encouraging them to store fat. The Stem Cell Technologies adipogenic differentiation supplement to Mesencult used in differentiation assay 3 did not state its constituent components, however, it is likely to contain similar biochemicals to those already described, although perhaps at different concentrations since the second adipogenic differentiation attempt was successful.

The myogenic differentiation medium used in differentiation assay 2 consisted of complete medium supplemented with 5 % (v/v) horse serum, 100 nM dexamethasone and 50  $\mu$ M hydrocortisone. The horse serum was likely to be expected to contain unknown factors thought to have an effect on myogenic differentiation, much the same as FCS contains unknown factors that readily support cell growth *in vitro*. As previously discussed, dexamethasone is a glucocorticoid steroid, and hydrocortisone is another member of the same family. Consequently, their role would be to induce gene transcription with each steroid presumably affecting different genes. However why they would induce differentiation into the myogenic lineage over any other lineage remains unclear, perhaps it is factors in the horse serum that are expected to drive the

differentiation in this direction. The myogenic differentiation medium used in the third differentiation assay took a different approach and consisted of complete medium supplemented with 10  $\mu\text{M}$  5-azacytidine. The cells were exposed to this differentiation medium for 24 hours before it was replaced with complete medium and the cells cultured under standard conditions for up to 4 weeks. 5-azacytidine is an analogue of cytosine and is consequently incorporated into DNA during the cell division cycle. However, unlike cytosine, it cannot be methylated. Methylation of cytosine is a common occurrence that changes how the DNA is read by protein synthesising ribosomes. Therefore, when 5-azacytidine is incorporated into new DNA, its transcription is altered, leading to changes in gene activation and therefore differentiation of the cells, although why this would induce differentiation into the myogenic lineage over any other lineage remains unclear.

The smooth muscle cell differentiation medium consisted of serum-free medium supplemented with 100  $\text{ng}\cdot\text{ml}^{-1}$  platelet-derived growth factor (PDGF). PDGF is a factor released by platelets upon clotting. It is a ubiquitous mitogen which has the function of stimulating the proliferation, differentiation and migration of predominantly connective tissue cells [Qiu & Ferguson, 1995]. Therefore its role in differentiating MSC into smooth muscle cells is clear. However, without FCS in the culture medium, the medium was not capable of fully supporting cell growth and the cells quickly became senescent and died before any analysis could be carried out.

The neurogenic differentiation medium used in differentiation assay three consisted of complete medium containing 20 % (v/v) FCS supplemented with 1 mM  $\beta$ -mercaptoethanol in the induction medium and 10 mM  $\beta$ -mercaptoethanol in serum free medium for the differentiation medium.  $\beta$ -mercaptoethanol is a reducing agent and free



radical scavenger that is used in the culture of some cell types to protect the cells. It is possible that its function here was to change the properties of cell surface proteins, thereby changing receptor sites and changing the binding of proteins, which could ultimately affect gene activation and therefore induce the cells' differentiation. Although why this would result in differentiation into the neurogenic lineage over any other lineage remains unclear.

Despite the method derived for isolating porcine MSC yielding fibroblastic cells morphologically similar to human MSC, attempts to differentiate them only produced adipogenic cells intentionally, with mixed results for the osteogenic assay and negative results for all the other assays. Since the methods for differentiating the cells were derived from papers in which the work was carried out on human MSC, the combination of the lack of success of the different differentiation assays, combined with the aberrant results of the osteogenic assays, suggested that porcine MSC did not respond in the same manner as human MSC when exposed to the same biochemical stimuli, or perhaps were not MSC to begin with. Thus it may be the case that pMSC require different combinations or concentrations of biochemical supplements in order to differentiate the cells into different lineages. However, the success of differentiating pMSC into adipogenic cells using a supplement designed for use on hMSC suggests that there are some similarities in cell signalling susceptibility.

Ringe *et al.* (2002) have since shown that it is possible to differentiate pMSC into cells of the osteogenic, adipogenic and chondrogenic lineages using methods commonly available for differentiating hMSC. It is difficult to say why they succeeded when this work failed. It is possible that the reagents they used to differentiate their cells were slightly different to the ones used here, being from different sources. However, it is

difficult to compare the work since the paper in which the work was published [Ringe *et al.*, 2002] did not give clear methods for differentiating the cells, but merely referenced to other papers for each differentiation assay attempted.

Mechanical stimulation has been shown to be capable of differentiating hMSC into a ligament cell lineage [Altman *et al.*, 2002], and is also known to affect other cell lineages such as fibroblasts and aortic endothelial cells [Streppeti *et al.*, 1993; Banes *et al.*, 1995; Sato *et al.*, 1996; Butt & Bishop (1997); Eastwood *et al.*, 1998; Hishikawa & Lüscher, 1997; Sirois *et al.*, 1998; Breen, 2000; Grinell, 2000]. Consequently, future experiments could try to differentiate pMSC into aortic valve interstitial cells by seeding undifferentiated cells onto a matrix and then exposing the construct to the mechanical conditions found in the heart by culturing the construct in a pulsatile-flow bioreactor without the need for biochemical stimuli. Other future experiments could further investigate the growth conditions of the cells *in vitro*. Some researchers have suggested that certain lots of foetal calf serum are more effective at supporting MSC [Lennon *et al.*, 1996]. Also, an investigation to create a more defined medium to culture the cells in could be carried out by replacing the serum component with other biochemicals such as ascorbate and insulin transferring sodium selenite, or adding specific growth factors to assess their effects on the cells. Also, the cells could be grown in a pellet culture as described later in Section 4.3.2 to try to aid their differentiation into the chondrogenic lineage and also to see what effect this system might have on the properties of the cells.

In conclusion, putative porcine MSC were isolated using methods adapted from techniques to isolate human and murine MSC. However, whilst the cells were morphologically similar to human MSC isolated by others, attempts to differentiate the

cells were only partially successful. As such, it was not possible to conclusively state that the cells isolated were in fact porcine MSC.

## **CHAPTER FOUR:**

### ***Isolation and Characterisation of Human Mesenchymal Stem Cells***

# CHAPTER 4:

## *Isolation and Characterisation of Human Mesenchymal Stem Cells*

### **4.1. Introduction**

In tandem with isolating and identifying porcine MSC as a potential cell source for inclusion in an animal model for tissue engineering an aortic heart valve, it was also important to investigate the potential of human MSC as this would ultimately be the cell source used in the clinical setting. If successful, the isolation, identification and reseeded of a decellularised matrix with hMSC and culturing the construct in a pulsatile-flow bioreactor would show the *in vitro* potential for using hMSC in a tissue engineering application.

Although much of the initial work on MSC was carried out on murine MSC, investigations of hMSC are now far more prevalent and have already led to clinical trials using hMSC for stem cell therapy purposes [Wakitani *et al.*, 1994; Grande *et al.*, 1995; Saito *et al.*, 1995; Kadiyala *et al.*, 1997; Yoo *et al.*, 1998; Young *et al.*, 1998; Wang *et al.*, 2000; Tomita *et al.*, 2002]. In these investigations, hMSC have been isolated and expanded in culture and then reintroduced back into the patient directly into an injury site. Local environmental cues were then thought to have differentiated the cells into the appropriate lineages required to repair and regenerate the damaged tissues.

Despite the positive findings of these experiments, identification of the isolated hMSC has frequently been based on the assumption that the cells must be MSC since no other

cells in the bone marrow are thought to be adherent. Many attempts have been made to produce a monoclonal antibody specific for hMSC to facilitate rapid identification of these cells, [Simmons & Torok-Storb, 1991; Barry *et al.*, 1999; Caplan & Haynesworth, 2000], however almost as soon as they were produced, the monoclonal antibodies were found to cross-react with other non-MSC lineages. Many groups have used panels of antibodies to identify and phenotype isolated hMSC [Galmiche *et al.*, 1993; Conget *et al.*, 1999; Fontes & Thomson, 1999; Pittenger *et al.*, 1999; Zvaifler *et al.*, 2000; Jones *et al.*, 2002; Lodie *et al.*, 2002], however, there is currently no agreed set panel that every group uses and each seems to have their own ideas regarding which markers are necessary to accurately identify hMSC. A group working at St James Hospital, Leeds, have used a panel of antibodies to identify hMSC isolated from bone marrow and synovial lining (CD13, CD45, D7FIB, CD105, CD10, LNGFR, CD55, BMP, AP) [Jones *et al.*, 2002].

Since there is no agreed panel of antibodies in general use to specifically distinguish hMSC from other cells, a more thorough identification of the cells had to be based on the differentiation potential of cell populations derived from a single source. In order to limit the possibility of multiple cell precursors being present in the bone marrow and to show the multipotentiality of the cells, it was decided that it would be necessary to differentiate the cells into at least three different lineages. It was decided to try to differentiate hMSC derived merely from the same source initially, with a view to then carrying out clonogenic analyses if multipotentiality was demonstrated from the results obtained.

## 4.2. *Aims & objectives*

The aims and objectives of this chapter were to develop and carry out methods to isolate and differentiate hMSC into cells of the adipogenic, myogenic, chondrogenic and neurogenic cell lineages. Successful differentiation into at least three of these cell lineages would strongly indicate these cells to be hMSC. Furthermore, the antigen profile of the isolated cells was to be examined using fluorescence activated cell sorting (FACS) with a panel of antibodies to CD45, CD13, CD10, CD55, CD105, D7FIB, low affinity nerve growth factor receptor (LNGFR), bone morphogenetic protein (BMP) and alkaline phosphatase (AP). Phenotypic analysis of the cells would enable a direct comparison with the work of Jones *et al.* (2002) and would further confirm the identity of the isolated cells.

## 4.3. *Materials & methods*

### 4.3.1. Isolation of MSC from human bone marrow

**Materials:** Ethical approval was sought and obtained from the local ethics committee (Leeds General Infirmary) to obtain waste bone marrow from hip replacement operations at Leeds General Infirmary for use in these investigations. A Percoll gradient was made by centrifuging 22.05 ml Percoll, (Sigma-Aldrich), 2.45 ml 1.5 M NaCl, (Sigma-Aldrich) and 10.5 ml Tyrode's balanced salt solution, (Sigma-Aldrich) at 20,000 g for 15 minutes. Polypropylene centrifuge tubes (50 cm<sup>3</sup>) were obtained from Fisher Scientific.

**Method:** Once obtained from the patient, the bone marrow was placed in transport medium and collected from the operating theatre within 4 hours of removal. The bone marrow and transport medium were then transferred to a 50 ml centrifuge tube. The tube was then shaken in order to disperse the bone marrow prior to being centrifuged at 600 g for 5 minutes. The supernatant and overlying fat layer were carefully removed

leaving approximately 5 ml of medium to avoid aspirating the pellet. The pellet was then resuspended by repeated pipetting in the remaining solution before being carefully layered onto a preformed Percoll gradient. The Percoll-marrow solution was centrifuged at 460 g for 15 minutes before 14 ml of the upper fraction was removed and added to 25 ml of complete medium as described in Section 2.4.1. The solution was then centrifuged at 600 g for 5 min, the supernatant removed, the pellet resuspended by repeat pipetting, and a further 20 ml of medium added. The tube was then centrifuged at 600 g for a further 5 minutes. The supernatant was then removed and the cells resuspended in 5 ml of complete medium. The cell suspension was then transferred to a 25 cm<sup>2</sup> tissue culture flask and the flask incubated under standard conditions as described in Section 2.4.1. Culture medium was first changed after five days and then every three to four days until the cells were cryopreserved for later use or used in the differentiation assays.

### **4.3.2. Human mesenchymal stem cell differentiation assay**

**Materials:** Adipogenic medium consisted of 50 ml of adipogenic differentiation supplement (Stem Cell Technologies) added to 450 ml of human Mesencult medium (Stem Cell Technologies).

Myogenic medium consisted of complete medium supplemented with 10  $\mu\text{M}$  5-azacytidine (Sigma-Aldrich).

Chondrogenic medium consisted of serum-free medium supplemented with 100  $\mu\text{g.ml}^{-1}$  sodium pyruvate, 40  $\mu\text{g.ml}^{-1}$  L-proline, 50  $\mu\text{g.ml}^{-1}$  L-ascorbic acid-2-phosphate, 1  $\text{mg.ml}^{-1}$  bovine serum albumin (BSA), 1 $\times$  insulin-transferrin-sodium selenite, 100 nM dexamethasone (all purchased from Sigma-Aldrich) and 10  $\text{ng.ml}^{-1}$  TGF $\beta$ 3 (Catalogue number 243-B3-002, R&D Systems). Mouse anti-collagen II antibody was purchased from Sigma-Aldrich.



Neurogenic induction medium consisted of complete medium containing 20 % (v/v) FCS (Invitrogen) supplemented with 1 mM  $\beta$ -mercaptoethanol (Sigma-Aldrich). Neurogenic differentiation medium consisted of serum-free DMEM supplemented with 10 mM  $\beta$ -mercaptoethanol (Sigma-Aldrich). Tyrode's balanced salt solution (TBSS) was purchased from Sigma-Aldrich. Cells were grown on multi-chamber slides purchased from Bibby Sterilin. Anti-tau, anti-neuron-specific enolase (NSE), anti-desmin, anti-myosin heavy chain-slow and anti- $\alpha$ -smooth muscle-actin antibodies were all purchased from Vector Laboratories. Antibodies were diluted as described in Section 2.6.5 prior to use. Oil Red O stain was purchased from Sigma-Aldrich. Methanol/acetone solution was made from 50:50 methanol:acetone which were both purchased from VWR International. 15 ml polypropylene centrifuge tubes were purchased from Fisher Scientific.

**Method:** For the adipogenic, myogenic and neurogenic differentiation assays,  $5 \times 10^4$  cells (passage 3) were cultured in each well of four multi-chamber slides each in complete medium for 24 hr to enable the cells to become fully attached.

For adipogenic differentiation, complete medium was then removed and replaced with adipogenic medium. Cells were then fed adipogenic differentiation medium twice weekly for up to four weeks with two slides fixed at 2 weeks, and two slides fixed at 4 weeks in formalin solution for 10 minutes. Slides were then stored at  $-20^\circ\text{C}$ . Lipid vesicles could be visualised by eye as the cells were growing and were also stained using Oil Red O once fixed.

The myogenic differentiation assay was derived from Wakitani *et al.* (1995). Briefly, complete medium was replaced with myogenic medium for 24 hr before this was replaced with complete medium again. Cells were then fed complete medium twice weekly for up to four weeks with two slides fixed at 2 weeks, and two slides fixed at 4 weeks in methanol/acetone for 2 minutes. Slides were then stored at  $-20^\circ\text{C}$  prior to

immunofluorescent evaluation. Immunofluorescent staining as described in Section 2.6.4 using antibodies to  $\alpha$ -smooth muscle actin, myosin heavy chain-slow and desmin was used to show if differentiation had taken place.

The neurogenic differentiation assay was derived from Woodbury *et al.* (2000). Briefly, complete medium was first replaced with neurogenic induction medium for 24 hr. The cells were then rinsed in TBSS, before this was replaced with neurogenic differentiation medium. Slides were then fixed for 2 minutes in methanol/acetone at 2, 4, 6 and 8 hours after differentiation medium was introduced before being stored at  $-20\text{ }^{\circ}\text{C}$  prior to immunofluorescent evaluation. Immunofluorescent staining using antibodies to tau and NSE were used to show if differentiation had taken place.

The chondrogenic differentiation assay was derived from Jones *et al.* (2002). Briefly,  $2.5 \times 10^5$  cells were centrifuged at 500 g for 5 min in each of 6 (15 ml) polypropylene centrifuge tubes. Four tubes of cells were then fed with chondrogenic differentiation medium three times a week for up to four weeks and incubated in the polypropylene tube with a loosened cap to allow gaseous exchange. The remaining cells were fed with complete medium three times a week for up to four weeks as a comparison. Cell pellets were harvested at 2 and 4 weeks and cryo-embedded for frozen sectioning prior to storage in liquid nitrogen until required for toluidine blue staining and immunofluorescent evaluation using an antibody to collagen II.

### 4.3.3. Toluidine blue staining

**Materials:** Methanol and isopropanol were purchased from VWR International. Toluidine blue was purchased from Sigma Aldrich.

**Method:** Cryo-embedded chondrogenic differentiation pellets were cryo-sectioned at 5  $\mu\text{m}$  and fixed onto super frost slides by immersing them in 100 % methanol for 2 minutes. Sections were then stained by immersion for 30 minutes in 1 % (w/v) toluidine

blue solution in 50 % (v/v) isopropanol at 37 °C. Slides were then rinsed extensively in isopropanol prior to being dehydrated and mounted through graded alcohols and xylene as previously described in Section 2.5.4.

#### 4.3.4. Fluorescence activated cell sorting (FACS) analysis

**Materials:** FACS tubes were purchased from Beckton Dickinson. Anti-mouse IgG, anti-low affinity nerve growth factor receptor (LNGFR), anti-CD105, anti-CD45, anti-CD13, anti-D7FIB and anti-CD10 antibodies were directly conjugated to phycoerythrin (PE), a red fluorescent marker. Anti-CD55 and anti-bone morphogenetic protein (BMP) were biotinylated and so were visualised using a secondary marker, streptavidin (STR), which was conjugated to PE and readily binds to biotin. Anti-alkaline phosphatase antibody (AP) was not conjugated and so was visualised using a goat anti-mouse (GAM) secondary antibody also conjugated to PE. Anti-mouse IgG, anti-LNGFR, anti-STR, and anti-CD55 antibodies were obtained from BD Pharmingen. Anti-CD105 and GAM antibodies were purchased from Serotec. Anti-BMP was obtained from RnD Systems. Anti-CD45, anti-CD13, anti-D7FIB, anti-CD10 and anti-AP antibodies were gifts from Elena Jones. All antibodies were diluted as described in Table 5.1 using TBS azide BSA as described in Section 2.6.1. Foetal calf serum (FCS) was obtained from Invitrogen.

**Methods:** A confluent flask of hMSC (passage 6) was trypsinised. Cells ( $1 \times 10^5$ ) were then added to each of 12 FACS tubes. FCS (1 ml) was then added to each tube to inactivate the trypsin. The tubes were then centrifuged at 200 g for 10 minutes. The supernatant was discarded and the cells resuspended in the residual fluid. Antibody solution (10  $\mu$ l) was then added to the respective FACS tubes and the tubes incubated for 15 minutes at room temperature. HBSS (5 ml) was then added to each tube and the cells were centrifuged at 200 g for 10 minutes. The supernatant was then discarded and

tubes containing antibodies directly conjugated to PE were then resuspended and analysed. Tubes containing antibodies requiring a secondary PE-conjugated antibody were also resuspended and 10  $\mu$ l of the respective secondary antibody was added to the tubes. These tubes were incubated for a further 15 minutes at room temperature before 5 ml of HBSS was added and the tubes centrifuged at 200 g for 10 minutes. The supernatant was then discarded and the cells resuspended ready for FACS analysis. Each tube was then introduced into the FACS machine and 10,000 cells counted and analysed for the presence of the respective markers on the F2 channel.

MARKER	CONJUGATE	SPECIFICITY	DILUTION
IgG	PE	Mouse immunoglobulin G negative control	1/2
CD45	PE	Leukocyte marker	Neat
CD13	PE	Monocyte, granulocyte and myelocyte marker	Neat
D7FIB	PE	Fibroblast marker	1/5
CD105	PE	Vascular endothelial cell marker	Neat
CD10	PE	Fibroblast and granulocyte marker	1/5
LNGFR	PE	Low-affinity nerve growth factor receptor, found on adventitial reticular cells of the bone marrow	Neat
STR	PE	Streptavidin negative control	1/200
CD55	BIO	Synovial lining and fibroblast marker	Neat
BMP	BIO	Bone morphogenetic protein marker	1/50
GAM	PE	Goat anti-mouse negative control	1/10
AP	None	Alkaline phosphatase marker for bone differentiation	Neat

**Table 4.1. Antibody dilutions used for FACS analysis. PE: phycoerythrin; BIO: biotin.**

### 4.3.5. Clonogenic analysis

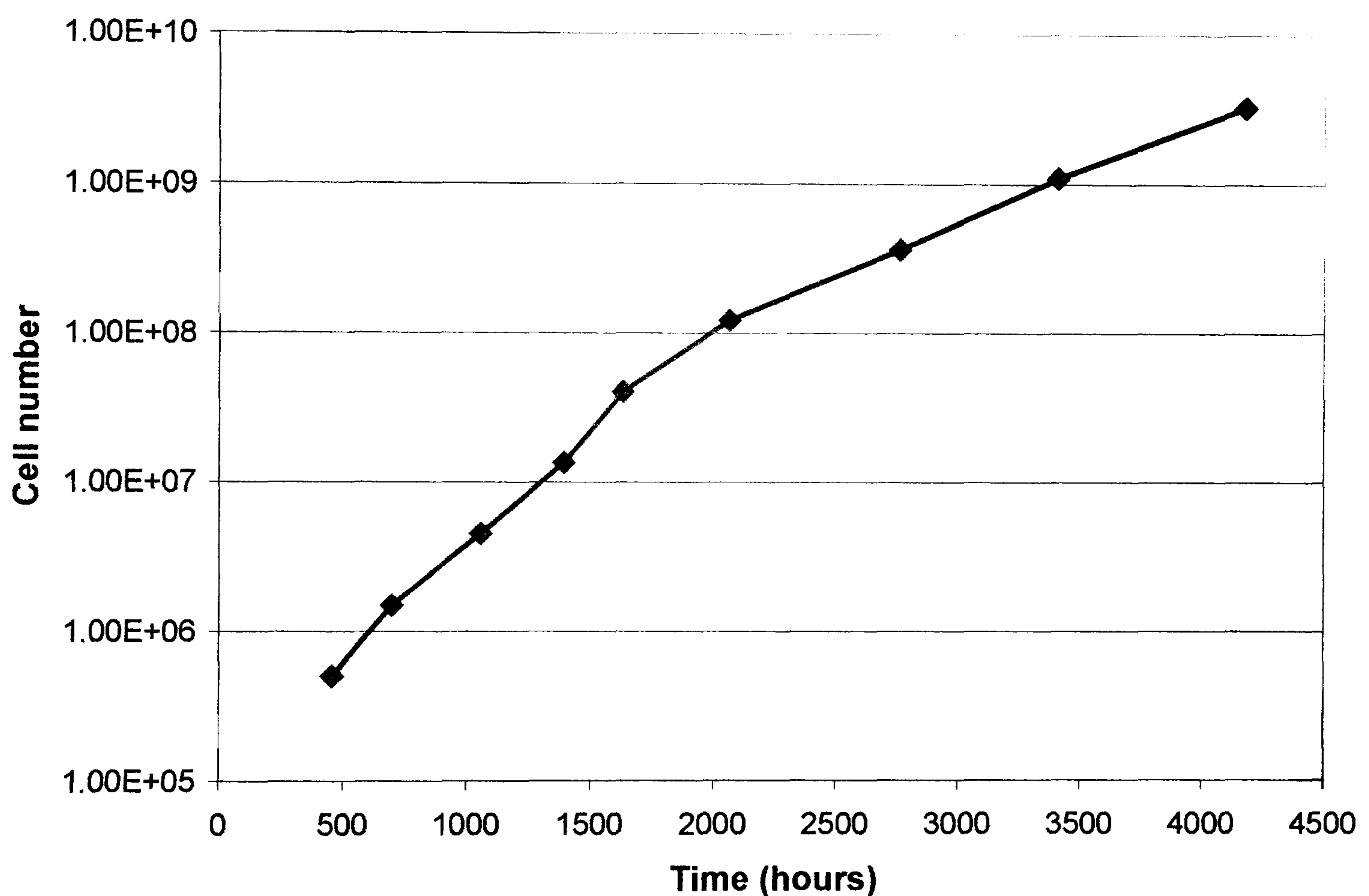
**Materials:** Tissue culture treated petri dishes (60 mm diameter) were purchased from Fisher Scientific. Cloning rings were purchased from Sigma-Aldrich. Whilst growing sufficient hMSC for various other experiments, the waste medium removed when the cells were fed was retained and mixed 50:50 with fresh complete medium to make hMSC conditioned medium.

**Methods:** Sample hBM6 was used for the clonogenic assay as this sample was harvested from the youngest patient available and, therefore, represented the potentially healthiest source of cells with the highest potential to proliferate for longer. A confluent flask of hMSC was trypsinised and the cells counted as described in Section 2.4.2. Approximately 30 cells were then reseeded into each of twenty 60 mm tissue culture treated petri dishes. Cells were returned to the incubator and allowed to attach to the dish for a minimum of four hours (fed on conditioned medium). The petri dishes were then viewed on an inverted microscope. A cloning ring was placed over each single cell that could be seen to have no other cells nearby. Once all the single cells on a dish were ringed, the tissue culture medium was removed from the dish, and then from within each of the rings. The individual cells were then trypsinised using 100  $\mu$ l of HBSS to wash the cells, and then 50  $\mu$ l of trypsin-EDTA solution to detach the cells. Once the cells had detached, 100  $\mu$ l of conditioned medium was added to each ring before the contents of each ring were transferred to a separate well of 96-well tissue culture plate. A further 100  $\mu$ l of conditioned medium was then added to each well and the cells cultured under standard conditions. The cells were then fed twice weekly with 200  $\mu$ l of conditioned medium. Once the cells became confluent, they were trypsinised and transferred to a 48 well plate, followed by 24-well and 12-well plates.

## 4.4. Results

### 4.4.1. Cultured human mesenchymal stem cells

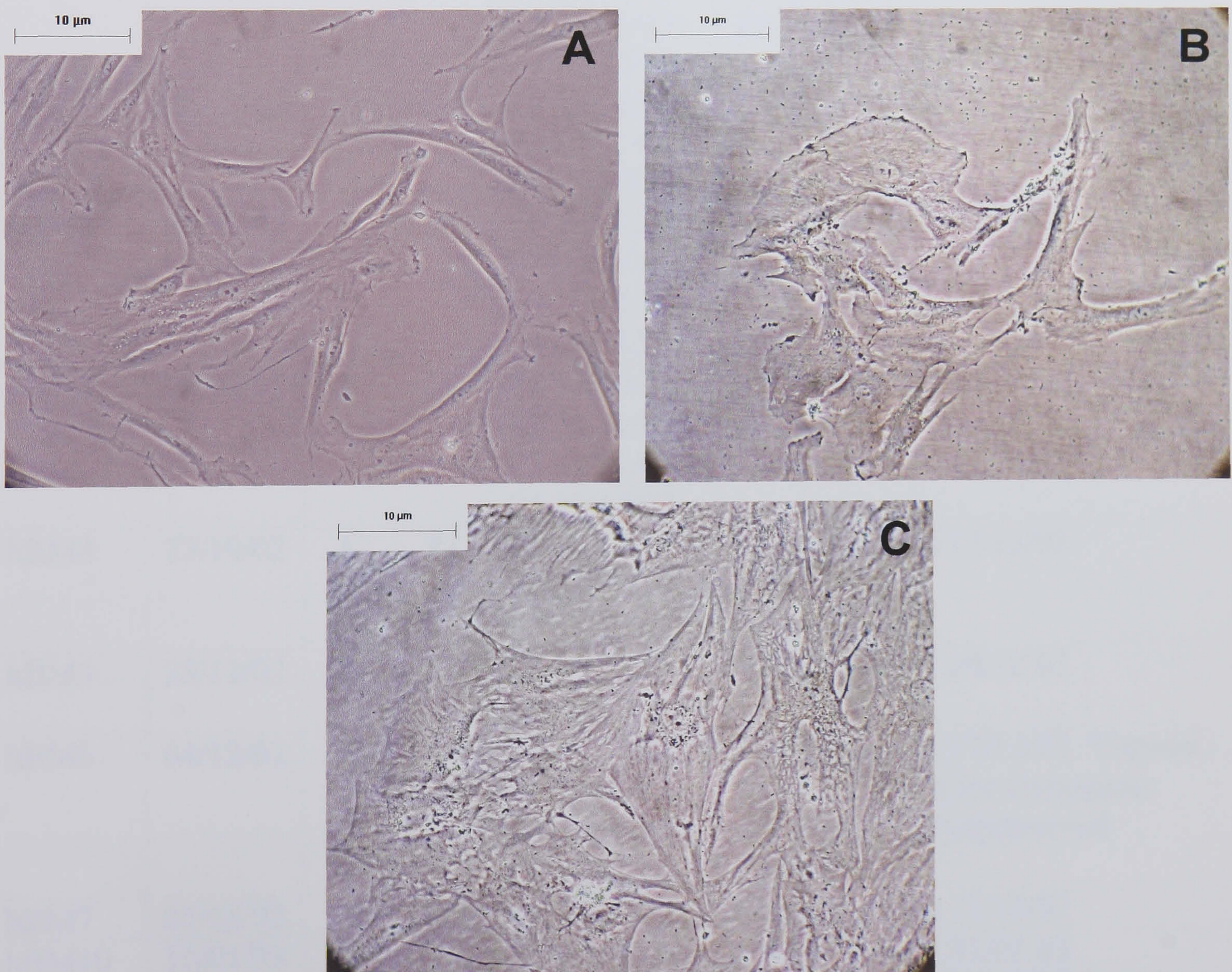
A growth curve was plotted for sample hBM1 (Figure 4.1) and the cell doubling time (CDT) was calculated as described in Section 2.4.3. hMSC between passages 1 and 2 were found to have a CDT of approximately 151 hours. However, between passages 8 and 9, this time had increased to approximately 438 hours.



**Figure 4.1. Growth curve of human mesenchymal stem cells.** Each point on the line represents a cell passage of hBM1. The original cell number could not be calculated due to difficulties in seeing the cells through other bone marrow debris.

The apparent reduction in proliferation rate was matched by a change in cell morphology (Figure 4.1). The cells were seen to be fibroblastic in appearance. Microscopic observation of cell morphology at different passages showed that the cells could generally be categorised into three age-related morphologies based on their appearance:

1. Cells one week after isolation (passage 0) are shown in Figure 4.2a. Cells were relatively small and highly proliferative, becoming confluent in approximately 2 weeks.
2. Cells at an intermediate stage (passage 5) whereby their rate of proliferation had decreased slightly and the cells were beginning to take on a more flattened and spread morphology are shown in Figure 4.2b.
3. At this stage, cells were largely quiescent and divided very slowly, if at all (passage 9). Cells became highly flattened and spread with prominent filaments visible in the cell cytoplasm, and typically took over 4 weeks to become confluent. Cell morphology can be seen in Figure 4.2c.



**Figure 4.2. Human mesenchymal stem cells at different ages.** A: Proliferating primary morphology (P0); B: Intermediate secondary morphology (P5); and C: Quiescent tertiary morphology (P9).

A number of attempts to isolate putative hMSC were made during the course of the investigation. These are listed in Table 5.2. The yield of cells was observed to vary from sample to sample, however it was impossible to accurately count the number of cells initially isolated because of the large amount of debris present after the isolation process. The cryopreserved cells were stored in liquid nitrogen as a cell bank for future studies.

SAMPLE	DATE	SEX	AGE	PASSAGES	NOTES
hBM1	28/08/02	F	71	P1 16/09/02 P2 26/09/02 P3 11/10/02 P4 25/10/02 P5 19/11/02 P6 20/01/03 P7 30/01/03 P8 17/02/03 P9 18/03/03 P10 14/04/03 P11 16/05/03	Used in differentiation assay at P3 for adipogenic and myogenic. P4 for neurogenic. P8 for chondrogenic. Cryopreserved 25/10/02. Cryopreserved 19/11/02. P5 thawed 06/01/03. Used for FACS at P6
hBM2	28/08/02	F	54	P1 16/09/02 P2 30/09/02 P3 22/10/02 P4 08/11/02	Cryopreserved 08/11/02
hBM3	25/09/02	F	61	P1 14/10/02 P2 01/11/02 P3 25/11/02 P4 20/12/02	Cryopreserved 20/12/02
hBM4	23/10/02	F	81	P1 10/12/02 P2 02/12/02 P3 17/12/02	Cryopreserved 17/12/02
hBM5	28/11/02	M	72	P1 10/12/02 P2 20/12/02	Cryopreserved 20/12/02
hBM6	04/12/02	M	40	P1 17/12/02 P2 18/07/03 P3 04/08/03 P4 27/08/03	Cryopreserved 17/12/02. Thawed 11/07/03. Used for clonogenic assay at P3. Cryopreserved 27/08/03
hBM7	05/12/02	M	67	P1 17/12/02	Cryopreserved 17/12/02
hBM10	15/01/03	F	71	P1 31/01/03	Cryopreserved 31/01/03
hBM11	16/01/03	?	55	P1 27/01/03	Cryopreserved 27/01/03
hBM12	16/01/03	F	54	P1 03/02/03	Cryopreserved 03/02/03
hBM13	16/01/03	M	69	P1 27/01/03	Cryopreserved 27/01/03
hBM14	02/06/03	F	62	P1 21/02/03	Cryopreserved 21/02/03
hBM16	26/02/03	?	?	P1 21/03/03	Cryopreserved 21/10/03
hBM17	27/02/03	M	79	P1 14/03/03	Cryopreserved 14/03/03



hBM19	12/03/03	F	80	P1 11/04/03	Cryopreserved 11/04/03
hBM20	12/03/03	F	74	P1 11/04/03	Cryopreserved 11/04/03
hBM21	12/03/03	F	75	P1 11/04/03	Cryopreserved 11/04/03
hBM22	20/03/03	M	47	P1 31/03/03	Cryopreserved 31/03/03
hBM24	02/04/03	F	68	P1 02/05/03	Cryopreserved 02/05/03
hBM26	24/04/03	F	67	P1 09/05/03	Cryopreserved 09/05/03
hBM27	07/05/03	F	68	P1 30/05/03	Cryopreserved 30/05/03
hBM28	07/05/03	F	79	P1 30/05/03	Cryopreserved 30/05/03
hBM29	08/05/03	F	52	P1 30/05/03	Cryopreserved 30/05/03
hBM30a	08/05/03	M	68	P1 30/05/03	Cryopreserved 30/05/03
hBM30b	08/05/03	M	68	P1 30/05/03	Cryopreserved 30/05/03
hBM32	31/07/03	F	95	P1 27/08/03 P2 05/09/03	Cryopreserved 05/09/03
hBM33	31/07/03	F	87	P1 27/08/03	Cryopreserved 27/08/03
hBM36	28/08/03	F	74	P1 15/09/03	Cryopreserved 15/09/03
hBM38	11/09/03	F	85	P1 03/10/03	Cryopreserved 03/10/03

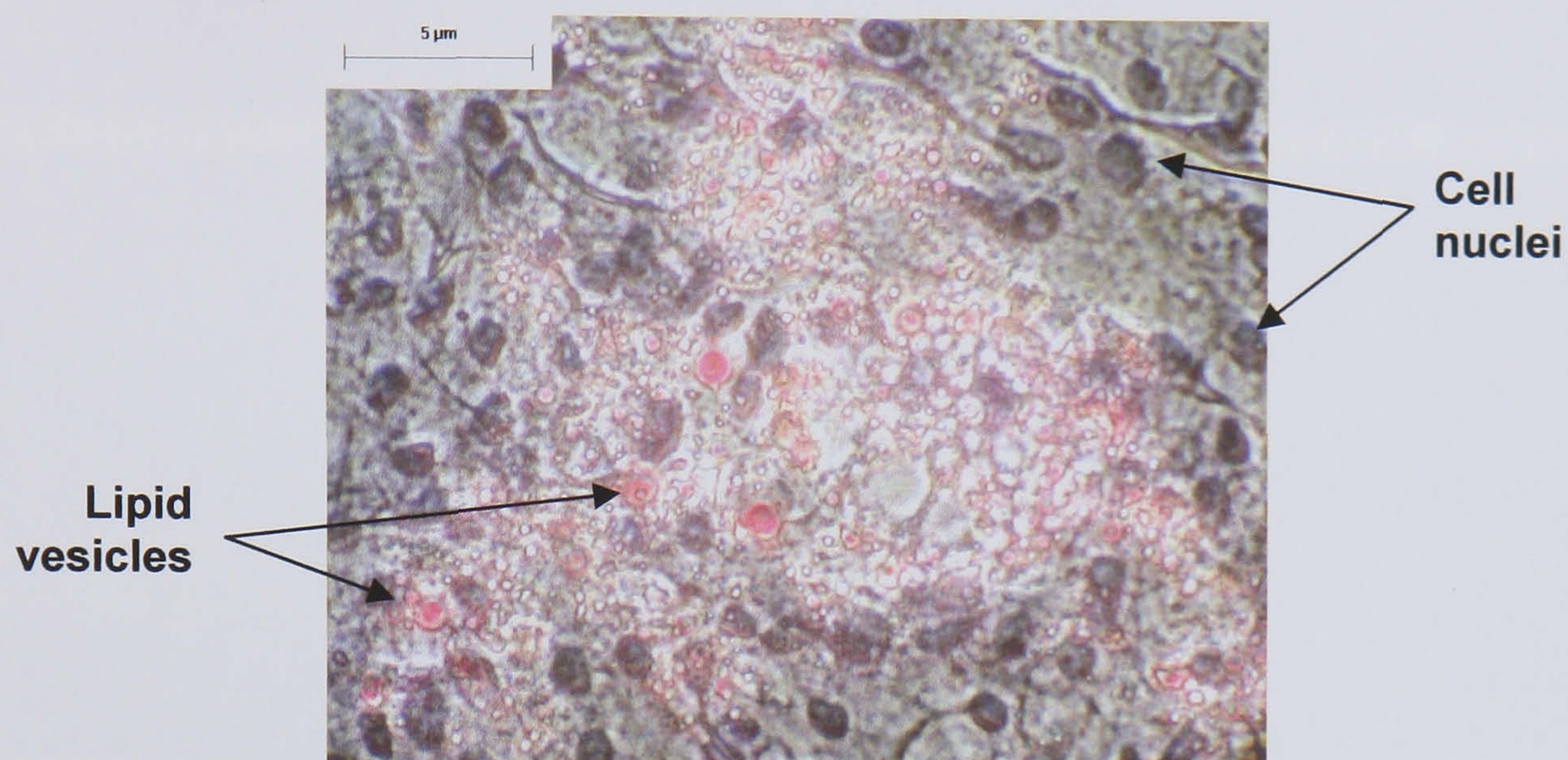
**Table 4.2. Putative human mesenchymal stem cell isolations.** Each isolation attempted to isolate putative hMSC from waste bone marrow obtained from a hip replacement operation at Leeds General Infirmary. Requested patient details were not always given.

#### 4.4.2. Human mesenchymal stem cell differentiation

Once putative hMSC had been isolated, it was necessary to try to differentiate the cells *in vitro* into multiple cell lineages in order to demonstrate the multipotentiality of the cells. When the bone marrow samples were first received, the supply was somewhat irregular as the operating theatre staff were not familiar with what was being requested of them. Consequently, the differentiation attempts were carried out on the first sample received, hBM1. Once the differentiation assays were started, it was necessary to continue to use the same cell population throughout the experiments in order to show that differentiation into multiple lineages was possible from one cell population in order to rule out different cell precursors being present in different cell populations.

#### 4.4.2.1. Adipogenic differentiation

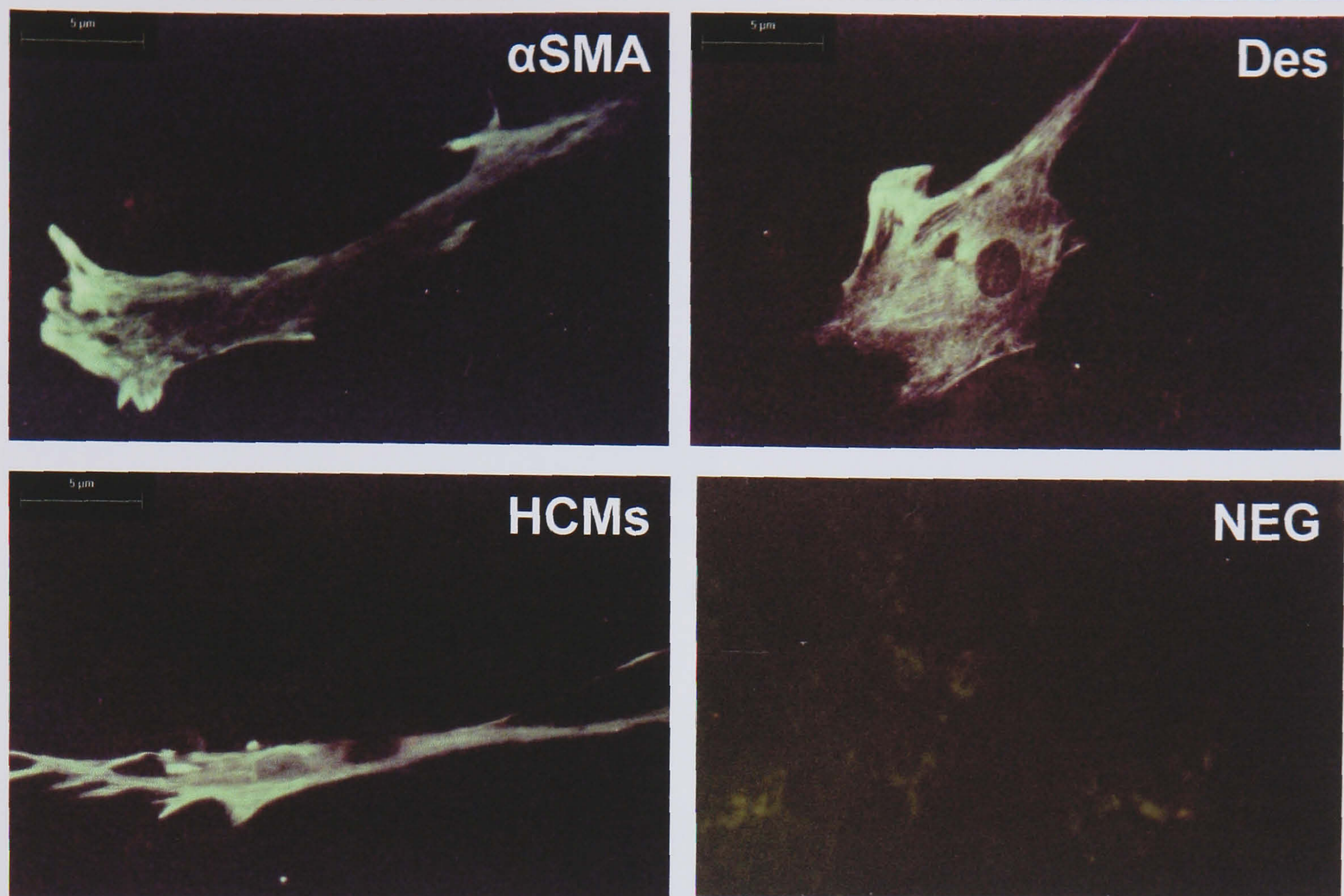
The assay was carried out sample hBM1 at passage 3. Oil Red O is a lipid soluble dye; therefore the areas stained pink mark lipid deposits made by the cells (Figure 4.3). It was observed that only approximately 20 % of cells differentiated.



**Figure 4.3. Differentiation of human mesenchymal stem cells into adipogenic lineage. Oil red O staining of lipid vesicles.**

#### 4.4.2.2. Myogenic differentiation

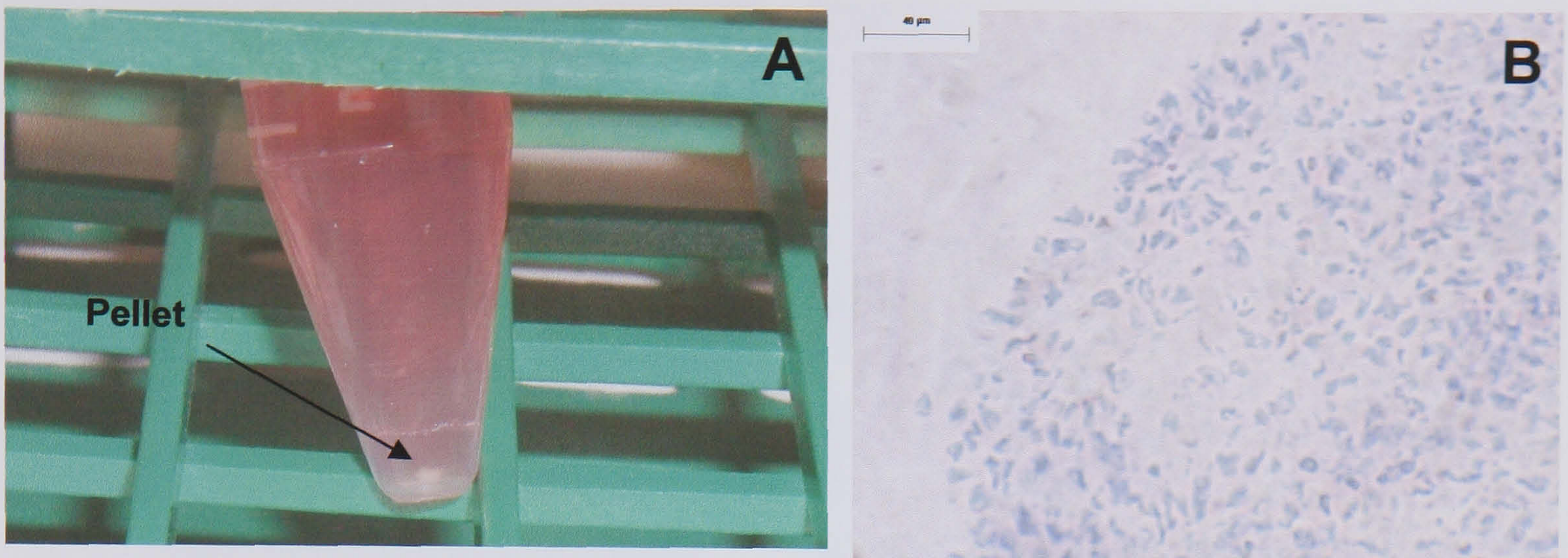
The assay was carried out on sample hBM1 at passage 3. Once the differentiation procedure was complete, the cells were fixed and stained with fluorescent antibodies to  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), desmin and heavy-chain myosin slow (HCMs, Figure 4.4). Approximately 10 % of cells were seen to weakly express these markers, with the  $\alpha$ SMA being greatly reduced from its normally high expression on freshly isolated cells. Porcine heart muscle was used as a positive control for the antibodies as no human heart could be obtained.



**Figure 4.4. Differentiation of human mesenchymal stem cells into myogenic lineage.** Fluorescent antibody staining to  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), desmin (Des), heavy-chain myosin slow (HCMs), negative control (NEG).

#### 4.4.2.3. Chondrogenic differentiation

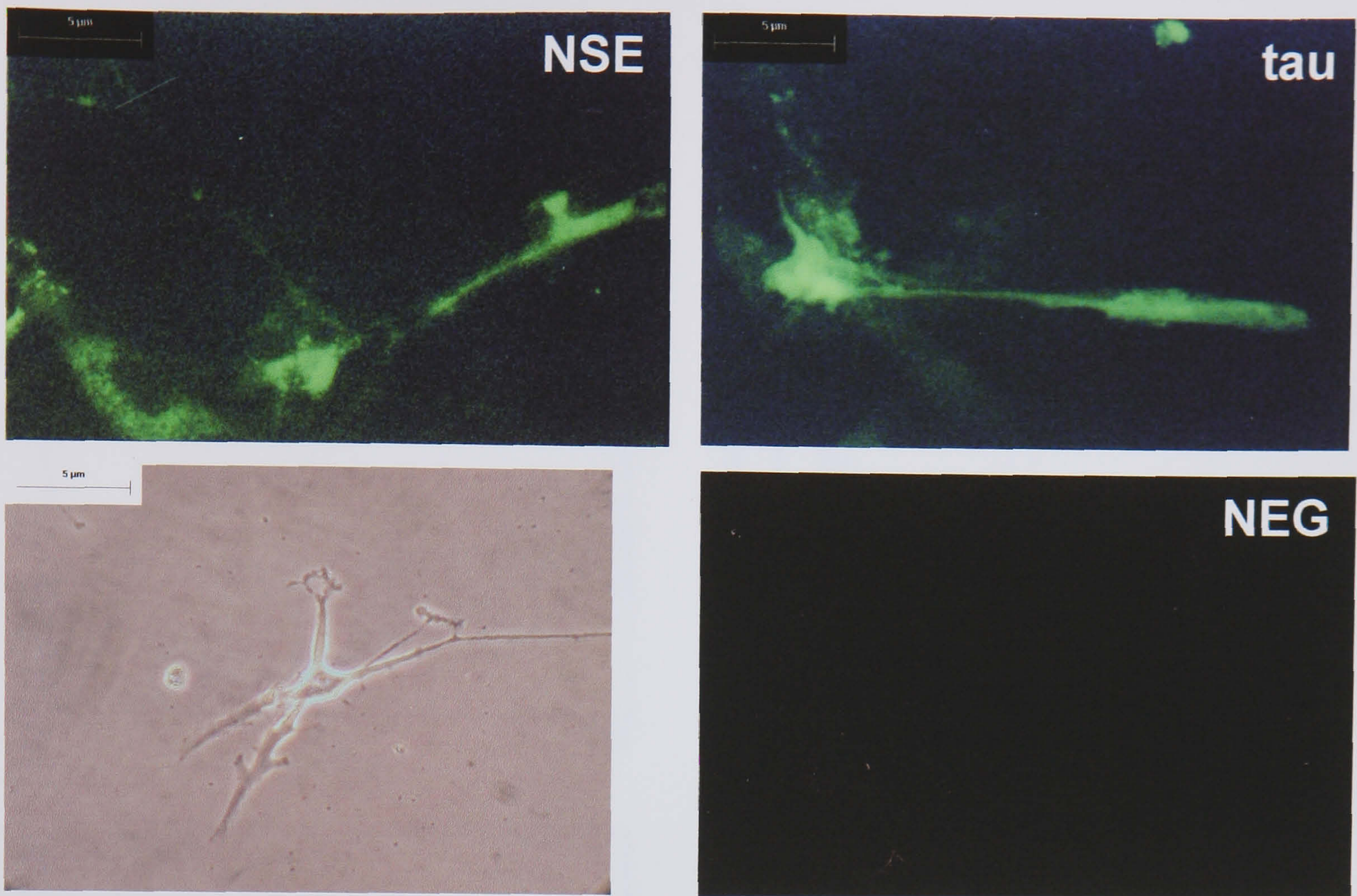
The assay was carried out on sample hBM1 at passage 8. After 24 hours of culture, the cells formed a spherical cell pellet that became detached from the centrifuge tube (Figure 4.5A). Blue regions of toluidine blue staining after 4 weeks of culture indicated extracellular matrix production by the cells (Figure 4.5B). The main marker for chondrogenic differentiation is collagen II, however, immunoperoxidase staining of the pellet indicated no collagen II production by the cells. Human cartilage was used as a positive control for the collagen II antibody.



**Figure 4.5. Differentiation of human mesenchymal stem cells into chondrogenic lineage.** A: cell pellet in culture; B: toluidine blue staining of the pellet.

#### 4.4.2.4. Neurogenic differentiation

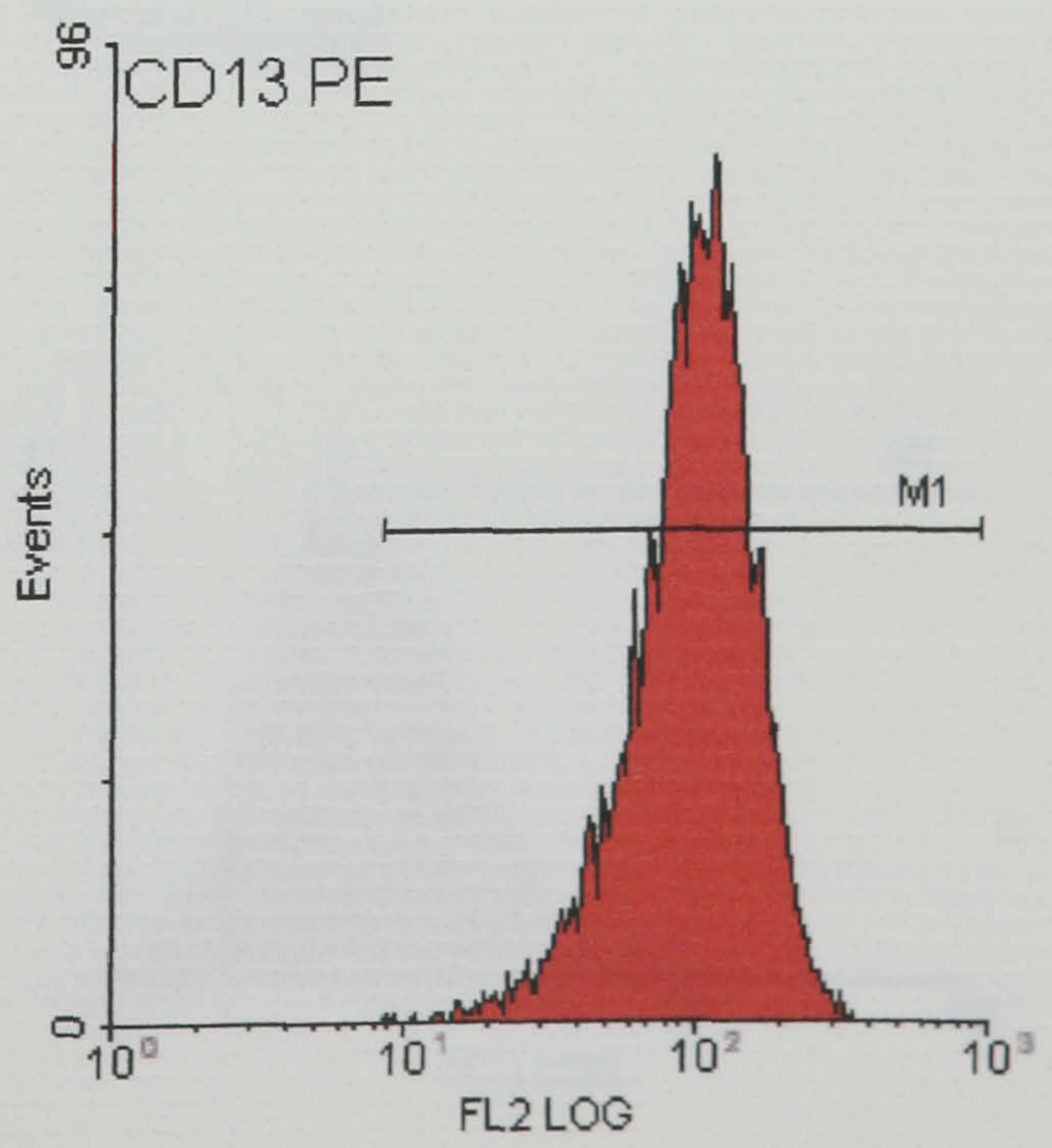
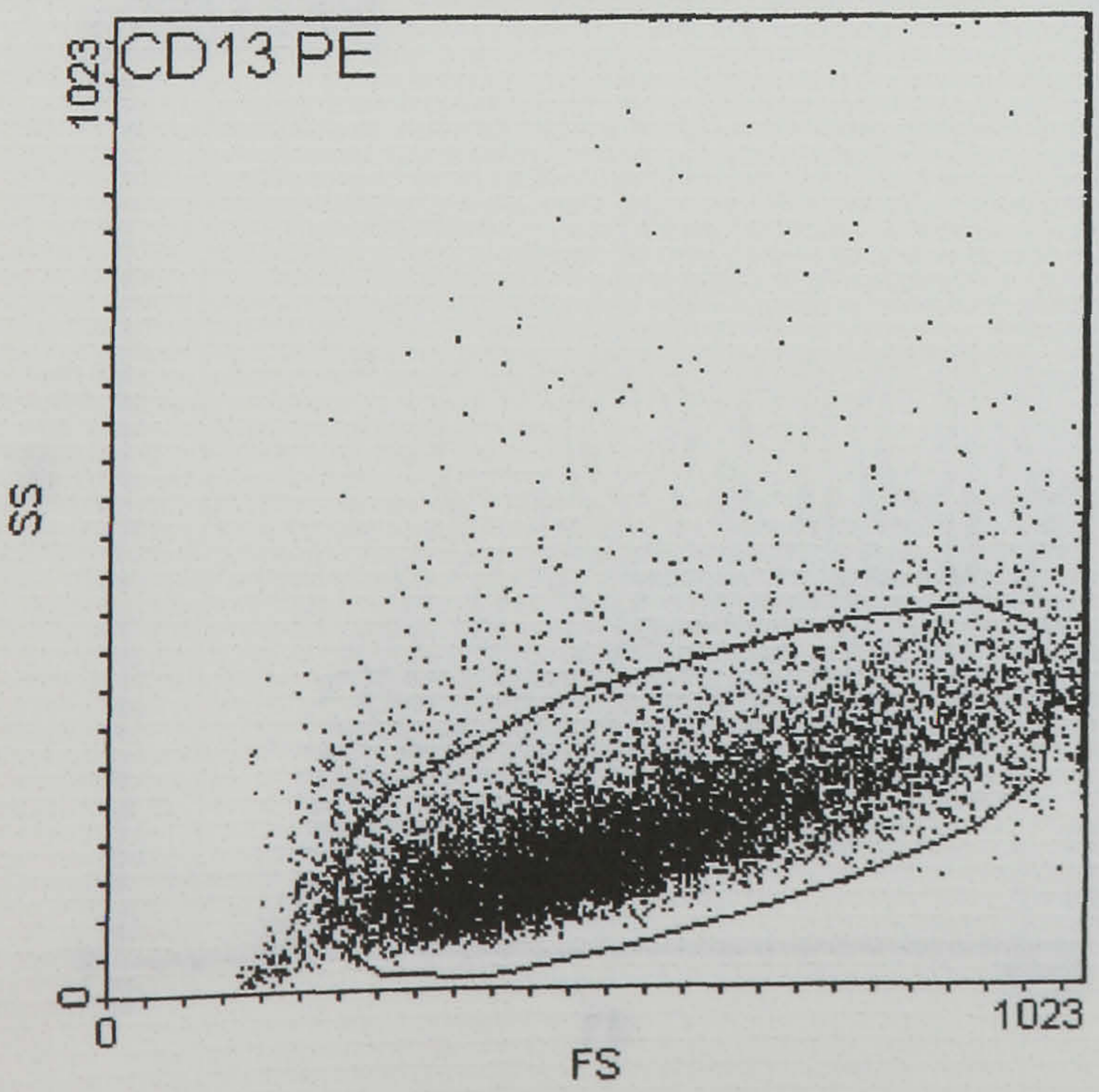
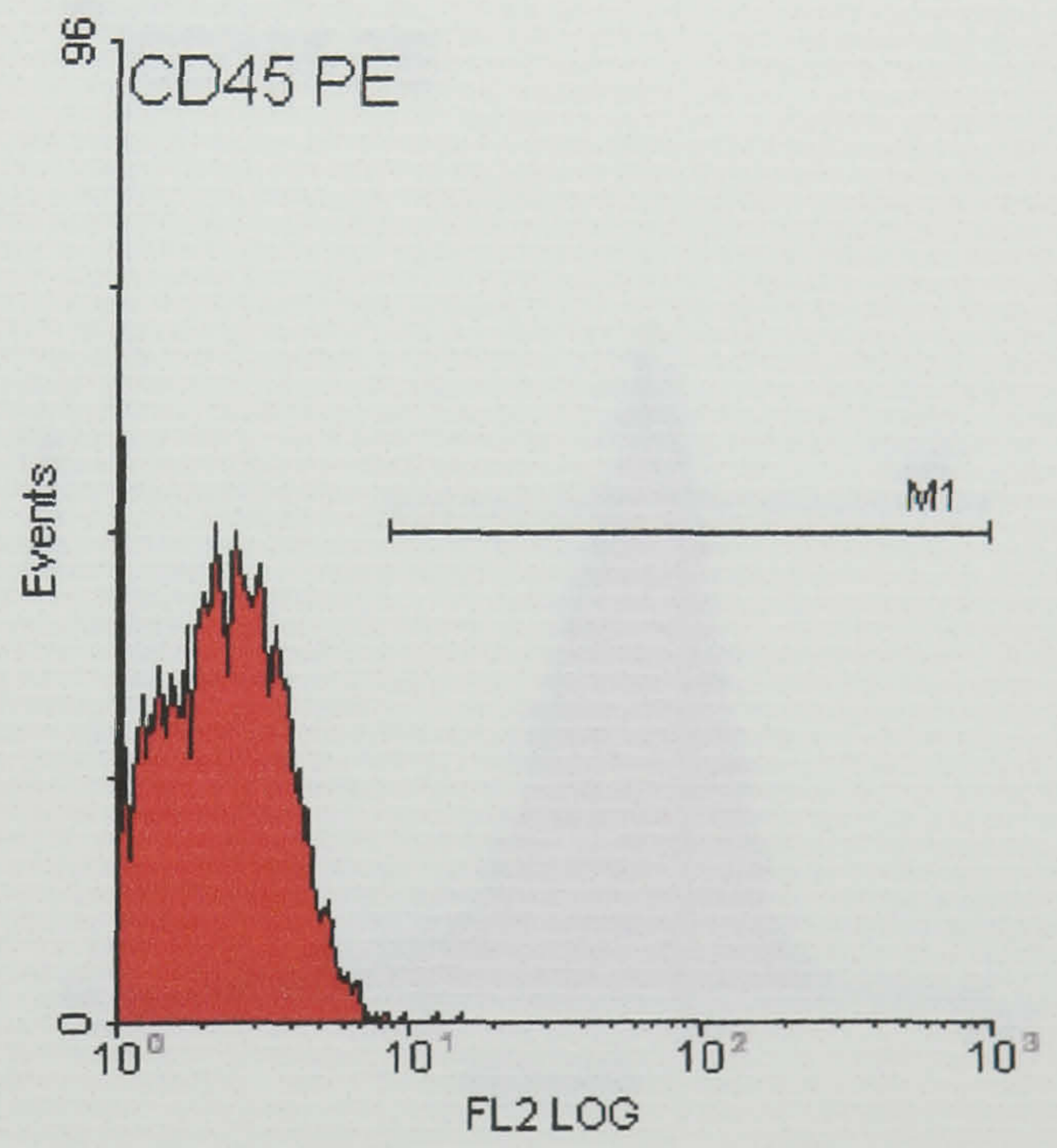
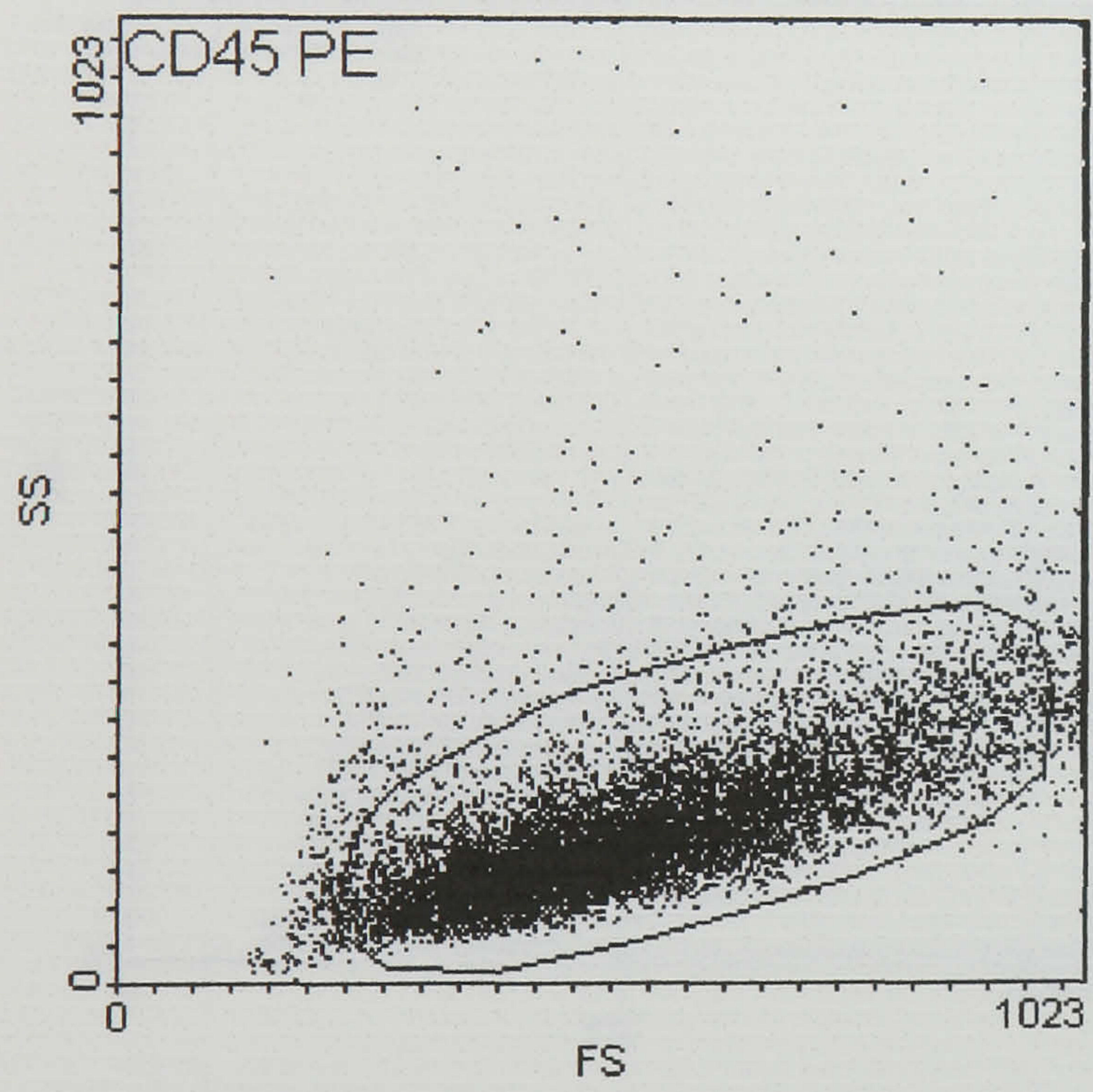
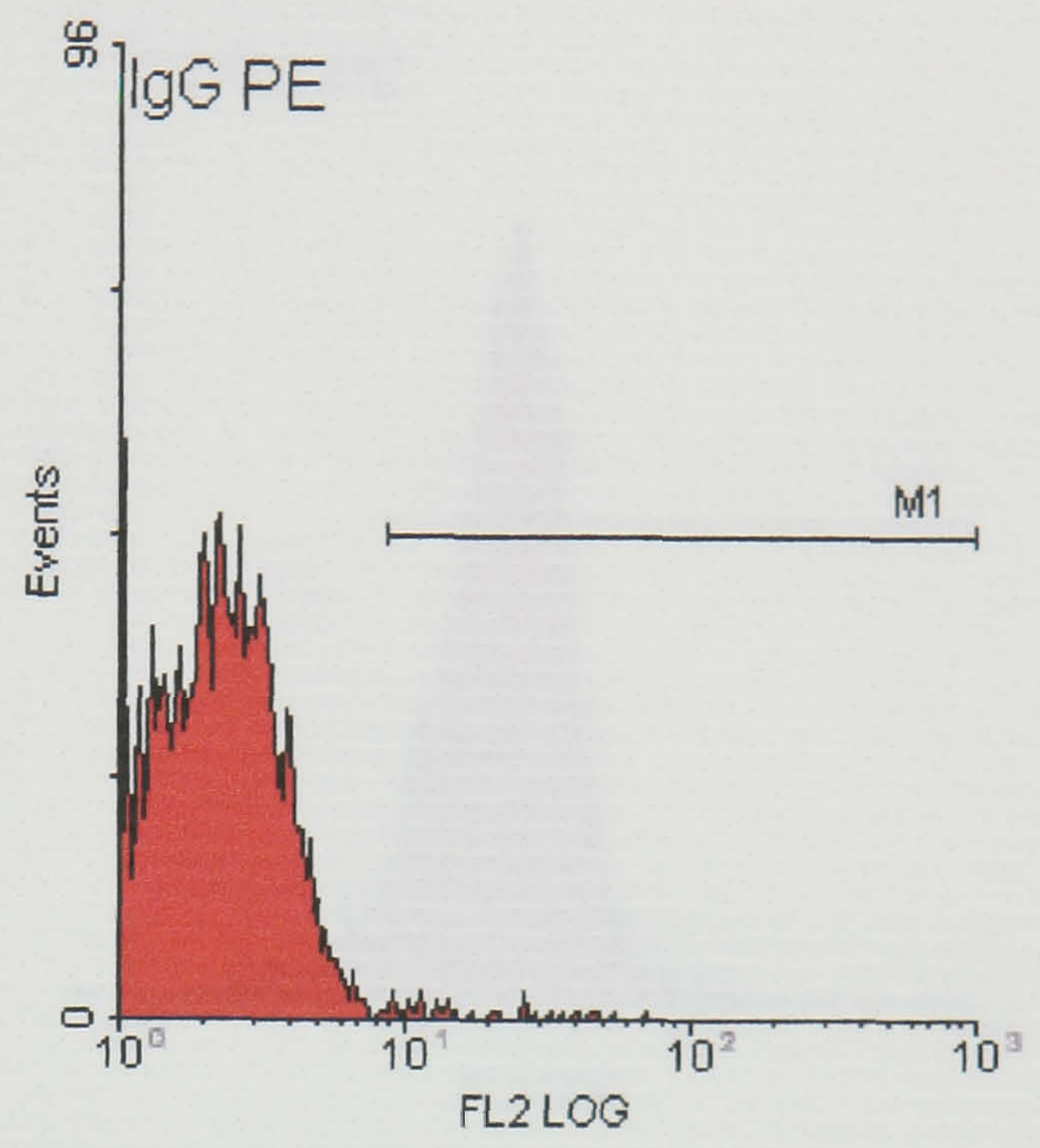
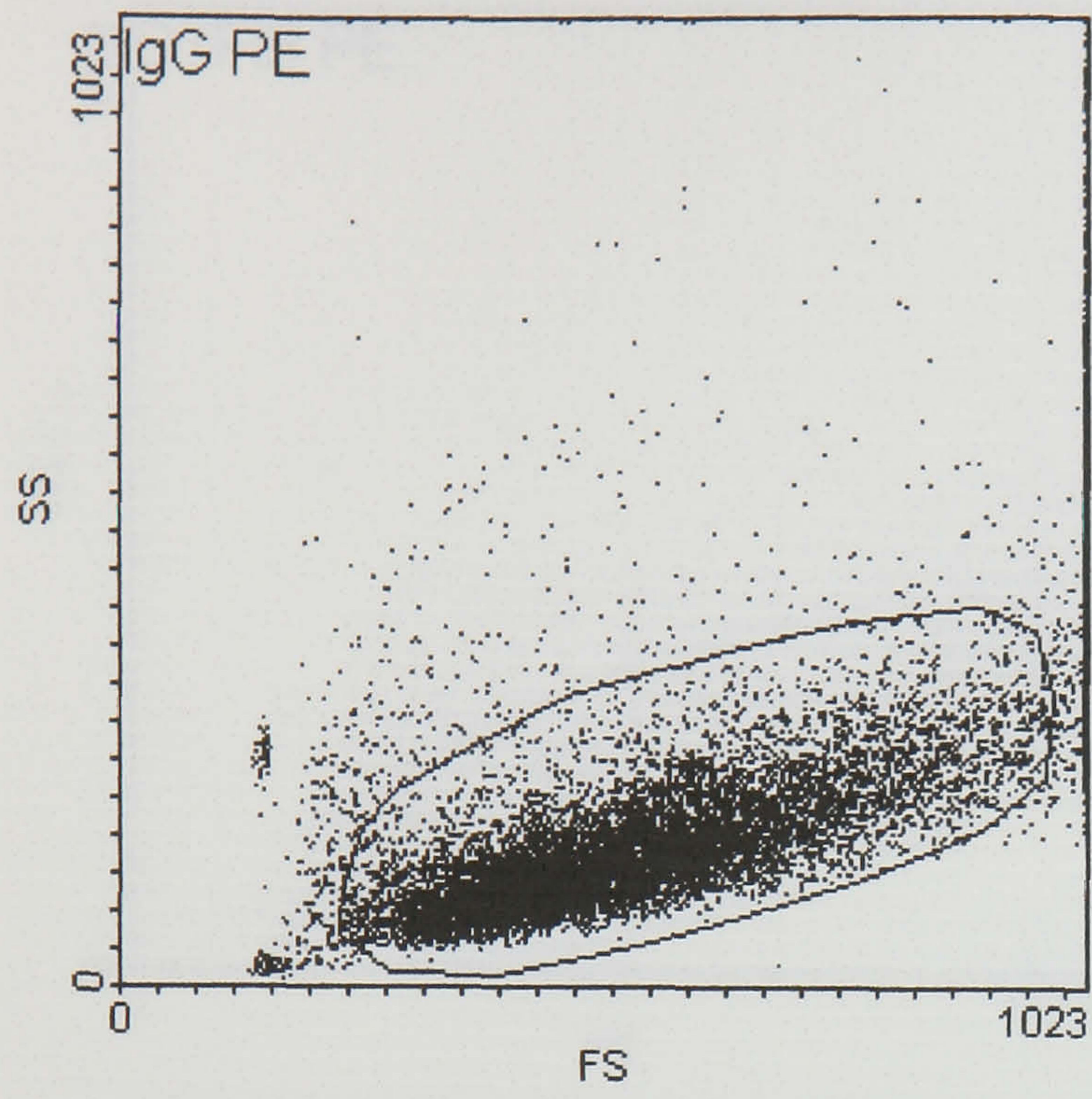
The assay was carried out on sample hBM1 at passage 4. Once the culture period was complete, cells were fixed and stained using fluorescent antibodies to tau and neurone-specific enolase (NSE), both markers of the neurogenic lineage (Figure 4.6). The cell morphology was also seen to change to an appearance resembling a neurone-like morphology. Again, approximately 10 % of cells were positively stained. Porcine optic nerve was used as a positive control for the antibodies.

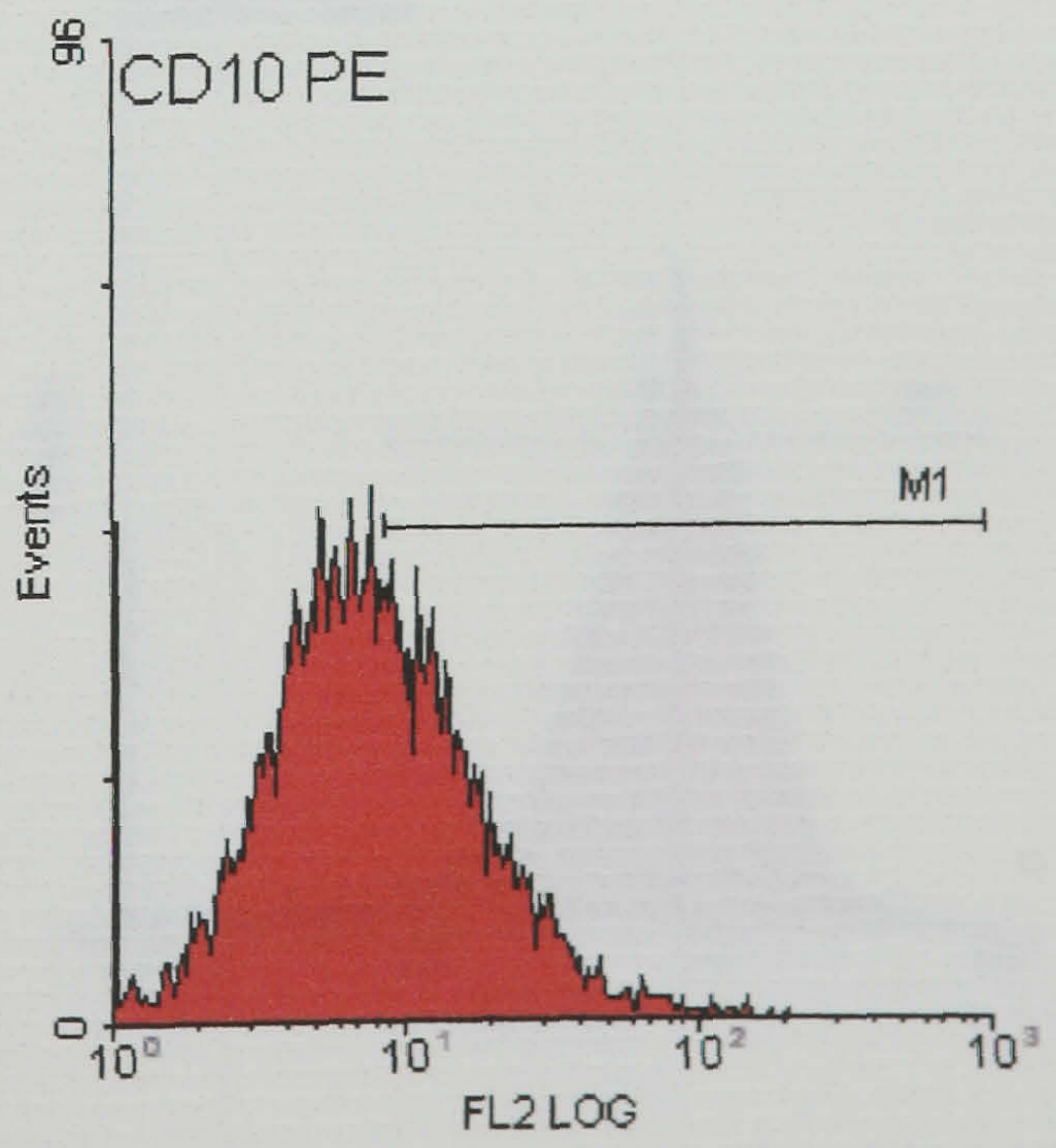
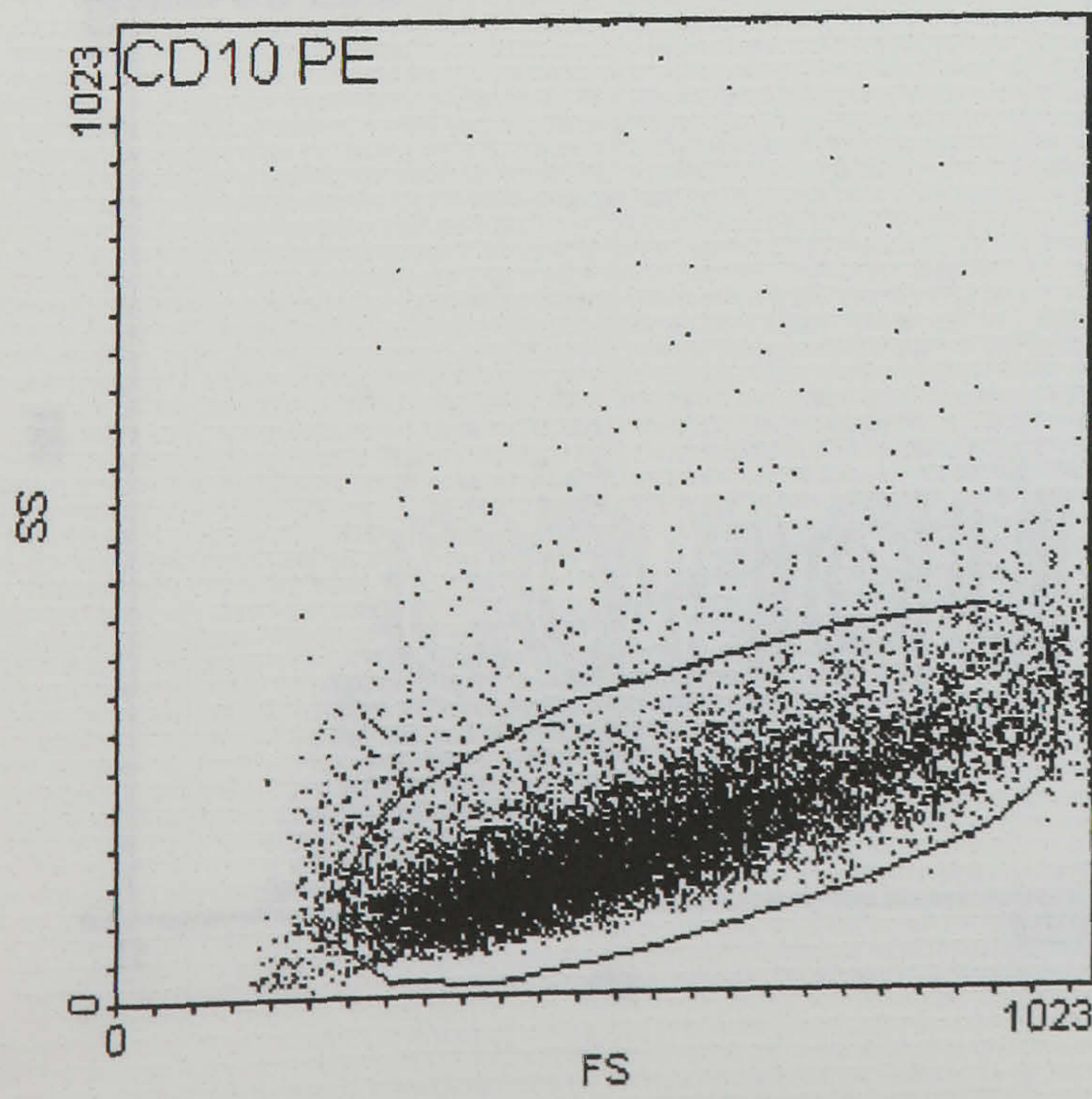
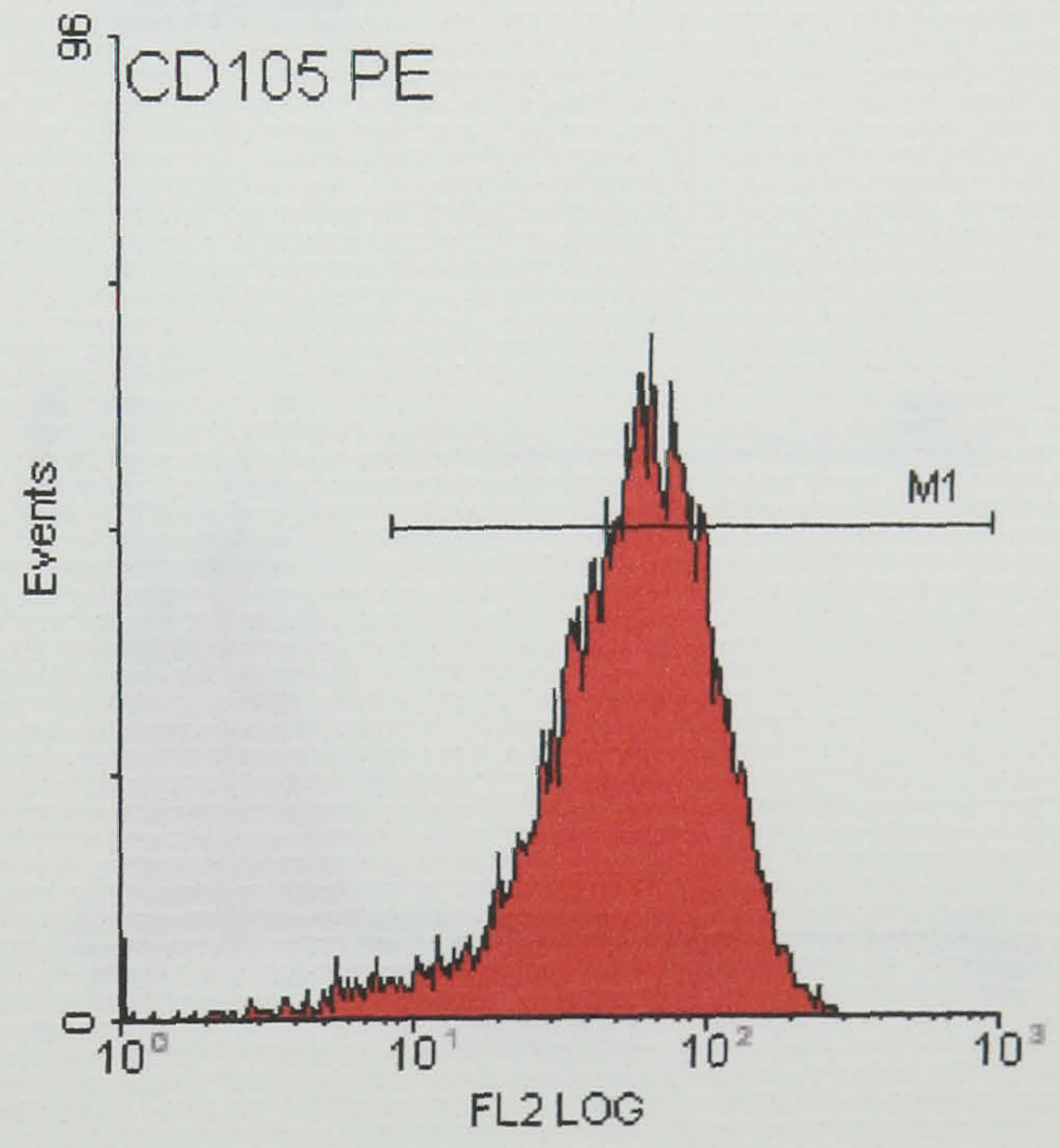
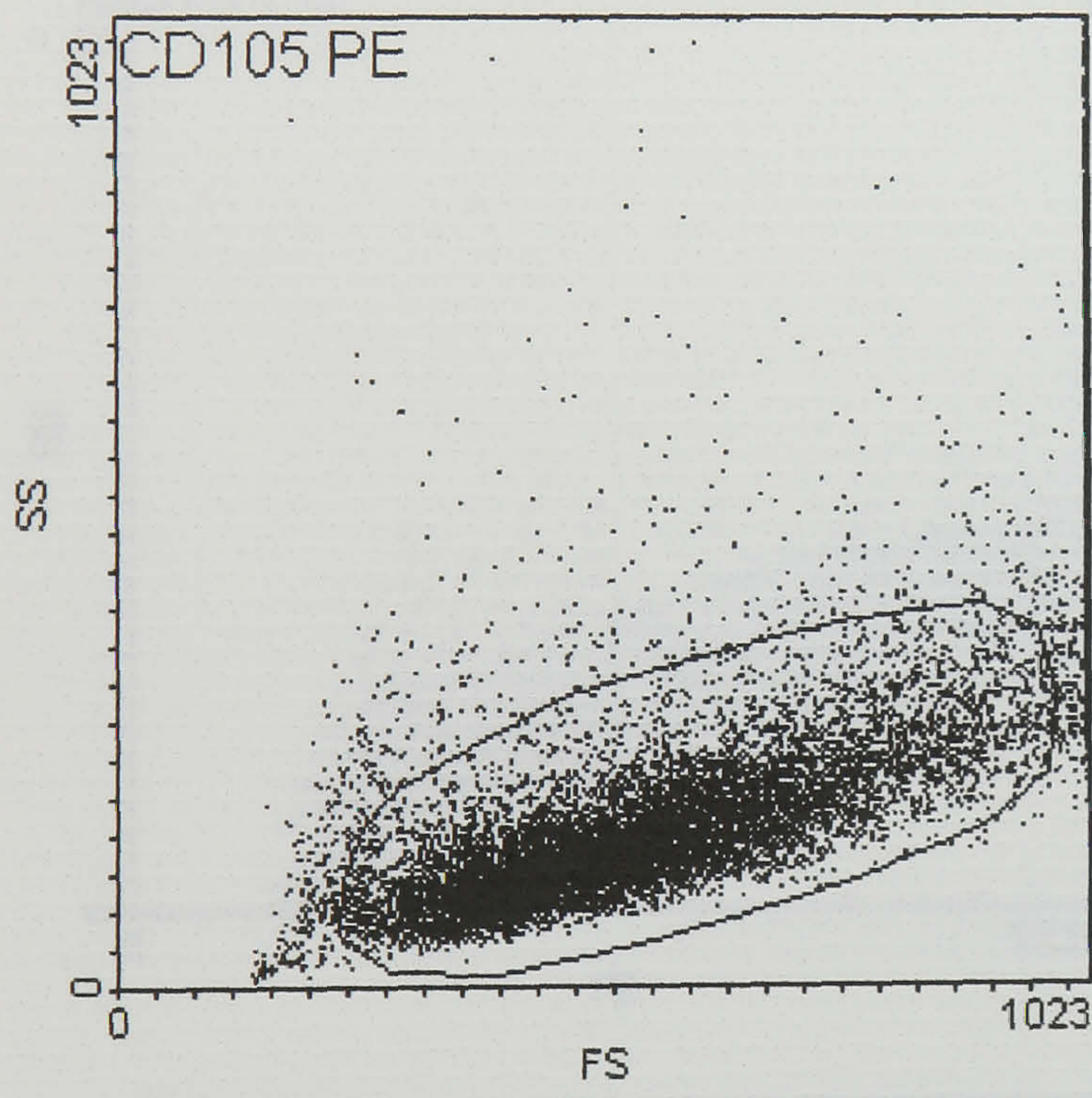
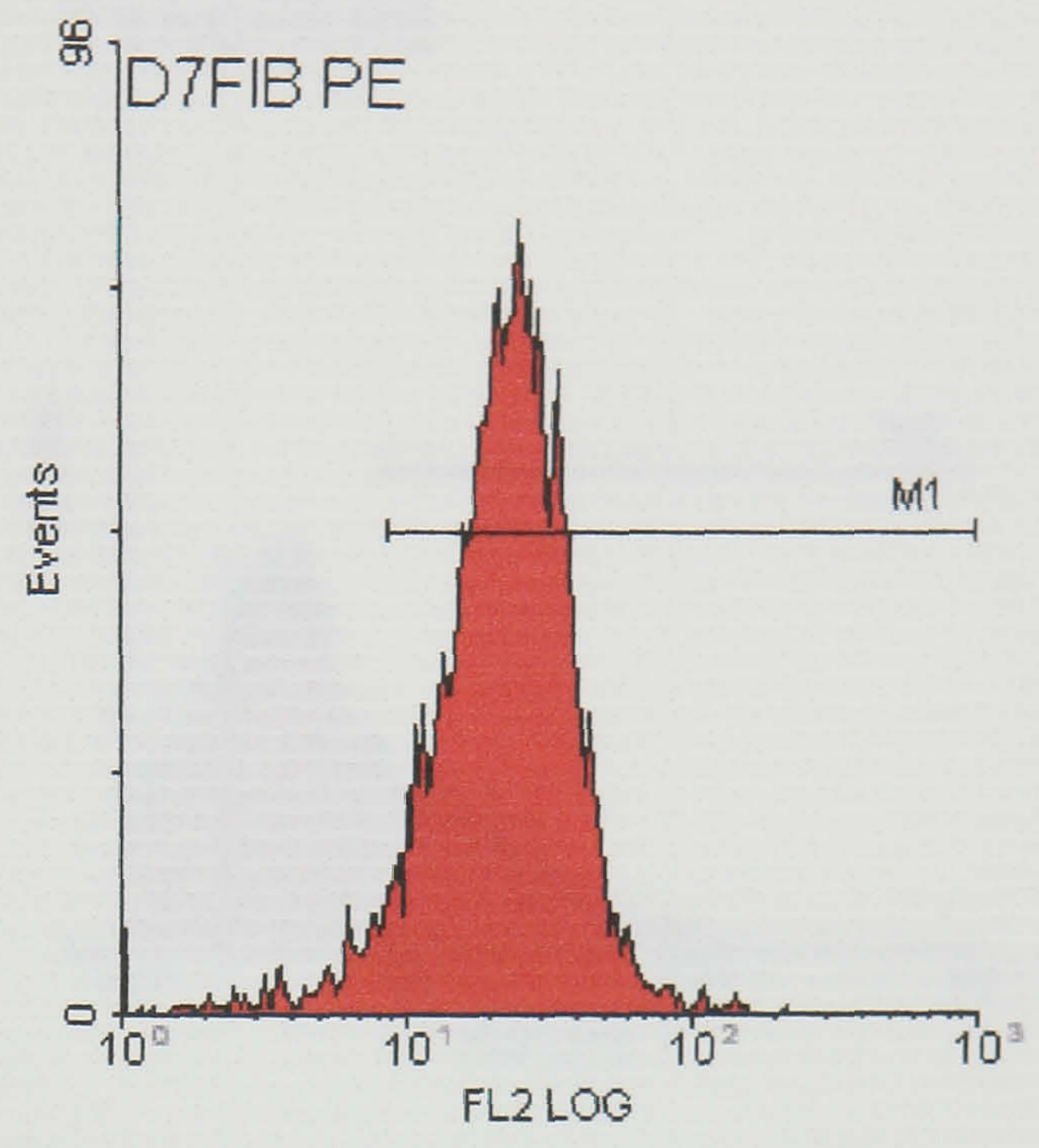
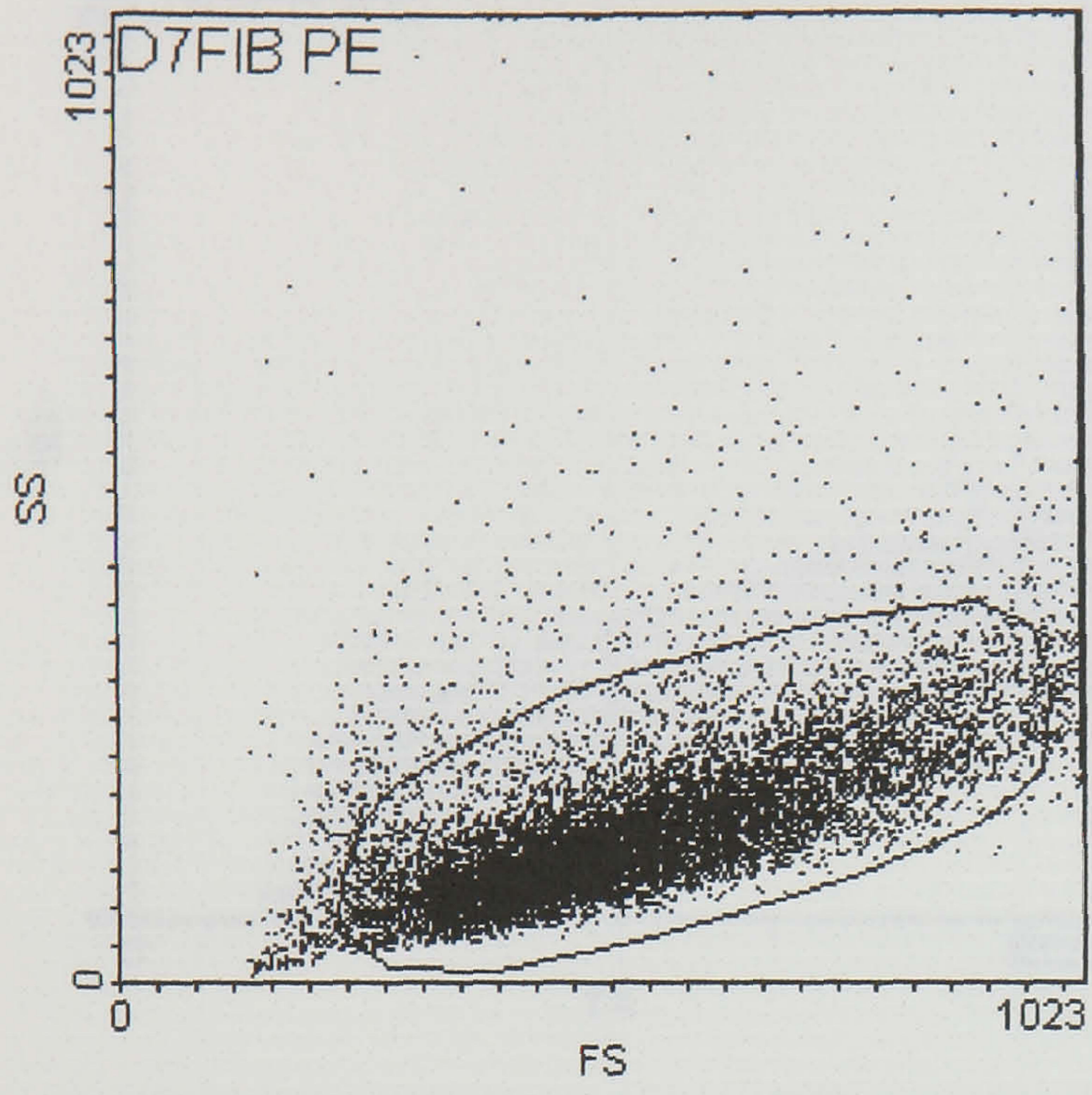


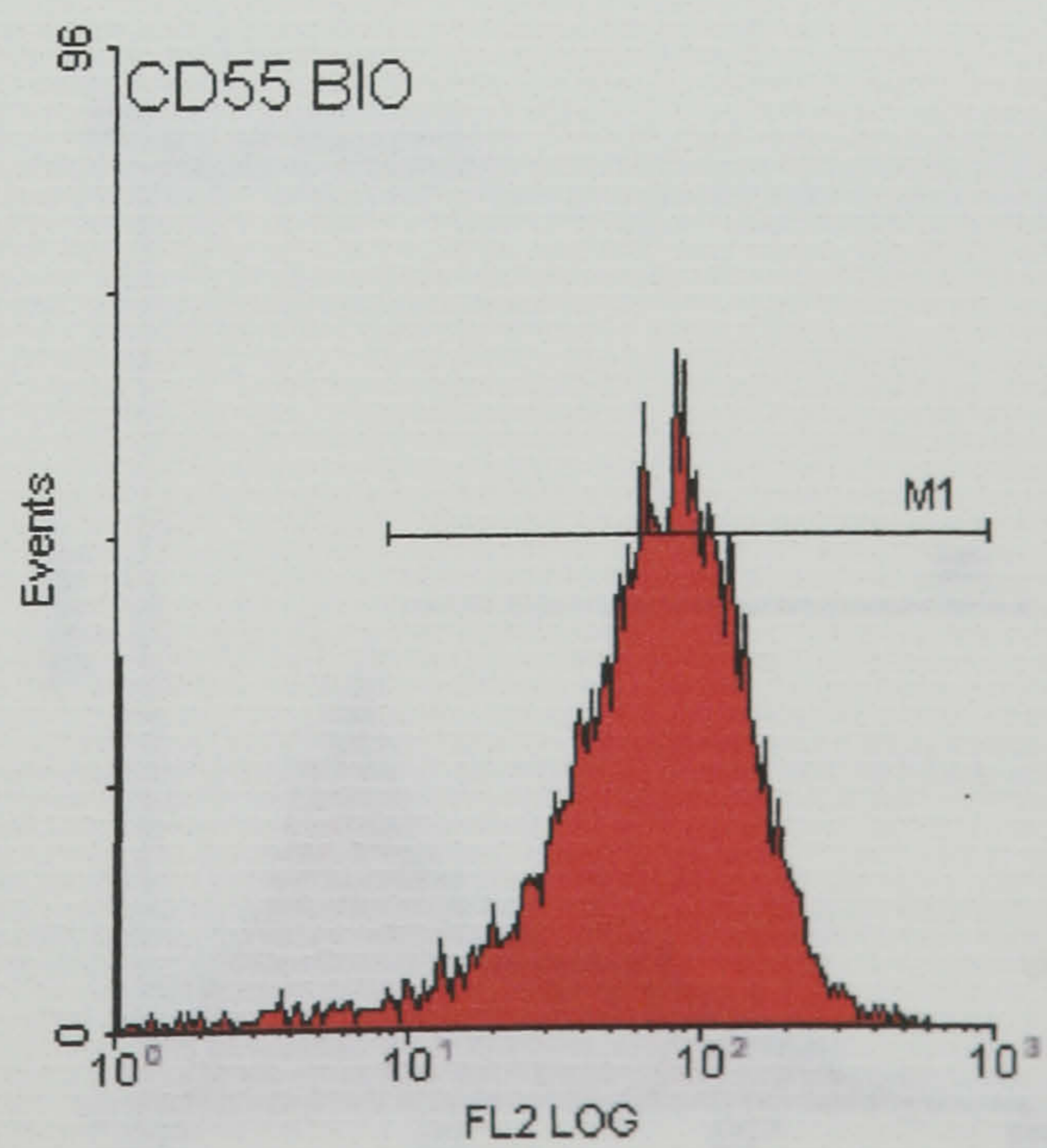
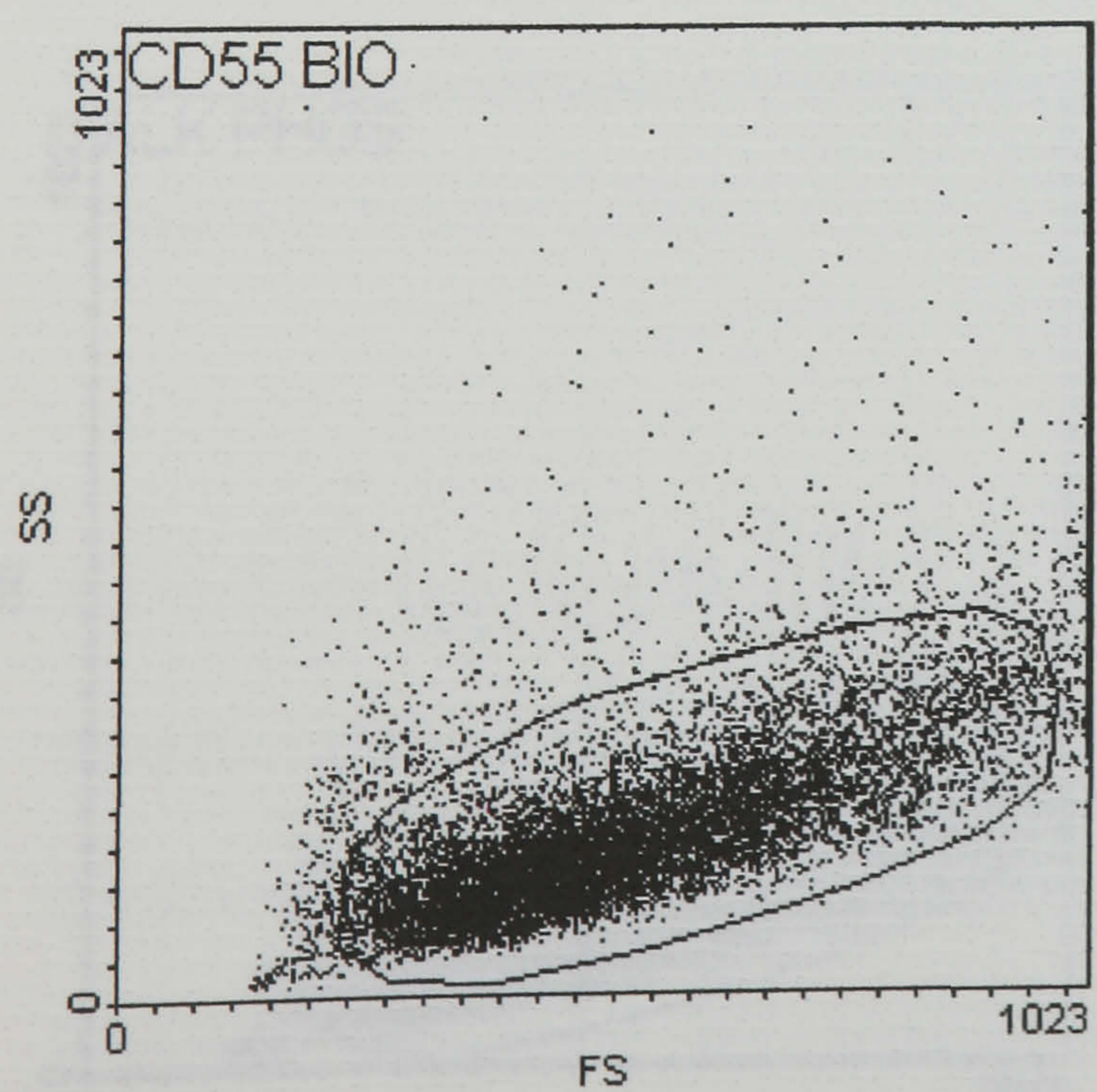
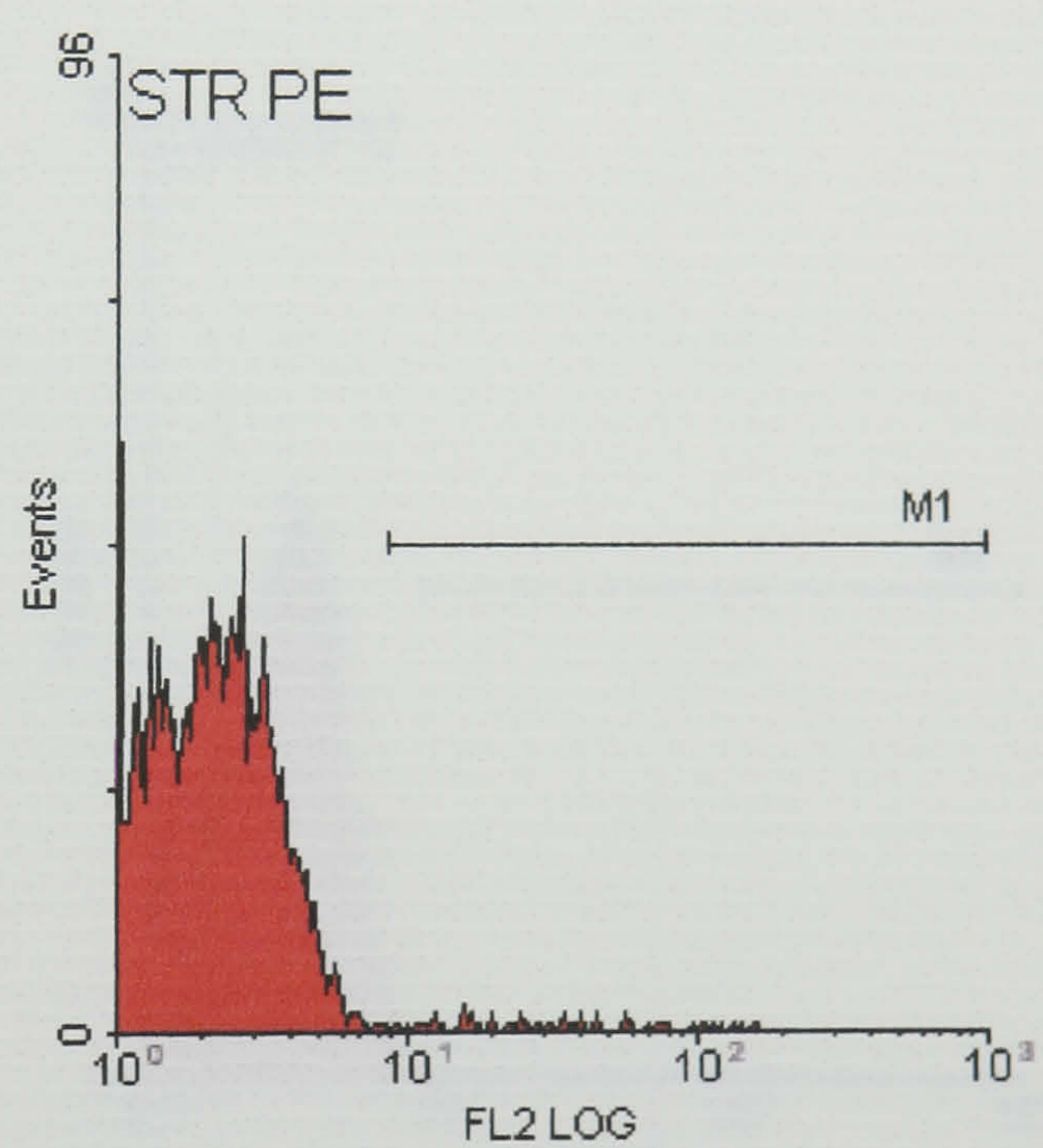
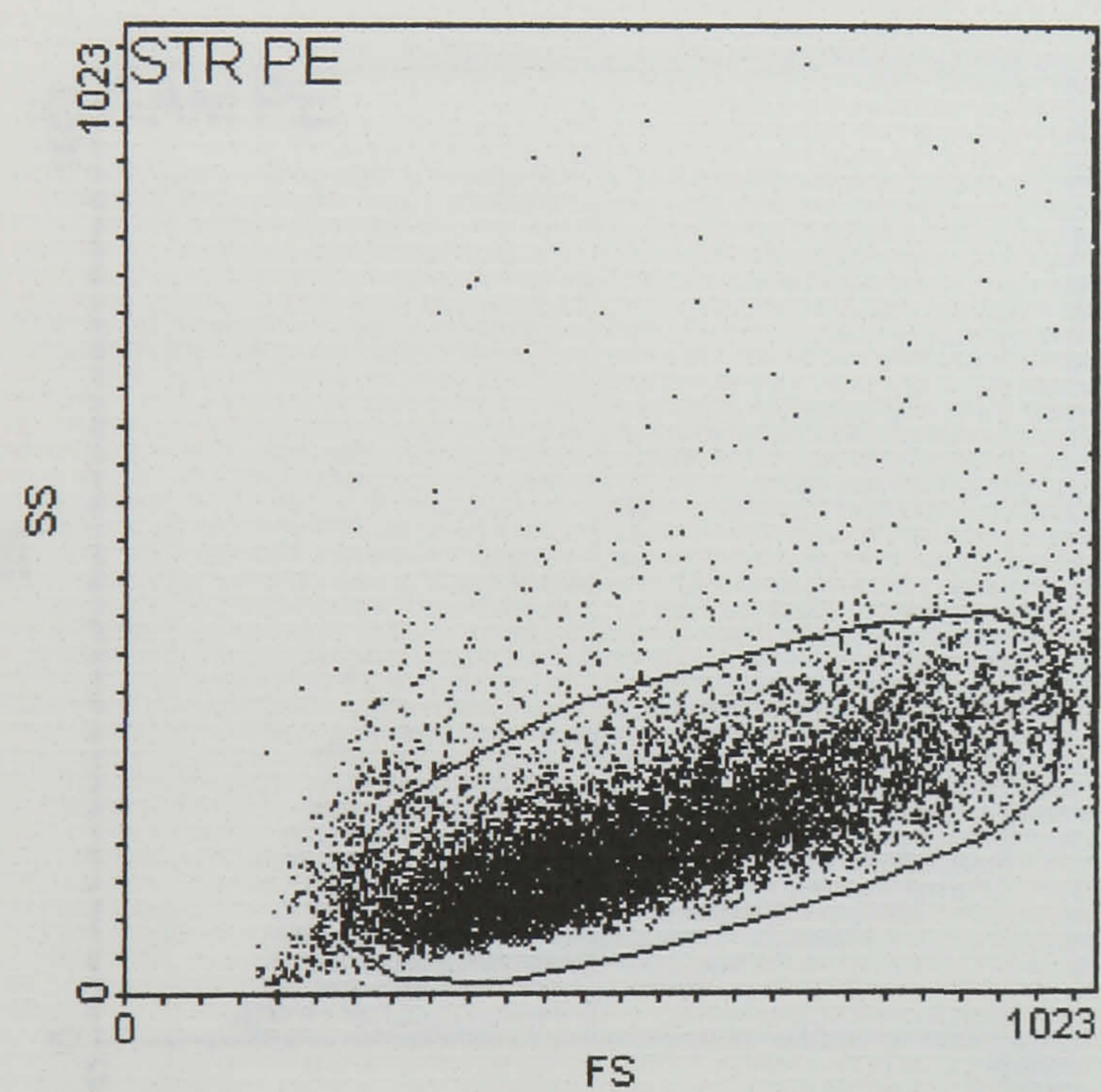
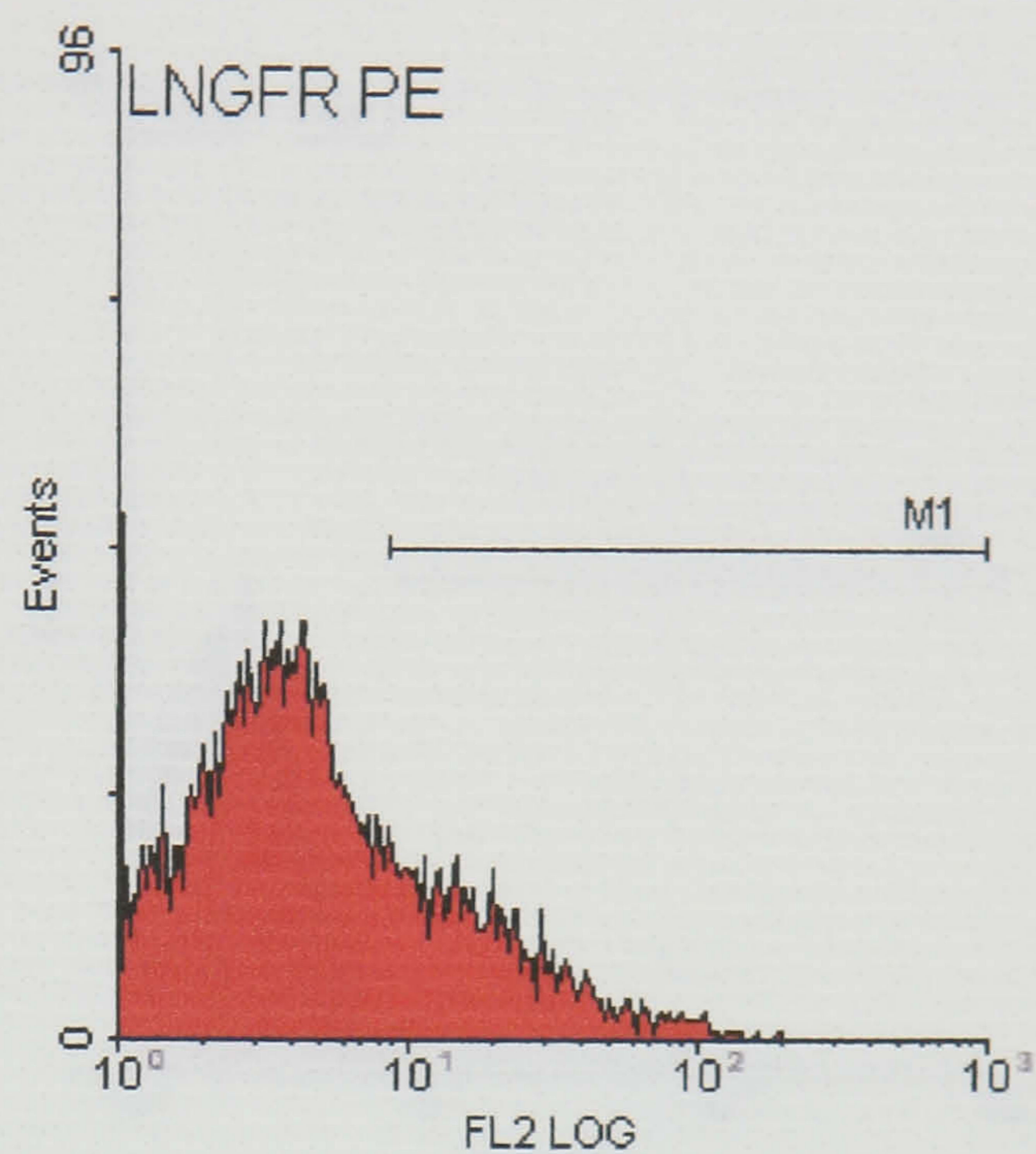
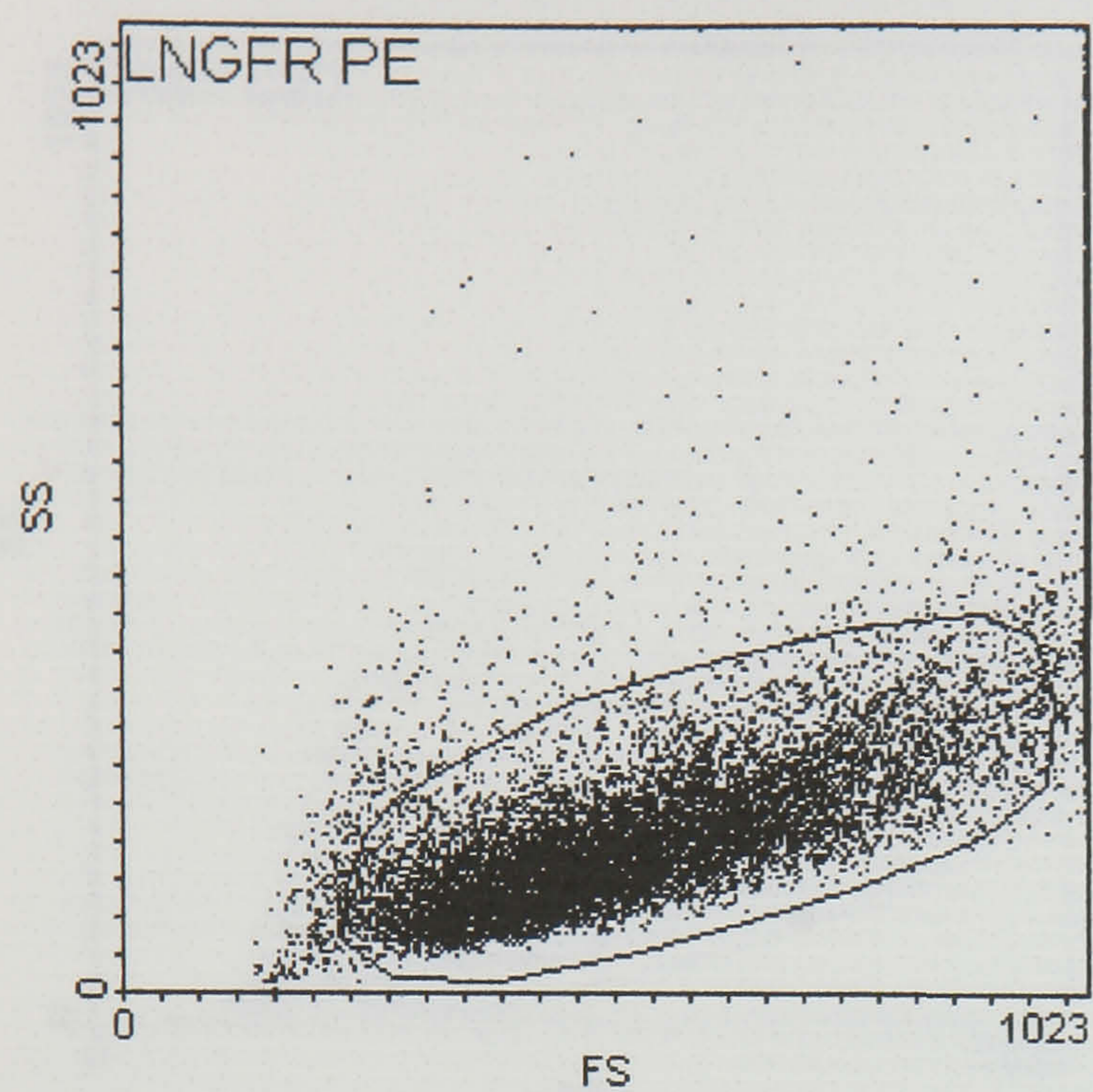
**Figure 4.6. Differentiation of human mesenchymal stem cells into neurogenic lineage.** Neurone-specific enolase (NSE) and tau are markers of the neurogenic lineage. The phase-contrast image shows cell morphology after 8 hours. NEG shows a negative control.

#### 4.4.3. Fluorescence activated cell sorting (FACS) analysis

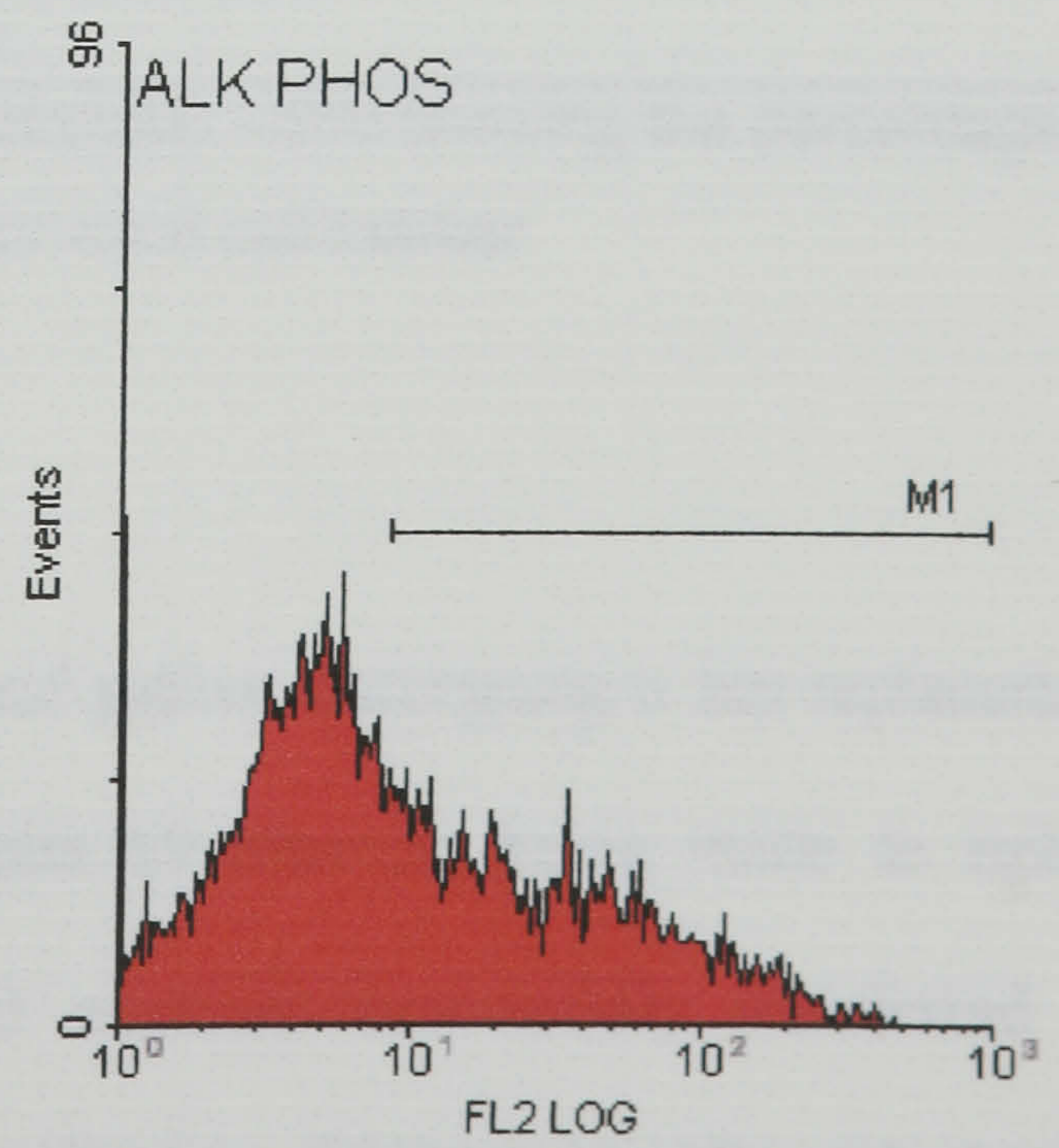
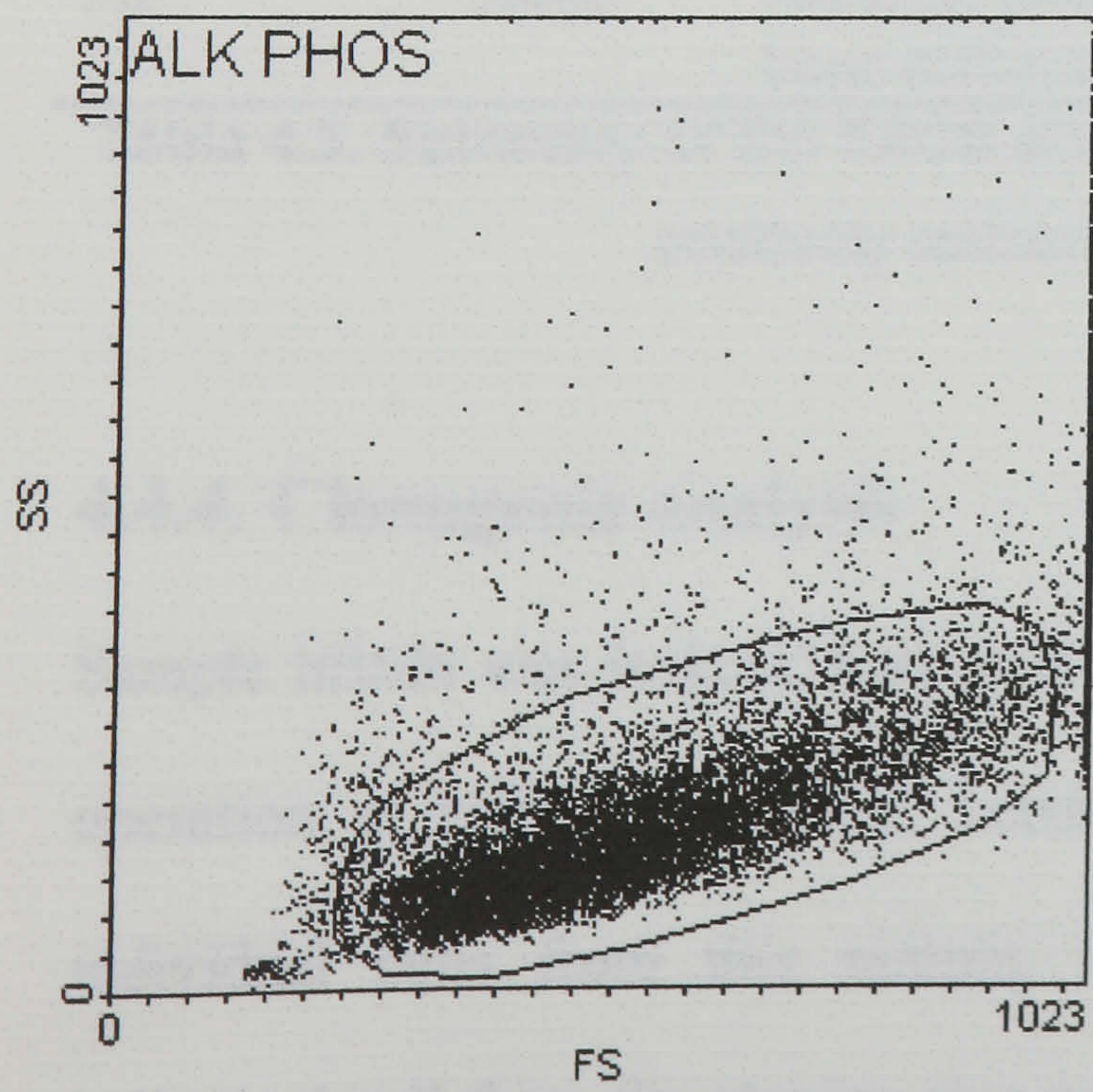
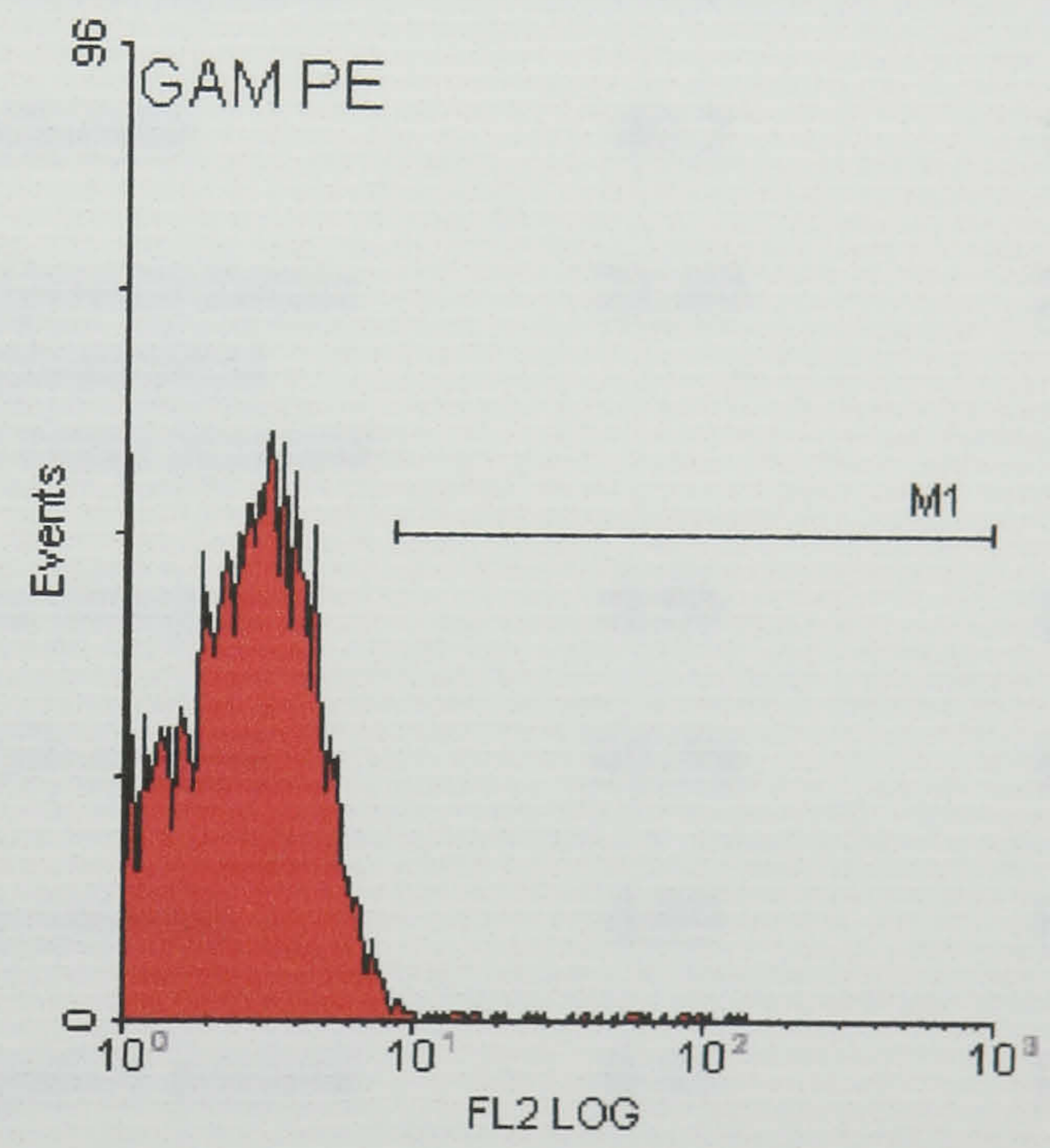
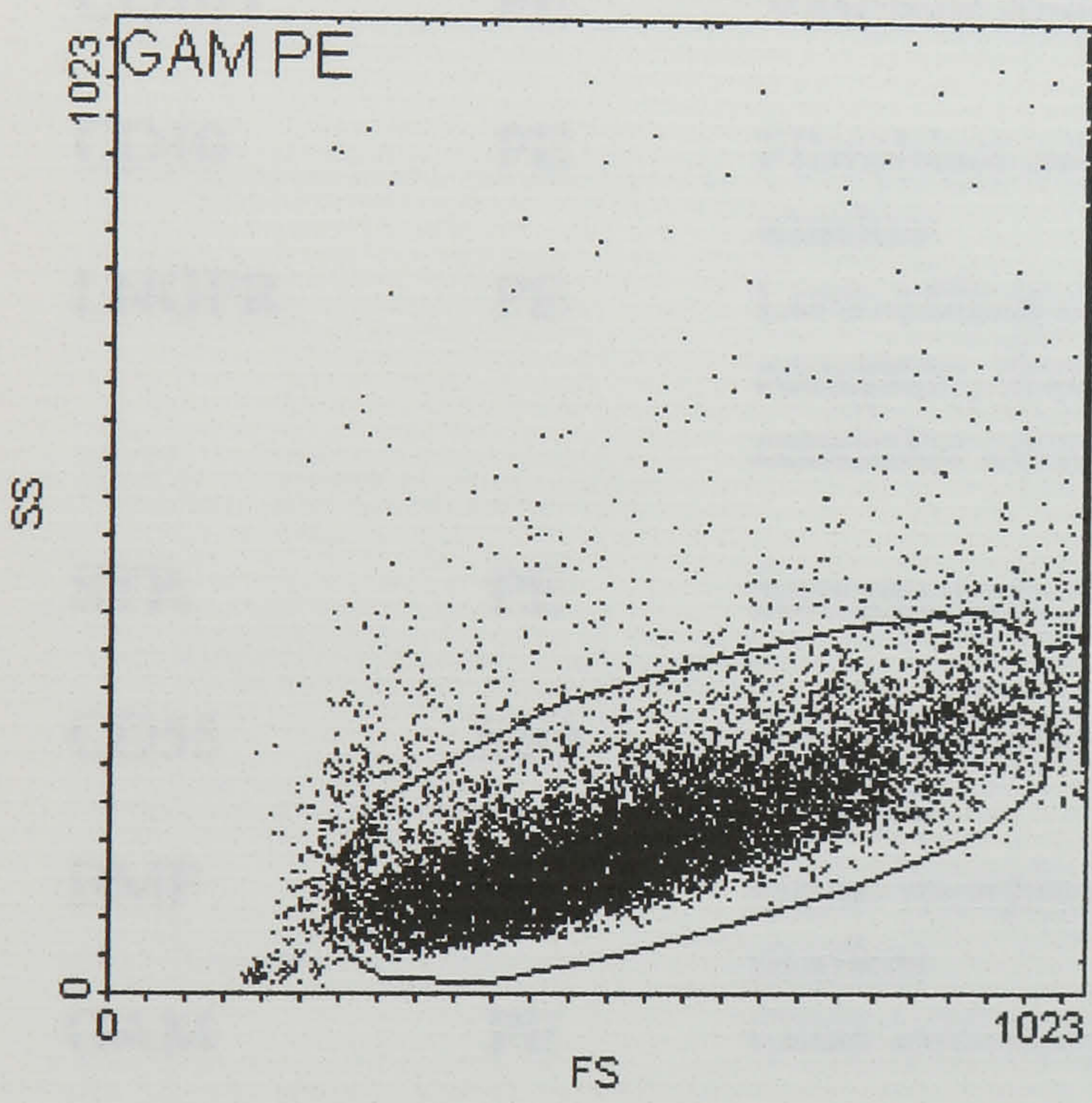
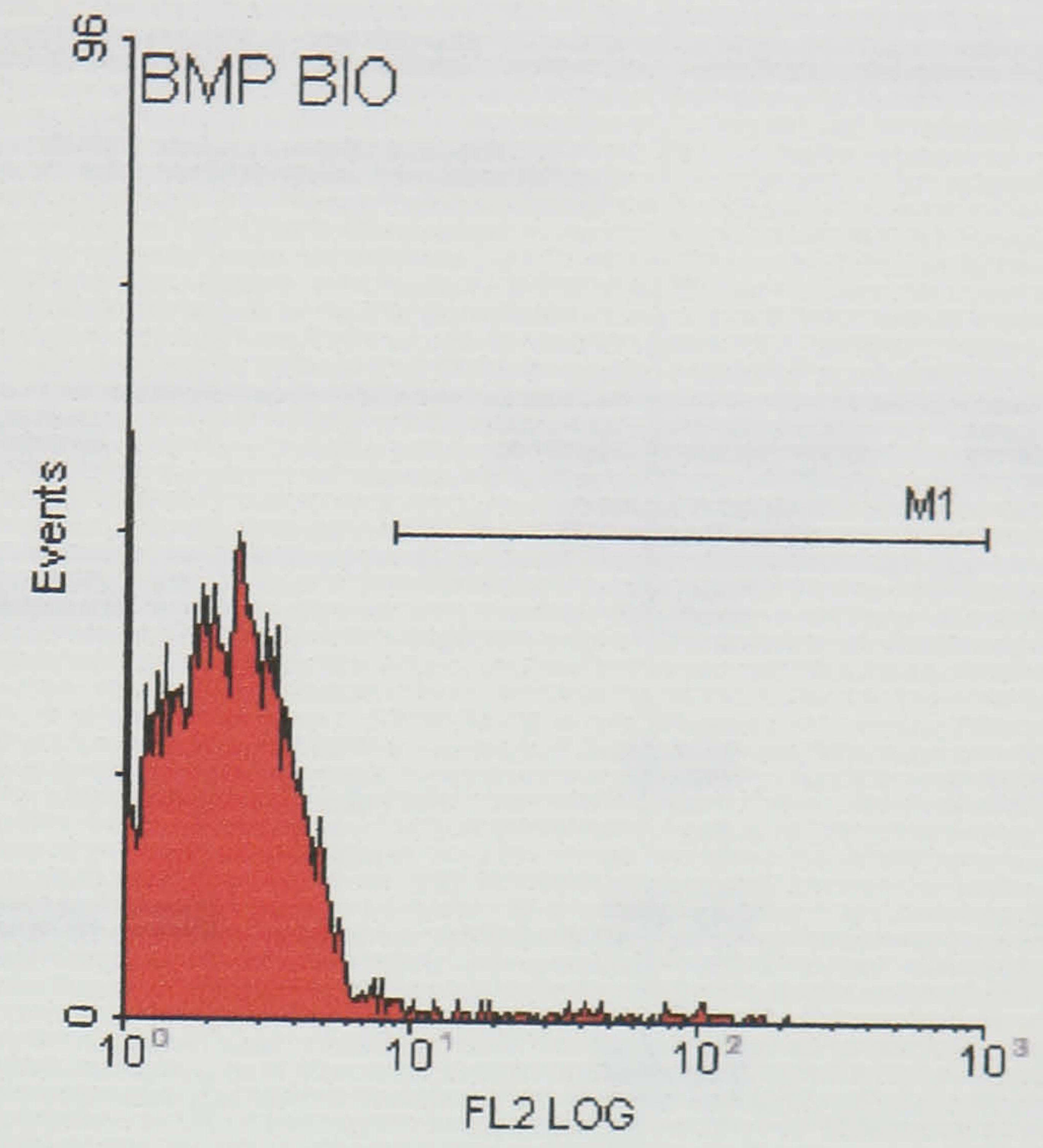
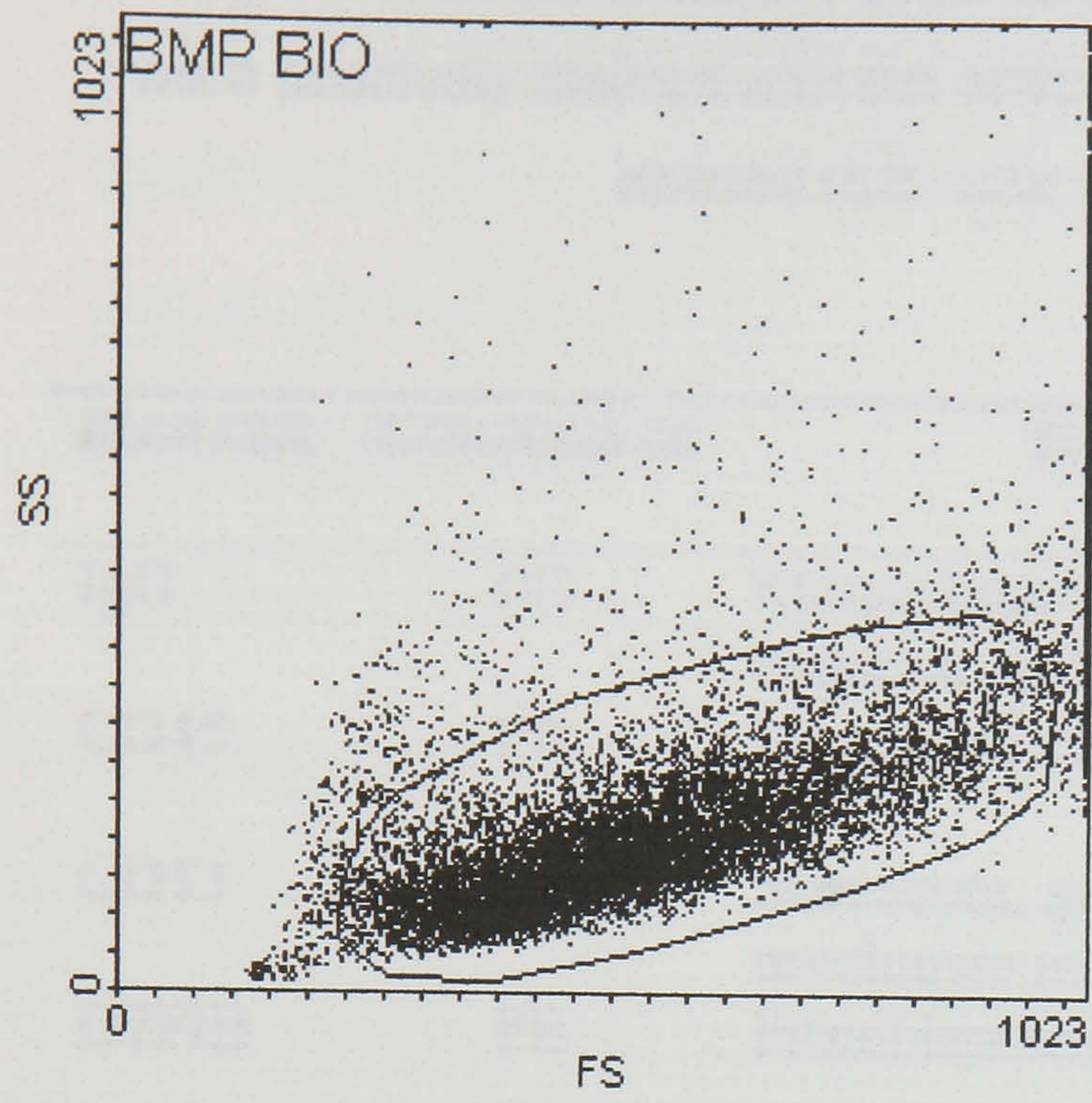
The analysis was carried out on sample hBM1 at passage 6. The dotplots for each marker were gated using the region shown in order to discount cell clumps and other artefacts. These regions were then used to make the corresponding histograms (Figure 4.7). The negative controls (IgGPE, GAMPE and STRPE) were used to establish the M1 region. Peaks that fell within this region were caused by fluorescently labelled cells. The percentage of cells within this region was calculated and the results for each marker can be seen in Table 4.3. All the cells were negative for CD45 and BMP, but positive for D7FIB, CD13, CD55 and CD105. CD10 was partly positive and partly negative, with the peak falling between the positive and negative regions. LNGFR and AP showed 2 peaks merged together on the histogram, falling on either side of the M1 region.











**Figure 4.7. FACS analysis of isolated hMSC.** Each of the pairs of dot-plot and histogram shows the marker used. Cells that fell within the M1 region of the histogram were positively stained with the antibody, and outside of that region were negatively stained (SS: side scatter; FS: forward scatter).

MARKER	CONJUGATE	SPECIFICITY	PERCENTAGE POSITIVE	PEAKS
IgG	PE	Mouse immunoglobulin G negative control	0.48	1
CD45	PE	Leukocyte marker	0.04	1
CD13	PE	Monocyte, granulocyte and myelocyte marker	99.99	1
D7FIB	PE	Fibroblast marker	95.47	1
CD105	PE	Vascular endothelial cell marker	98.14	1
CD10	PE	Fibroblast and granulocyte marker	40.2	1
LNGFR	PE	Low-affinity nerve growth factor receptor, found on adventitial reticular cells of the bone marrow	21.09	2
STR	PE	Streptavidin negative control	0.66	1
CD55	BIO	Synovial lining and fibroblast marker	97.58	1
BMP	BIO	Bone morphogenetic protein marker	0.99	1
GAM	PE	Goat anti-mouse negative control	0.51	1
AP	None	Alkaline phosphatase marker for bone differentiation	41.12	2

**Table 4.3. Summary of the FACS analysis results.** Table showing the percentage of positively stained cells for each cell marker.

#### 4.4.4. Clonogenic analysis

Sample hBM6 was isolated from a 40 year old patient undergoing a hip replacement operation at Nuffield Hospital, Leeds. Despite 60 attempts being made to isolate individual cells from this sample, only 11 attempts successfully transferred an individual cell from the cloning ring to a 96-well plate. Of these 11 cells, only 3 cells proliferated sufficiently for enough cells to be grown to necessitate transfer to larger

wells. Two wells of cells reached a 24-well plate stage and one well of cells reached a 12-well plate stage. However, at this point the cells stopped dividing and appeared to become senescent. By this time, the cells that had reached the 12-well stage had undergone 13 cell doublings to give over 8400 cells from the original single cell. Interestingly, these 13 cell doublings had occurred in 50 days, giving a CDT of only approximately 92 hours compared to the CDT of 151 hours calculated from the normally cultured cells. However, in order for differentiation analysis to be carried out, over 21 cell doublings would have been required to give sufficient cells for analysis. Furthermore, for investigation by FACS analysis, 10,000 cells were required per marker assessed, therefore a further 120,000 cells would have been required. Consequently, no further analyses could be carried out.

#### **4.5. Discussion**

Attempts were made to isolate hMSC using the same method that was adapted for isolating pMSC in Chapter 4. This method yielded fibroblast-type cells of a similar appearance to cells isolated by other groups. The CDT calculated for hBM1 was shown to gradually slow with prolonged culturing, however, the CDT calculated during the clonogenic assay was shown to be considerably shorter. Since the cells used in the clonogenic assay were from a younger patient (hBM6, Male aged 40) than those used in the differentiation assays (hBM1, Female aged 71) it is possible that hMSC from older patients have a reduced proliferation capacity and may show other age related degeneration such as reduced differentiation capacity. However, full details of patient medication or reasons for surgery were not available and may provide a further explanation. Conget *et al.* (1999) stated the CDT of hMSC to be approximately 33 hours, significantly faster than the findings here. However, their cells were sourced from healthy individuals undergoing bone marrow harvests for allogeneic

transplantation. Although the paper did not specifically state the age of the donors, it is likely that they would have been much younger than those used to obtain human bone marrow for this investigation. Consequently, this again could represent an age related effect on hMSC proliferation capacity.

The change in morphology and decreasing proliferation rates of the hMSC as they aged suggested that the cells might not be immortal, which is thought to be a prerequisite in the identification of any stem cells. This compared well with reports by others, which showed that hMSC, do not possess telomerase activity [Banfi *et al.*, 2002], which is thought to be an important enzyme involved in maintaining telomere length and consequently cell immortality. However, it may well be that the *in vitro* conditions used to grow the cells cannot sustain the immortal capacity of the cells and instead exert a selective environment that preferentially encourages the cells to become terminally differentiated. This also associated well with findings of the FACS analysis of the cells (discussed later) using an antibody to alkaline phosphatase (AP). AP is a marker that is thought to be specific to osteogenic cells. The histogram from the FACS analysis showed two peaks, one positive and one negative. This means that the cells were a mixed population of AP<sup>+</sup> and AP<sup>-</sup> cells. This could be interpreted as being the result of the cells initially being AP negative, but gradually becoming positive as the cells aged, i.e. hMSC gradually differentiated into osteogenic cells *in vitro*. It may be that conditions for growing and maintaining cells in their undifferentiated state need to be improved beyond the standard techniques and conditions currently implemented by the majority of groups working on hMSC. Also, the different morphologies of the cells could account for the frequently described heterogeneity of the cells when they are initially isolated from the bone marrow. The different cells seen could all be hMSC at different stages of differentiation as shown in Figure 1.11 earlier, which describes the

progression of the cells from long-term replicating MSC through to mature terminally differentiated cells.

A number of attempts to differentiate the cells were made using biochemical supplements to complete medium and serum-free medium. The methods were derived from Stem Cell Technologies, Wakitani *et al.* (1995), Woodbury *et al.* (2000), and Jones *et al.* (2002). The adipogenic differentiation medium used consisted of an adipogenic differentiation supplement to Mesencult, the hMSC growth medium produced by Stem Cell Technologies. Although the contents were not stated, it is likely to have contained insulin as a major differentiation factor as insulin promotes the storage of glucose as glycogen and consequently the production of fatty acids and therefore lipids.

The myogenic differentiation medium used 5-azacytidine to alter gene transcription as described in Section 3.5, however; again why myogenic differentiation would occur rather than differentiation into any other lineage is unclear.

The chondrogenic differentiation medium consisted of serum-free medium supplemented with  $100 \mu\text{g.ml}^{-1}$  sodium pyruvate,  $40 \mu\text{g.ml}^{-1}$  L-proline,  $50 \mu\text{g.ml}^{-1}$  ascorbate-2-phosphate,  $1 \text{ mg.ml}^{-1}$  bovine serum albumin (BSA), and  $1\times$  insulin transferrin sodium selenite (ITSS) to replace the FCS and then  $100 \text{ nM}$  dexamethasone and  $10 \text{ ng.ml}^{-1}$  transforming growth factor beta-3 ( $\text{TGF}\beta 3$ ) as the differentiation agents. The more critical part of this method was culturing the cells in a three-dimensional pelleted micromass culture rather than the 2-dimensional culture system used to attempt the chondrogenic differentiation of pMSC in Chapter 3. It is known that when chondrocytes are cultured in a 2-dimensional environment they rapidly de-differentiate

and stop producing a collagen matrix. Consequently, by trying to differentiate the hMSC whilst in a 3-dimensional micromass pellet, this method would be more likely to work than others where the cells are cultured in a tissue culture flask.

Neurogenic differentiation medium used  $\beta$ -mercaptoethanol as a reducing agent, as described in Section 3.5, however, again why it would induce neurogenic differentiation over any other lineage is unclear.

The success of three of the four differentiation assays, along with the demonstration of increased AP expression with cell age suggests that the isolated cells were in fact hMSC. The failure of the chondrogenic assay to give cells that produced collagen II may well be related to the age of the patient from which the cells were isolated as a number of groups have reported decreased cell proliferation and differentiation capacity as donor age increases [Caplan, 1994; Digorolamo *et al.*, 1999; Mendes *et al.*, 2002]. Also, the cells used for this assay were at passage 8, therefore the cells had undergone more cell doublings than those used for previous differentiation assays and may have had a reduced differentiation capacity as a result of this. However, despite these findings, it is still possible that the bone marrow contains multiple cell precursors and the differentiation media simply select for these cells.

The results of the FACS analysis were comparable to those discussed in Jones *et al.* (2002), having a phenotype of CD45<sup>-</sup>, CD13<sup>+</sup>, D7FIB<sup>+</sup>, CD105<sup>+</sup>, CD10<sup>-</sup>, LNGFR<sup>+/-</sup>, CD55<sup>+</sup>, BMP<sup>-</sup>, and AP<sup>+/-</sup>. Caplan & Haynesworth (2000) described CD105 as being specific to hMSC. It has since been found to also be present on vascular endothelial cells, and therefore is not specific to hMSC. However, since the cells were shown to be positively stained for this antigen, it further demonstrated another indication of the

hMSC phenotype. It is thought that CD10 is present on hMSC when first isolated, but that it is gradually lost with subsequent cell ageing. This associated well with results here that 40% of cells were positive for the marker as the cells used for this analysis were at passage 6. The histogram for LNGFR also showed two peaks, which indicated two populations of cells, one with and the other without this marker being present on the cell surface. This also suggested that this marker is gradually lost as the cell population ages. The alkaline phosphatase (AP) marker also produced two peaks on the histogram. AP is known to be specific to osteogenic cells, and the appearance of two peaks suggested that AP expression gradually increased with cell age. This indicated that the osteogenic lineage may be the default pathway for hMSC, i.e. if the cells do not receive signals to differentiate into an alternate lineage, they become osteogenic cells. Conversely, it may be that current culture conditions are selective for the osteogenic lineage or provide some form of signalling that encourages the cells to become osteogenic. All of the results of the FACS analysis were analogous with the findings of Jones *et al.* (2002). However, in their work, the FACS analysis had been carried out on cell populations which had been isolated and grown from a single cloned cell. Therefore, comparable results here gave further support to the prospect that the cells isolated could be mesenchymal stem cells.

The problem of the cells gradually differentiating into cells of the osteogenic lineage could also have been the reason behind the failure of the clonogenic assay. Although some of the single cells isolated went on to proliferate, they all became senescent before sufficient cell numbers were grown to assess their differentiation capacity. The low number of single cells that proliferated combined with other observations indicated that below a certain cell density, hMSC were not receiving sufficient cell signals and other factors to encourage their proliferation. Although some of these factors would have

been provided by the conditioned growth medium used to grow the cells, permitting some of the cells to proliferate, clearly the signals present were not sufficient for many of the cells. The presence of these factors could be increased by using a higher proportion of used cell growth medium in the conditioned medium used to feed the cells. Alternatively, other means could be used to support the cell growth more effectively such as using an irradiated hMSC feeder layer to grow the cells on. Also, as previously mentioned, the problem may lie with some other part of the culture conditions which may actively encourage the cells to become terminally differentiated, leading to their rapid senescence before any further investigations could be carried out.

The large numbers of cells isolated and cryopreserved during the course of the investigation were stored as a cell bank for future experiments. For example, the varied age of the patients from whom the cells were harvested would enable investigation of whether the proliferation and/or differentiation capacities of the cells are affected by the age of the patient. The cells would also be a cell source for future clonogenic studies and reseeded experiments.

Clearly, any future investigation of hMSC must first focus on providing the optimal culture conditions to consistently maintain their multipotential differentiation and immortality capacities. Some researchers have suggested that certain lots of foetal calf serum are more effective at supporting MSC [Lennon *et al.*, 1996]. Also, a chemically defined medium could be established, as described in Section 3.5. Furthermore, an irradiated feeder layer may assist in providing the correct chemical signals to maintain MSC in an undifferentiated state. Other experiments could investigate the use of mechanical stimulation to differentiate hMSC as previously discussed in Section 3.5.



In conclusion, putative hMSC were successfully isolated using a common method frequently described in the literature. These cells were morphologically comparable to those of others and their differentiation into multiple cell lineages was successfully achieved. Furthermore, FACS analysis of the cells enabled a direct positive comparison with clonally isolated hMSC as described by Jones *et al.* (2002), further adding to the evidence that the cells were hMSC. However, an attempt to grow the cells in a clonogenic manner in order to show that cells from a single source were capable of differentiating into multiple cell lineages was unsuccessful as the cells became senescent before their differentiation capacity could be investigated. As a result of these findings, the cells were confidently, though not conclusively, believed to be hMSC and their potential as a cell source for use in tissue engineering an aortic heart valve was investigated.

## **CHAPTER FIVE:**

# ***Biochemistry and Biocompatibility of Decellularised Valve Leaflets***

# CHAPTER 5:

## *Biochemistry and Biocompatibility of Decellularised Valve Leaflets*

### **5.1. Introduction**

The basic principle of tissue engineering involves seeding cells into a matrix scaffold which then interact to produce a viable replacement tissue. However, there are a number of ways of creating a suitable matrix scaffold. Essentially, the matrix can be one of two varieties: either synthetic or natural. Synthetic scaffolds can be made from a number of materials, such as polyglycolic acid (PGA), fibrin and collagen. However, whilst these have their advantages in that they can be readily mass produced and can be highly defined in composition, they frequently lack the mechanical strength found in natural tissues. Developing a heart valve tissue substitute from a naturally occurring decellularised biological matrix is an alternative strategy that would provide a ready-made scaffold, which already possesses optimal mechanical and biochemical qualities and characteristics. There is also an immediate distinct advantage in that there should be minimal host response to a naturally occurring extracellular matrix provided decellularisation is adequately carried out [Badylak, 2002]. Methods for producing acellular matrices typically use a combination of enzymes and detergents to remove the cells from an allogeneic or xenogeneic tissue [Bader *et al.*, 1998; Booth *et al.*, 2002]. This approach could offer an unlimited source of valve matrices, which could then be reseeded with autologous cells from the valve recipient to give a non-immunogenic living product. The difficulty with this technique, however, lies in the decellularisation process: all of the cells must be completely removed without damaging the tissue matrix so that mechanical and structural properties are fully maintained. Any chemicals used in

this process must be completely removed to ensure biocompatibility with cells and host tissue upon implantation. Remaining cell proteins could result in rejection of the tissue, and remaining fragments could lead to calcification, resulting in rapid stiffening and irreparable damage to any implanted tissues [Dohmen *et al.*, 2002b].

A patented method has been developed at the University of Leeds for the decellularisation of porcine aortic heart valve tissue using the detergent sodium dodecyl sulphate (SDS). Histological analysis has shown matrix components to remain intact [Booth *et al.*, 2002], whilst mechanical testing has demonstrated that the SDS treatment produced valves with equal strength, competence under physiological pressures (120mmHg) and greater extensibility than untreated fresh valves [Korossis *et al.*, 2002]. However, histological assessment alone is only a qualitative means of showing that cells have been completely removed whilst the matrix is maintained. Consequently, it was deemed necessary to further confirm these findings by quantifying the collagen, glycosaminoglycan (GAG) and DNA content of the tissues before and after decellularisation by the use of quantitative biochemical assays.

Collagen exists in at least 12 different forms and is the major protein in the tissue matrix. Types I, II, and III are the most abundant. The amino acids in the chains are predominantly glycine, proline and hydroxyproline. Hydroxyproline is critical for collagen stability and consequently it can be used to determine the levels of collagen in tissues using a conversion factor of 7.14 [Harding & Wesley, 1968]. Glycosaminoglycans (GAGs) are also an important component of the valve leaflet as they make up the large majority of the spongiosa layer [Schoen, 1999]. They are long, unbranched, highly negatively charged polysaccharides with extended conformation, high viscosity and low compressibility; properties that confer excellent lubrication and

shock absorbance. This enables the fibrosa and ventricularis layers to slide easily over one another and also absorb and dissipate the forces endured by the leaflet during different stages of the cardiac cycle. The GAG content can be assessed by colourimetrically measuring the amount of sulphated proteoglycans present in the tissue. Furthermore, analysis of the DNA content of the leaflets before and after decellularisation would further indicate that all cellular material had been removed during the process. This can be carried out by using a fluorescent marker specific to DNA.

It was important to determine whether the decellularised matrix would induce an inflammatory and/or immune response once implanted *in vivo* from either remaining cell debris or remaining reagents used in the decellularisation process. Many bioprosthetic valve replacements calcify after extended periods *in vivo*, which leads to their deterioration and eventual failure [Butany & Leask, 2001]. In the case of these implants, it is thought that the gluteraldehyde cross-linking of cellular elements provides focal points in the tissue from which calcification can develop and spread throughout the tissue. It was possible that inadequate removal of cellular fragments during the decellularisation process and/or failure to wash out the reagents used could also lead to calcification. However, if it is carried out adequately, no calcification should occur. Similarly, only a minimal immunogenic response should occur, as the constituent components of porcine heart valve tissue are all common to human tissues.

## **5.2. Aims & objectives**

The aims of this chapter were to analyse the major biochemical components and assess the biocompatibility of a decellularised porcine valve tissue matrix for use as a scaffold for stem cell seeding. The specific objectives were to a) determine the levels of

collagen, GAGs and DNA both before and after decellularisation of valve tissue; and b) implant the decellularised porcine tissue matrix into mice and determine the calcification and inflammatory/immunogenic potential.

### **5.3. *Materials & methods***

#### **5.3.1. Biochemical analysis**

##### **5.3.1.1. Acid hydrolysis of tissues<sup>1</sup>**

Fresh and decellularised tissues were hydrolysed using strong acid in order to break down the tissue so that the hydroxyproline could dissolve into solution and be measured by means of a colourimetric assay.

**Materials:** Porcine hearts were obtained from a local abattoir within 4 hours of slaughter. Hydrochloric acid and sodium hydroxide were purchased from VWR International. An Edwards Freeze Drier Modulyo was used to freeze-dry tissues. The bench-top autoclave (Duromatic Pressure Cooker) was purchased from SLS.

**Method:** Heart valves were excised and the valve leaflets dissected out. Leaflets were decellularised as described in Section 2.8.1. Whole fresh (n=6) and decellularised (n=6) leaflet samples (approx. 2×1cm) were freeze-dried to constant weights (-45 °C, vacuum) and the dry weights determined prior to being finely chopped using a scalpel and placed in 5 ml of 6 M HCl. The samples were then autoclaved for 2 hours in a bench top autoclave. After cooling, an equal volume of 6 M NaOH was added to each sample solution and the pH was finely adjusted to 7.0 using 1 M NaOH. Samples were then centrifuged for 20 minutes at 2000 g and the supernatant aliquoted and stored at -20 °C.

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<sup>1</sup> The biochemical assays were carried out by Ms Natalie Beveridge, undergraduate student, under my supervision.

### 5.3.1.2. Enzymatic digestion of tissues

Tissue samples for the DNA assay had to be digested enzymatically as strong acid would destroy the DNA. This then enabled the DNA to go into solution where it was free to react with a colourimetric marker.

**Materials:** All reagents were purchased from Sigma-Aldrich except hydrochloric acid, which was obtained from VWR International. The water-bath was purchased from Fisher Scientific. Papain solution was made from 125  $\mu\text{g}\cdot\text{ml}^{-1}$  papain in sterile PBS with 5 mM cysteine-HCl and 5 mM sodium ethyldiaminotetraacetic acid adjusted to pH 6.0 using hydrochloric acid.

**Method:** Papain solution (5 ml) was added to weighted and recorded freeze-dried leaflets (fresh, n=6; decellularised, n=6) and incubated for 16 hours in a 60 °C water-bath. Samples were centrifuged for 20 minutes at 2000 g and the supernatant aliquoted and stored at -20 °C.

### 5.3.1.3. Hydroxyproline assay

**Materials:** Trans-4 hydroxyproline, *p*-dimethylaminobenzaldehyde and chloramine T were purchased from Sigma-Aldrich. All other reagents were purchased from VWR International.

Hydroxyproline buffer solution consisted of 13.3 g citric acid, 3.2 ml glacial acetic acid, 32 g sodium acetate, 9.1 g sodium hydroxide, 80 ml propan-1-ol made up to 300 ml with distilled water. This solution was then adjusted to between pH 6.0 and pH 6.5 using 0.2 M sodium hydroxide before being adjusted to a final volume of 400 ml using distilled water.

Oxidizing solution consisted of 1.41 g chloramine T dissolved in 100 ml of distilled water.

Ehrlich's reagent was made by adding 7.5 g *p*-dimethylaminobenzaldehyde to 30 ml propan-1-ol and 13 ml 62 % (v/v) perchloric acid. This was then made up to 50 ml using distilled water.

**Method:** This method was derived from Edwards & O'Brien (1980). Standard solutions of 0, 2, 4, 6 and 8  $\mu\text{g}\cdot\text{ml}^{-1}$  of hydroxyproline dissolved in hydroxyproline buffer were prepared. Standard solutions (50  $\mu\text{l}$ ,  $n=6$ ) and test solutions (50  $\mu\text{l}$ ,  $n=6$ ) were aliquoted into wells of a flat-bottomed 96-well plate. Oxidising solution (100  $\mu\text{l}$ ) was then added to each test well and the plate was gently shaken for 5 minutes. Ehrlich's solution (100  $\mu\text{l}$ ) was then added to each well and mixed thoroughly, before the plate was incubated at 60 °C for 45 minutes. The absorbance of the wells was then measured on a plate reader set at 570 nm. A curve of hydroxyproline ( $\mu\text{g}\cdot\text{ml}^{-1}$ ) versus absorbance was constructed using the standards and the hydroxyproline content ( $\mu\text{g}\cdot\text{ml}^{-1}$ ) of the test samples interpolated from the standard curve. The levels of hydroxyproline ( $\mu\text{g}\cdot\text{ml}^{-1}$ ) were divided by their dry weight (mg) of each tissue sample and data for each sample expressed as  $\mu\text{g}\cdot\text{mg}^{-1}$ .

#### 5.3.1.4. Sulphated proteoglycan assay

**Materials:** All reagents were purchased from Sigma-Aldrich except ethanol, which was obtained from VWR International.

Phosphate buffer was made by adding 137 ml of 0.1 M sodium di-hydrogen phosphate to 63 ml of 0.1 M di-sodium hydrogen phosphate.

Dimethylene blue dye solution was made by dissolving 16 mg of 1,9-dimethylene blue in 5 ml ethanol. This was then added to 2 ml formic acid and 2 g sodium formate before being made up to 1000 ml using distilled water.

**Method:** This method was derived from Farndale *et al.* (1986). Standard solutions of 0, 3.125, 6.25, 12.5, 25, 50, 100, 150 and 200  $\mu\text{g}\cdot\text{ml}^{-1}$  chondroitin sulphate were made by



dissolving chondroitin sulphate in phosphate buffer. Standard or test solution (80  $\mu\text{l}$ ,  $n=6$ ) and dimethylene blue dye solution (500  $\mu\text{l}$ ) were added to 1 ml glass cuvettes and the absorbance read at 525 nm on a spectrophotometer within a fixed time of 60 seconds. A curve of sulphated proteoglycans ( $\mu\text{g}\cdot\text{ml}^{-1}$ ) versus absorbance was constructed using the standards and the sulphated proteoglycan content ( $\mu\text{g}\cdot\text{ml}^{-1}$ ) of the test samples interpolated from the standard curve. The levels of sulphated proteoglycans ( $\mu\text{g}\cdot\text{ml}^{-1}$ ) were divided by their dry weight (mg) of each tissue sample and data for each sample expressed as  $\mu\text{g}\cdot\text{mg}^{-1}$ .

#### 5.3.1.5. Fluorometric DNA assay

**Materials:** All reagents were purchased from Sigma-Aldrich, except hydrochloric acid, which was obtained from VWR International. Plastic cuvettes were purchased from Fisher Scientific.

Hoechst dye was stored as a stock solution at 1  $\text{mg}\cdot\text{ml}^{-1}$  in distilled water. A working solution was made immediately before use by diluting this solution to 0.1  $\mu\text{g}\cdot\text{ml}^{-1}$  in dye buffer.

Dye buffer consisted of 10mM Tris, 1 mM sodium ethyldiaminotetraacetic acid, and 0.1 M sodium chloride, adjusted to pH 7.4 using hydrochloric acid.

**Method:** This method was derived from that described by Kim *et al.* (1988). Standard solutions of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 100  $\mu\text{g}\cdot\text{ml}^{-1}$  calf thymus DNA dissolved in PBS were made. Test sample or standard (100  $\mu\text{l}$ ,  $n=6$ ) was aliquoted into a plastic cuvette and mixed manually with 2 ml Hoechst-33258 dye solution by repeated pipetting. The fluorescence intensity was recorded with excitation set at 365 nm and emission at 458 nm. A curve of DNA ( $\mu\text{g}\cdot\text{ml}^{-1}$ ) versus absorbance was constructed using the standards and the DNA content ( $\mu\text{g}\cdot\text{ml}^{-1}$ ) of the test samples interpolated from

the standard curve. The levels of DNA ( $\mu\text{g.ml}^{-1}$ ) were divided by the dry weight (mg) of each tissue sample and data for each sample expressed as  $\mu\text{g.mg}^{-1}$ .

### 5.3.1.6. Analysis of data

Data for hydroxyproline, GAG and DNA content in the tissues before and after decellularisation were analysed using the students t-test.

## 5.3.2. Biocompatibility study

### 5.3.2.1. Preparation of tissue samples

**Materials:** Hypotonic buffer consisted of 10 mM Tris with 10 KIU aprotinin and 0.1 % EDTA adjusted to pH 8.0 using HCl. Porcine hearts were obtained from a local abattoir.

**Method:** Porcine hearts were dissected and the valve leaflets removed. The following tissue samples were prepared in blinded groups:

- 1) Hypotonic-buffered leaflets;
- 2) Decellularised leaflets.

Samples were prepared by Dr Helen Wilcox according to methods described in Section 2.8.1. Hypotonic-buffered treatments involved harvesting the tissues and storing them in hypotonic buffer prior to implantation. Once prepared, tissue samples were cut into  $3\text{mm}^2$  pieces for implanting.

In the initial experiment  $n=3$  for each group. At this point it was the intention that when the samples were excised the sections would be cut in three using a scalpel. This would provide replicates and would enable one tissue piece to be used for haematoxylin and eosin staining and Von Kossa's calcification staining, one for cell phenotype analysis using immunoperoxidase labelling and one could be digested and used for atomic absorption spectroscopy to quantitatively analyse the amount of calcium present.

The experiment was later repeated, this time using  $n=4$  for each group. Of the four implants for each group it was intended that 3 be used for paraffin embedding as described in Section 2.5.1. These samples could then be used for haematoxylin and eosin staining and Von Kossa's calcification staining and also for cell infiltration analysis using antibodies to CD3, CD4, CD34 and F4/80. The fourth sample was cryo-embedded, as described in Section 2.5.5 in the event of problems with the antibodies working effectively on paraffin embedded tissues.

#### **5.3.2.2. Subcutaneous implantation of tissues into mice**

The biocompatibility of the tissues was tested in a growing mouse subcutaneous implant model. Short term anaesthesia was induced using a mixture of halothane and oxygen. The mid dorsum of the mouse was aseptically prepared using 70 % methanol. Tissue samples ( $3 \text{ mm}^2$ ) were implanted subcutaneously between the mid-dorsal line with the aid of a sterile trochar. Mice were allowed to recover in an incubator and returned to their cages. Mice were fed food and water ad libitum and left for a period of 12 weeks. Mice were then killed by schedule (1) and the implanted tissues retrieved for analysis. All animal procedures were carried out by Professor E Ingham under Home Office licences PPL 40/2409 and PIL 50/00027; I assisted.

#### **5.3.2.3. Haematoxylin and eosin staining**

Haematoxylin and eosin staining of implanted tissues was carried out as described in Section 2.5.7.

#### **5.3.2.4. Von Kossa's calcification staining**

**Materials:** Silver nitrate and sodium thiosulphate were purchased from Sigma-Aldrich. Safranin O stain was purchased from Raymond A Lamb.

**Method:** Tissue sections were dewaxed and rehydrated as described in Section 2.5.3. Slides were then transferred to 200ml 1 % (w/v) silver nitrate solution and left on a window ledge for 1 hour. Slides were then rinsed by immersing the slides in deionised water for 3×1 minute prior to being transferred to 200 ml 2.5 % (w/v) sodium thiosulphate for 5 minutes. Slides were then rinsed by immersing the slides in deionised water for 3×1 minute. Slides were counterstained by immersion in Safranin O solution for 1 minute prior to being dehydrated and mounted as described in Section 2.5.4.

#### **5.3.2.5. Cell infiltration phenotyping**

**Materials:** CD3, CD4, CD34, F4/80, and biotinylated rabbit anti-rat, goat anti-rat and donkey anti-rat antibodies were purchased and used as described in Section 2.6.5.

**Methods:** Immunoperoxidase was carried out on the cryo-embedded explanted tissues using the antibodies described above to examine the T-cell (CD3, CD4), endothelial cell (CD34) and macrophage (F4/80) infiltration into the tissue by the host immune system. Images were then taken at 400× magnification of the capsule around the implant, at the edge of the implant and in the centre of the implant. The numbers of positively labelled cells were then counted in a known area with 6 replicates for each area. A 7-point scoring system was then developed (Table 5.1) and the scores used to assess the infiltration of the cells into the implant.

CELL NO.	SCORE
0	0
1-5	1
6-10	2
11-15	3
16-20	4
21-25	5
26<	6

**Table 5.1. Scoring system for positively labelled cells in implanted tissues.**

### 5.3.2.6. Absorption of secondary antibodies

Despite attempting the immunoperoxidase labelling with all three secondary antibodies listed above, all produced high amounts of background staining which made it impossible to gain any useful results. Consequently, the antibodies were absorbed against pig tissues as follows.

**Materials:** Porcine hearts were obtained from a local abattoir within 4 hours of slaughter and the aortic valve leaflets dissected out. Secondary antibodies were acquired from companies as described in Section 2.6.5. A blood tube rotator was purchased from Fisher Scientific. Centrifugation was carried out using an MSE Micro Centaur microcentrifuge (Fisher Scientific).

**Methods:** One valve leaflet per antibody per day was finely minced using a scalpel. This was then added to the antibody after 1:5 dilution (to give a volume of 5 ml) using TBS azide BSA buffer as described in Section 2.6.1. Antibody/leaflet samples were then placed on a blood tube rotator for 24 hours at 4 °C. After this point, samples were centrifuged at 200 g for 10 minutes in a microcentrifuge and the supernatant was removed. Further minced leaflets were then added to each antibody sample and returned to the rotator for a further 24 hours. This was then repeated for a third incubation. The samples were then centrifuged again and the supernatant removed. Antibodies were then stored at 4 °C prior to use in immunoperoxidase labelling, as described in section 2.6.1.

### 5.3.2.7. Atomic absorption spectroscopy analysis

Samples were acid hydrolysed as described in Section 5.3.1.1 before being sent for atomic absorption spectroscopy analysis by Dr Ken Newton at Leeds General Infirmary Pathology Department.

## 5.4. Results

### 5.4.1. Biochemical analysis

#### 5.4.1.1. Hydroxyproline assay

Decellularised and fresh leaflet samples were hydrolysed and assayed for their hydroxyproline content. The results indicated a slight, proportional increase in the amount of hydroxyproline present in decellularised tissue (Table 5.1).

The results were compared using a student's t-test, which showed there was no significant difference between the hydroxyproline content of cellular and acellular porcine aortic valve leaflets (Figure 5.1).

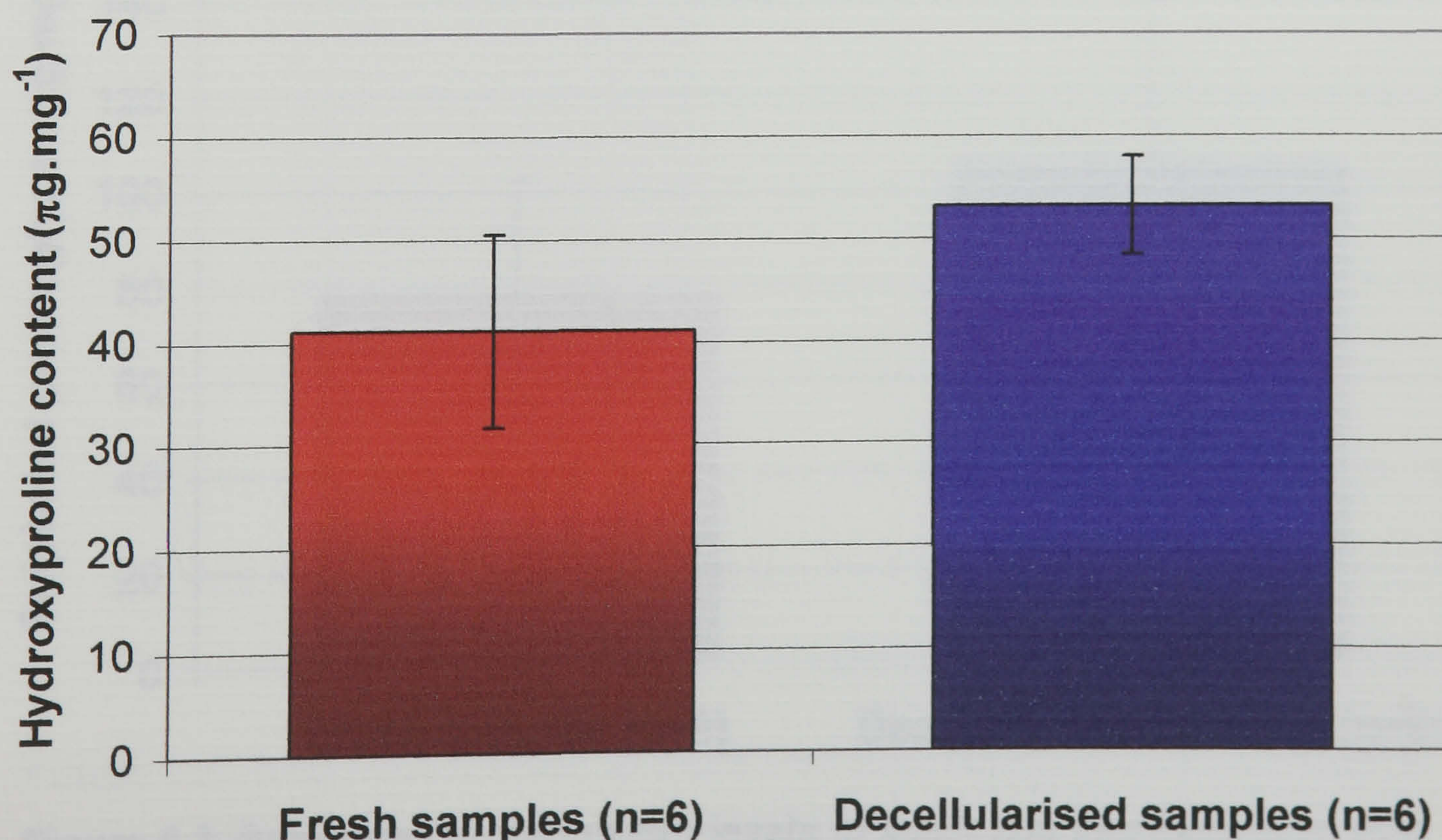


Figure 5.1. Hydroxyproline content of fresh and decellularised acid hydrolysed porcine aortic tissue. Bars denote 95 % confidence limits

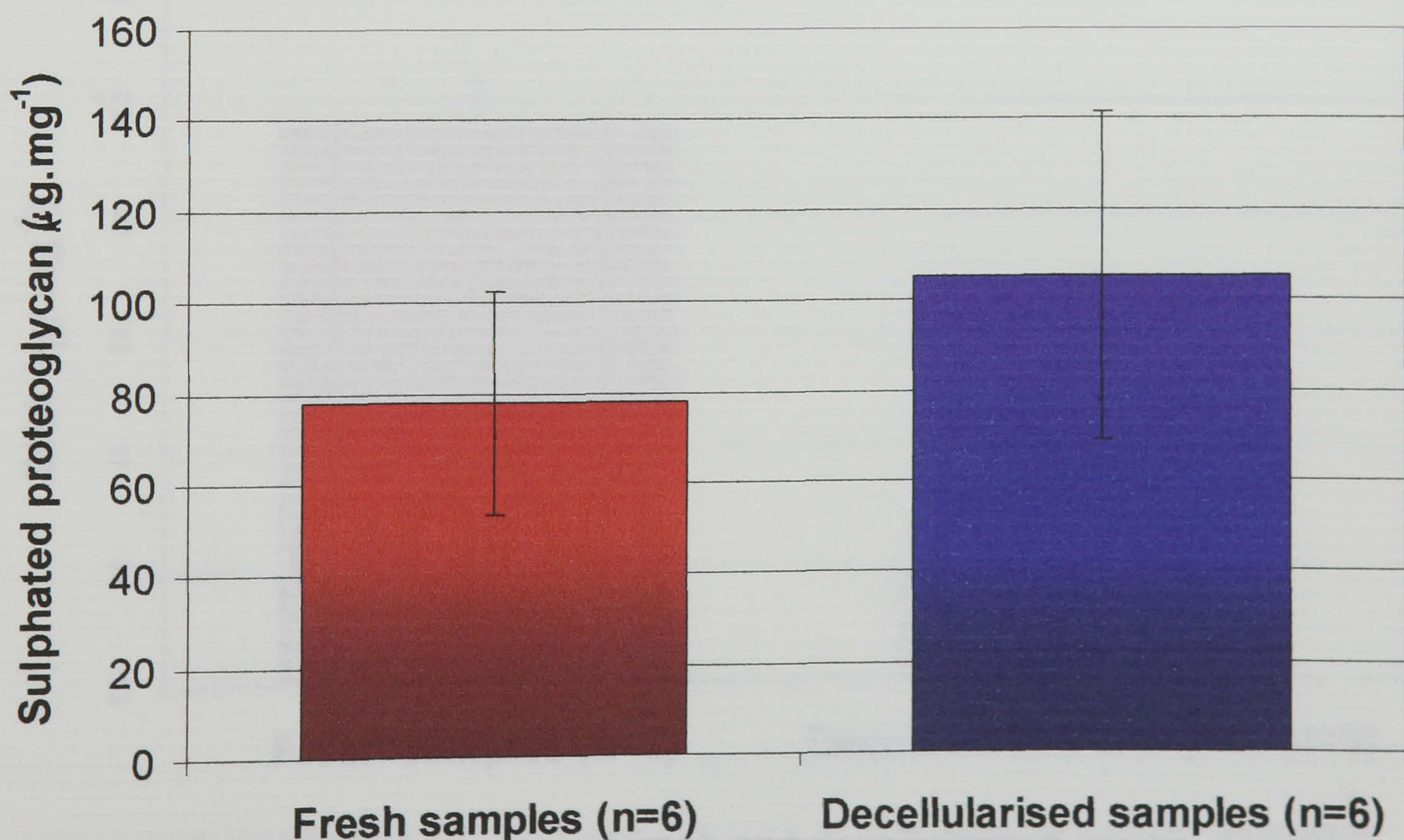
	DRY WEIGHT ( $\mu\text{g}$ )	MEAN ABSORBANCE (470nm)	MEAN HYDROXYPROLINE ( $\mu\text{g}\cdot\text{mg}^{-1}$ )
FRESH SAMPLES (n=6)	5.86e-4	0.9445	41.075
DECELLULARISED SAMPLES (n=6)	3.98e-4	0.8235	53.095

**Table 5.2. Hydroxyproline content of fresh and decellularised acid hydrolysed porcine aortic tissue.**

#### 5.4.1.2. Sulphated proteoglycan assay

Decellularised and fresh leaflet samples were hydrolysed and assayed for their sulphated proteoglycan content. The results demonstrated a slight, proportional increase in the amount of sulphated proteoglycan present in decellularised tissue (Table 5.2).

The results were compared using a student's t-test this showed there was no significant difference between the levels of sulphated proteoglycans present in fresh and decellularised porcine aortic valve leaflets, (Figure 5.2).



**Figure 5.2. Sulphated proteoglycan levels of fresh and decellularised acid hydrolysed porcine aortic tissue. Bars denote 95 % confidence limits.**

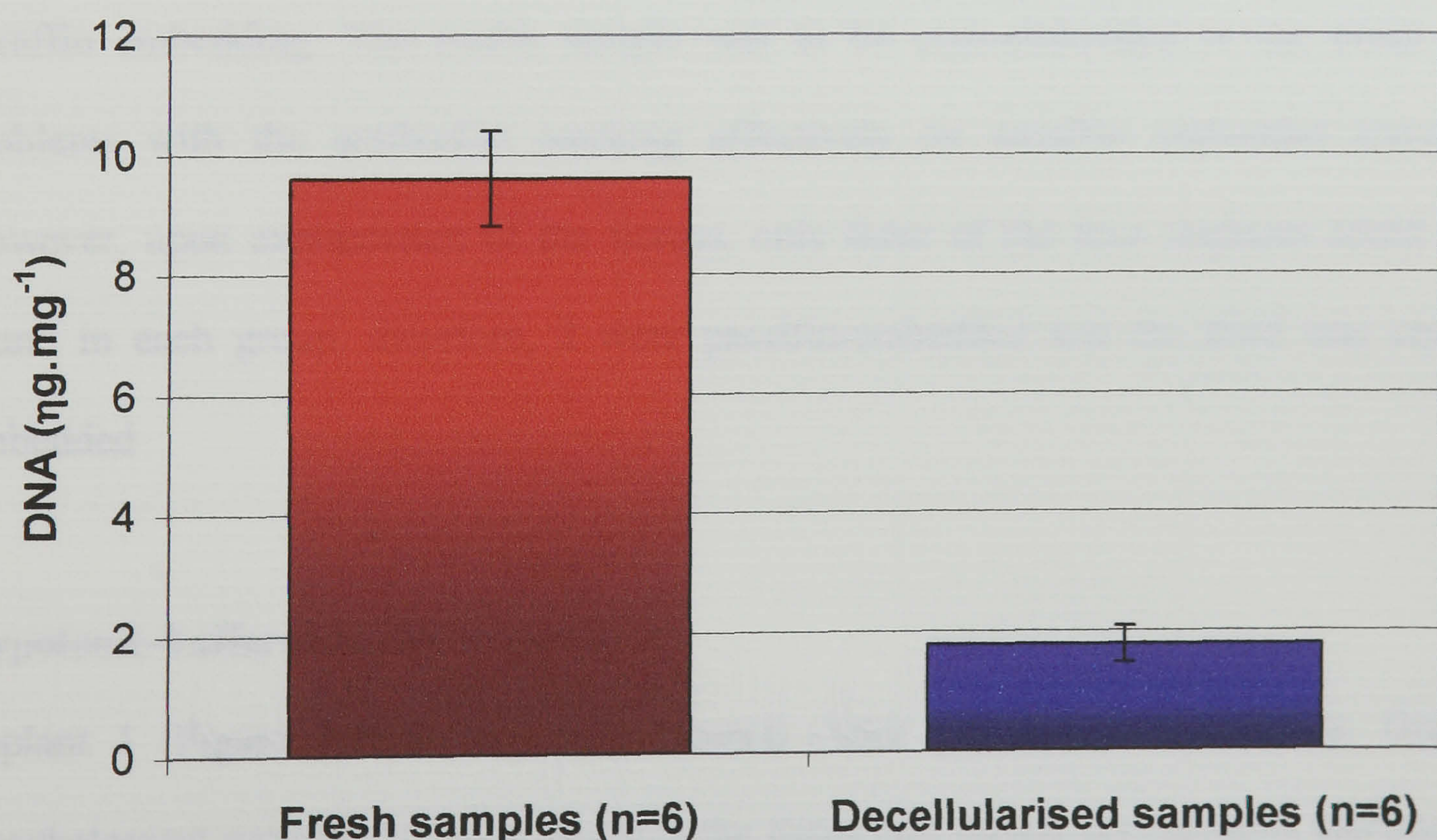
	DRY WEIGHT ( $\mu\text{g}$ )	MEAN ABSORBANCE (525nm)	MEAN SULPHATED PROTEOGLYCAN ( $\mu\text{g}\cdot\text{mg}^{-1}$ )
FRESH SAMPLES (n=6)	5.86e-4	0.735	77.80
DECELLULARISED SAMPLES (n=6)	3.98e-4	0.712	105.21

**Table 5.3. Sulphated proteoglycan levels of fresh and decellularised acid hydrolysed porcine aortic tissue.**

#### 5.4.1.3. Fluorometric DNA assay

Decellularised and fresh leaflet samples were digested with papain and assayed for their DNA content. The results showed a decrease in the amount of DNA present in the decellularised tissue (Table 5.3).

The results were compared using a student's t-test, which showed there was a significant difference between the DNA content of cellular and acellular porcine aortic valve leaflets, (Figure 5.3).



**Figure 5.3. Total DNA content of fresh and decellularised papain digested porcine aortic leaflets. Bars denote 95 % confidence limits.**



	<b>DRY WEIGHT (<math>\mu\text{g}</math>)</b>	<b>LUMINESCENCE (Excitation 365nm, Emission 458nm)</b>	<b>MEAN DNA (<math>\mu\text{g}\cdot\text{mg}^{-1}</math>)</b>
<b>FRESH SAMPLES (n=6)</b>	4.05	711.19	9.6
<b>DECELLULARISED SAMPLES (n=6)</b>	2.07	61.86	1.76

**Table 5.4. Total DNA content of fresh and decellularised papain digested porcine aortic leaflets.**

## **5.4.2. Biocompatibility study**

### **5.4.2.1. Morphological and histological examination of implants**

After the initial implantation period, it was realised that cutting each leaflet into three pieces for different analyses was problematic. Essentially, the tissue pieces were just too small for practical use. The pieces were difficult to manipulate for paraffin embedding and then were near impossible to section adequately. Consequently, the experiment was repeated using whole tissue samples (n=4) for sectioning and analysis. As described in Section 5.3.2.1., of the four implants for each group it was intended that 3 be used for paraffin embedding. The fourth sample was to be cryo-embedded in the event of problems with the antibodies working effectively on paraffin embedded tissues. However, upon explantation of the tissues, only three of the four implants could be found in each group, therefore, 2 were paraffin-embedded and the third was cryo-embedded

### **Hypotonic-buffered leaflet explants**

Implant 1 (Figure 5.4A) was easily located under the skin of the mouse. Gross morphological examination indicated that the tissue had shrunk considerably compared to the  $3\text{mm}^2$  tissue size implanted. Also, the tissue itself appeared white in colouring.

Haematoxylin and eosin staining revealed that the implanted tissue had become highly vacuolated and had a necrotic appearance. The remains of a fibrous capsule could be

seen around the edge of the implant. Relatively few cells were present in the centre of the implant. Those that had penetrated into the tissue appeared to have a varied morphology; however, the majority had a fibroblastic appearance.

Von Kossa's calcification staining indicated that histologically no calcification had occurred in this implant (Figure 5.5).

Implant 2 (Figure 5.4B) was also easily located under the skin of the mouse and was also noticeably smaller compared to the 3mm<sup>2</sup> tissue size implanted. Again the tissue was white in appearance.

Haematoxylin and eosin staining showed the remains of a fibrous capsule and that the tissue was also vacuolated, although not to the same extent as implant 1. This again gave the tissue a necrotic appearance, although in this implant the cell infiltrate was more prominent, with cells possessing fibroblastic, polymorphonuclear and mononuclear morphologies being present.

Again, Von Kossa's calcification staining indicated that histologically no calcification had occurred in this implant (Figure 5.5).

### **Decellularised leaflet explants**

Implant 1 (Figure 5.4C) could be visualised under the skin of the mouse. Gross morphological inspection indicated that the implant had shrunk considerably compared to the 3mm<sup>2</sup> tissue size implanted and it was also white in appearance.

Haematoxylin and eosin staining showed that there was no obvious fibrous capsule present around the implant and the tissue did not show any vacuoles. The matrix of the implant was healthy in appearance and had a prominent cell infiltrate which extended into the centre of the implant. Closer examination revealed the presence of cells with mononuclear and polymorphonuclear morphologies and also fibroblastic cells.

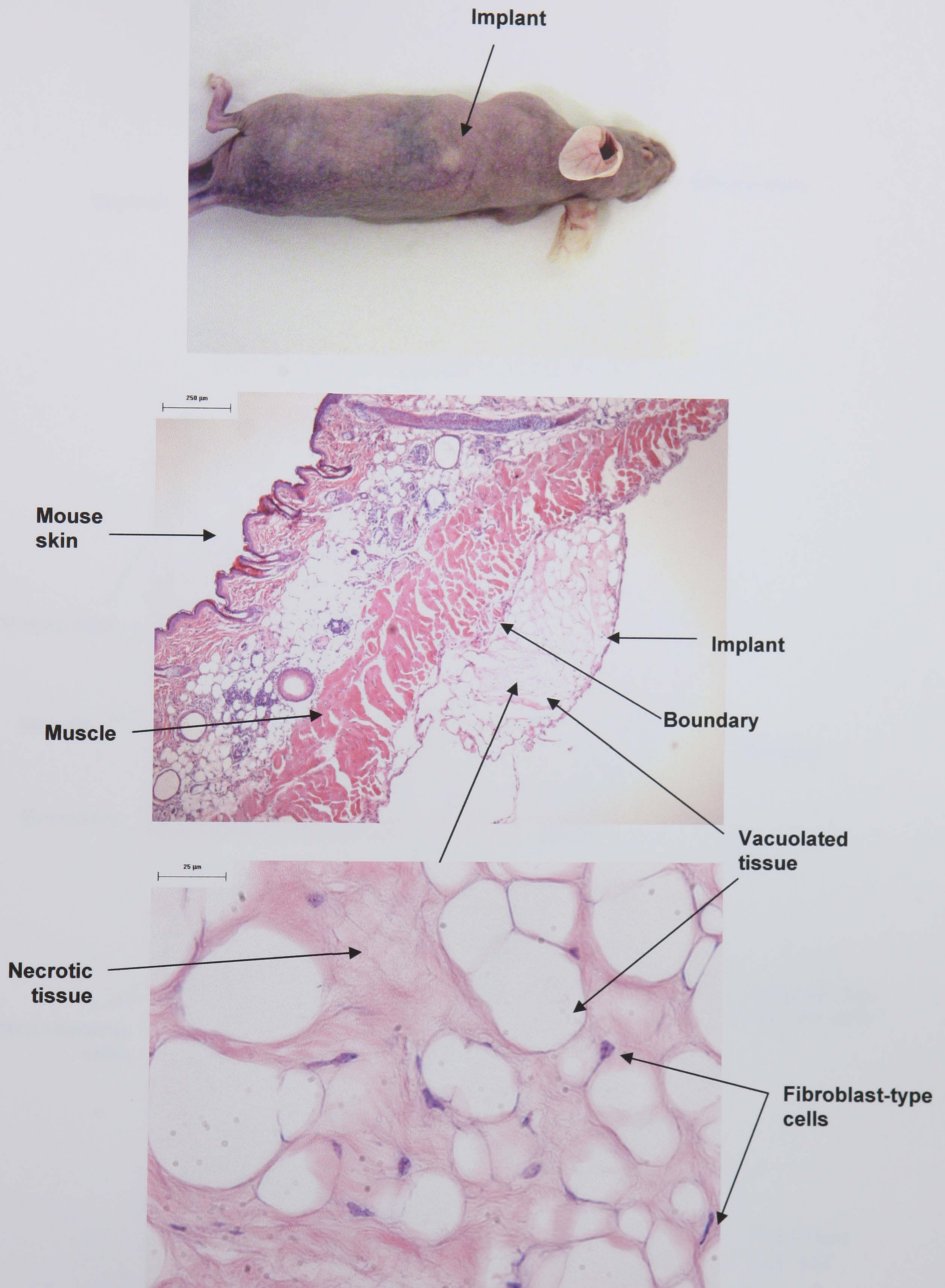
Furthermore, there was evidence of vascularisation of the tissue in the form of numerous blood vessels.

Von Kossa's calcification staining indicated that histologically no calcification had occurred in this implant (Figure 5.5).

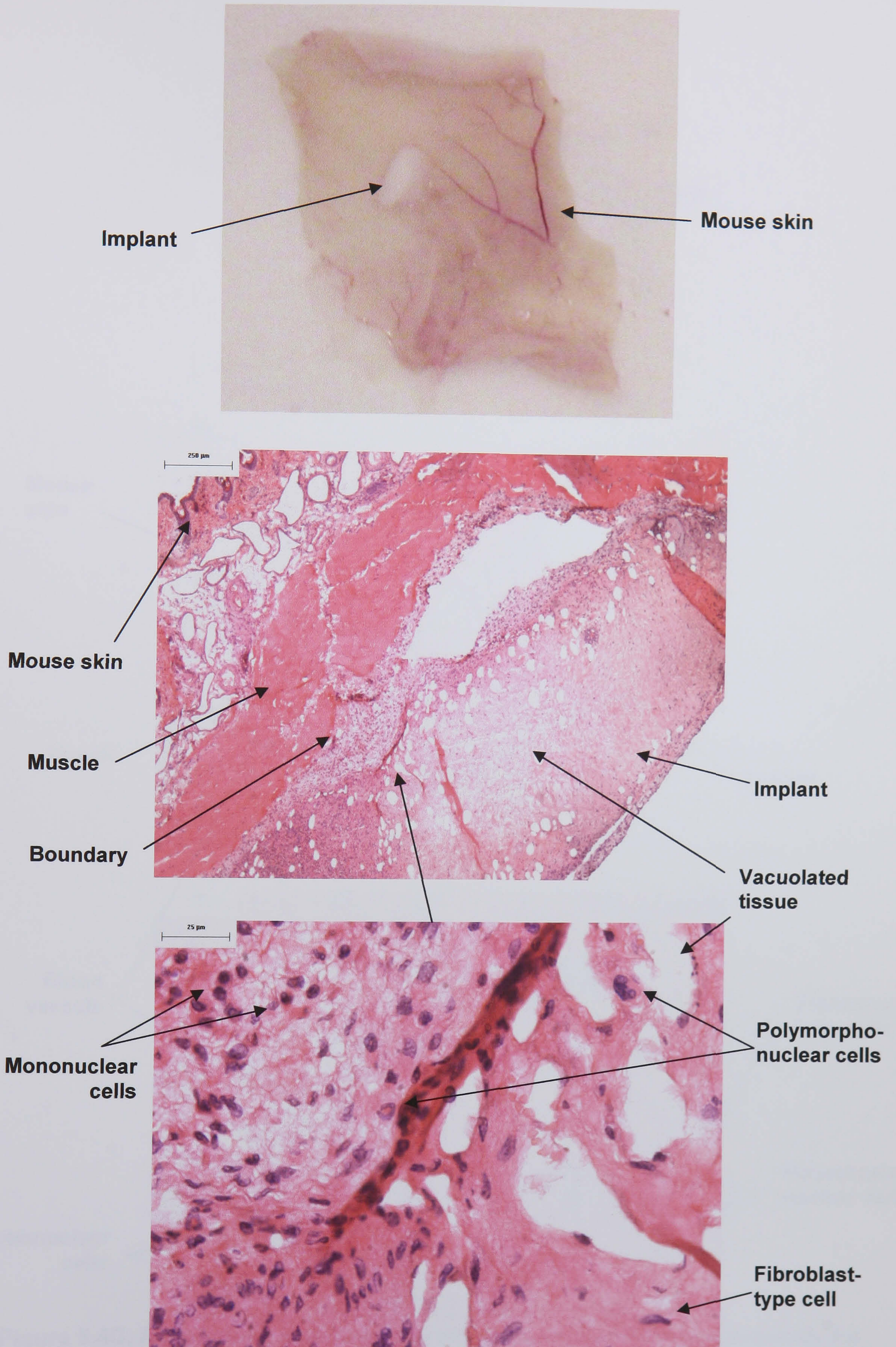
Implant 2 (Figure 5.4D) was also easily located under the skin of the mouse. Again, gross examination revealed that the implant itself was white in appearance and was visibly smaller in comparison to the size of the tissue implanted.

Haematoxylin and eosin staining showed that there was no fibrous capsule present around the edge of the implant and the tissue matrix appeared healthy, without any necrotic or vacuolated areas being present. Again cells had infiltrated into the centre of the tissue, although they were fewer in number compared to implant 1 and the cells appeared less dense. Both polymorphonuclear and fibroblastic-type cells could be observed. Again, vascularisation appeared to have resulted in the formation of some new blood vessels in the implant.

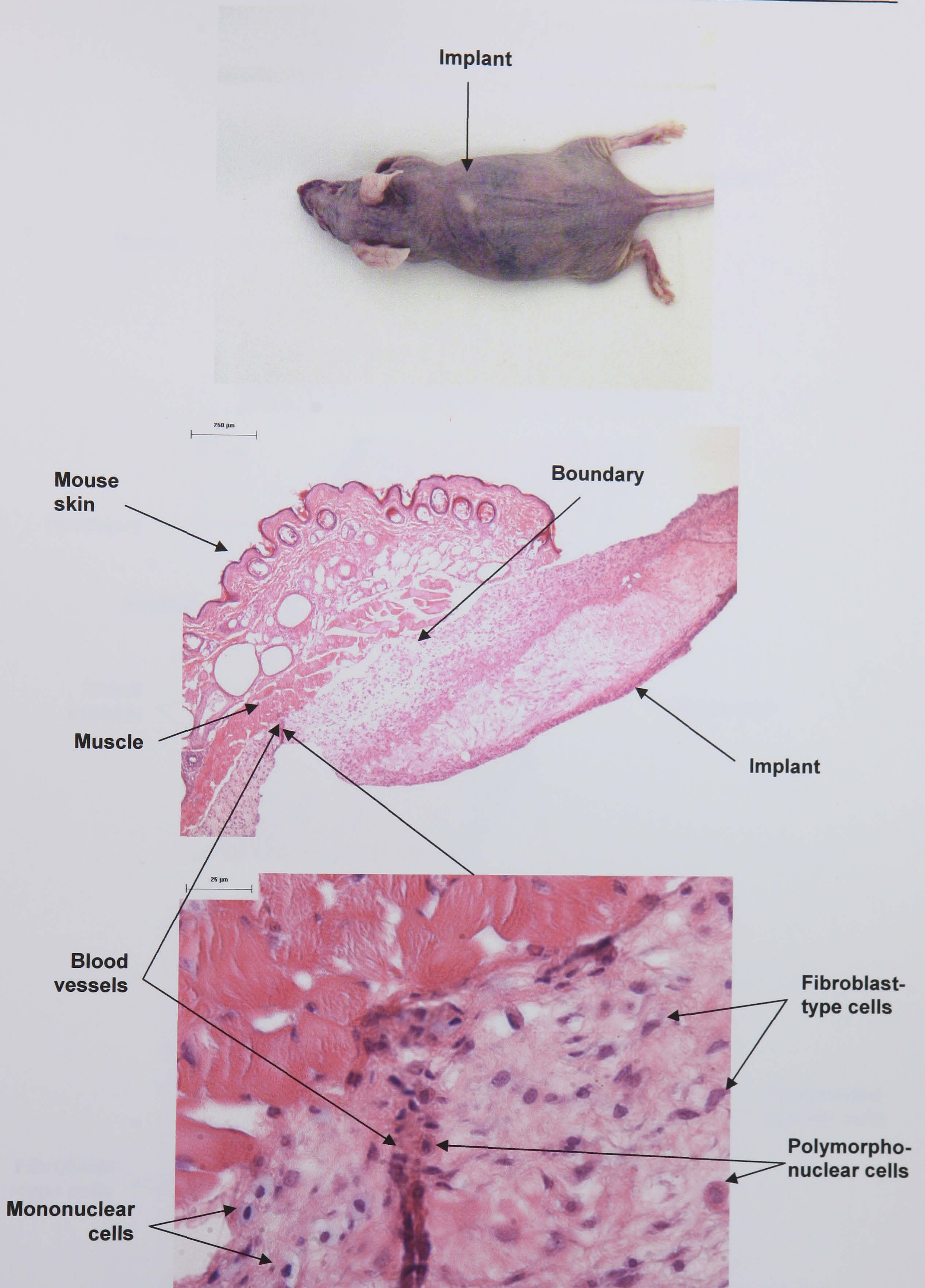
Von Kossa's calcification staining indicated that histologically no calcification had occurred in this implant (Figure 5.5).



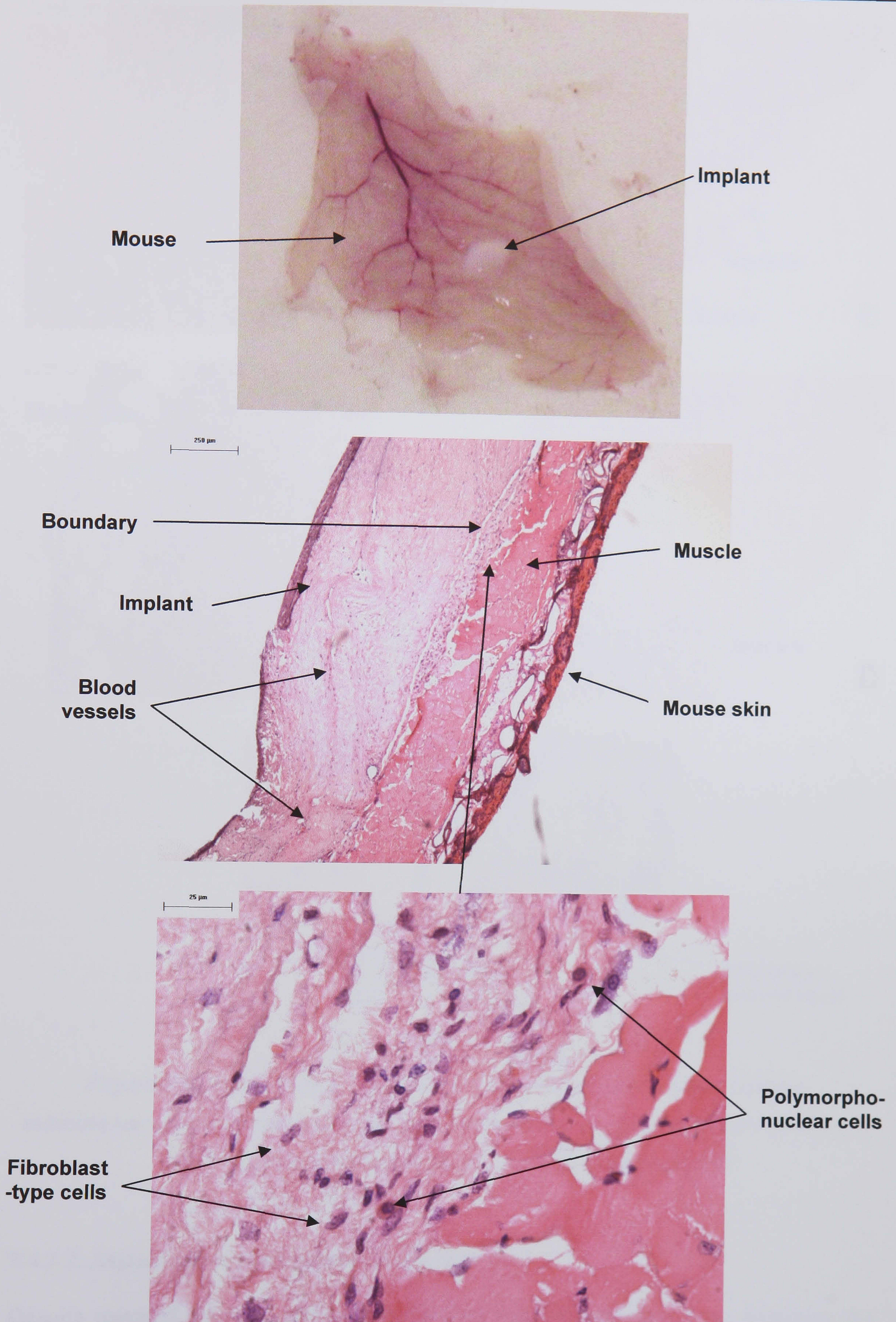
**Figure 5.4A. Morphology and histology of tissue implanted subcutaneously for 12 weeks. A: Hypotonic-buffered leaflet. Top image: location of implant at explantation; Lower images: haematoxylin and eosin staining.**



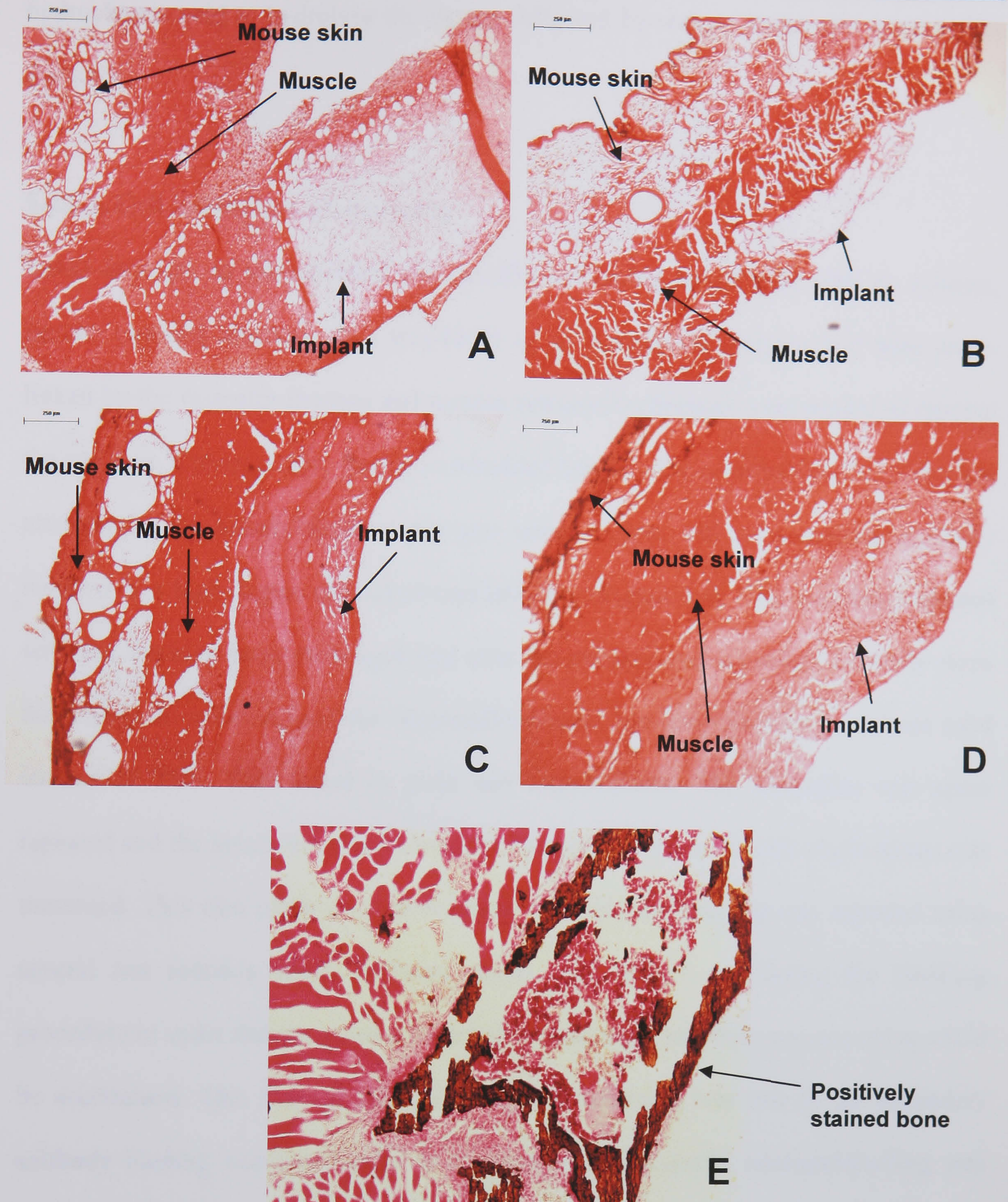
**Figure 5.4B. Morphology and histology of tissue implanted subcutaneously for 12 weeks. B: Hypotonic-buffered leaflet. Top image: gross morphology of implant at explantation; Lower images: haematoxylin and eosin staining.**



**Figure 5.4C. Morphology and histology of tissue implanted subcutaneously for 12 weeks. C: Decellularised leaflet. Top image: location of implant at explantation; Lower images: haematoxylin and eosin staining.**



**Figure 5.4D. Morphology and histology of tissue implanted subcutaneously for 12 weeks. D: Decellularised leaflet. Top image: gross morphology of implant at explantation; Lower images: haematoxylin and eosin staining.**



**Figure 5.5. Von Kossa's calcification staining of aortic valve tissues subcutaneously implanted into hairless mice. A & B: Hypotonic-buffered leaflet; C & D: Decellularised leaflet; E: mouse skull positive control**

#### 5.4.2.2. Atomic absorption spectroscopy analysis

Despite repeated attempts, no meaningful data could be obtained from analysing the hydrolysed tissue samples by atomic absorption spectroscopy. This was thought to be due to the high sodium content of the samples which was present as a result of using

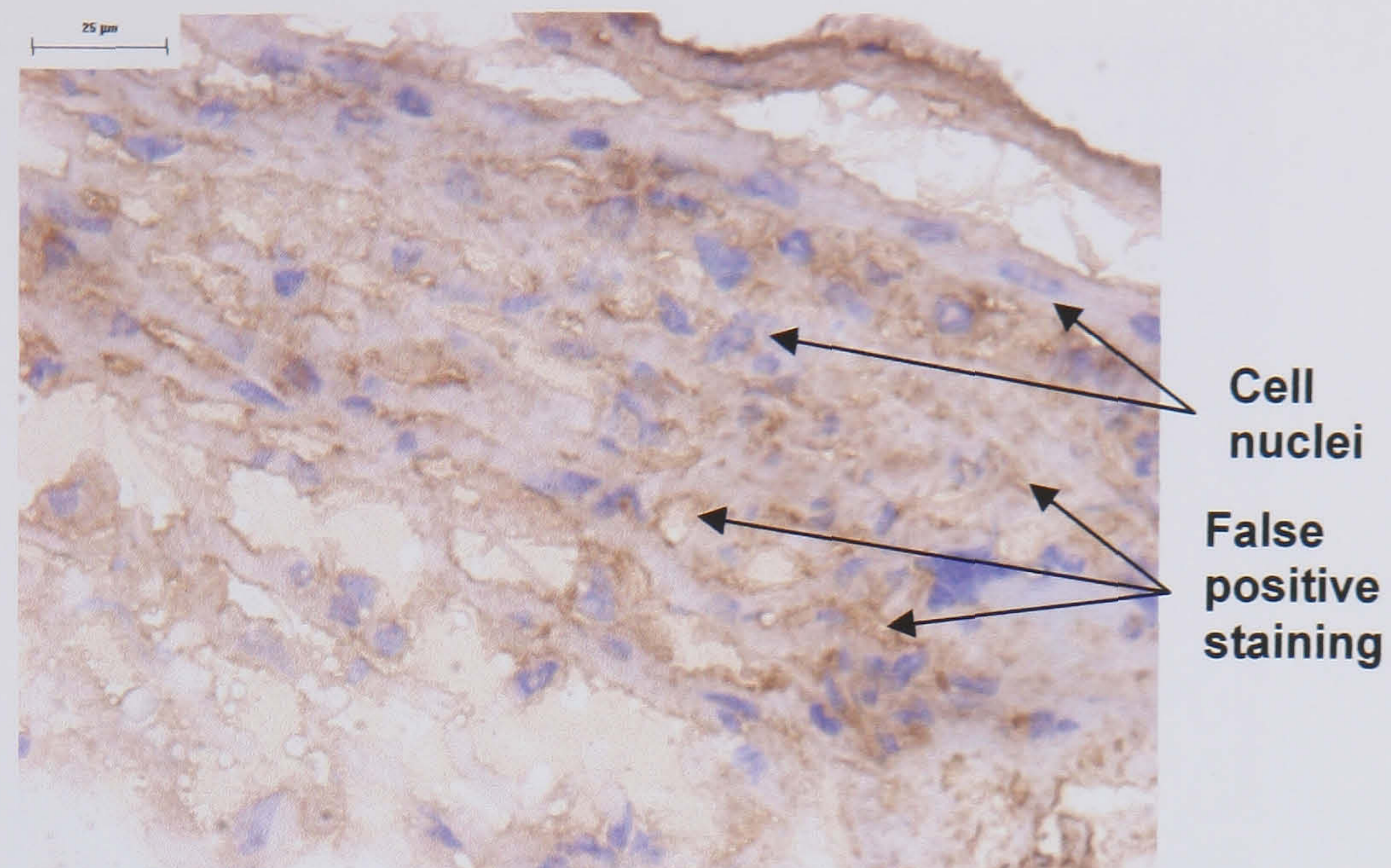


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hydrochloric acid to hydrolyse the tissues followed by sodium hydroxide to neutralise the acid.

#### **5.4.2.3. Cell infiltration phenotyping**

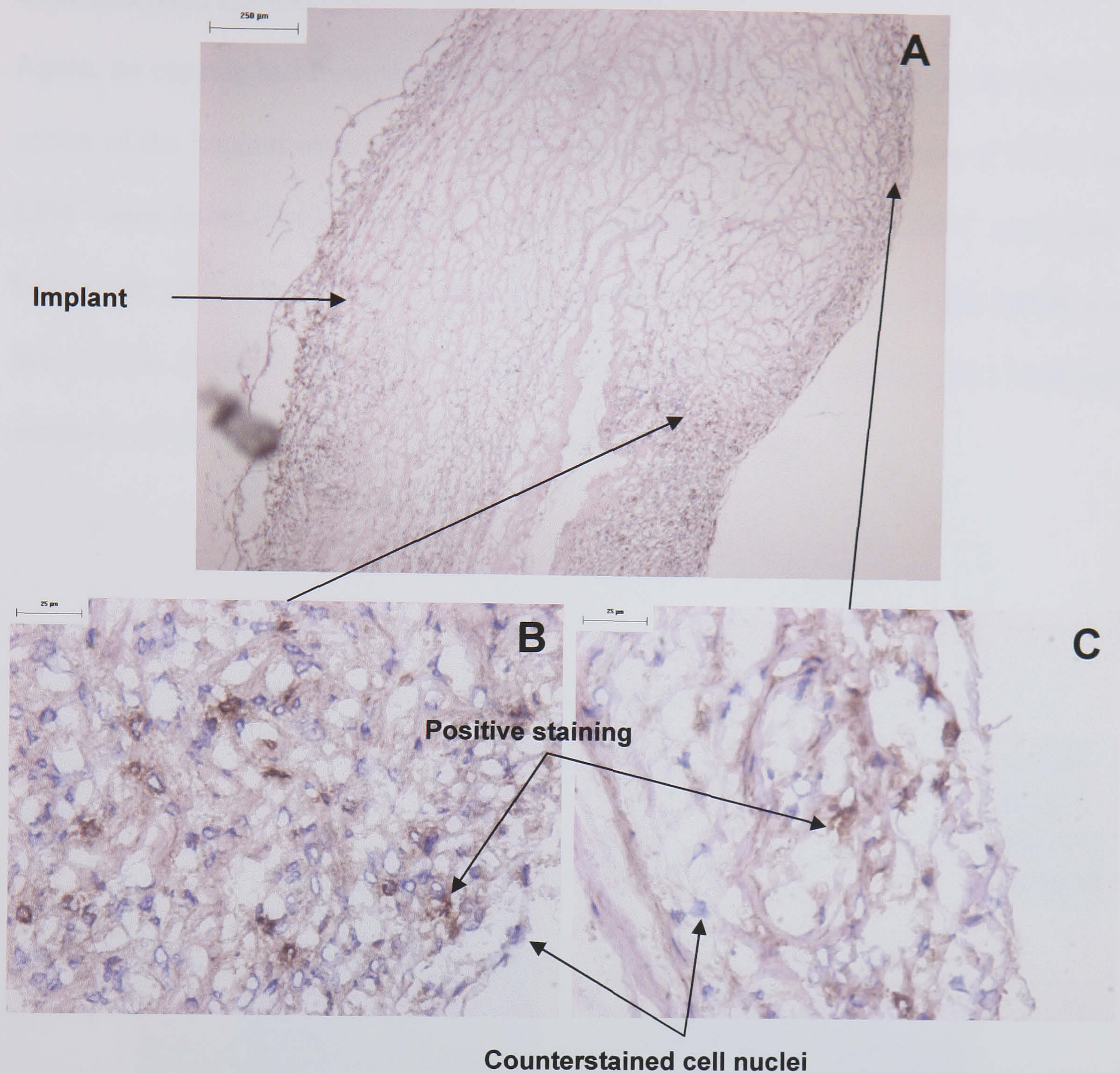
Initial attempts to immunolabel the paraffin embedded tissues met with no success, despite repeated attempts. This was likely to be because the antigens had been cross-linked by the formalin fixation and antigen retrieval techniques were unable to retrieve the antigens. Consequently, the cryo-embedded tissues were sectioned and cell labelling attempted. The initial labelling attempts using a goat anti-rat secondary antibody resulted in a high degree of background labelling (Figure 5.6). This was then repeated with the washing steps prolonged and after the primary and secondary antibody steps the Tween 20 solution used for immunofluorescent labelling (Section 2.6.4) was used instead of TBS. This failed to yield any improvement. The procedure was again repeated and the incubation period used for blocking the sections with whole serum was increased. This also made no improvement, consequently staining was repeated using several test sections where different stages were missed out during the labelling procedure in order that the point at which the background labelling was occurring could be ascertained. This showed that the background staining was due to the secondary antibody binding non-specifically to the tissue. Subsequently, immunolabelling was repeated using rabbit anti-rat then later donkey anti-rat secondary antibodies. However, the background staining remained using both secondary antibodies. Consequently, it was decided to try to absorb out this cross-reactivity by incubating the antibodies with minced valve tissue as described in Section 5.3.2.6. This effectively removed the non-specific binding, allowing full evaluation of the implanted tissues.



**Figure 5.6.** F4/80 immunoperoxidase negative labelling of un-fixed hypotonic-treated valve leaflet implanted in mice for 12 weeks. This negative control gave brown positive labelling when primary antibody was replaced with buffer.

Bar represents 25μm.

Cryo-embedded tissue sections were labelled with antibodies to CD3 (pan-T-cell marker), CD4 (T-helper cell marker), CD34 (endothelial cell marker) and F4/80 (macrophage marker). Images were then taken (example in Figure 5.7) and the cells counted and the count scored as described in Section 5.3.2.5 (Figure 5.8).



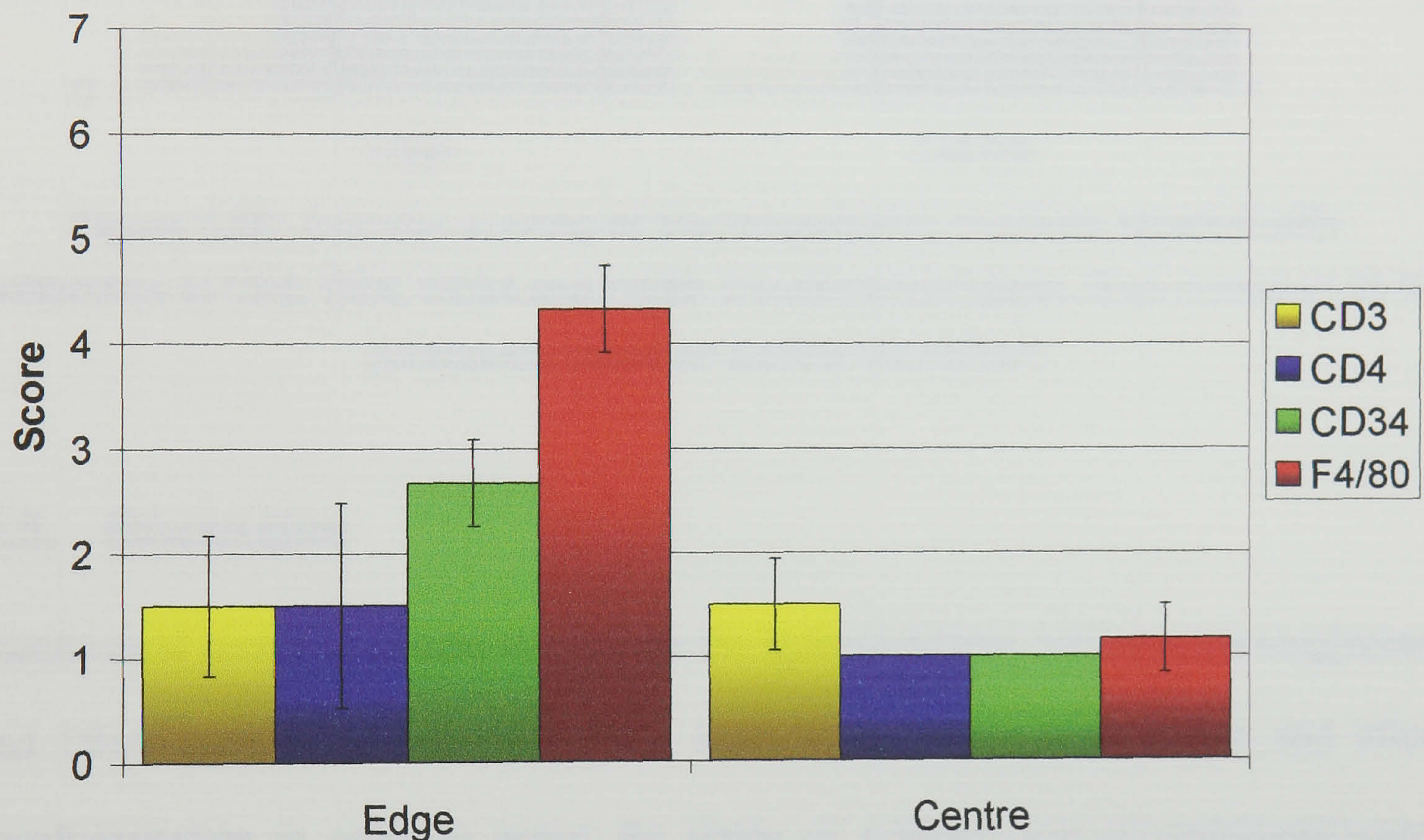
**Figure 5.7. Examples of images used for cell scoring.** A: overview of section; B: middle of tissue section; C: edge of tissue section. Six images were taken from each of these areas and the positively labelled cells counted and scored. Sections are from unfixed hypotonic-treated tissue labelled with antibodies to CD3. Secondary antibodies were absorbed as described in Section 5.3.12.

### Hypotonic-buffered leaflets (Figure 5.8A)

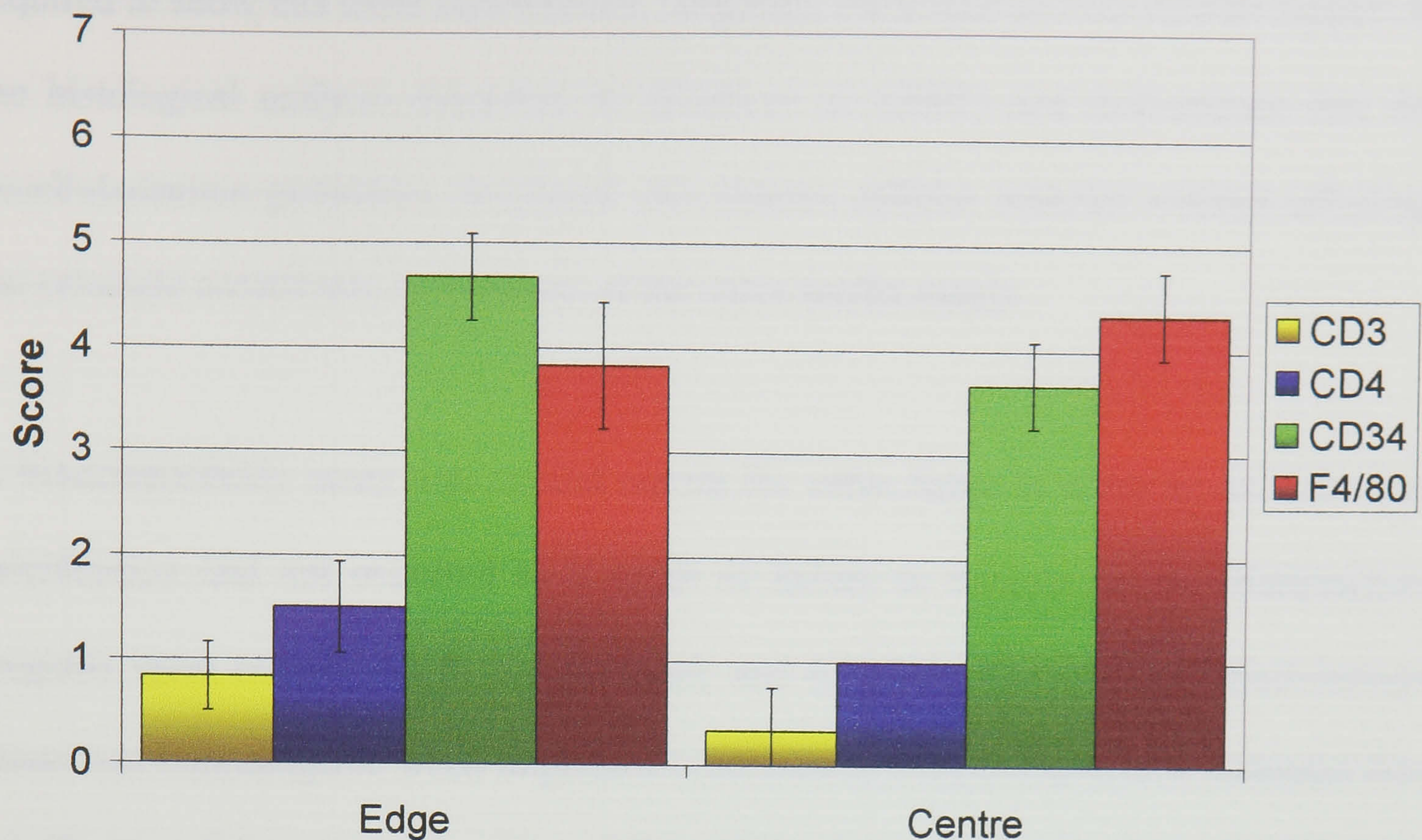
No capsule had formed around this implant therefore only counts at the edge and centre of the implant were made. At the edge,  $F4/80^+$  cells were in the highest proportion (score 4) with fewer  $CD34^+$  (score 2) and  $CD4^+$  cells (score 2). Only low numbers of  $CD3^+$  cells (score 1) were found here. In the centre, no  $CD3^+$  cells were noted with only low numbers of  $CD4^+$ ,  $CD34^+$  and  $F4/80^+$  cells (all score 1).

### Decellularised leaflets (Figure 5.8B)

Again, no capsule had formed around this implant therefore only counts at the edge and centre of the implant were made. At the edge of the leaflet, low numbers of CD3<sup>+</sup> and CD4<sup>+</sup> were found (both with score 1), but much higher numbers of CD34<sup>+</sup> and F4/80<sup>+</sup> cells (both with score 4). The proportion of cell types in the centre of the leaflet was also similar, this time with no CD3<sup>+</sup> cells, low numbers of CD4<sup>+</sup> cells (score 1) and high numbers of CD34<sup>+</sup> and F4/80<sup>+</sup> cells (again both with score 4).



**Figure 5.8A. Average scoring of biocompatibility implants labelled with antibodies to CD3, CD4, CD34 and F4/80. Hypotonic-buffered leaflet. Bars represent 95% confidence based on arcsine conversion.**



**Figure 5.8B. Average scoring of biocompatibility implants labelled with antibodies to CD3, CD4, CD34 and F4/80. Decellularised leaflet. Bars represent 95 % confidence based on arcsine conversion.**

## 5.5. Discussion

Biochemical assays were used to compare the hydroxyproline, sulphated proteoglycans and DNA content of digested porcine heart valve tissues both before and after decellularisation in order to assess the levels of collagen and glycosaminoglycans (GAG) respectively and to verify that all cell DNA had been removed from the tissue by the decellularisation procedure. It was found that there no was significant difference between the hydroxyproline and sulphated proteoglycan content before and after the decellularisation procedure, indicating that the decellularisation procedure had not affected the levels of these components of the tissue. Analysis of the DNA content of the leaflet before and after decellularisation revealed that the DNA had been reduced to considerably lower levels. It is possible that the apparent remaining DNA was the result of a background anomaly (perhaps due to elastin which is known to autofluoresce), however, further experiments with increased DNase treatment (time and/or concentration) or longer washing periods after the decellularisation itself would be

required to show this more conclusively. Together, these data provide further support to the histological analysis described by Booth *et al.* (2002) and demonstrate that the decellularisation procedure developed can remove cellular material without affecting the principle constituent components of the valve leaflet matrix.

A biocompatibility assay was carried out on the valve tissue in order to: a) show that calcification had not occurred as a result of failure to remove the decellularisation reagents upon completion of the protocol; and b) assess whether the decellularised tissue was immunogenic when implanted in an animal model. The results indicated that calcification did not occur in either of the implanted tissue samples, indicating that the decellularisation reagents were completely removed during the washing stages at the end of the procedure. Further experiments here could improve the acid-hydrolysis of the tissue in order to more precisely quantify the calcium content to compare the hypotonic-treated tissue with the decellularised leaflets by use of atomic absorption spectroscopy. This technique is capable of measuring very small quantities of calcium that might not be visible by histological evaluation and would further confirm that no calcification had occurred.

An analysis of the phenotype of the cells that had infiltrated into the implanted tissues indicated favourable results. Here, high numbers of CD3<sup>+</sup> and CD4<sup>+</sup> cells would indicate a specific T-cell mediated response equivalent to rejection of the tissue. F4/80<sup>+</sup> cells would indicate the presence of macrophages, which could, in the absence of T-cells indicate a wound-healing type response, as these cells would provide signals for CD34<sup>+</sup> endothelial cells, which are involved in vascularisation of the tissue. However, the numbers of macrophages should fall after a while as the tissue is incorporated and remodelled by the host. The principle tissue being investigated here, namely the

decellularised leaflet, showed a low T-cell infiltrate with high numbers of macrophages and endothelial cells, suggesting that the leaflet might be being remodelled by the mouse model. Furthermore, the haematoxylin and eosin staining revealed that the centre of the implanted decellularised tissues appeared to be living and healthy. There was a prominent cell infiltrate consisting of both fibroblasts and mononuclear cells and some regeneration had occurred, as indicated by the presence of blood vessels in the implanted tissues. In contrast, the centre of the implanted hypotonic-buffered tissues appeared to be highly vacuolated and necrotic with no vascularisation and the remains of a fibrous capsule could be seen around the implants. This indicated that the implanted decellularised tissue was in the process of being remodelled and integrated whereas necrosis of the hypotonic-buffered tissue indicated that the tissue was starved of oxygen and dying. Further experiments here would need to show definitively that the decellularised tissue was biocompatible using more replicates and that the numbers of macrophages falls in the later stages of remodelling to show that the host immune response was not a continued presence in the tissue in a non-specific manner. However, this would require an implantation period of up to 6 months rather than the 3 months investigated here.

In conclusion, biochemical analyses revealed that the decellularisation procedure efficiently removed cellular material without damaging the principle matrix components, namely collagen and glycosaminoglycans. An examination of the biocompatibility of the un-fixed decellularised tissue indicated that the decellularisation reagents were efficiently removed at the end of the procedure and the tissue appeared to be in the process of being remodelled and incorporated by the host tissues.

## **CHAPTER SIX:**

### ***Study of the Capacity of hMSC to Re-seed Decellularised Porcine Aortic Valve Matrices***



# CHAPTER 6:

## *Study of the Capacity of hMSC to Re-seed Decellularised Porcine Aortic Valve Matrices*

### 6.1. Introduction

The decellularised porcine aortic valve tissue had been found to be biocompatible and did not lead to an adverse inflammatory response or calcification *in vivo*. The matrix structure was also retained after the decellularisation procedure. The next phase was to determine whether it was possible to re-seed the aortic valve matrix with hMSC *in vitro*. Prior to reseeded, however, it was important to establish conditions for culturing the reseeded valve tissue. In order to determine whether the mass transfer of oxygen and nutrients into cells within valve tissue would be sufficient, freshly isolated porcine aortic valves were cultured *in vitro*. At this stage, a basic system of static culture was deemed the most appropriate to use, however, it was important to ascertain whether the cells could be maintained in this way and also what effect this system had on the matrix, i.e. whether it remained intact, or whether it degenerated since the *in vitro* environment lacked the mechanical and biochemical stimulation present *in situ*.

The next step was to reseed hMSC into a decellularised aortic valve leaflet to assess how effective the cells were at repopulating the tissue without biochemical or mechanical stimulation. As a comparison, human smooth muscle cells were also reseeded in order to establish whether either cell type was more effective for repopulating the leaflets. The density of the re-seeded cells was then compared to the cell density found in a human aortic homograft valve as a means of measuring the

success of the re-seeding attempts. Once the reseeded culture period was complete, it was also necessary to compare the phenotype of the reseeded cells with that of native valve cells to ensure that the cells retained an appropriate phenotype, and also to ensure that the reseeded hMSC had not differentiated into an undesirable phenotype.

## **6.2. *Aims & objectives***

The aims and objectives of this chapter were to firstly devise a method for culturing freshly isolated valve tissue. This system could then be applied to decellularised porcine aortic valve leaflets reseeded with both human mesenchymal stem cells (hMSC) and human smooth muscle cells (hSMC) in order to establish the optimal cell concentration required for cell adhesion and migration/infiltration into the decellularised tissue. The cell phenotype would then be analysed to establish whether the reseeded cells had maintained their phenotype and whether it was comparable to native homograft valve cells. The specific objectives were to:

- Determine the capacity of aortic valves to maintain cells and matrix in culture
- Determine the cell number and distribution in fresh valve leaflets
- Determine the degree of attachment and infiltration of the re-seeded cells
- Determine the phenotype of the re-seeded cells as compared to native valve interstitial cells.

## **6.3. *Materials & methods***

### **6.3.1. Intact static leaflet culture**

#### **6.3.1.1. Culture conditions**

**Materials:** Nunc 6-well tissue culture plates were obtained from Fisher Scientific. Steel rings and stainless-steel mesh were gifts from Mr Devon Darby. Prolene surgical sutures (size 4.0) were purchased from NHS Supplies.

**Method:** Aortic heart valve leaflets were dissected under aseptic conditions from pig hearts obtained from a local abattoir. Duplicate samples were then placed in individual wells of 6-well plates and 5 ml of complete medium was added. The leaflets were weighted down with sterile steel rings to ensure they were completely covered in medium. The medium was changed every two to three days, as required, and leaflets were harvested at 0, 1, 2, and 3, 8, 15 and 22 days in the first experiment. In a second experiment, leaflets were sutured to a stainless steel mesh for the duration of the culture period and were harvested at 0, 7, 14, 21, 28 and 35 days. Leaflets were then paraffin-embedded and sectioned as described in Section 2.5.

#### **6.3.1.2. Haematoxylin and eosin staining**

Haematoxylin and eosin staining was carried out as described in Section 2.5.7.

#### **6.3.1.3. Alcian blue staining**

**Materials:** Alcian blue and acetic acid were purchased from Sigma-Aldrich. Alcian blue stain consisted of 1 g of alcian blue dissolved in 100 ml of 2 % (v/v) acetic acid solution.

**Method:** Sections were first dewaxed and rehydrated using xylene and graded alcohols as described in Section 2.5.3. Slides were then briefly dipped in 2 % (v/v) acetic acid prior to being immersed in alcian blue stain for 20 minutes. Slides were then carefully blotted dry using tissue paper and counterstained by immersion in haematoxylin solution for 1 minute. Slides were then rinsed in running tap water for 5 minutes prior to being dehydrated and mounted as described in Section 2.5.4.

#### **6.3.1.4. Antibody labelling of fresh-cultured leaflets**

**Materials:** Ki67, collagen I, and elastin antibodies were purchased and used as described in Section 2.6.5.

**Methods:** Immunoperoxidase labelling of fresh-cultured valve leaflets was carried out as described in Section 2.6.1.

#### **6.3.1.5. Assessment of cell density**

Cell density was calculated by calibrating the haematoxylin and eosin images shown and placing a circle of known area randomly at different points (n=6). The number of cells in these areas was then counted and an average taken. The standard deviation and 95 % confidence limits were then calculated. This was performed at the beginning and end of the culture period for both free-cultured and statically cultured leaflets.

### **6.3.2. Re-seeding of decellularised leaflets**

#### **6.3.2.1. Isolation of human smooth muscle cells from saphenous vein**

This was carried out according to the methods described in Section 2.7.1.

#### **6.3.2.2. Source of hMSC**

All hMSC used for osteogenic cell phenotyping and re-seeding of decellularised matrices were from sample hBM1 at passage 8.

#### **6.3.2.3. Osteogenic cell phenotyping**

There was concern since the FACS analysis in Chapter 4 had revealed that a proportion of the hMSC were alkaline phosphatase positive. Consequently, it was decided to assess the phenotype of the hMSC seeded into the matrix in relation to the osteogenic phenotype.

**Materials:** Osteonectin, osteopontin and alkaline phosphatase monoclonal antibodies were purchased and used as described in Section 2.6.1. Multi-spot slides were purchased from C.A. Hendley. Methanol and acetone were purchased from VWR.

**Method:** hMSC (hBM1) at P8 were seeded onto multi-spot slides and cultured for 3 days. The cells were then fixed for 2 minutes in methanol:acetone (50:50, v/v) prior to being stored at  $-20\text{ }^{\circ}\text{C}$ . Slides were thawed when required and immunoperoxidase labelling carried out as described in Section 2.6.1.

#### 6.3.2.4. Assessment of adhesion and cell infiltration

**Materials:** Nunc 6-well tissue culture plates were obtained from Fisher Scientific. Multi-spot slides were purchased from C. A. Hendley. Methanol and acetone were purchased from VWR. Porcine heart valves were obtained from a local abattoir and had been decellularised and cryo-preserved as described in Section 2.8.

**Methods:** Previously decellularised porcine aortic heart valve leaflets (10 leaflets per cell concentration, per cell type) were thawed from cryopreservation as described in Section 2.8.3. Leaflets were then disinfected as described in Section 2.8.4 prior to being washed for  $3\times 30\text{min}$  in PBS on a shaker at room temperature. Leaflets were then placed into 6-well tissue culture plates, laid flat and 2 ml complete medium added to each well to keep the leaflets hydrated whilst cells were counted and prepared. The relevant cells were then counted using a haemocytometer as described in Section 2.4.2 and sufficient cells were carefully added drop-wise in 2 ml of complete medium (as described in Section 2.4.1) at three concentrations of  $1\times 10^4$ ,  $5\times 10^4$  and  $1\times 10^5\text{ cells.cm}^{-2}$  after removal of the medium used to keep the leaflets hydrated. The hSMC used were seeded at passage 5 and the hMSC used were seeded at passage 8 and cultured under standard conditions as described in Section 2.4.1. After 24 hours, a further 1 ml of complete medium was added to each well. After 3 days of culture, leaflets were transferred to

new 6-well plates so those cells that had adhered to the tissue culture plastic rather than the leaflets did not consume the nutrients in the medium. Leaflets were then carefully fed twice weekly with 3 ml of complete medium. Two leaflets were harvested at 24 hours for scanning electron microscopy (SEM) analysis and then 2 each at 1, 2, 3 and 4 weeks after seeding for each cell type and were embedded and sectioned for visualisation using haematoxylin and eosin staining, as described in Section 2.5.7. The length of the free edge of the leaflets cultured for 4 weeks was measured before and after the culture period to ascertain the degree of leaflet contraction. This was then converted to a percentage reduction in area of the leaflet and this value was used to calculate the true cell density without contraction in order for a direct comparison with the native homograft leaflets to be made.

#### **6.3.2.5. Preparation of samples for scanning electron microscopy (SEM)**

**Materials:** A series 3 330BM CamScan scanning electron microscope was purchased from Obducat CamScan. Acetone was purchased from VWR.

**Methods:** Samples were fixed in formalin solution (Section 2.3.3) for 24 hours prior to being dehydrated in ascending acetone concentrations of 20, 40, 60, 80, and 100 % (v/v) for 30 minutes each. Samples were then critical-point dried before being sputter-coated in gold and viewed by SEM. The images captured were digitally scanned for computer manipulation using a Minolta Dimage Image Scanner attached to a personal computer using Adobe Photoshop 5.5 software.

#### **6.3.2.6. Antibody labelling of re-seeded leaflets**

Antibody labelling of the re-seeded leaflets was carried out in order to show that seeded hMSC had not differentiated once within the tissue into any of the lineages previously described.

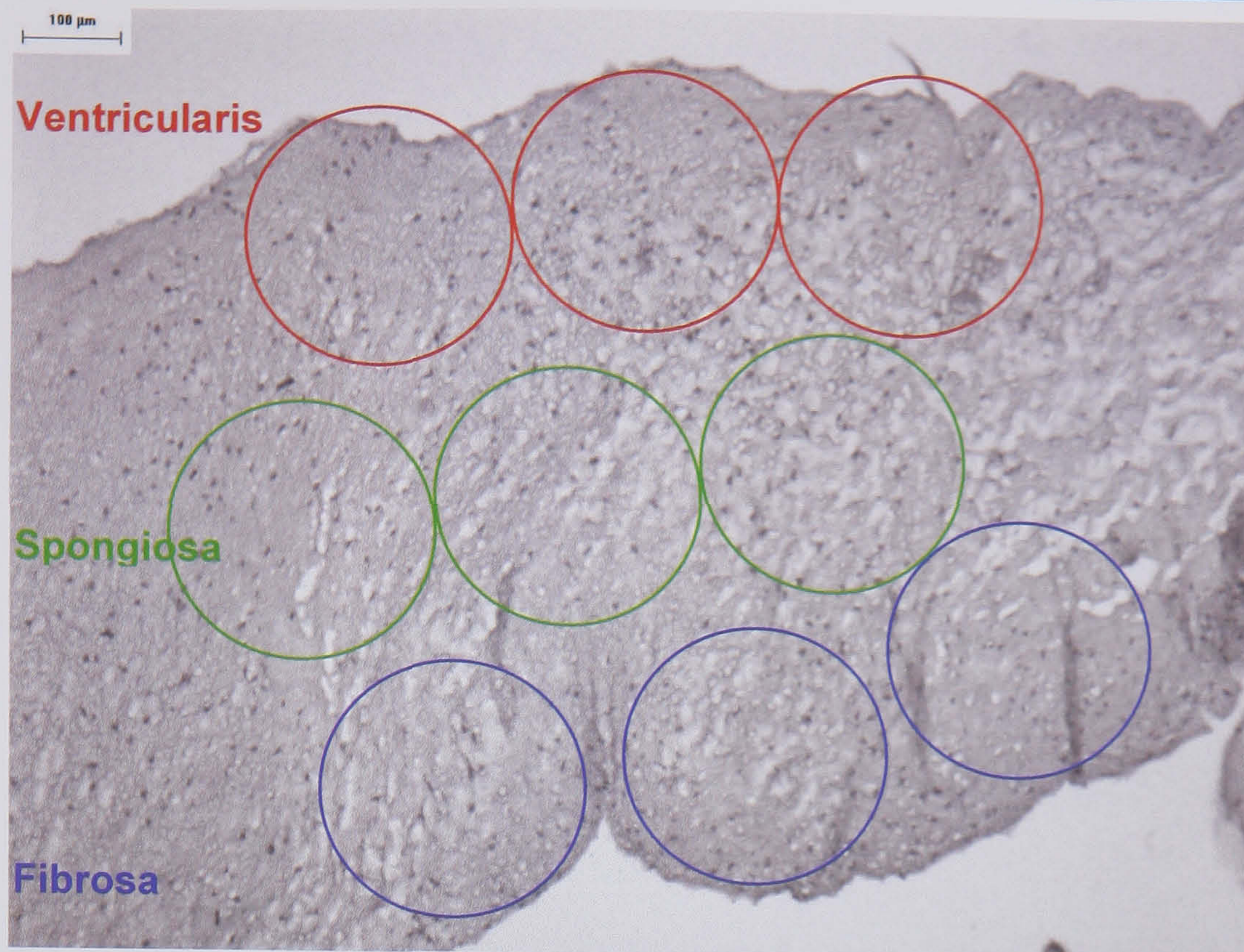
**Materials:** Antibodies were purchased and used as described in Section 2.6.1.

**Methods:** Both aortic homografts and hMSC reseeded leaflets were labelled using antibodies to  $\alpha$ SMA, vimentin, desmin, and HCMs, which are all markers of the smooth muscle cell phenotype, and tau, NSE, collagen II, alkaline phosphatase, osteonectin and osteopontin.

### 6.3.2.7. Cell counting of aortic homografts and reseeded leaflets

**Materials:** Two aortic homograft valves that were no longer suitable for clinical use had subsequently become available for research purposes and were obtained from the National Blood Service. Image Pro Plus software was supplied by MediaCybernetics UK

**Methods:** The leaflets were dissected from the homograft valves, and paraffin embedded as described in Section 2.5.1. Decellularised leaflets reseeded with both human smooth muscle cells (hSMC) and human mesenchymal stem cells (hMSC) and cultured for four weeks were also harvested and paraffin embedded. Serial sections of each of the leaflets with a thickness of 4 $\mu$ m were then made and every 15<sup>th</sup> section for the homograft leaflets and every 10<sup>th</sup> section for the reseeded leaflets were mounted onto a slide. Once 50 sections from each leaflet had been mounted, the sections were stained with haematoxylin and eosin and photographed. Image Pro Plus software was then used to calibrate the images and draw three circles in each of the three tissue layers. The area of the circles was then measured, and the number of cells within each counted. This is illustrated in Figure 6.1. The number of cells per square micrometer was then calculated for each tissue layer in each image, and the average cell number per area was calculated for each of the tissue layers in each leaflet. A comparison of cell number between the homografts and the reseeded tissues was then made as a measure of the success of the reseeded experiments.



**Figure 6.1. Diagram showing areas used for cell counting in the homografts and re-seeded leaflets.** Three circles of known area for each tissue layer were placed over the image and the number of cells in each circle counted. The count was then divided by the area of the circle and the mean number of cells per square micrometer throughout each leaflet established. Bar represents 100  $\mu\text{m}$ .

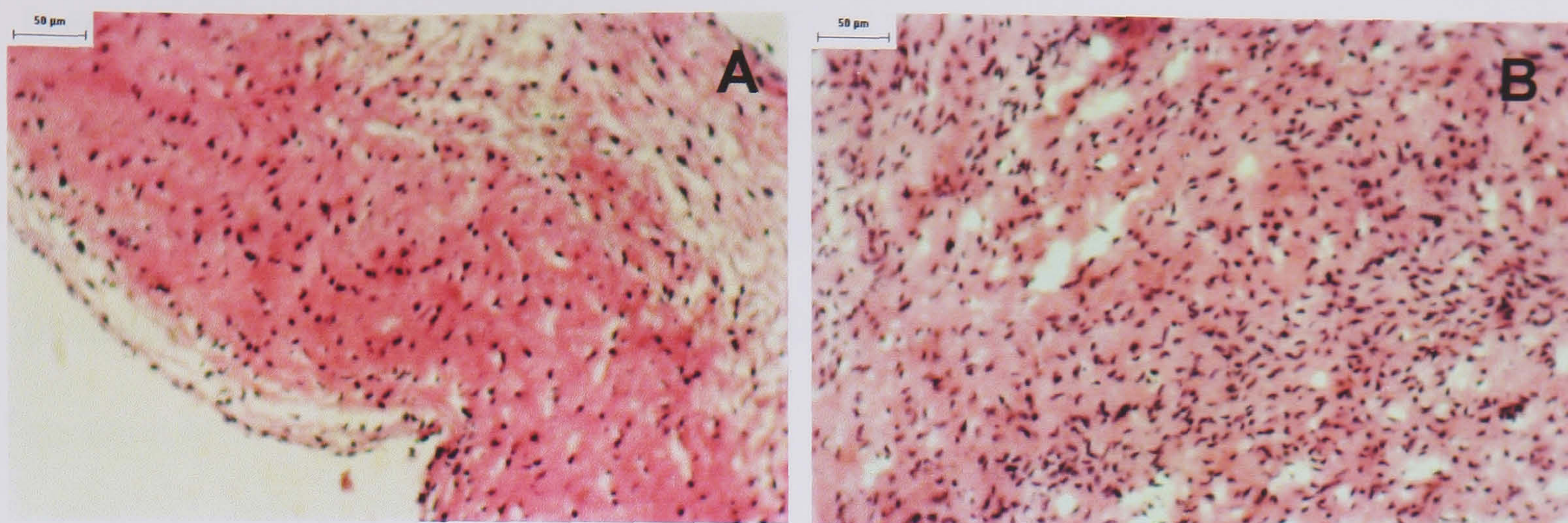
## 6.4. Results

### 6.4.1. Assessment of cultured porcine aortic valve leaflets

#### 6.4.1.1. Haematoxylin & eosin staining of free-cultured valve leaflets

Freshly dissected porcine aortic heart valve leaflets were statically cultured in complete medium for up to 22 days. The leaflets were then embedded and stained with haematoxylin and eosin, as described in Section 2.5.7 in order to assess the overall tissue structure (Figure 6.2). By day 22 it appeared as though the cells had proliferated considerably as the cell density appeared visually to be much higher when compared to a time zero tissue sample. This was later verified in a comparison of cell densities at the beginning and end of the culture period (Figure 6.5), which showed that there was a significant difference in the cell density at the beginning and end of the culture period.

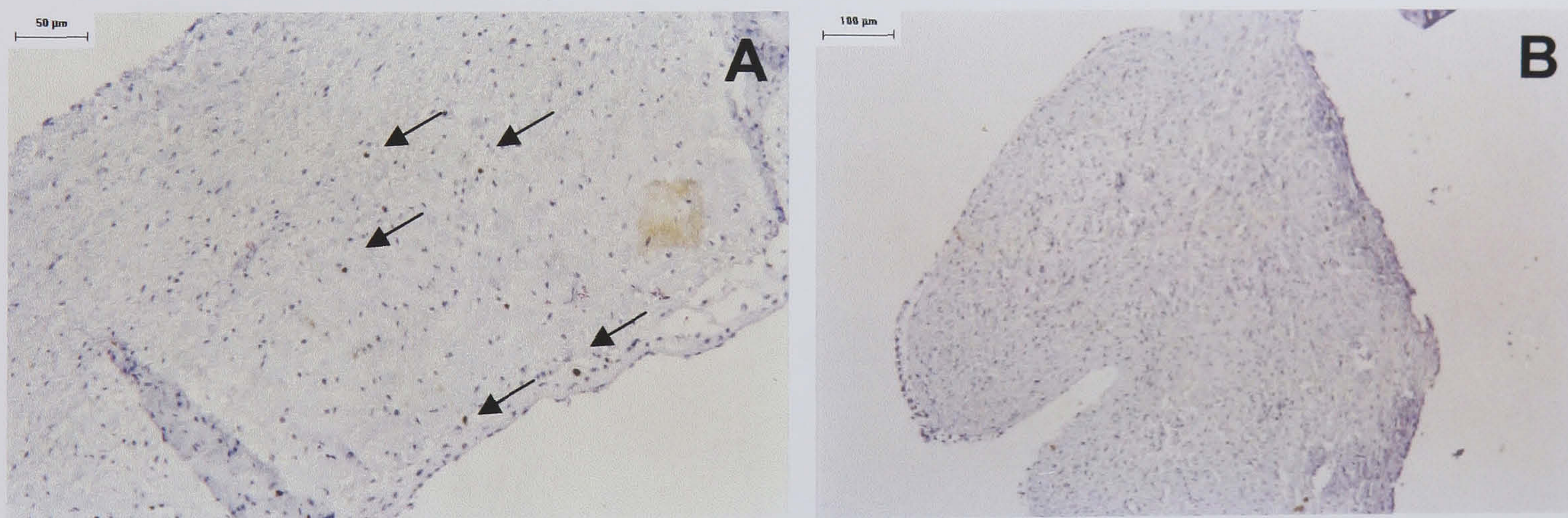




**Figure 6.2. Haematoxylin and eosin staining of statically cultured heart valve leaflets.** A: t=1 day; B: t=22 days. Purple shows the haematoxylin-stained cell nuclei, pink shows the eosin-stained cytoplasm/matrix ( $\times 200$ ).

#### 6.4.1.2. Ki-67 Labelling of free-cultured valve leaflets

The cells were stained using an antibody specific for the Ki67 antigen (Figure 6.3). Histological examination revealed only a low number of positive cells. The number of nuclei positively labelled was highest at time zero, but after 5 weeks, few positively stained cells could be seen on the leaflets.

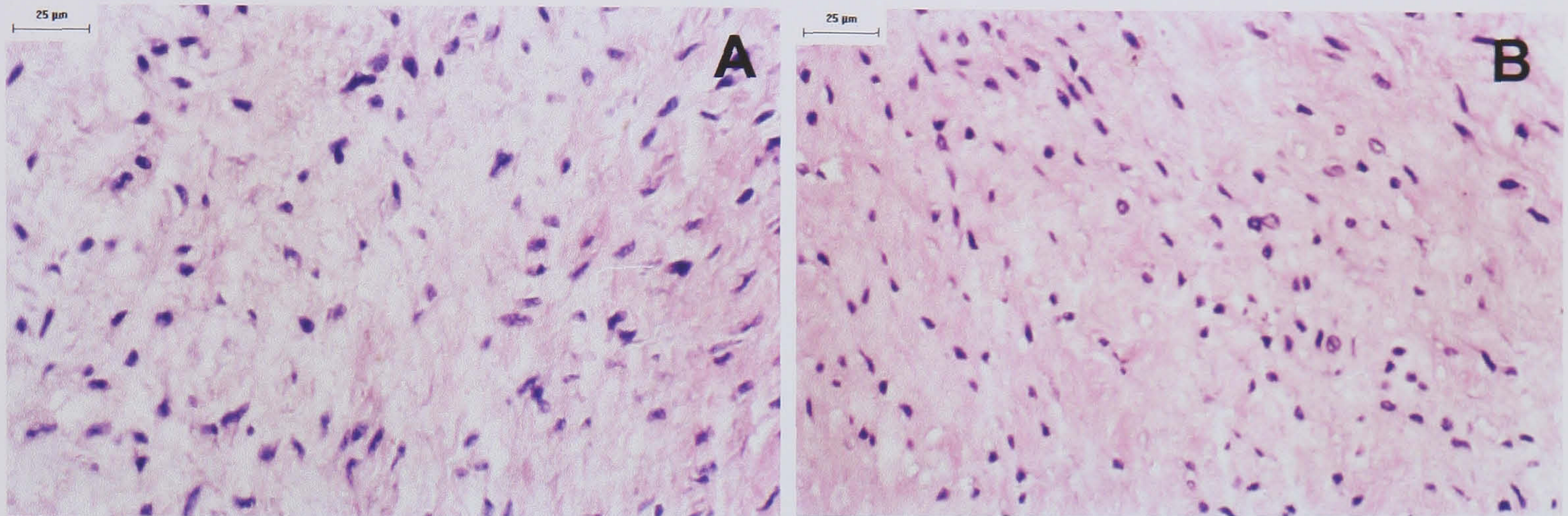


**Figure 6.3. Ki67 antibody labelling of statically cultured heart valve leaflets.** A: t=0 ( $\times 200$ ); B: t=22 days ( $\times 100$ ). Arrows highlight the brown Ki67 positive nuclei, purple corresponds to haematoxylin counterstain.

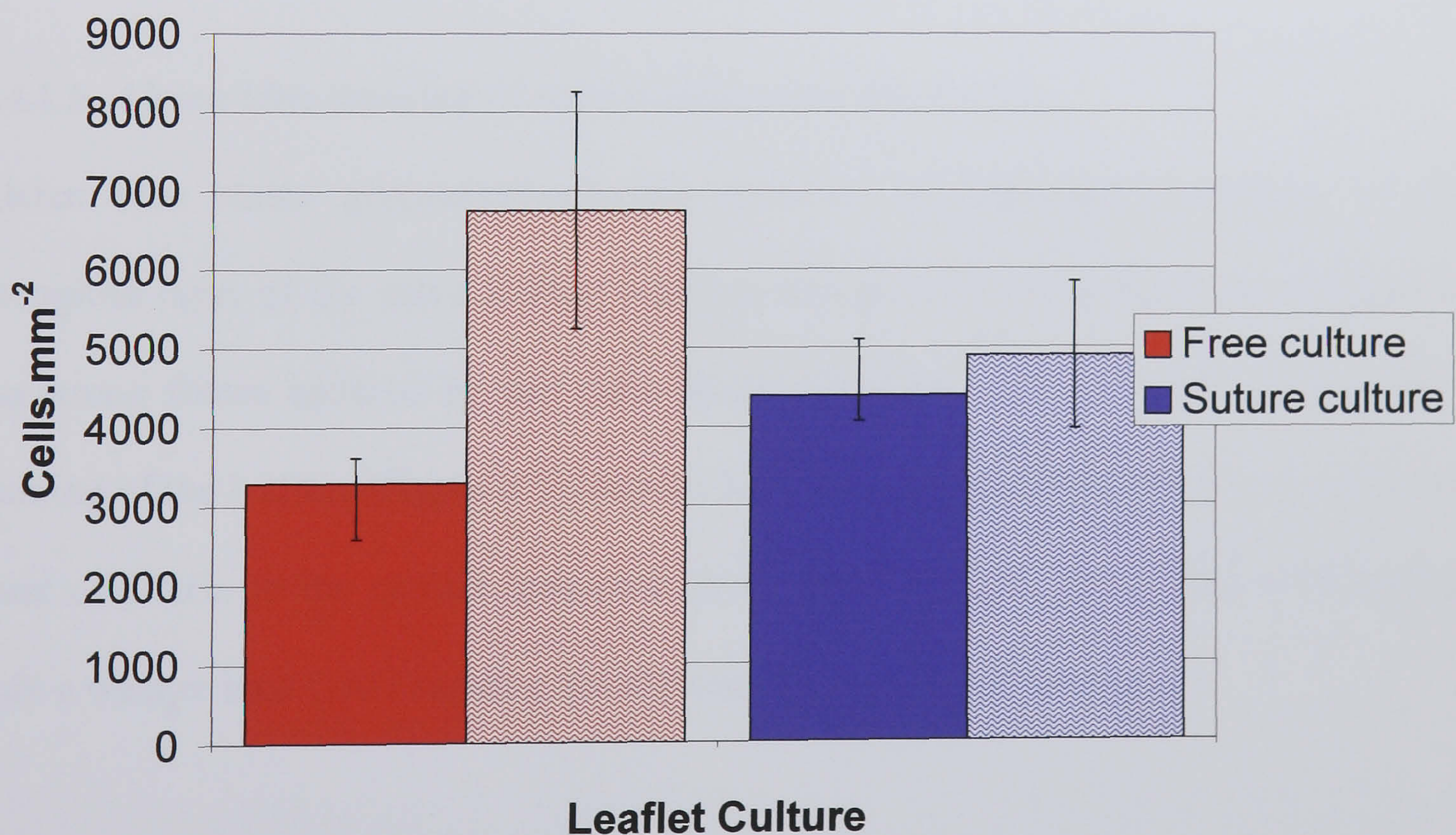
#### 6.4.1.3. Haematoxylin & eosin staining of suture-cultured valve leaflets

In order to counteract the shrinkage of the leaflets observed previously, the leaflet culture was repeated, but this time the leaflets were cultured statically under standard conditions whilst sutured to a steel mesh. Tissues were then harvested at time zero, 7, 14, 21, 28 and 35 days. The morphology of the cultured leaflets was assessed using

haematoxylin and eosin staining (Figure 6.4). In this case, the nuclei appeared to remain as spread as the time zero sample and no shrinkage of the leaflet was observed. To investigate this further, a comparison of the cell densities of the images shown demonstrated that the suture culture method had maintained the cell density at a constant level (Figure 6.5).



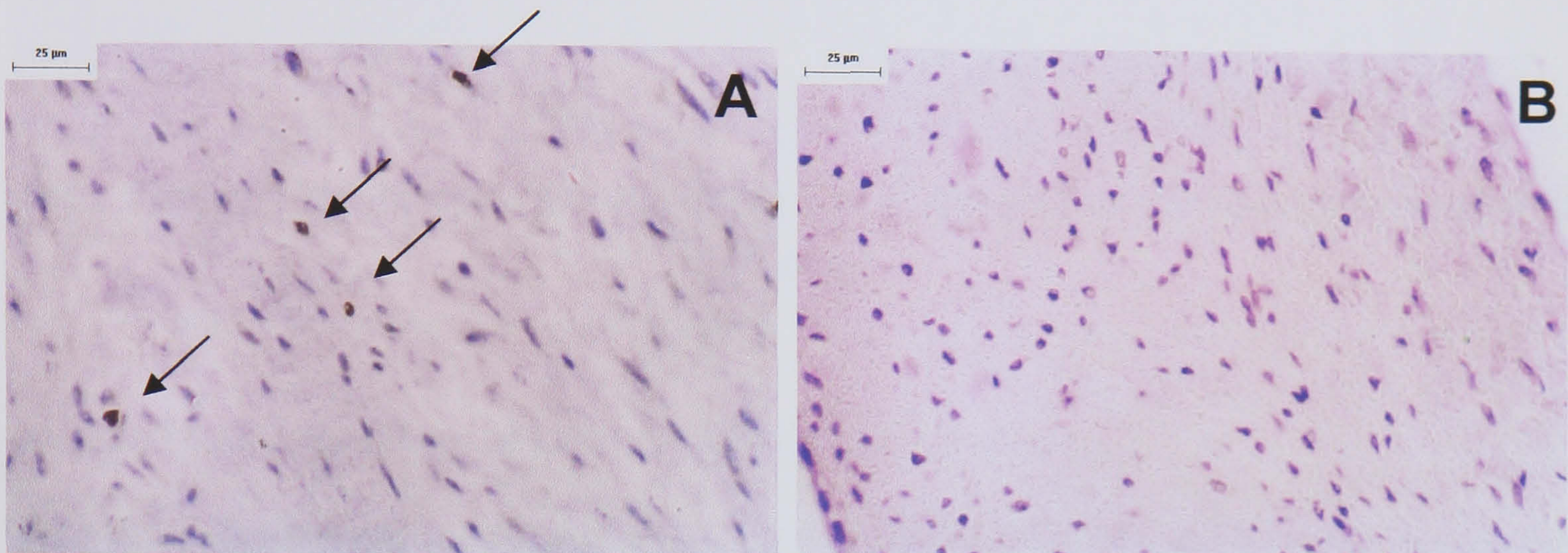
**Figure 6.4.** Haematoxylin & eosin staining of suture-cultured valve leaflet. A: t = 0wk; B: t = 6wk.



**Figure 6.5.** Comparison of cell density from before and after culture of leaflets cultured freely and sutured to steel mesh. Solid bar shows density at beginning of culture, patterned bar shows density at end of culture. Error bars show 95 % confidence limits.

#### 6.4.1.4. Ki67 Labelling of suture-cultured valve leaflets

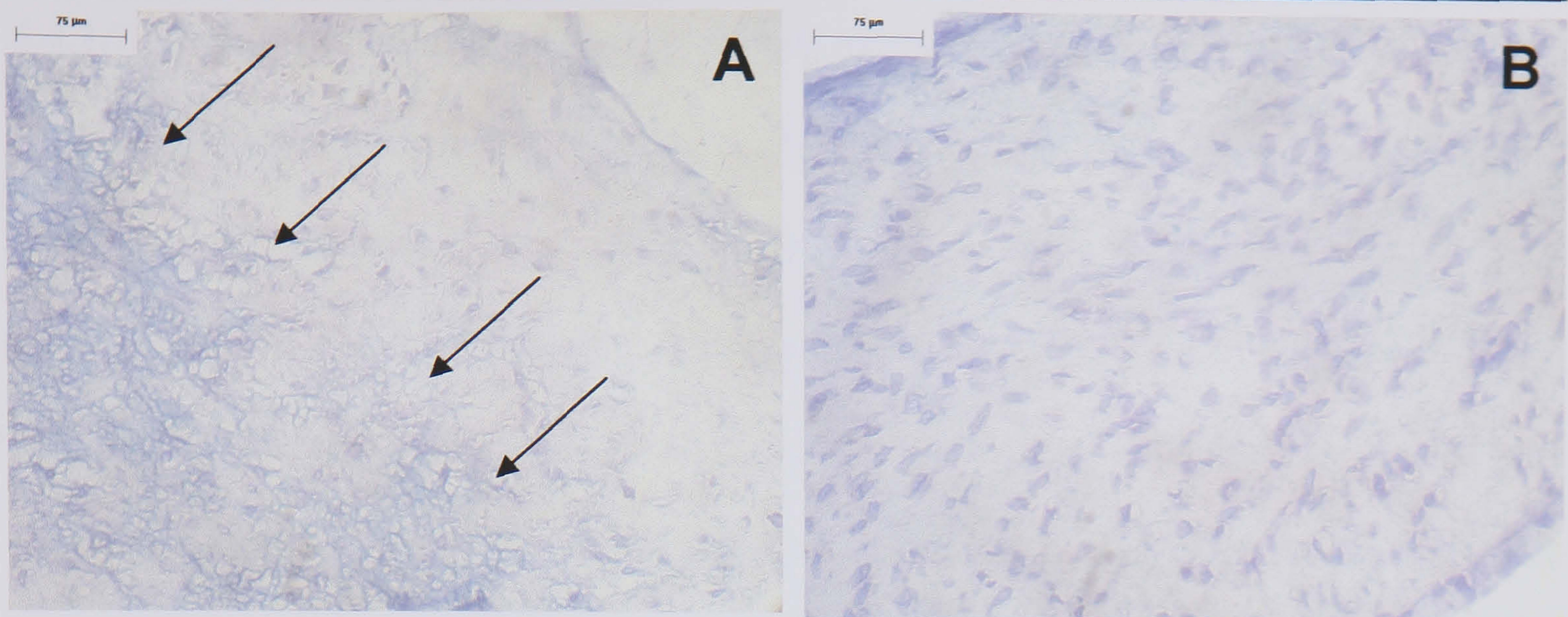
The cells were again stained using an antibody specific for the Ki67 antigen in order to establish whether the cells were actively proliferating (Figure 6.6). Labelling again revealed that only a small number of cells were positive for the antigen and by 5 weeks no positively stained cells could be found at all.



**Figure 6.6. Ki-67 labelling of suture-cultured valve leaflets.** A:  $t = 0\text{wk}$ ; B:  $t = 5\text{wk}$ . ( $\times 200$ ). Arrows highlight the brown Ki67 positive nuclei, purple corresponds to haematoxylin counterstain.

#### 6.4.1.5. Alcian blue staining of suture-cultured valve leaflets

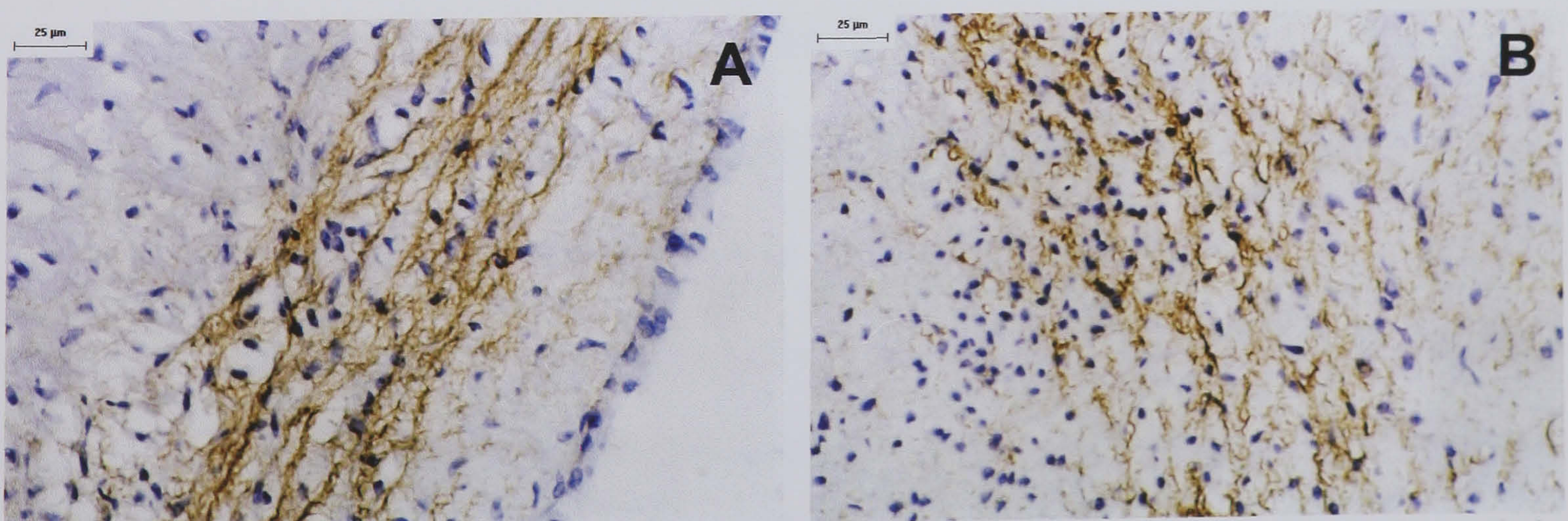
Alcian blue stains glycosaminoglycans (GAGs), an important constituent of the spongiosa layer of the valve leaflets which is thought to be responsible for dissipating the strong forces endured by the valve leaflet [Schoen, 1999]. The blue extracellular staining of the leaflets (Figure 6.7) showed how although initially there was a prominent band of GAGs in the central spongiosa layer, after 5 weeks this disappeared leaving only a weaker blue GAG staining throughout all the valve leaflet layers.



**Figure 6.7. Alcian blue staining of suture-cultured valve leaflets.** A:  $t = 0$ wks; B:  $t = 5$ wks. Note prominent localised blue staining in A (arrows) compared to diffuse staining in B ( $\times 200$ ).

#### 6.4.1.6. Elastin labelling of suture-cultured valve leaflets

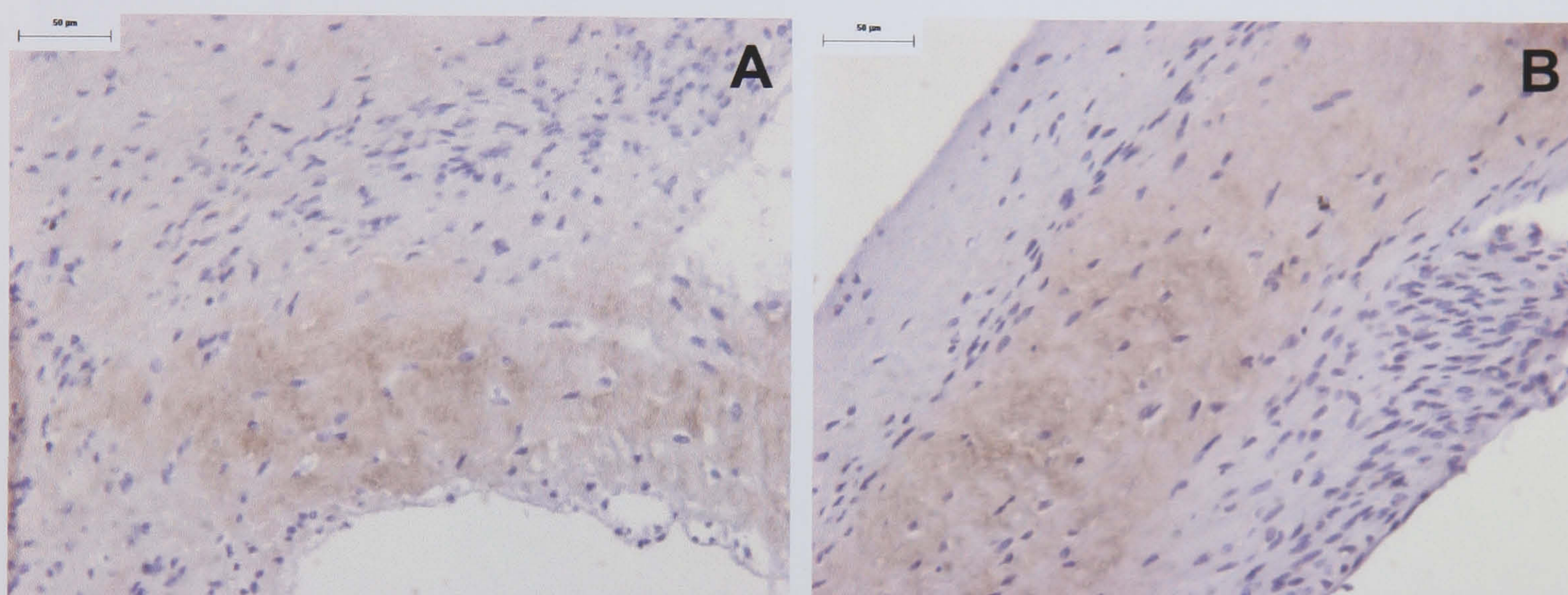
Elastin, present in the ventricularis layer, has a vital role in providing leaflet tissue with an elastic property which allows the leaflets to be stretched against the aorta when the valve is open, and pulls the leaflets back to occlude the valve when it closes. Consequently an antibody to elastin was also used to stain the leaflets to see whether this important flexibility-giving protein had been maintained during the culture period. The brown labelling of the leaflets (Figure 6.8) shows long elastin chains at time zero which degraded and fragmented during the course of 5 weeks of static culture.



**Figure 6.8. Elastin labelling of suture-cultured valve leaflets.** A:  $t = 0$ wk; B:  $t = 5$ wk ( $\times 200$ ). Brown staining shows elastin chains that became fragmented by 5 weeks.

### 6.4.1.7. Collagen I labelling of suture-cultured valve leaflets

Collagen I is the major strength-giving component in aortic heart valve leaflets where it features most prominently in the fibrosa layer. Here it forms macroscopically crimped, densely packed fibres which are arranged parallel to the leaflet's free edge. These crimps expand during backpressure to ensure the valve is fully occluded. Thus it was also necessary to assess how the collagen I was affected during static culture. The brown staining (Figure 3.8) indicated that the collagen was unaffected during the culture period and remained histologically intact.



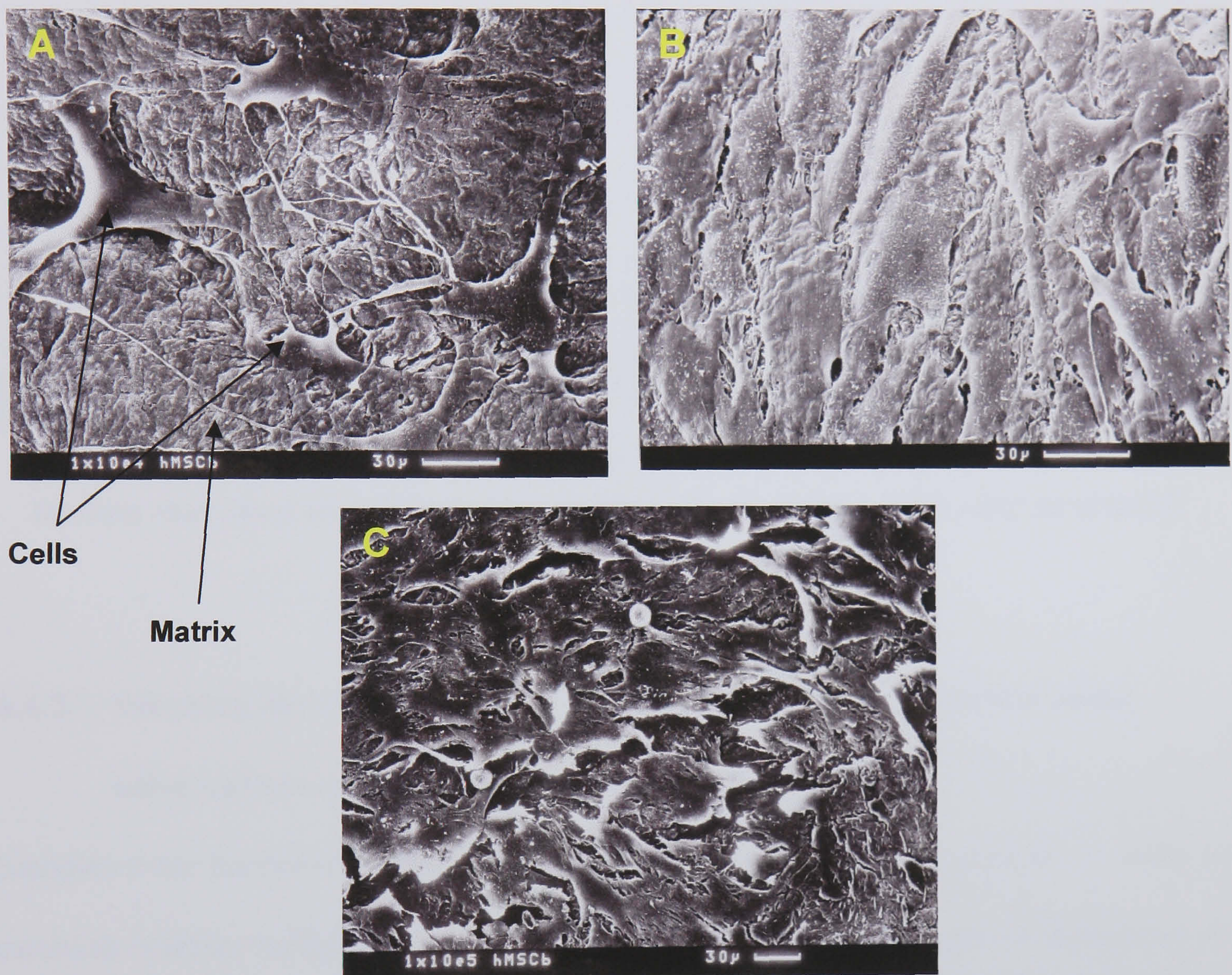
**Figure 6.9. Collagen I labelling of suture-cultured valve leaflets.** A: 3 week culture; B: 6 week culture. Brown staining shows presence of collagen I, purple shows counterstained cell nuclei. Bar represents 50  $\mu\text{m}$ .

## 6.4.2. Re-seeding of decellularised leaflets

### 6.4.2.1. Scanning electron microscopy (SEM) analysis of decellularised aortic valve leaflets reseeded with human mesenchymal stem cells.

Twenty-four hours after seeding was carried out, samples were harvested for SEM analysis to establish whether sufficient cells had been applied to the tissue to give a monolayer of cells on the surface of the leaflet. The results of the SEM analysis indicated that at  $1 \times 10^4 \text{ cells.cm}^{-2}$ , the cells were spread out over the surface of the leaflet and were frequently not in physical contact with neighbouring cells. At a

concentration of  $5 \times 10^4$  cells.cm<sup>-2</sup> a monolayer of cells was observed which were mostly in direct contact with neighbouring cells. At a concentration  $1 \times 10^5$  cells.cm<sup>-2</sup>, the cells could be seen to be piled on top of one another with the many cells not in direct contact with the matrix itself. Consequently, a concentration of  $5 \times 10^4$  cells.cm<sup>-2</sup> was deemed the most appropriate concentration to use (Figure 6.10).



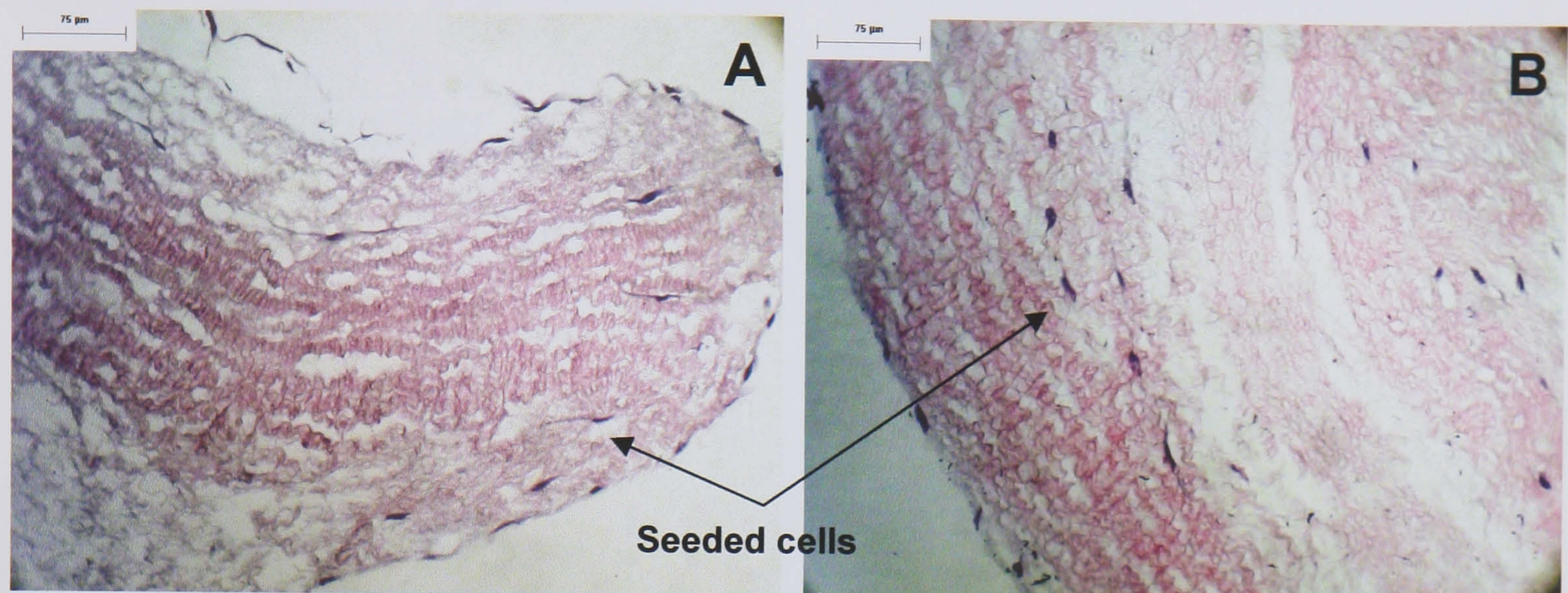
**Figure 6.10. Scanning electron microscopy (SEM) of decellularised aortic valve leaflets reseeded with human mesenchymal stem cells. A: cells seeded at  $1 \times 10^4$  cells.cm<sup>-2</sup>; B: cells seeded at  $5 \times 10^4$  cells.cm<sup>-2</sup>; C: cells seeded at  $1 \times 10^5$  cells.cm<sup>-2</sup>.**

#### **6.4.2.2. Haematoxylin and eosin staining analysis of decellularised aortic valve**

##### **leaflets reseeded with human mesenchymal stem cells**

Samples were harvested after 1, 2, 3, and 4 weeks, paraffin embedded, sectioned and stained using haematoxylin and eosin as described in Section 2.5 in order to assess whether the cells had migrated into the leaflet. The results indicated that some cell migration into the tissue had occurred (Figure 6.11). Measurement of the length of the

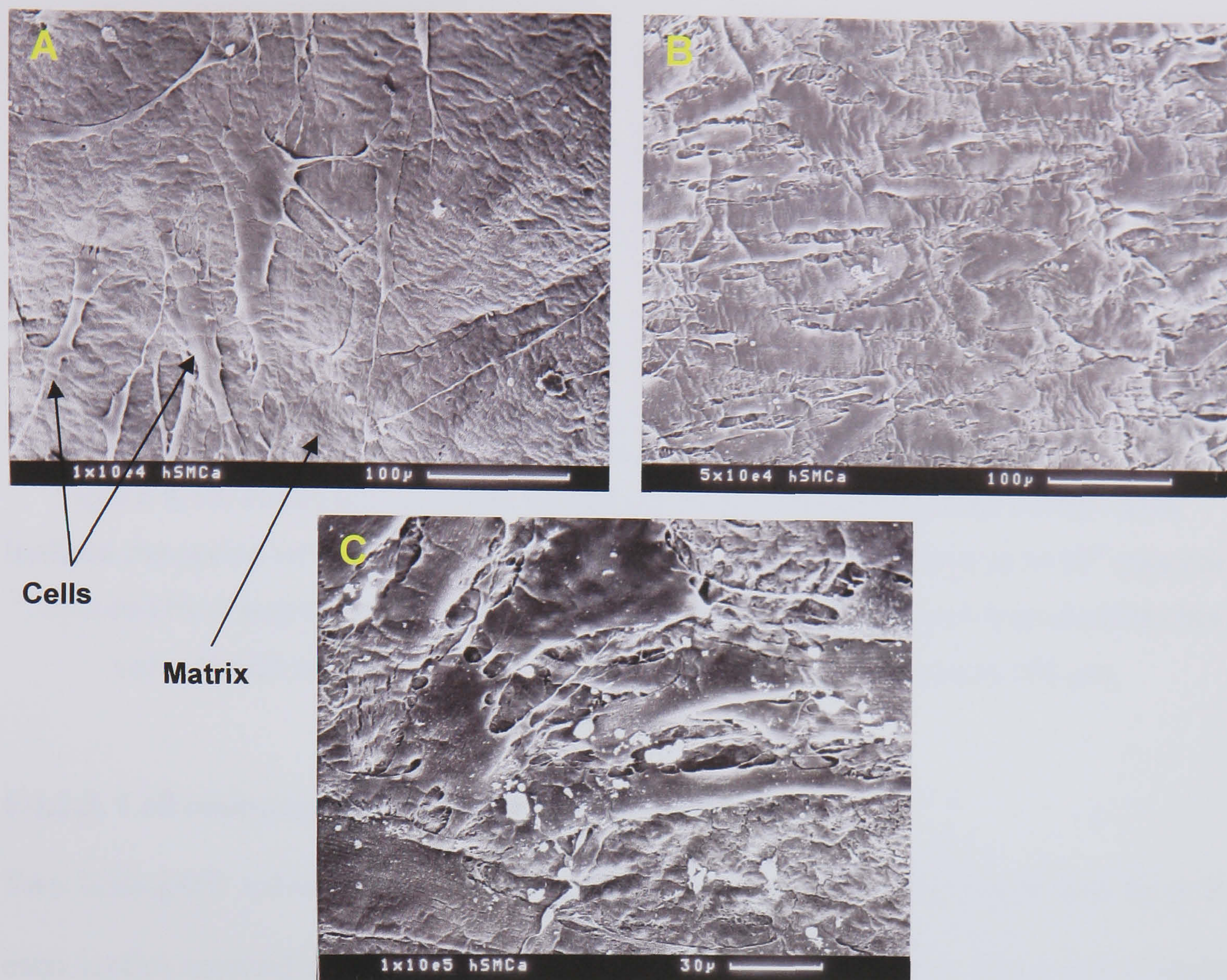
free edge of the leaflets cultured for 4 weeks both before and after culture indicated that the free edge had shrunk from 21.5 mm to 10 mm on leaflet A, and from 20 mm to 9 mm on leaflet B. This corresponded to an area reduction by 80 % for leaflet A and 78 % for leaflet B.



**Figure 6.11. Haematoxylin and eosin staining of decellularised aortic valve leaflets reseeded with human mesenchymal stem cells. Cells seeded at  $5 \times 10^4$  cells.cm<sup>-2</sup>, cultured for 4 weeks.**

#### **6.4.2.3. Scanning electron microscopy (SEM) analysis of decellularised aortic valve leaflets reseeded with human smooth muscle cells.**

Samples were harvested twenty-four hours after seeding for SEM analysis in order to establish whether sufficient cells had been applied to the tissue to give a monolayer of cells on the surface of the leaflet. The results of the SEM analysis indicated that at  $1 \times 10^4$  cells.cm<sup>-2</sup>, the cells were spread out over the surface of the leaflet and were frequently not in physical contact with neighbouring cells. At a concentration of  $5 \times 10^4$  cells.cm<sup>-2</sup> a monolayer of cells was observed which were all in direct contact with neighbouring cells. At a concentration  $1 \times 10^5$  cells.cm<sup>-2</sup>, the cells could be seen to be piled on top of one another with the many cells not in direct contact with the matrix itself. Consequently, a concentration of  $5 \times 10^4$  cells.cm<sup>-2</sup> was deemed the most appropriate concentration to use (Figure 6.12).

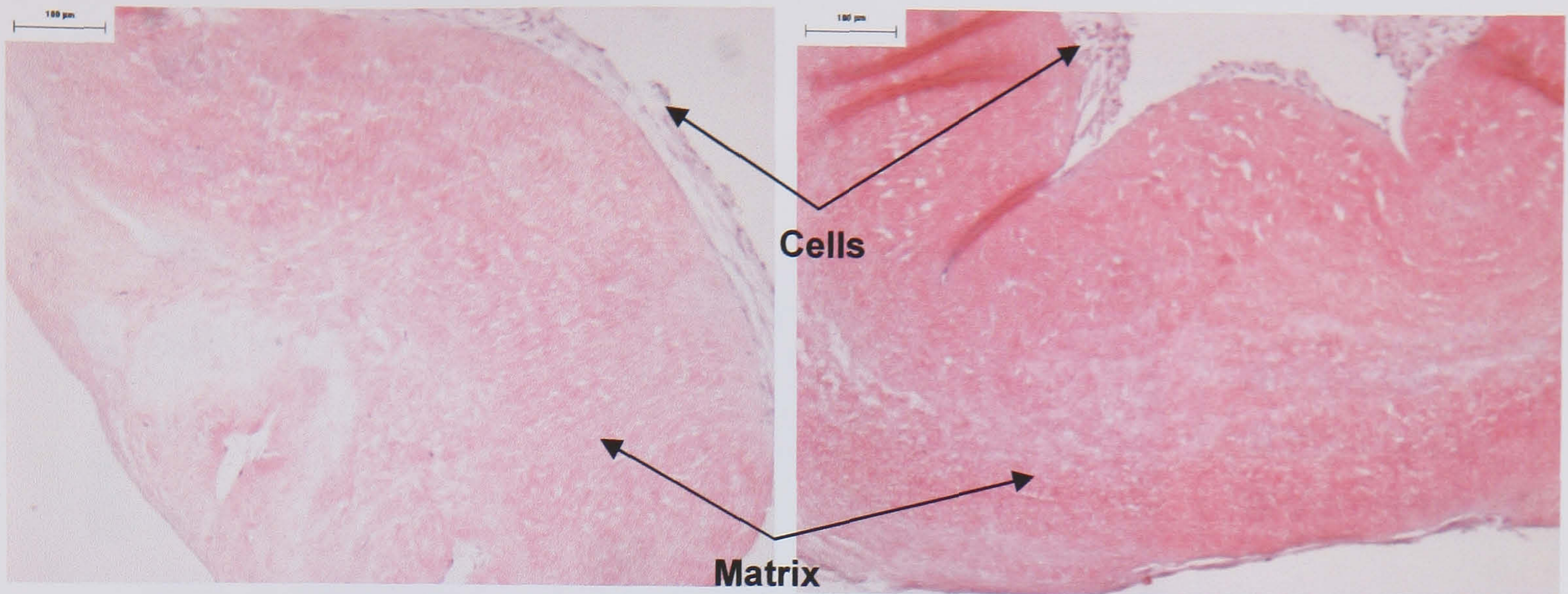


**Figure 6.12. Scanning electron microscopy (SEM) of decellularised aortic valve leaflets reseeded with human smooth muscle cells. A: cells seeded at  $1 \times 10^4$  cells. $\text{cm}^{-2}$ ; B: cells seeded at  $5 \times 10^4$  cells. $\text{cm}^{-2}$ ; C: cells seeded at  $1 \times 10^5$  cells. $\text{cm}^{-2}$ .**

#### **6.4.2.4. Haematoxylin and eosin staining analysis of decellularised aortic valve leaflets reseeded with human smooth muscle cells**

Samples were harvested after 1, 2, 3, and 4 weeks and stained using haematoxylin and eosin as described in Section 2.5.4 in order to assess whether the cells had migrated into the leaflet. The results indicated that no cell migration into the matrix had occurred and instead, the cells had remained on the surface of the leaflets (Figure 6.13). Measurement of the length of the free edge of the leaflets cultured for 4 weeks both before and after culture indicated that the free edge had shrunk from 24 mm to 12 mm on leaflet A, and from 21 mm to 11 mm on leaflet B. This corresponded to an area reduction of 75 % for leaflet A and 73 % for leaflet B.

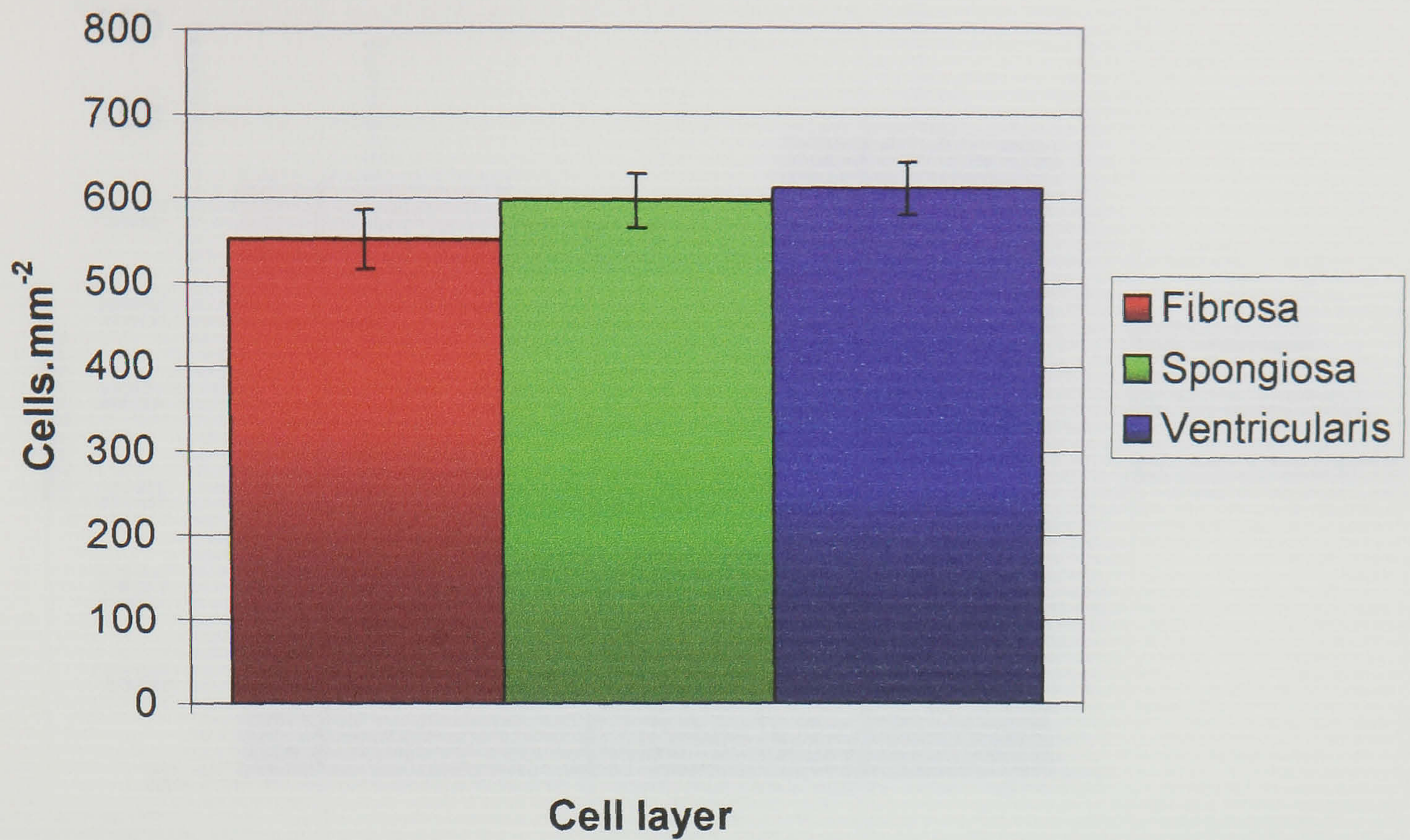




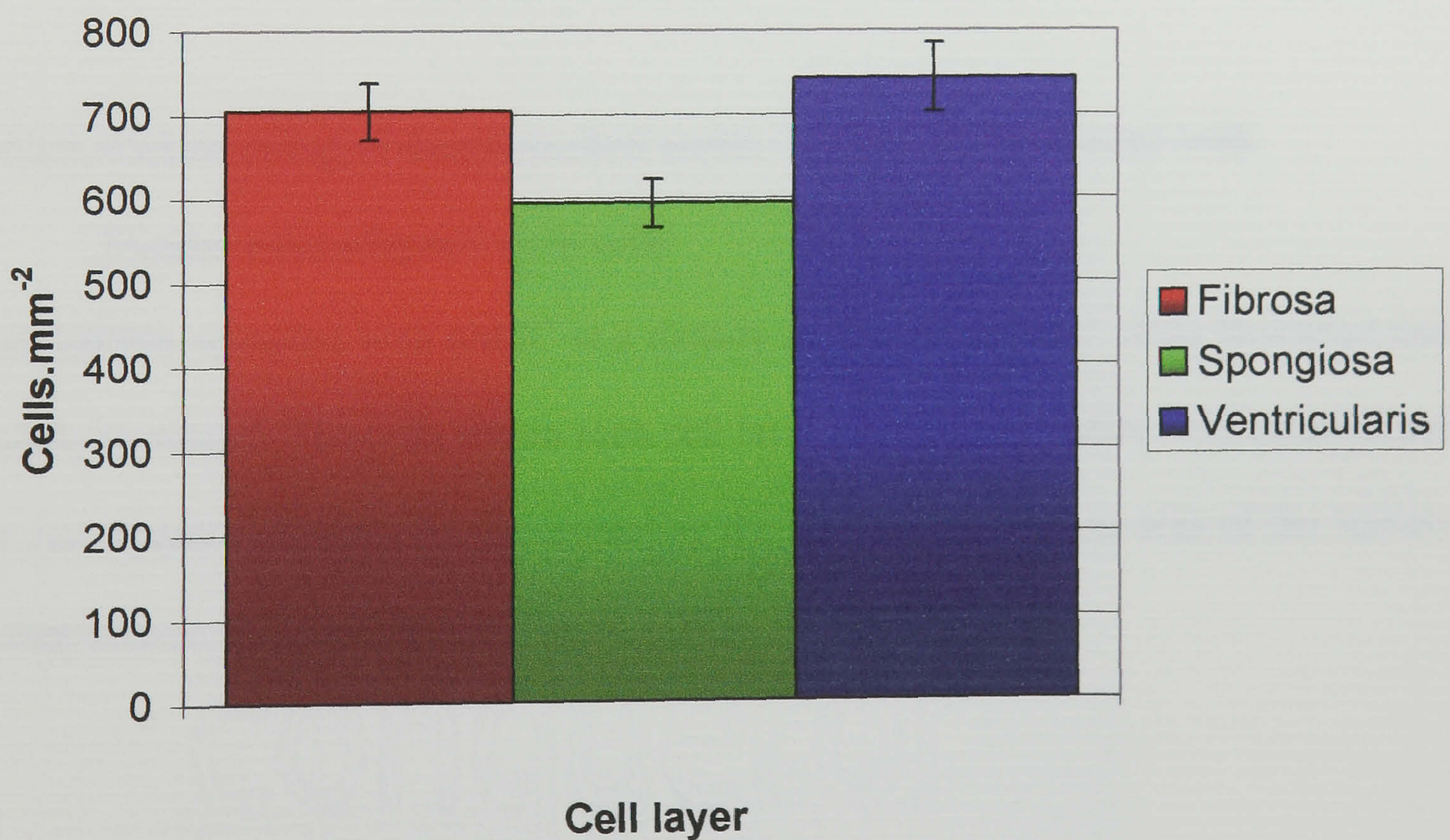
**Figure 6.13. Haematoxylin and eosin staining of decellularised aortic valve leaflets reseeded with human smooth muscle cells.** Cells seeded at  $5 \times 10^4$  cells. $\text{cm}^{-2}$ , cultured for 4 weeks. In both images, no cells can be seen to have migrated into the valve tissue and instead remain on the surface. Bar represents 100  $\mu\text{m}$ .

#### 6.4.2.5. Cell counting of human aortic homograft valves

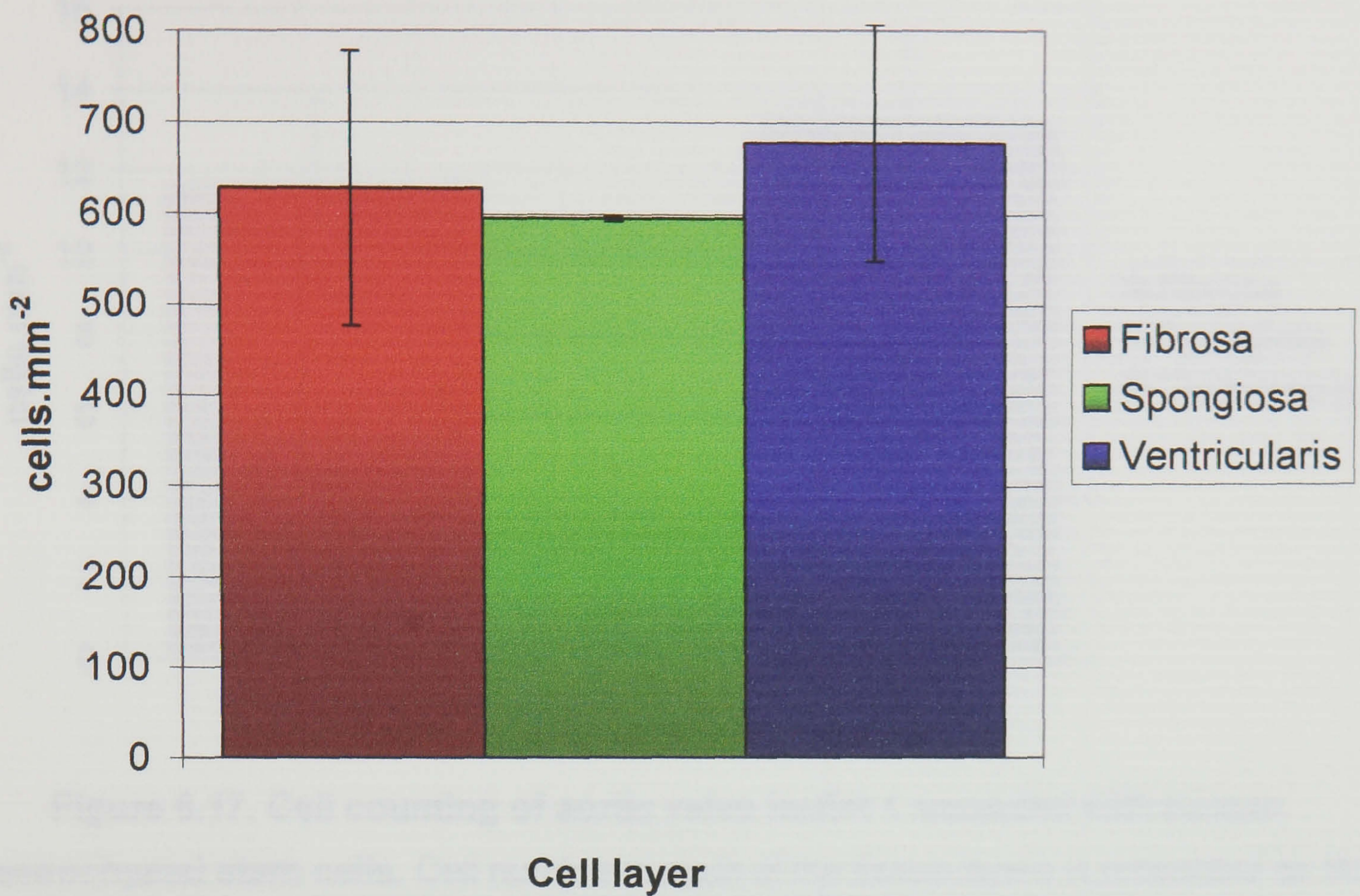
Two homograft valves were serially sectioned and the cells within each cell layer in each leaflet counted and compared (Figures 6.14, 6.15 and 6.16). There was a slight overall difference between the cell numbers between the two valves: however, patient details were only available for the first valve, which was taken from a 46 yr old immunosuppressed male.



**Figure 6.14. Cell counting of human aortic homograft valve 1.** Cell number in each of the tissue layers is presented as the mean ( $n=150$ )  $\pm$  95 % confidence limits.



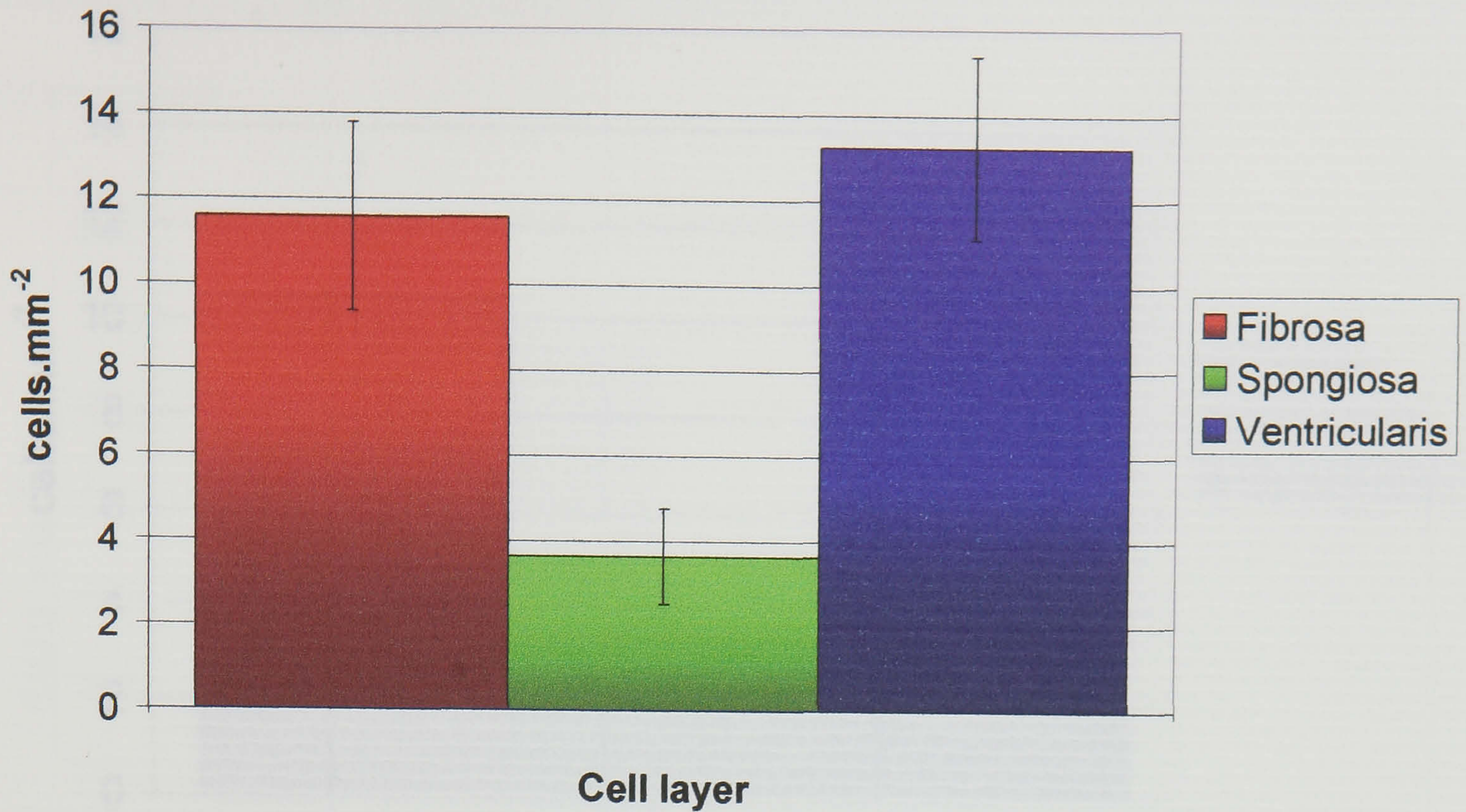
**Figure 6.15. Cell counting of human aortic homograft valve 2.** Cell number in each of the tissue layers is presented as the mean ( $n=150$ )  $\pm$  95 % confidence limits.



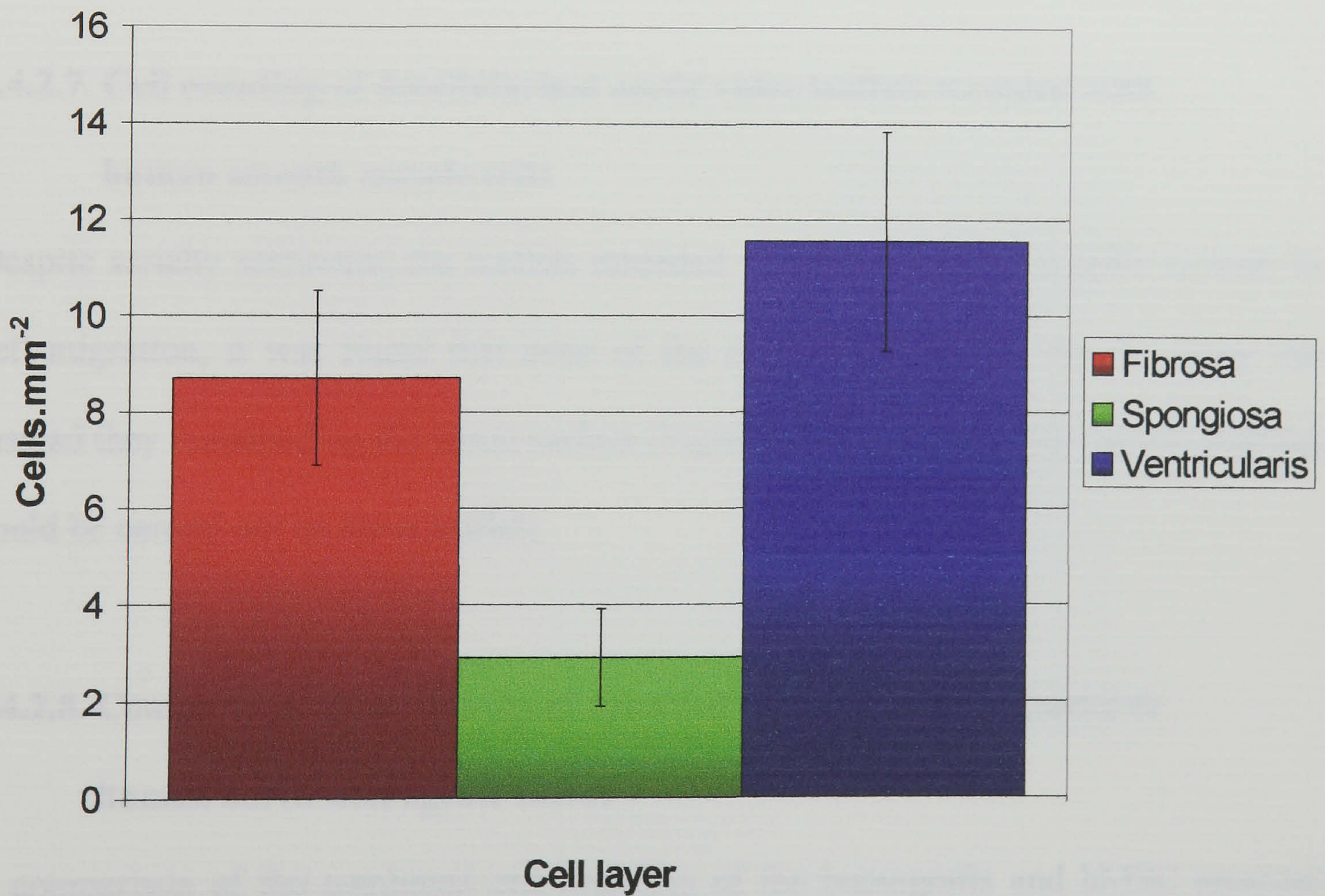
**Figure 6.16. Combined cell counting of both human aortic homograft valves.** Cell number in each of the tissue layers in the two valves combined is presented as the mean ( $n=2$ )  $\pm$  95 % confidence limits.

#### 6.4.2.6. Cell counting of decellularised aortic valve leaflets reseeded with human mesenchymal stem cells

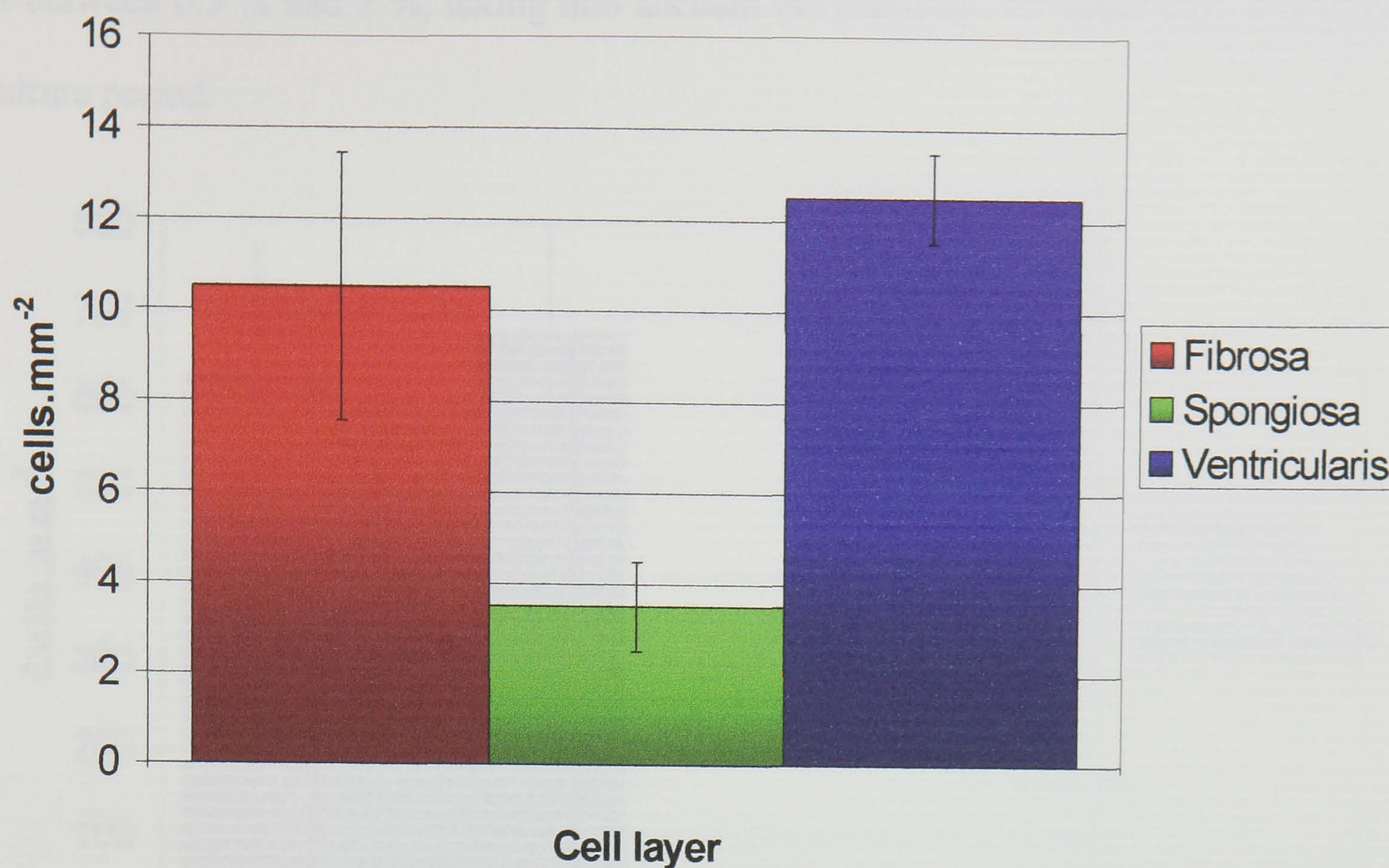
Two leaflets reseeded with hMSC at a density of  $5 \times 10^4$  cells.cm<sup>-2</sup> were harvested and serially sectioned. The cells within each cell layer within each leaflet were counted and the cell densities determined, incorporating the reduction in surface area of the leaflets during culture discussed earlier (Figure 6.19).



**Figure 6.17. Cell counting of aortic valve leaflet 1 reseeded with human mesenchymal stem cells.** Cell number in each of the tissue layers is presented as the mean ( $n=150$ )  $\pm$  95 % confidence limits.



**Figure 6.18. Cell counting of aortic valve leaflet 2 reseeded with human mesenchymal stem cells.** Cell number in each of the tissue layers is presented as the mean ( $n=150$ )  $\pm$  95 % confidence limits.



**Figure 6.19. Cell counting of two aortic valve leaflets reseeded with human mesenchymal stem cells.** Cell number in each of the tissue layers is presented as the mean ( $n=2$ )  $\pm$  95 % confidence limits.

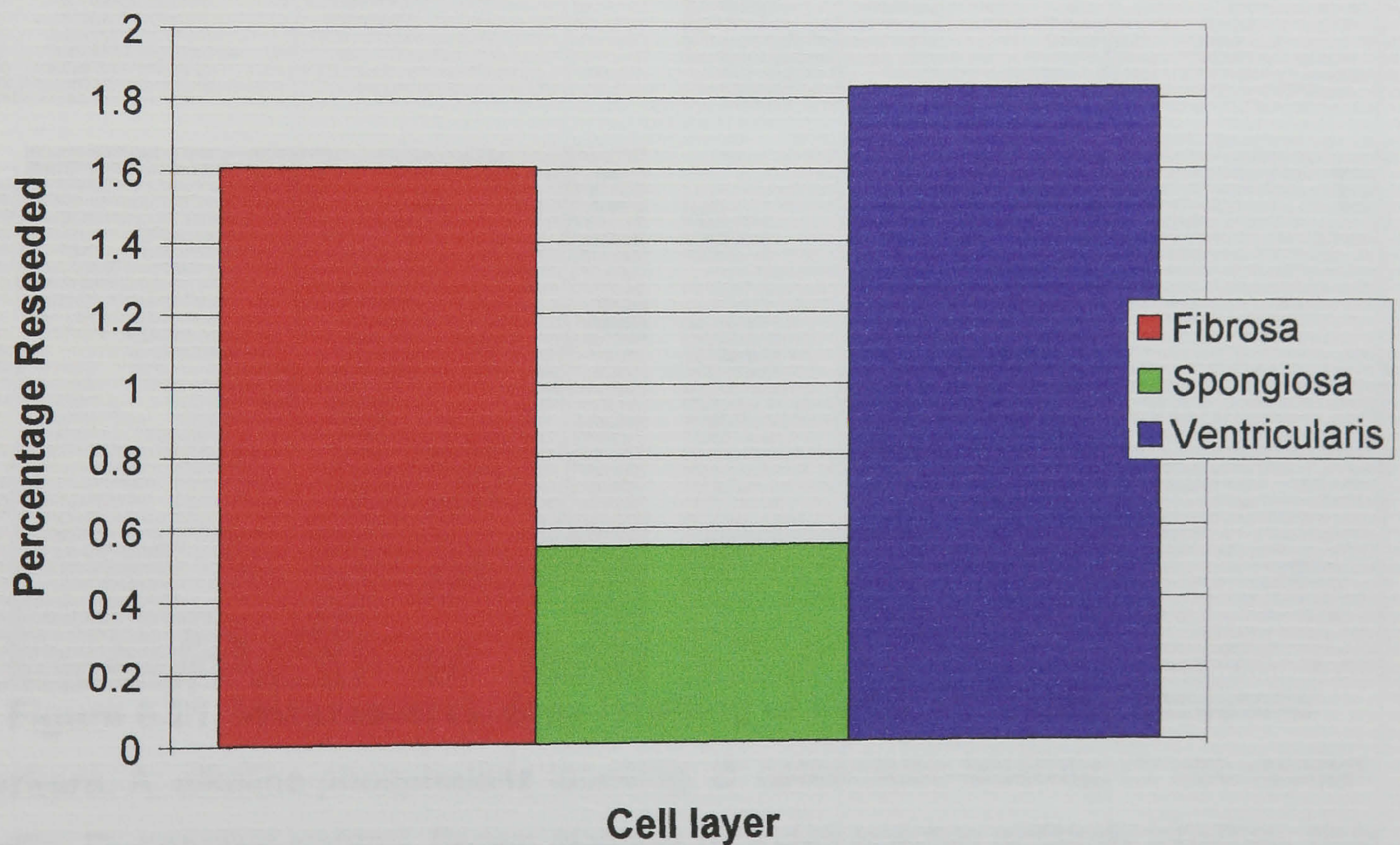
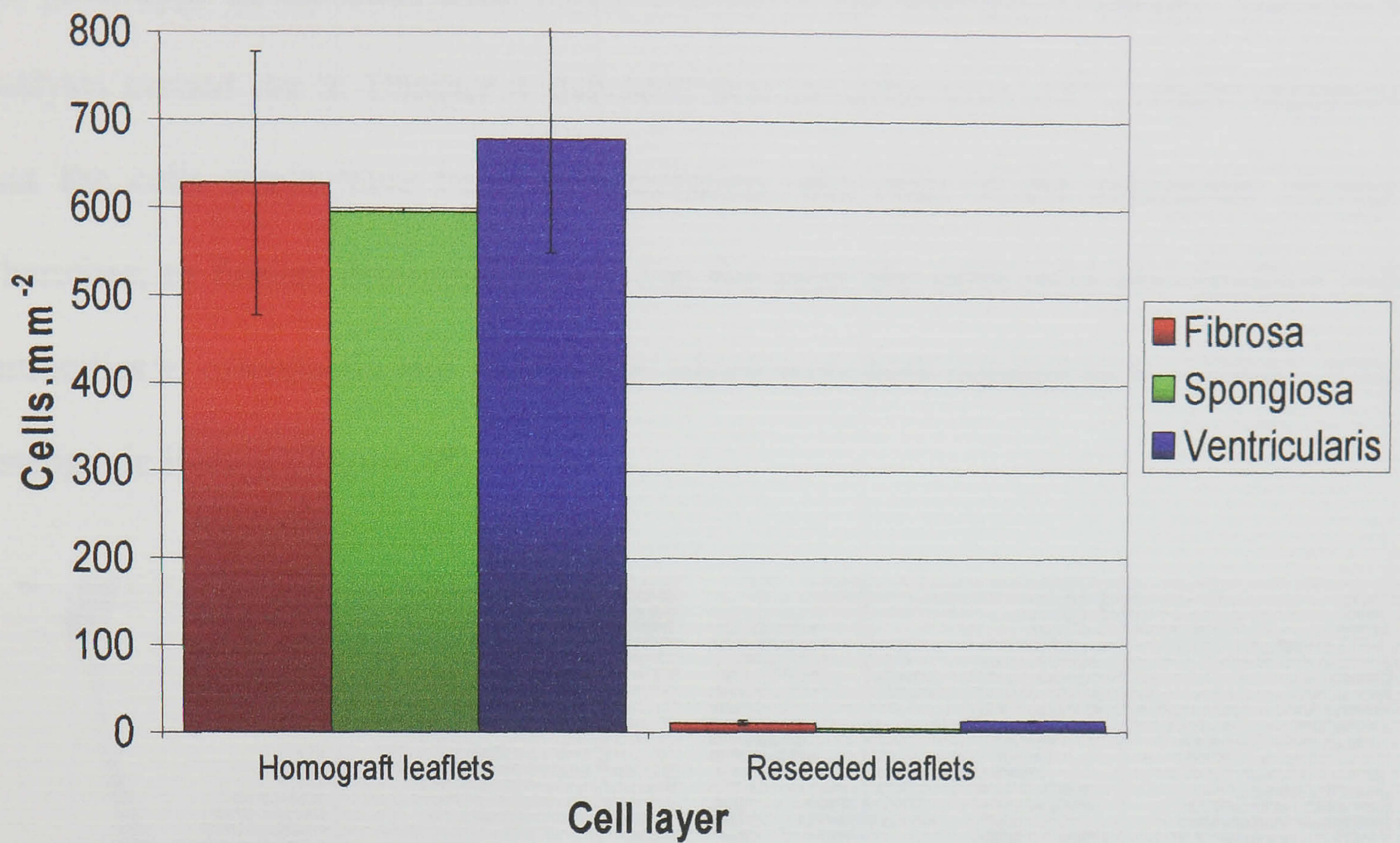
#### 6.4.2.7. Cell counting of decellularised aortic valve leaflets reseeded with human smooth muscle cells

Despite serially sectioning the leaflets reseeded with human SMC in order to look for cell migration, it was found that none of the cells had migrated into the tissue and instead they remained on the tissue surface (Figure 6.13). Consequently no cell counting could be carried out on these leaflets.

#### 6.4.2.8. Comparison of cell density of reseeded valve leaflets compared to human aortic homograft valves

A comparison of the combined cell densities of the homografts and hMSC reseeded valve leaflets was made (Figure 6.20). The percentage reseeded was then calculated by setting the mean cell density in the respective valve layers of the homografts as 100 % and comparing the reseeded valve leaflet layers to this level. This gave a reseeded level

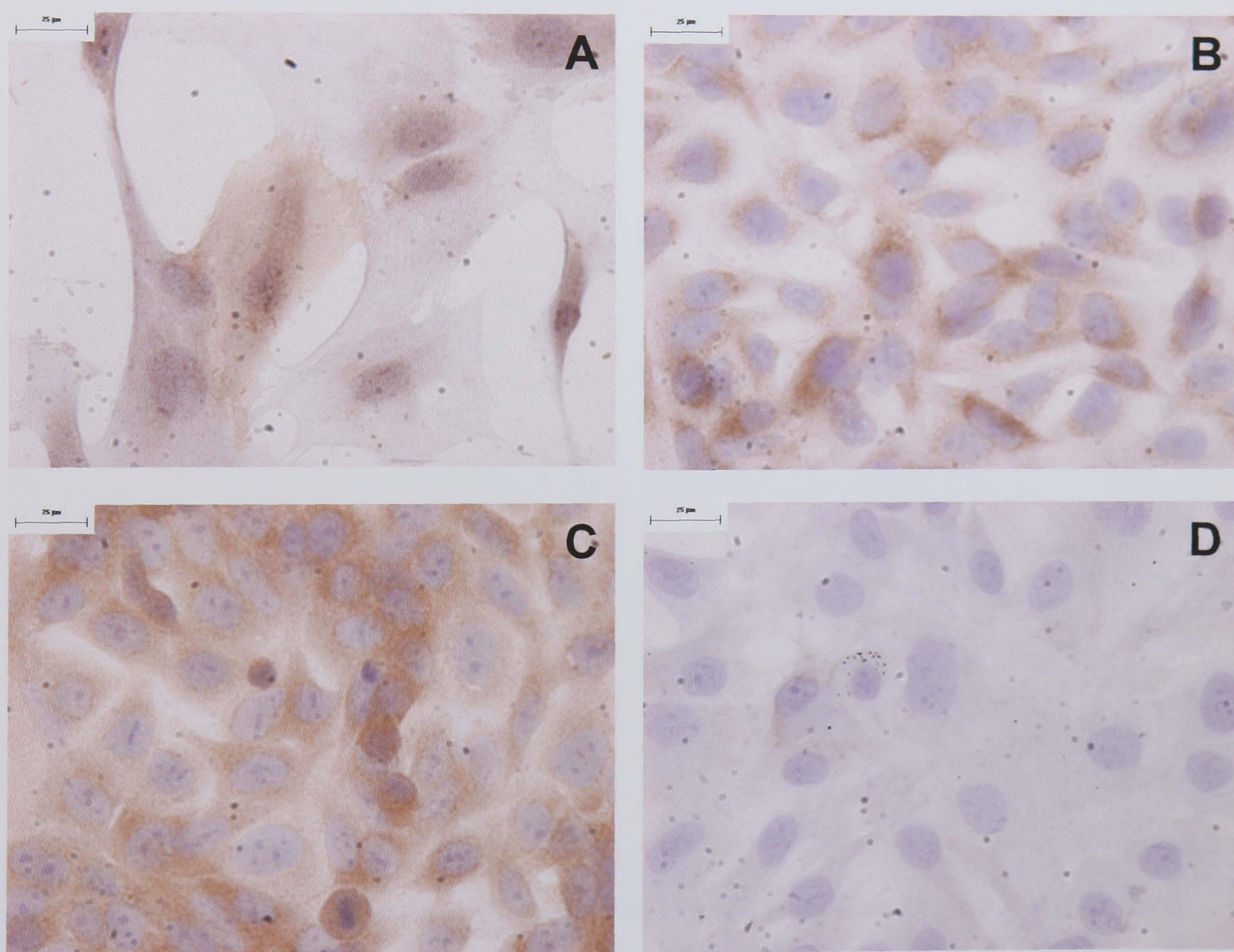
of between 0.5 % and 2 %, taking into account the reduction in leaflet area during the culture period.



**Figure 6.20. Comparison of cell density of reseeded valve leaflets compared to human aortic homograft valves.** Top graph: comparison of cell densities between homografts and hMSC reseeded tissues Cell number in each of the tissue layers is presented as the mean ( $n=150$ )  $\pm$  95 % confidence limits; Lower graph: percentage of cells reseeded relative to homograft valve.

#### 6.4.2.9. Antibody labelling of hMSC used for re-seeding

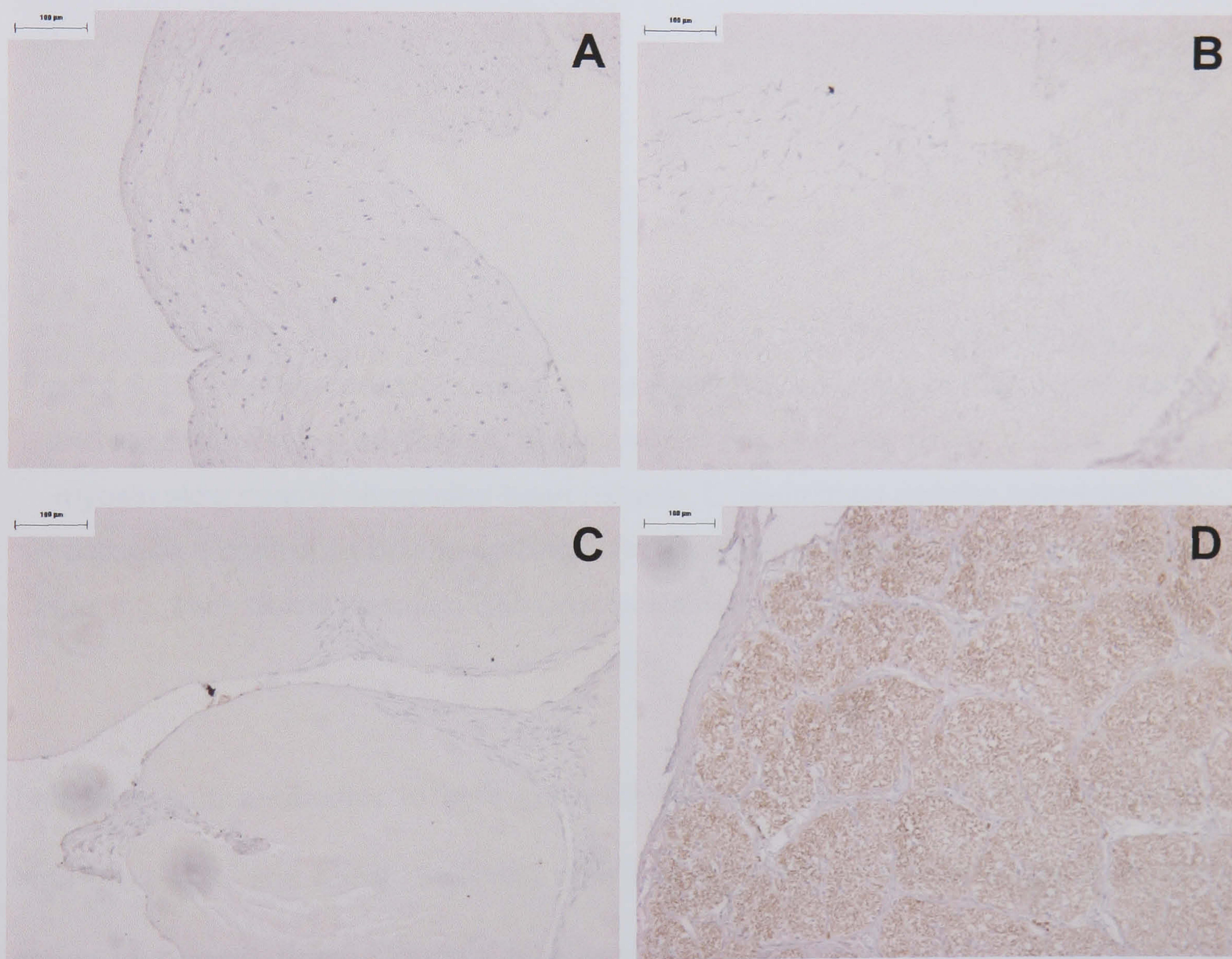
Prior to re-seeding the decellularised matrices, it was first deemed necessary to establish the phenotype of the cells used with reference to the osteogenic lineage. The FACS analysis carried out in Chapter 4 indicated that the cells were AP<sup>+</sup>, which suggested that the cells could have been differentiating into cells of the osteogenic lineage. Therefore, to further determine if this was the case, the cells were also labelled with antibodies to osteonectin and osteopontin which were both deemed to be markers of the osteogenic lineage (Figure 6.21).



**Figure 6.21. Immunoperoxidase labelling of hMSC at P8 with osteogenic markers.** A: alkaline phosphatase labelling; B: osteonectin labelling; C: osteopontin labelling; D: negative control. Brown staining indicates positive antibody labelling, blue shows haematoxylin counterstaining of cell nuclei. Bar represents 25 µm.

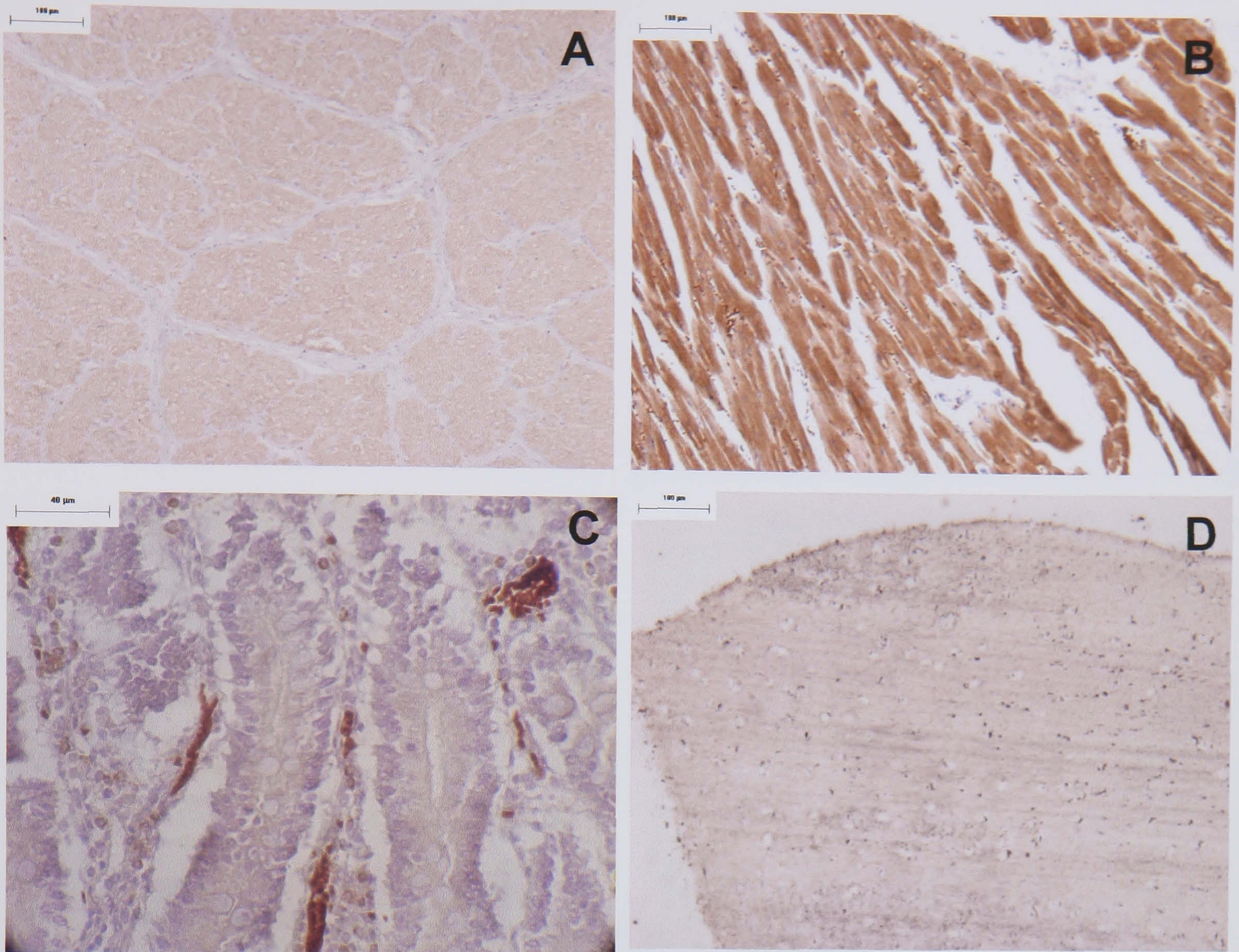
#### 6.4.2.10. Antibody labelling of reseeded valve leaflets

Both the homografts and reseeded tissues were labelled with various antibodies to compare the cell phenotype and also to ensure that the seeded cells had not differentiated into undesirable lineages. The cells in the tissues were negative for neurone specific enolase (Figure 6.22), tau, heavy chain myosin slow, desmin, and collagen II (Figure 6.23. shows positive controls for these antibodies).



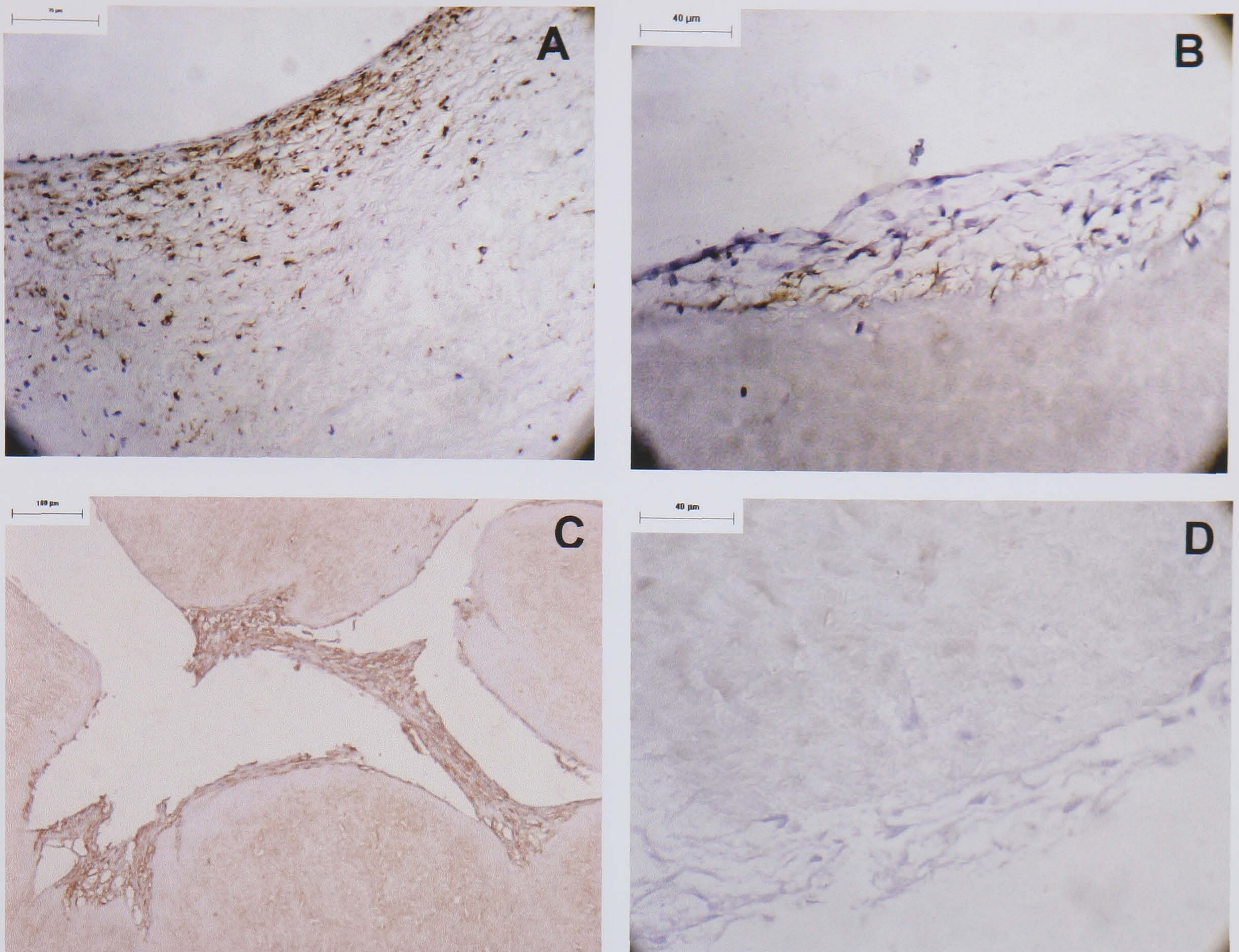
**Figure 6.22. Neurone specific enolase labelling of a homograft leaflet and decellularised valve leaflets reseeded with hMSC and hSMC. A: homograft; B: leaflet reseeded with hMSC; C: leaflet reseeded with hSMC; D: Porcine optic nerve positive control. Brown staining indicates positive antibody labelling, blue shows haematoxylin counterstaining of cell nuclei. Bar represents 100 µm.**



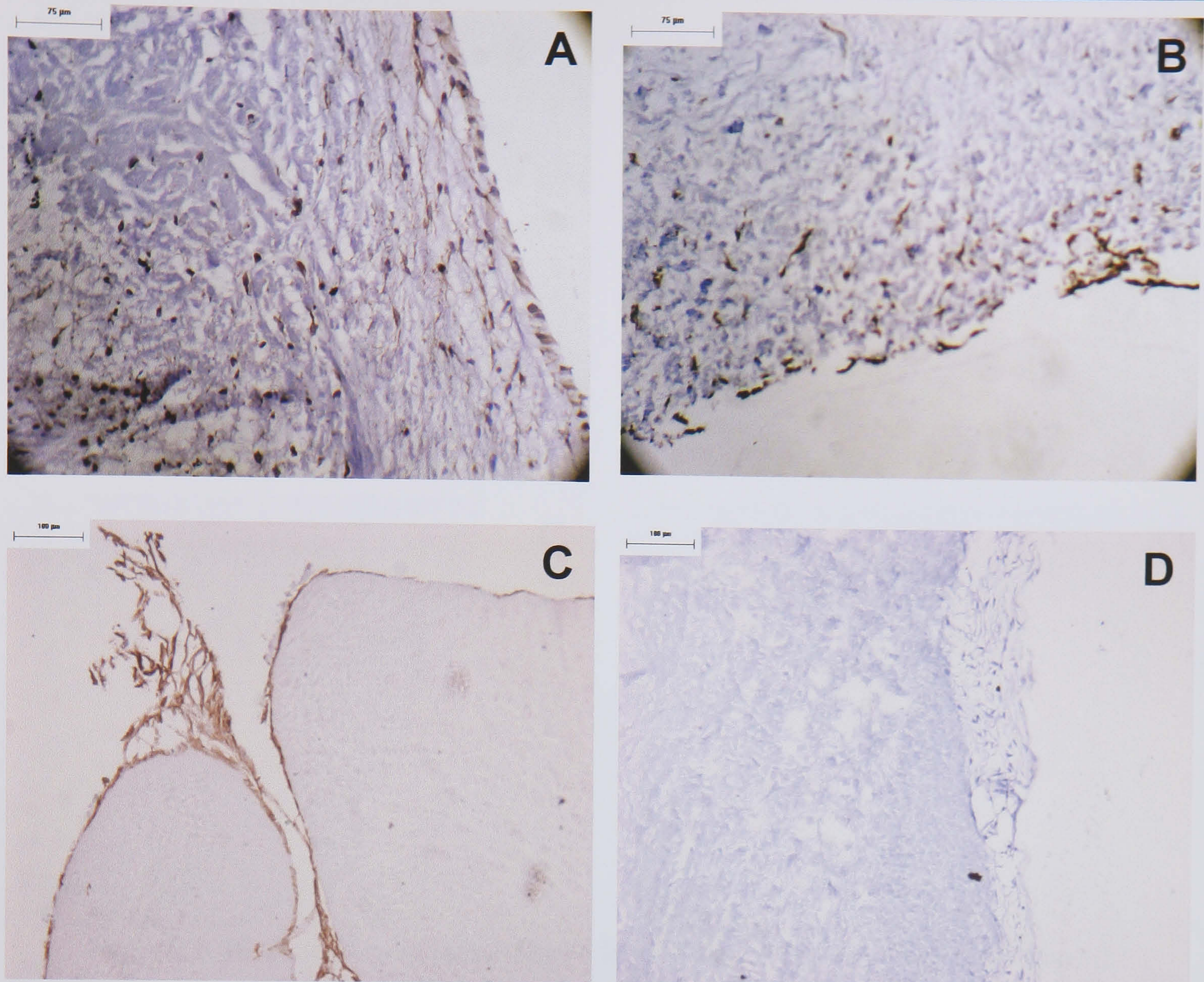


**Figure 6.23. Positive controls used to for antibody labelling of homograft leaflets and reseeded valve leaflets.** A: tau control on porcine optic nerve; B: heavy chain myosin slow control on porcine heart muscle; C: desmin on porcine bowel wall; D: collagen II control on bovine cartilage. Brown staining indicates positive antibody labelling, blue shows haematoxylin counterstaining of cell nuclei. Bar represents 100  $\mu\text{m}$ .

Labelling with antibodies to both  $\alpha$ -smooth muscle actin and vimentin revealed that both the hMSC and hSMC reseeded cells had a phenotype similar to the cells of the native homograft tissue (Figures 6.24, and 6.25.). However,  $\alpha$ SMA labelling of the reseeded hMSC revealed a mixed population of  $\alpha$ SMA<sup>+/-</sup> cells.

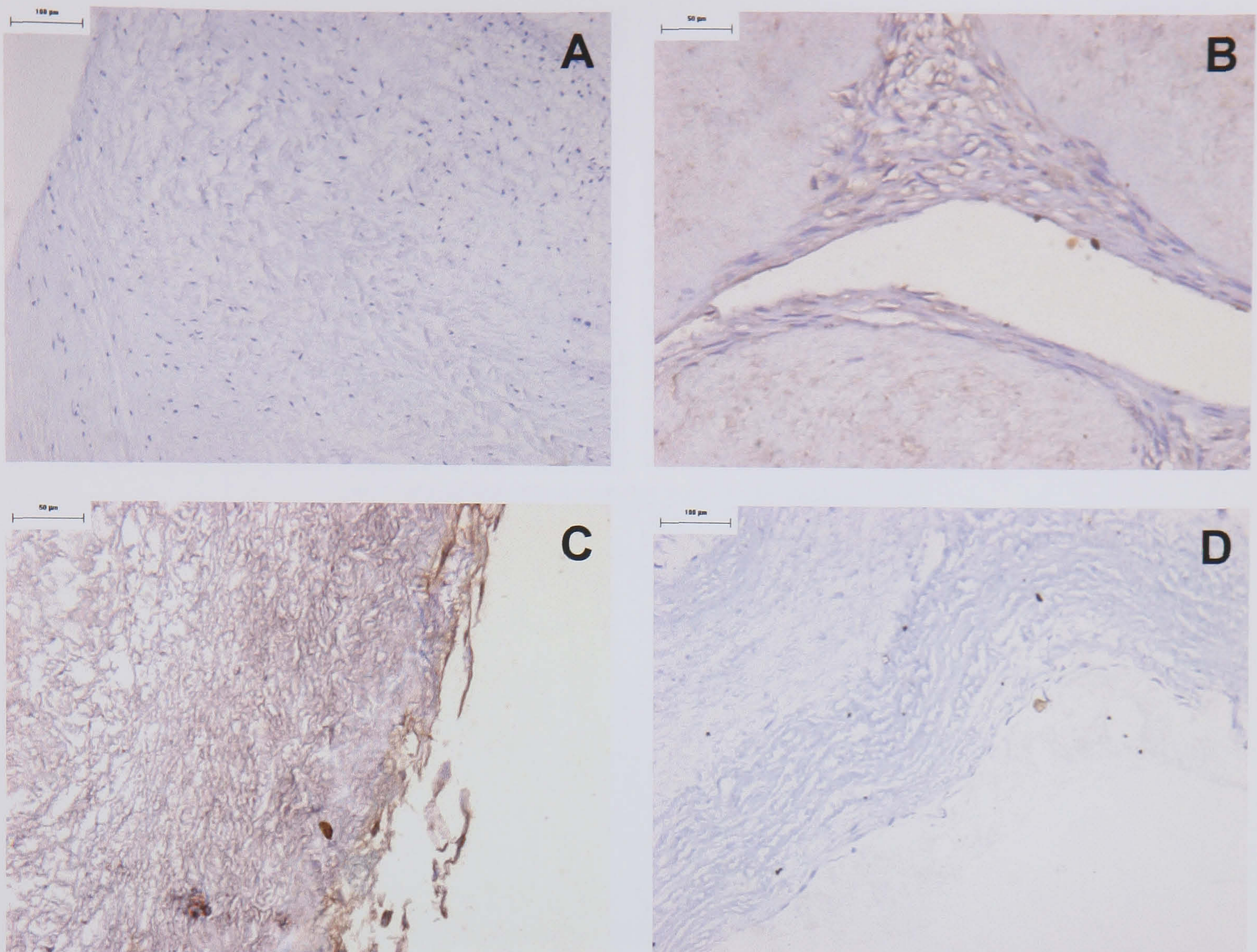


**Figure 6.24.  $\alpha$ -smooth-muscle actin labelling of a homograft leaflet and valve leaflets reseeded with hMSC and hSMC. A: homograft; B: leaflet reseeded with hMSC; C: leaflet reseeded with hSMC; D: negative control. Brown staining indicates positive antibody labelling, blue shows haematoxylin counterstaining of cell nuclei.**

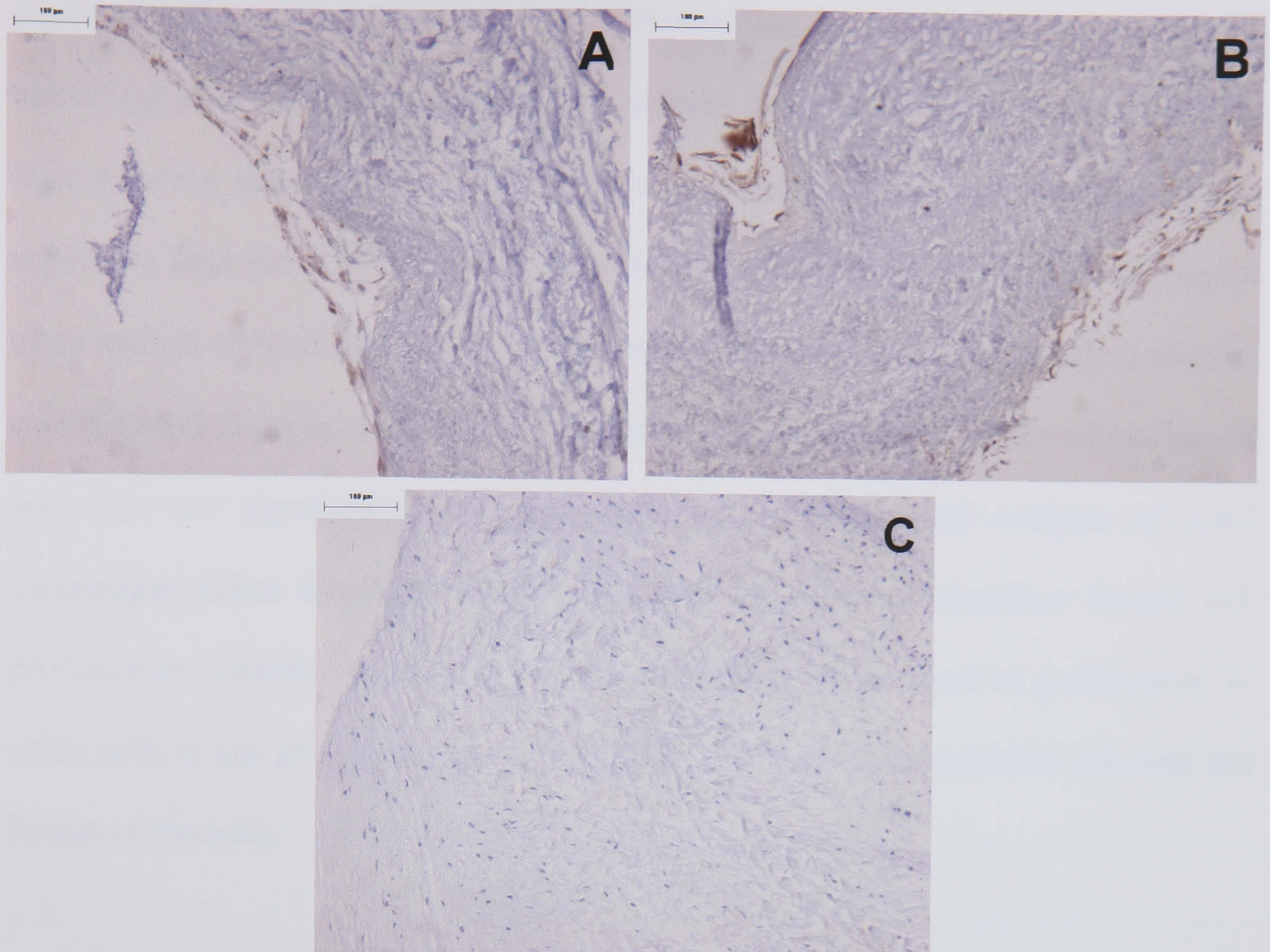


**Figure 6.25. Vimentin labelling of a homograft leaflet and valve leaflets reseeded with hMSC and hSMC.** A: homograft; B: leaflet reseeded with hMSC; C: leaflet reseeded with hSMC; D: Negative control. Brown staining indicates positive antibody labelling, blue shows haematoxylin counterstaining of cell nuclei.

Immunoperoxidase labelling with an antibody to alkaline phosphatase (AP) revealed that although the homograft cells and the reseeded hSMC were AP<sup>-</sup>, as would be expected, the reseeded hMSC were shown to be AP<sup>+</sup> (Figure 6.26). Further analysis of the osteogenic phenotype of the cells was then carried out using antibodies to osteonectin and osteopontin. The reseeded cells appeared to be uniformly positive for osteonectin and osteopontin (Figure 6.27).



**Figure 6.26. Alkaline phosphatase labelling of a homograft leaflet and decellularised valve leaflets reseeded with hSMC and hMSC. A: homograft; B: leaflet reseeded with hSMC; C: leaflet reseeded with hMSC; D: negative control. Brown staining indicates positive antibody labelling, blue shows haematoxylin counterstaining of cell nuclei.**



**Figure 6.27. Osteonectin and osteopontin immunoperoxidase labelling of decellularised leaflet reseeded with hMSC. A: osteonectin; B: osteopontin labelling; C: negative control. Brown staining indicates positive antibody labelling, blue shows haematoxylin counterstaining of cell nuclei.**

## 6.5. Discussion

Leaflets were initially statically cultured by being weighted down in complete medium and cultured under standard conditions. However, with nothing restraining the tissue, shrinkage of the leaflets occurred. This gave the appearance that the cells had multiplied rapidly. However, qualitative histological analysis of the leaflets using an antibody to Ki67 indicated that few cells were positive for this marker. Ki67 protein expression is thought to be linked to cell proliferation as it has been found to be expressed during all the active phases of the cell cycle ( $G_1$ , S,  $G_2$  and mitosis) but not the resting phase ( $G_0$ ) [Scholzen & Gerdes, 2000]. Consequently, it was realised that shrinkage of the leaflet had merely brought the cells closer together and had given the appearance of an

increased cell number. This was later demonstrated by comparing the cell density before and after the culture period, which showed a significant increase in cell density after 4 weeks culture. Consequently, the experiment was repeated with the leaflets sutured to steel gauze to try to prevent shrinkage. Haematoxylin and eosin staining of these leaflets showed that shrinkage was being prevented and that the cells remained evenly spread throughout the leaflet. The cell density before and after the culture period was also not significantly different. Furthermore, histological analysis of Ki67 immunoperoxidase labelling revealed few positive cells and therefore limited cell proliferation. Whether or not this is a typical finding of the level of proliferation of valve cells is not possible to assess as no-one else in the literature has assessed this feature of the cells.

Alcian blue stain was used to stain the glycosaminoglycans (GAGs) present in the valve tissue and the results indicated that, although the centrally located spongiosa was stained most heavily before culturing, after 5 weeks the stain was more diffuse across all three layers of the valve tissue. This indicated that the predominantly GAG-based spongiosa had undergone some degradation. This may have been due to a lack of mechanical stimulation resulting in reduced GAG synthesis by the spongiosa cells. Similarly, maintenance of the elastin in the ventricularis layer may have been affected by the lack of mechanical stimulation and this corresponded well with elastin immunoperoxidase labelling which showed that the long elastin chains gradually degraded and fragmented as the culture period progressed. An antibody to collagen I, however, indicated that histologically the collagen appeared not to have been affected during the culture period.

In conclusion, it was deemed that the conditions used to culture the freshly isolated valve leaflets were suitable for culturing reseeded valve tissue. Although leaflet shrinkage was prevented by suturing the leaflets to steel gauze, both elastin and GAGs appeared to have degraded during the culture period. Therefore this method was not used for the reseeded leaflets. It is likely that mechanical stimulation would have been necessary in order to provide the correct conditions to induce the cells to maintain and remodel the tissue to ensure that the elastin and GAGs were maintained. However, this would form part of future detailed studies.

Both human mesenchymal stem cells (hMSC) and human smooth muscle cells (hSMC) were seeded onto decellularised aortic valve leaflets at concentrations of  $1 \times 10^4$ ,  $5 \times 10^4$  and  $1 \times 10^5$  cells.cm<sup>-2</sup>. After 24 hours of culturing, SEM analysis for both cell types showed that a seeding density of  $5 \times 10^4$  cells.cm<sup>-2</sup> resulted in a confluent monolayer forming across the surface of the leaflet. A monolayer was preferable at this stage because observations have indicated that hMSC grow poorly at low cell density and rapidly develop a flat, spread morphology with little cell proliferation taking place. In contrast, a high cell density would result in a more rapid consumption of nutrients and many cells would not be in contact with the matrix itself, which would be likely to result in cell death, leading to the release of toxins, which could harm other cells.

Although cells were only seeded onto one side of the valve leaflets, histological analysis revealed cells on both side of the leaflets, indicating that the cells had migrated around the edge of the leaflet. However, although low numbers of hMSC had migrated into the tissues varying distances over the course of the culture period, reseeded hSMC remained entirely on the surface of the tissue and did not penetrate into the tissue at all. This indicated another potential advantage of hMSC over terminally differentiated cells

such as hSMC for tissue engineering purposes as this result suggests that hMSC may be more versatile at adapting to growth in tissue matrices; being capable of developing a migratory phenotype to assist cell penetration and repopulation of acellular tissues.

A comparison of the cell counts of the leaflets reseeded with hMSC with the homograft tissue revealed that the leaflet had only been repopulated to between 0.5 and 2%. Cells had mainly only penetrated into the fibrosa and ventricularis layers with relatively few cells reaching the central spongiosa layer. Future experiments here could try to improve the repopulation of the tissue by treating the surface of the tissue to try to improve migration of seeded cells into the tissue. For example, the tissue could be briefly treated with enzymes or perhaps physically disrupted by sonication to try to open up the matrix in order to make it easier for the cells to migrate into it. Alternatively, it may be the case that the tissue has to be repeatedly reseeded with cells in order to encourage the cells to penetrate and also in order to achieve a desirable cell density comparable to the native valve, or at least sufficient cells to maintain the tissue whilst host cells infiltrate the tissue once it is implanted *in vivo*. A further option would be to seed a layer of endothelial cells onto the tissue after the hMSC in order to protect the tissue and assist in the differentiation of the cells already in place. Furthermore, biochemical and/or biomechanical stimulation may also assist in increasing the amount and rate of cell migration into the tissue.

A major advantage of this work in comparison to the works of others who have carried out cell reseeded is the number of cells used. Relatively few cells were used, which keeps the *in vitro* cell expansion and hence cell age to a minimum. In contrast, a number of others who have re-seeded both synthetic matrices and decellularised matrices have used huge numbers of cells that would not be feasible in the clinical setting. For



example, Zünd *et al.* (1998) re-seeded a synthetic scaffold measuring  $1 \times 1 \times 0.3$  cm with  $3.4 \times 10^6$  cells 8-10 times every 90 minutes. Seeding strategies for whole valve constructs have used similarly large cell numbers, for example Schenke-Layland *et al.* (2003) used  $3 \times 10^7$  fibroblasts followed by  $9 \times 10^6$  endothelial cells on day 16. Significantly, both failed to make any quantitative comparison with the cell density in the native tissue, frequently only stating that histologically the re-seeded tissue appeared similar to the native valve, and therefore an accurate measurement of the success of the re-seeding cannot be made. It is likely that a quantitative cell count would reveal that fewer cells were present in the construct than were initially seeded. This information could then be used to optimise the techniques and reduce the number of cells required for the initial re-seeding.

Immunoperoxidase labelling of the tissues reseeded with both hMSC and hSMC in comparison to a native human aortic homograft valve revealed that both cell types had a phenotype comparable to the native homograft valve cells. However, in the case of hMSC,  $\alpha$ SMA labelling revealed that the cells had become a mixed population, some positive and some negative for this antigen. This suggested that the hMSC may have been in the process of differentiating into a new phenotype. This is supported by the finding that the cells were  $AP^{+/-}$  prior to seeding and became uniformly  $AP^+$  in the tissue. Consequently, it is possible that the cells were in the process of developing an osteogenic phenotype. This was verified by labelling the hMSC prior to seeding with markers to osteonectin and osteopontin, which revealed the cells to be osteonectin<sup>+/-</sup> and osteopontin<sup>+</sup>. However, after reseeded, the cells became osteonectin<sup>+</sup> and osteopontin<sup>+</sup>. This could be explained by the age of the cells used for the reseeded (passage 8), and gives further support to the suggestion that osteogenic differentiation is the default pathway for hMSC that are not instructed to differentiate into another lineage.

Moreover, it may be a further reflection of inadequate culture conditions, which perhaps induced differentiation by a currently unknown mechanism. However, if the reseeded tissue is exposed to mechanical stimulation in a pulsatile-flow bioreactor, it might be possible that this differentiation could be prevented and the environment created could instead encourage the cells to differentiate into an appropriate interstitial cell phenotype.

However, although originally defined as bone-specific markers, both osteonectin and osteopontin have since been found to be expressed on other tissues. Osteonectin is principally associated with tissue mineralisation and hence bone formation, but it has also been found to be expressed on certain tumours and is also related to angiogenesis and some wound healing responses [Yan & Sage, 1999]. Similarly, osteopontin was originally identified as a major component of the non-collagenous organic bone matrix, but has also since been found to be expressed in relation to cell adhesion and migration, being present in the gastrointestinal tract, gall bladder, pancreas, urinary and reproductive tracts, lung, salivary and sweat glands [Brown *et al.*, 1992]. Furthermore, alkaline phosphatase has been found to be present in relation to the liver [Lawson *et al.*, 1985] and has been reported to be present on bone marrow reticulum cells [Westen & Bainton, 1979] and even embryonic stem cells [Draper *et al.*, 2002]. Therefore, rather than its presence on MSC being regarded as a negative finding, it could actually be an encouraging finding as it would compare favourably with other findings in relation to stem cells. Another aspect of this is that osteogenic differentiation is likely to have many stages and therefore, different labelling with different markers may represent cells at different stages of differentiation for different periods of time. However, a full analysis would require an investigation of the temporal markers of osteogenesis. Overall, this indicates that the assessment of osteogenic lineage on the MSC is not conclusive for showing that the cells have developed an osteogenic phenotype. Further

characterisation would be required to aid the analysis of the osteogenic default progression of hMSC, perhaps using an antibody to another bone marker such as osteocalcin.

The lengths of the free edge of the reseeded leaflets were measured both before and after culturing the reseeded leaflets for 4 weeks. Both seeded cell types were observed to reduce the surface area of the leaflet in static culture conditions by up to 80%. It is possible that this occurred because without mechanical stimulation the seeded cells began to digest and degrade the matrix without replacing it. It is possible that given the correct mechanical stimulation the cells would instead remodel the tissue in an ordered manner and would hopefully produce an appropriate matrix to maintain the tri-laminar structure of the leaflet. Here, the use of a pulsatile-flow bioreactor would provide an environment comparable to a native heart valve and would therefore be the ideal means to test this theory. However, a more preliminary study could investigate what happens when cells are seeded onto a matrix that is kept under tension, or perhaps under controlled biaxial strain. The matrix components of the leaflet could then be measured both histologically and biochemically (as described in Chapter 6) in order to quantify how the environment created by the bioreactor influenced the cells to maintain and/or remodel the valve tissue. Similarly the phenotype of the cells could be monitored by use of FACS analysis.

Overall, the most important factor in aiding the cell migration and differentiation is likely to be the effect of culturing the reseeded tissue in a pulsatile-flow bioreactor. The physical environment created would closely mimic the natural conditions of a heart valve and it is likely that the mechanical stimulation will greatly assist in the differentiation of the cells and may also encourage the cells to migrate into the tissue

and to assume a native interstitial cell role of repairing and maintaining the tissue matrix. By these means, it may be possible to produce a tissue-engineered valve with a cell density close to that of the native valve where the cells have differentiated into cells capable of producing and maintaining the appropriate tri-laminar structure and withstanding the forces found in the native valve.

**CHAPTER SEVEN:**  
*General Discussion*

# CHAPTER 7:

## *General Discussion*

The aims of this study were to develop methods for the isolation and characterisation of mesenchymal stem cells as a cell source for re-seeding a decellularised aortic heart valve matrix in order to create a living valve that is capable of growth, repair and regeneration. In addition, a previously developed decellularised porcine valve matrix was further characterised and its biocompatibility assessed prior to being reseeded with putative human mesenchymal stem cells. Overall, although the decellularised matrix was found to be biocompatible and biochemically equivalent to native valve tissue, mesenchymal cells isolated from both porcine and human bone marrows could not be conclusively labelled as stem cells.

The most recent definition of a stem cell is that of a cell with the following capabilities [Verfaillie, 2002]:

1. The ability to self renew;
2. The ability for a single cell to differentiate into cells of the tissue of origin and into at least one cell type different from the tissue of origin; and
3. The ability to functionally differentiate *in vivo* into cells of the tissue of origin and at least one cell type of a tissue other than the tissue of origin.

During the course of this investigation, neither the porcine or human putative mesenchymal stem cells isolated conclusively matched all of these criteria. No *in vivo* analyses were carried out, ruling out the third requirement, furthermore, it became apparent during the course of the investigation that although the cells were capable of

renewal, it was only for a limited time-span *in vitro* and the cells ultimately appeared to become senescent. Attempts were made to differentiate both porcine and human putative MSC into multiple cell lineages. For the pMSC, attempts were made to differentiate the cells into cells of the osteogenic, chondrogenic, myogenic, adipogenic, neurogenic and smooth muscle cell lineages. However, despite multiple attempts using different reagents and concentrations, only adipogenic differentiation was achieved. Furthermore, attempts were made to differentiate hMSC into cells of the adipogenic, myogenic, neurogenic and chondrogenic lineages. Of these attempts, adipogenic, myogenic and neurogenic differentiation was achieved in up to 20 % of the cells. However, despite repeated attempts, the hMSC could not be cloned. This failed to rule out the possibility that the cell populations used contained multiple cell precursors and that the biochemical cocktail used to “differentiate” the cells merely selected for cells of the appropriate lineage whilst perhaps discouraging growth of other cells in the population. Despite these findings, FACS analysis of hMSC indicated the cells to have a similar phenotype to those described by Jones *et al.* (2002) who were able to clone their cells prior to differentiation. Whilst this is far from conclusive, it does suggest that further time and investigation may have yielded successful cloning and further differentiation of both cells sources *in vitro*. Further areas of investigation here could be to use an irradiated cell feeder layer to culture the cloned individual MSC upon in order that they could continue to receive adequate cell signalling that would not be present when cells are otherwise cultured individually. Further differentiation attempts could vary the quantities of reagents used or use different differentiation supplements as used by other researchers.

Comparing the work of one person with that of others, however, is increasingly difficult. Whilst there are currently no markers available specific for MSC of any

source, the majority of investigators rely on a panel of antibodies to phenotype their cells and rule out the possibility of other cell types being present. However, whilst this is a common method, the choice of antibodies to use in the panel is not. Each group of investigators has their own opinion as to which antibodies need to be used, and very little consensus exists behind the rationale for choosing these markers. Until a specific marker can be generated, if indeed one exists, it is of vital importance for a widely agreed antibody panel to be devised. Not only would this enable an easier comparison of work from different laboratories, but it would also help to clarify the true nature of the cells, ultimately ascertaining whether all the different names currently used by different researchers to name these cells (as discussed in Section 1.10) are in fact the same thing, or whether it is possible to isolate stem cells at different stages of lineage commitment as some groups claim to be doing [Reyes & Verfaillie, 1999]. Furthermore, this could then aid the possibility of isolating stem cells from animal tissues. This would enable the use of animal models for tissue engineering and other purposes, which is a vital developmental step for any application that is ultimately to result in a product suitable for human implantation.

Another problem is that the cells do not appear to be immortal, as the theory suggests they should be. Different groups claim to have propagated the cells for differing lengths of time from anywhere between 8 or 9 to 120 cell doublings; however, almost all ultimately indicated that the cells became senescent [Bruder *et al.*, 1998; DiGirolamo *et al.*, 1999; Grompe, 2002; Jiang *et al.*, 2002]. This may be because *in vitro* culturing conditions are simply not close enough to the natural *in vivo* environment. Other cell sources have been shown to de-differentiate when transferred to *in vitro* conditions [Bennet *et al.*, 1991; Ma *et al.*, 2003], therefore it may be that something similar happens to stem cells which could be overcome by the addition of the correct growth



factors to the culture medium, or perhaps by using an irradiated cell feeder-layer to supply growth factors and other signals to the cells. Indeed, the culture of embryonic stem (ES) cells has required feeder layers and also leukaemia inhibition factor (LIF) supplements to maintain cell pluripotency [Evans & Kaufman, 1981; Thomson *et al.*, 1998]. Another possibility is that standard culture medium is too good for keeping the cells in an undifferentiated state. Culture media are typically designed to carry a full, balanced range of nutrients and salts in such concentrations that would not necessarily be found in tissues *in situ* where competition for nutrients would greatly reduce availability for any given cell and local changes in blood flow and blood oxygenation could lead to cells living in a less than optimum environment. Therefore, a more simplified culture medium could be preferential for maintaining a multipotent differentiation capacity. Alternatively, it could be something unknown present in the complete culture medium used by most groups which is influencing the cells by either gradually selecting for osteogenic cells, or in some other way encouraging the cells to differentiate into the osteogenic lineage. Foetal calf serum is routinely added to growth medium as a “growth” factor containing poorly defined components capable of sustaining cells. The variance from animal to animal of the serum used is frequently cited by a number of groups who actively screen sera in order to find samples capable of promoting proliferation whilst maintaining the multipotential capacity of their cells [Lennon *et al.*, 1996; Conget & Minguell, 1999; Caterson *et al.*, 2002]. If the sera could be more accurately analysed, a highly defined serum-free alternative could be devised which would enable consistent concentrations of factors to be used which could consistently provide the optimal conditions for the cells and may help to prevent unwanted differentiation. Furthermore, a defined serum-free growth medium would be highly preferential for use in tissue engineered applications where the use of bovine

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serum remains highly problematic due to the potential for disease transmission such as bovine spongiform encephalopathy (BSE).

Studies have shown that the number of hMSC and differentiation capacity of the cells decreases with the age of the patient from which they are harvested [Mendes *et al.*, 2002]. One limitation of this study has been that the hMSC used have been obtained from the bone marrow of patients undergoing hip replacement operations. This operation typically occurs in those aged over 60, and therefore the cells used may already have been restricted in their potential as a result of the patient's age. It is possible that if hMSC from younger patients had been available then differentiating and cloning the cells may have met with more success. Furthermore, cell longevity may have been improved. However, in combination with the apparent default to differentiate into the osteogenic lineage with *in vitro* cell ageing and the currently limited cell proliferation, it is possible that no *in vitro* environment could maintain the cells adequately. Therefore, rather than concentrating on trying to improve the conditions so that multipotency and longevity can be maintained, it may simply be easier to use cells that have undergone only limited cell doublings prior to being used in a tissue engineering application. The application of adequate biochemical and/or mechanical stimulation could then encourage the cells to differentiate into an appropriate lineage for the tissue engineering application rather than into an inappropriate default lineage. However, this could only be carried out if the cells were appropriately analysed with an antibody panel prior to use so that the cells were adequately characterised and purified.

Other tissue engineering applications have met with similar problems in relation to the poor identification of the hMSC. However, since the cells seem to differentiate to the osteogenic lineage, this is providing a useful cell source for those researching

osteogenesis and related areas [Haynesworth *et al.*, 1992; Allay *et al.*, 1997; Jaiswas *et al.*, 2000]. Due to the similar location and the relation in functioning of the tissues *in situ*, differentiation of the cells into the chondrogenic lineage also seems to be relatively straightforward. As such people have made use of this cell source for investigating cartilage and the replacement of cartilage defects [Wakitani *et al.*, 1994; Catterson *et al.*, 2001]. In other tissues where the potential of MSC has been investigated as a cell source, investigators frequently fail to carry out any analysis of the MSC at all prior to use in their system. Young *et al.* (1998) investigated the use of MSC in a collagen matrix for Achilles tendon repair. However, their paper fails to mention any analysis to establish that the cells isolated were MSC and instead appears to assume that merely isolating the cells by a common method means that the cells must be MSC. Furthermore, they again failed to clone the cells and must therefore be using a poorly defined heterogeneous population. This assumption that the cells must be MSC and lack of analysis of the cells is extremely common in the tissue engineering literature. This is a very poor scientific approach and only serves to add to the confusion about the phenotype and capabilities of MSC rather than clarifying the common issues and is something that must be addressed if the full potential of these cells is ever to be realised.

During the course of this investigation, other researchers have attempted to tissue-engineer cardiovascular constructs using mesenchymal stem cells. Perry & Roth (2003) have assessed the use of ovine mesenchymal stem cells for tissue engineering heart valves. They identified their MSC primarily using SH2 antibody (which is known to not be specific for bone marrow stem cells as it cross-reacts with endothelial cells; Fleming *et al.*, 1998) and smooth muscle actin, calponin and desmin, which in combination are more specific for myogenic cells by the researcher's own admittance. Furthermore, the only differentiation assay attempted was the adipogenic lineage; however without first

cloning the cells, this fails to show whether they were merely selecting for adipogenic cells, or whether there were adipogenic progenitors present rather than MSC and does not provide any evidence of multipotency. Moreover, no cell characterisation was carried out after the culture on a PGA scaffold, therefore the effects of the culture system on the cell phenotype were not assessed, leaving the possibility that the cells could have differentiated into an undesirable lineage. Furthermore, a large number of cells were used ( $2 \times 10^6$  cells.cm<sup>-2</sup>) making it clinically unfeasible. Kadner *et al.* (2002) have also investigated the use of MSC for cardiovascular tissue engineering, and found that the cells labelled positively for vimentin and smooth muscle actin and showed layered tissue formation of extracellular matrix with deposition of collagen fibrils. They concluded that MSC demonstrated myofibroblast-like characteristics and represented a promising cell source for cardiovascular tissue engineering. However, as is frequently the case, they failed to show that the cells they were calling MSC showed any stem cell properties as they did not attempt differentiation of the cells, and the FACS analysis carried out used only a few antibodies which were poorly chosen, using only CD14 (expressed on the surface of cells of the myelomonocytic lineage such as monocytes and macrophages) and CD31 (expressed on the surface of platelets, monocytes, granulocytes and B-cells), which would only serve to rule out contamination by haematopoietic cells.

It is vital that more basic, fundamental investigations are carried out on MSC to more accurately identify and characterise them. There is a need to optimise the culture conditions and establish more precisely the biochemical factors involved in the maintenance of pluripotency and differentiation in order to fully control these cells and encourage them to do exactly what is required by the researcher in a manner comparable to how they are controlled *in vivo*. Areas to investigate here could use chemically defined media supplemented with growth factors to culture the cells and the resulting

effects could be examined by assessing changes in gene transcription using reverse-transcriptase polymerase chain reaction (rt-PCR) or cell surface markers using FACS analysis. True control of stem cells at this level will make their application in tissue engineering strategies considerably easier and will have implications for the understanding of all cells throughout the body. Indeed, if the means by which the body recruits the normally quiescent, resting stem cells to injury sites could be ascertained, then enhancement of this signal could allow improved harvesting of the cells for *in vitro* manipulation, or perhaps could even abrogate the need for tissue engineering entirely in some applications as enhanced cell recruitment to specific locations could enable the body to repair itself more efficiently without the need for surgical or other intervention. Only by carrying out this fundamental research can the true potential of these cells be realised and their potential as an adequate competitor to embryonic stem cells, without the ethical nightmare associated with ESC, be assessed outright.

With respect to the heart valve scaffold, the matrix developed by Booth *et al.* (2002) would appear to be extremely promising for use in tissue engineering a living aortic heart valve. Histological analyses have shown matrix components to remain intact [Booth *et al.*, 2002], whilst mechanical testing has demonstrated that the SDS treatment produced valves with equal strength, competence under physiological pressures (120 mm<sup>3</sup> Hg) and greater extensibility than untreated fresh valves [Korossis *et al.*, 2002]. However, histological assessment alone is only a qualitative means of assessing the procedure. Consequently, investigations were carried out in this study in order to make a quantitative biochemical comparison between fresh and decellularised porcine aortic heart valve leaflets in order to ensure that the matrix was unaffected by the procedure. This was found to be the case as the biochemical analyses showed no significant changes in GAG or hydroxyproline content of the leaflet. Furthermore, analysis of DNA

content indicated that the DNA had been removed by the decellularisation procedure. The biocompatibility of the decellularised tissue was assessed by subcutaneous implantation in a murine animal model for twelve weeks. This revealed that the decellularised tissue did not calcify and that the inflammatory infiltrate was favourable, consisting mainly of macrophages and endothelial cells. Furthermore, histological examination indicated that the implanted decellularised tissue did not become necrotic and instead remained healthy in appearance. One possible further investigation here would be to implant tissues for a longer period. This is because although the presence of macrophages at 12 weeks seen here was deemed beneficial in order to attract endothelial cells required for remodelling of the tissue, if the tissue is fully accepted by the host, the number of macrophages should later decrease as a sustained persistence of macrophages in the tissue would suggest that the tissue was inflammatory.

Decellularised valve leaflets were later re-seeded with hMSC and these were shown to penetrate into the tissue to up to 2 % of a native valve. Although poor when compared to a native homograft, there are multiple options to explore which may enhance this re-cellularisation phase of the investigation. Mechanical stimulation of the re-seeded tissues alone may provide adequate conditions to improve cell infiltration into the tissue. Alternatively, treatment of the tissue by, for example, coating the tissue with fibronectin, could improve cell attachment and migration. The tissue could also be disrupted by use of, for example, sonication in order to provide more openings for the cells to migrate through. Analysis of the wound healing response in heart valve leaflets could possibly yield factors which could encourage cells to infiltrate into the tissue and once *in vivo* could aid the migration of host cells into the tissue engineered matrix. Other options include multiple cell seedings to try to increase the cell density. However, this would have to be balanced against the practicalities of growing sufficient cells

within an appropriate time frame to deliver the product to the patient. Furthermore, too many cell doublings *in vitro* could lead to cells with limited proliferation capacity unless the culture conditions could be adequately improved to increase the longevity of the cells. Alternatively, it may be possible to stimulate the seeded cells to divide *in situ* by the addition of growth factors. However, these would have to be carefully screened to ensure that they did not have an effect on the differentiation of the cells, and also to ensure that once the stimulation is removed cell division would slow again to normal levels as too many cells could affect the mechanical properties of the tissue. It may also be the case that a layer of endothelial cells may be required to protect the tissues and re-seeded cells: The factors released by these cells may aid the migration and differentiation of the hMSC whilst protecting the tissues and preventing blood clot formation on the valve leaflets.

Prior to re-seeding, the hMSC phenotype was assessed using osteogenic markers as it was suspected that the cells would default to the osteogenic lineage if no other cell signals were received. It appeared as though the cells were becoming osteogenic and this was further highlighted when the phenotype of the cells was analysed after re-seeding into the decellularised matrix using markers for alkaline phosphatase, osteonectin and osteopontin. However, the combination of results obtained from labelling cells with vimentin,  $\alpha$ SMA, HCMs and desmin indicated that the re-seeded cells had a phenotype similar to valve interstitial cells and it was later found that the osteogenic markers used were not sufficiently specific to show conclusively that the cells were osteogenic in nature. Further analysis using a more specific bone marker such as osteocalcin could give further indication to whether osteogenic differentiation did occur. However, it is likely that dynamic culturing of the leaflets in a re-seeded whole valve using a pulsatile-flow bioreactor would provide adequate signalling to prevent this

differentiation and would instead encourage the cells to develop an interstitial cell phenotype. The effects of mechanical stimulation on MSC have already been demonstrated by Altman *et al.* (2002) who have shown mechanical stimulation to cause a change in gene expression, resulting in cells of a ligament cell lineage. Similar results have also been achieved by stimulating fibroblasts and aortic endothelial cells, as described in Section 1.11.3. Furthermore, as already discussed, using cells that have undergone fewer cell doublings may also circumvent this problem.

The matrices currently being researched by other groups are most commonly of synthetic origin. Synthetic matrices have the problem that the matrix is usually mechanically weak in comparison to the native valve and relies upon rapid remodelling by seeded cells and host tissues *in vivo* to provide improved mechanical strength. In contrast, although remodelling will eventually occur in any tissue that is implanted, the use of a decellularised valve matrix would ensure that the mechanical requirements would be optimal at the point of implantation so there would be no period of remodelling that is essential for adequate valve function. Synthetic matrices that have been tested in animal models have met with some promising results [Shinoka *et al.*, 1996; Kim *et al.*, 2001; Rothenburger *et al.*, 2002], however, the investigators frequently failed to fully characterise the valves upon explantation, usually relying on basic histology alone for analysis, making it difficult to fully assess the degree of success. One use of decellularised porcine tissue has been in the acellular SYNERGRAFT<sup>TM</sup> valve developed by Cryolife Inc. However, as discussed in Chapter 1, this failed very quickly and catastrophically in human trials [Simon *et al.*, 2003], suggesting a hyper-acute immune rejection. Others have investigated a decellularised porcine aortic heart valve matrix re-seeded with autologous endothelial cells and implanted into an ovine model [Dohmen *et al.*, 2001]. The focus of this particular



research was to investigate the calcification potential of the implant and it was found to be comparable to a non-implanted valve tissue-engineered in the same manner.

There are, however, two fundamental problems with using porcine tissue: firstly is the issue of adequate disinfection of the tissue prior to implantation. In the studies presented here, the tissue was disinfected with an antibiotic cocktail prior to reseeded with cells. This means of disinfection was not intended to be a permanent choice, but was merely an *in vitro* tool to produce a matrix for study. This study was part of a larger programme that aims to re-seed decellularised valves with terminally differentiated cells, and this programme is in collaboration with the National Blood Service Tissue Services (NBS) as a means of ultimately delivering the final product to patients through the NHS. Because of their familiarity with the legislation involved in delivering products to patients, the NBS took on the role of developing an appropriate sterilisation technique. Consequently, the antibiotic cocktail has now been superseded by the use of peracetic acid treatment of the tissues (0.1 % v/v for 3 hours) as a means of sterilising the tissue. The sterilising capabilities of peracetic acid have been known about for over 100 years and it has been widely used as a cold disinfectant for heat labile medical equipment and is also used widely in the food and agriculture industries [Lomas *et al.*, 2003; Kitis, 2004]. It is believed to be active against bacteria, viruses, bacterial spores and protozoan cysts [Kitis, 2004]. Despite this treatment, the issue of porcine endogenous retroviruses (PERVs) potentially remains a problem [Patience *et al.*, 2001; Blusch *et al.*, 2002]. Pigs are known to carry these retroviruses asymptotically, but there are concerns that they could be transmitted to humans with unknown effects through tissue engineered products. Indeed, they have been readily detected in native porcine heart valves [Moza *et al.*, 2001]. Since viruses are obligate intracellular parasites, if the porcine cells are completely removed, then so should any virus material that may be present. However,

the presence of PERVs needs to be fully assessed before and after decellularisation to be certain as they have been shown to infect human cells *in vitro* [Patience *et al.*, 1997; Martin *et al.*, 1998; Wilson *et al.*, 1998; Specke *et al.*, 2001] and poorly judged assumption could lead to catastrophic consequences for any recipient patient. In order for the efficacy of peracetic acid against these viruses to be tested, ideally, tissues would have to be spiked with known concentrations of the virus prior to measuring the effects of the peracetic acid treatment and assessment of whether any infectious virus material remained.

A second important potential problem with the transplantation of animal tissues into humans is the issue of the Gal $\alpha$ 1-3Gal ( $\alpha$ -gal) epitope. This is a sugar residue known to be present on the surface proteins and glycoproteins of all animal cells except humans and old-world non-human primates [Teranishi *et al.*, 2002]. Humans have developed a high-titre antibody response to antigens present on bacteria found in the human gut which cross-react with the  $\alpha$ -gal epitope found on animal cells [Galili *et al.*, 1987]. Therefore, should an  $\alpha$ -gal positive organ be implanted into a human, antibody would bind to the antigen expressed on endothelial cells resulting in the rapid activation of the complement cascade which would lead to clotting and blockage of the vessels resulting in hypoxia followed by necrosis of the tissue in a process known as hyper-acute rejection. If an  $\alpha$ -gal positive matrix were to be implanted into a human, again the antibody would bind to the antigen, but in this situation the subsequent inflammatory response would lead to a cascade of events that would give rise to the attraction of neutrophils and macrophages to the area, ultimately resulting in inflammation and the degradation of the implanted matrix. Indeed,  $\alpha$ -gal has been shown to be implicated in the hyper-acute rejection of porcine organs transplanted into human recipients [Maruyama *et al.*, 1999]. Whilst this in theory would not be a problem provided

complete decellularisation of the matrix is achieved, it is something that needs to be fully verified through research as the consequence of implanting tissues where this has not been investigated and dealt with accordingly could be devastating to the patient. Monoclonal antibodies against  $\alpha$ -gal are now commercially available and therefore the presence of this antigen can be readily assessed by immunolabelling, thereby allowing techniques to remove the antigen to be developed.  $\alpha$ -gal knock-out pigs have now been bred [Lai *et al.*, 2002], however, in order to provide a tissue-engineered living heart valve to the NHS through the NBS as a suitable alternative to currently available valve replacements, it would also have to be economically viable. Overcoming patents and similar legal issues relating to the knock-out pigs would greatly increase the overall cost of delivering a tissue engineered valve to a patient. Thus it is vital firstly to assess whether indeed this would be a problem in heart valves, as it has been suggested that it is not [Chen *et al.*, 2003], and secondly, if a problem exists, to develop a modification to the decellularisation procedure which could adequately remove  $\alpha$ -gal residues.

## 7.1 Future studies

A vital issue is the cloning of the hMSC to more accurately demonstrate their multipotential capacity and to rule out the possibility of multiple cell precursors being present in the bone marrow. A method needs to be developed which enables this to be carried out in a relatively straightforward manner in order that it can be routinely carried out to ensure that all research is carried out on purified cell populations. Options here include the use of an irradiated cell feeder layer to culture the cells with in order that the individually cloned cells receive adequate stimulation from neighbouring cells which might prevent premature senescence. Alternatively, growth factors may be required to maintain the cells in an undifferentiated state in a way comparable to the culture of ES cells where LIF has been used to prevent differentiation [Evans & Kaufman, 1981;

Thomson *et al.*, 1998]. One possibility would be fibroblast growth factor-2 (FGF-2), which is known to stimulate endothelial cell proliferation [Bikfalvi *et al.*, 1997] and has been shown to have encouraging effects on MSC from various sources [Tsutsumi *et al.*, 2001]. The continued use of unpurified cell populations in research is particularly unhelpful for comparison between different laboratories, and also fails to show which cell population of the bone marrow is being used in the application, leaving a great deal of vagueness about the exact science of the whole process.

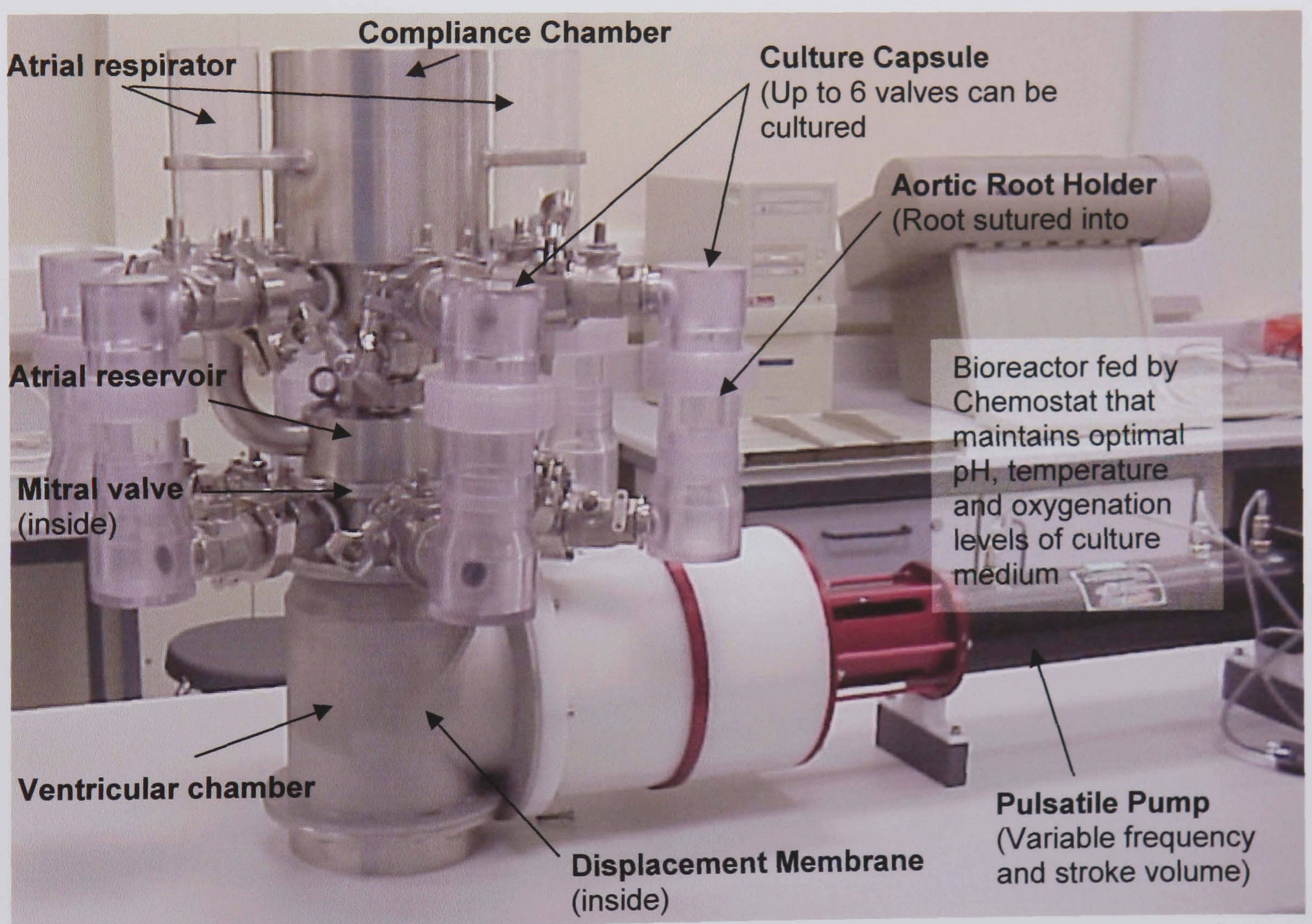
Another interesting approach would be to investigate how mesenchymal stem cells change as the patient from which they are harvested ages and how this could affect their usefulness for tissue-engineering and other therapies. Whilst in younger patients MSC would be in optimal condition, making them ideal for tissue engineering applications, the same may not be true for cells of older patients. It is known that the differentiation capacity of MSC is reduced with patient age (Caplan, 1994; Digorolamo *et al.*, 1999; Mendes *et al.*, 2002) and as discussed in Chapter 1, there is the possibility of a link between neoplasia and stem cells and the accumulation of a life-time of age-related degeneration and cell damage would be likely to reduce the regulatory control the body has over stem cells. Therefore the application of MSC from older patients in a tissue-engineered product, even if they are differentiated *in vitro*, could potentially create more problems than the tissue-engineered product might solve.

Furthermore, as discussed previously, it is of absolute critical importance to investigate the means to remove both PERVs and  $\alpha$ -gal residues if porcine tissue is to become a viable option for tissue engineering products for human implantation. Without this work, the progression to human studies simply cannot occur and it will hold back any tissue-engineered products using un-fixed animal tissues.

However, without doubt, the most important and exciting future study carrying on from this research will be the use of a pulsatile-flow bioreactor to culture the re-seeded valves *in vitro*. A number of groups have already created bioreactors for creating tissue-engineered heart valves. Both Hoerstrup *et al.* (2000) and Zeltinger *et al.* (2001) have created similar pulsatile-flow bioreactors. They are both relatively simple in design, being capable of culturing one valve construct at a time by re-circulating culture medium in a closed loop system at varying speeds and volumes to cause the valves to open and close. This simplistic approach, whilst providing mechanical stimulation, is undermined by the fact that only one valve can be cultured at a time, meaning that no replicates can be cultured simultaneously, making it impossible to carry out any statistically relevant analyses of the outcomes when the allotted culture time is completed.

As part of the heart valve project, the construction of a pulsatile-flow bioreactor has recently been completed and is currently undergoing early-stage evaluation (Figure 7.1) [Ingham & Fisher, 2003]. The bioreactor has the capacity to culture between 1 and 6 valves simultaneously, which are sutured into frames that sit within transparent chambers to enable visual monitoring of the valves during culture. There is a central mitral valve incorporated into the system and the pump is capable of producing physiological pressures of up to 120 mm<sup>3</sup> Hg with a varying stroke rate. There is a medium reservoir and a conditioning system able to maintain appropriate conditions by monitoring temperature, oxygenation and pH of the culture medium. It is hoped that with a primary static seeding stage to allow the cells to attach to the leaflet, reseeded valves could then be introduced into the bioreactor and the pressure and flow of the medium gradually increased to physiological conditions so as to avoid washing off the

cells. It is thought that the mechanical environment created during the culturing of re-seeded valves for up to 6 weeks would aid in the cell migration and differentiation and would encourage the cells to produce an appropriate matrix to create a fully functional tissue-engineered valve replacement. Re-seeding decellularised matrices with porcine smooth muscle cells and then implanting the pulsatile-cultured constructs back into a porcine animal model would show the potential for using the system for creating a valve re-seeded with human cells for use in clinical trials.



**Figure 7.1. Pulsatile-flow bioreactor for tissue engineering a living heart valve**

[Ingham & Fisher, 2003].

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# *Appendix*

# APPENDIX

## *Suppliers*

**Amersham Pharmacia Biotech UK Ltd**, Amersham Place, Little Chalfont, Bucks HP7 9NA.

**Appleton Woods Ltd**, Lindon House, Heeley Road, Selly Oak, Birmingham, B29 6EN.

**BD Biosciences**, (*formerly* Becton Dickinson), 21 Between Towns Road, Cowley, Oxford, OX4 3LY.

**Beckman Coulter UK Ltd**, Oakley Court, Kingsmead Business Park, London Road, High Wycombe, Buckinghamshire, HP11 1JU.

**Bibby Sterilin Ltd**, Tilling Drive, Stone, Staffordshire, ST15 0SA.

**Bio-Rad Laboratories Ltd**, Life Science Research Division, Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD.

**Biowhittaker UK Ltd**, 1 Ashville Way, Wokingham, Berkshire, RG41 2PL.

**BOC Ltd**, The Priestly Centre, 10 Priestly Road, The Surrey Research Park, Guilford, Surrey, GU2 5XY.

**Bright Instruments Co Ltd**, Margarets Way, Huntingdon, Cambridgeshire, PE29 6EU.

**C. A. Hendley (Essex) Ltd**, Oakwood Hill Industrial Estate, Loughton, Essex, IG10 3TZ.

**Caltag-MedSystems Ltd**, PO Box 6139, Silverstone, Towcester, NN12 8GN.

**Chemicon Europe Ltd**, The Science Centre, Eagle Close, Chandlers Ford, Hampshire, SO53 4NF.

**Corning Costar UK Ltd**, 1 The Valley Centre, Gordon Road, High Wycombe, Bucks, HP13 6EQ.

**DAKO Ltd**, Denmark House, Angel Drove, Ely, Cambridgeshire, CB7 4ET.

**Developmental Studies Hybridoma Bank (DSHB)**, Department of Biological Sciences, University of Iowa, 028 BBE, Iowa City, IA 52242-1324.

**DYNEX Technologies Ltd**, Columbia House, Columbia Drive, Worthing, West Sussex, BN13 3HD.

**European Collection of Cell Cultures (ECACC)**, Centre for Applied Microbiology and Research, Salisbury, Wiltshire, SP4 0JG.

**Fisher Scientific UK Ltd**, Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG.

**Harlan UK Ltd**, Shaw's Farm, Blackthorn, Bicester, Oxon, OX25 1TP.

**ICN Pharmaceuticals Ltd**, Cedarwood, Chineham Business Park, Crockford Lane, Basingstoke, RG24 8WD.

**Invitrogen**, (*formerly* Life Technologies Ltd), 3 Fountain Drive, Inchinnan Business Park, Paisley, Scotland, PA4 9RF.

**Jencons (Scientific) Ltd**, Cherrycourt Way, Stanbridge Road, Leighton Buzzard, Bedfordshire, LU7 4UA.

**Kendro Laboratory Products**, (*formerly* Heraeus Instruments and Sorvall), Sortford Hall Park, Bishops Stortford, Herts, CM23 5GZ.

**Leica UK Ltd**, (*formlery* Leitz), Davy Avenue, Knowhill, Milton Keynes, MK5 8LB.

**MediaCybernetics UK**, Falcon Business Park, 40 Ivanhoe Road, Finchampstead, Berkshire, RG40 4QQ.

**Molecular Probes Europe BV**, PoortGebouw, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.

**National Blood Service (NBS)**, Trent Regional Centre, Longley Lane, Sheffield, S5 7JN.

**NHS Supplies Authority**, NE Division, Foxbridge Way, Normanton Industrial Estate, Normanton, WF6 1TL.



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**Nunc Inc**, 2000N Aurora Road, Naperville, IL, USA 60566.

**Olympus Optical Company UK Ltd**, Great Western Industrial Park, Dean Way, Southall, Middlesex, UB2 4SB.

**Oxoid (Unipath) Ltd**, Wade Road, Basingstoke, Herts, RG24 0PW.

**John Penny & Son Abattoir**, Low Green Farm, 40 Leeds Road, Rawdon, West Yorkshire, LS19 6NU.

**Pharmacia Biotech**, 23 Grosvenor Road, St Albans, Herts, AL1 3AW.

**Raymond A. Lamb Ltd**, Units 4 & 5 Parkview Industrial Estate, Alder Close, Lottbridge Drove, Eastbourne, East Sussex, BN23 6QE.

**R&D Systems Europe Ltd**, 19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB.

**Roche Diagnostics Ltd**, Bell Lane, Lewes, East Sussex, BN7 1LG.

**Sanyo Gallencamp PLC**, Monarch Way, Loughborough, LE11 5XG.

**Schleicher & Schuell UK Ltd**, Unit 11, Brunswick Industrial Estate, London, NW11 1JL.

**Scientific Laboratory Supplies Ltd**, 26-27 Nottingham, South & Wilford Industrial Estate, Nottingham, NG11 7EP.

**Serotec Ltd**, 22 Bankside, Station Approach, Kidlington, Oxford, OX5 1JE.

**Sigma-Aldrich Company Ltd**, Fancy Road, Poole, BH17 3NH, Stevenage, Herts, SG1 4QN.

**Starlab (UK) Ltd**, 4 Tanners Drive, Milton Keynes, MK14 5NA.

**Stem Cell Technologies**, PO Box 35727, London, E14 9YE.

**Thermo Life Sciences UK**, Unit 5, The Ringway Centre, Edison Road, Basingstoke, Hampshire, RG21 6YH.

**Triangle Biomedical Sciences Ltd**, Unit 5, Gardiners Place, West Gillibrands, Skelmersdale, Lancashire, WN8 9SP.

**Vector Laboratories Ltd**, 3 Accent Park, Bakewell Road, Orton Southgate,

Peterborough, PE2 6XS.

**VWR Internaltional Ltd**, (*formely* Merck Eurolab), Hunter Boulevard, Magna Park,

Lutterworth, Leicestershire, LE17 4XN.

**Whatman International Ltd**, Whatman House, St Leonard's Road, 20/20 Maidstone,

Kent, ME16 0LS.

**Wilkinson's**, 8/24 Albion Street, Leeds, West Yorkshire, LS1 6HX.