

DIVERSITY AMONG MONOCYTE DERIVED STROMAL CELLS

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## ABSTRACT

Fibrocytes are monocyte-derived cells that morphologically look like fibroblasts, but express both stromal and haematopoietic markers, and have been reported as being involved in wound healing and fibrosis. Fibrocytes can be differentiated *in-vitro* in both the presence and absence of serum and we wanted to study the relationship between them; which potentially could be involved at different time points of a wound-healing site.

Differences between these two potential cell types were found in how they differentiated, and the way they reacted to serum. However they were both found to differentiate from two populations of monocytes. The relationships between these two fibrocytes, as well as macrophages were also examined using transcriptome analysis, clustering the samples based on all the genes, and identifying those that were significantly different between the populations. This demonstrated that both fibrocyte populations are distinct from each other, as well as from macrophages.

These data demonstrate that these two fibrocytes have different characteristics, suggesting that they may have different roles at a wound-healing site. Potentially with serum-containing derived fibrocytes emerging first to assist other immune cells, and begin the wound healing process, and serum-free later, when the environment changes, to continue with the process of repair.

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# TABLE OF CONTENTS

<b>1.0 INTRODUCTION.....</b>	<b>1</b>
<b>1.1 Haematopoiesis.....</b>	<b>1</b>
<b>1.2 Cross talk between cells.....</b>	<b>9</b>
<b>1.3 Monocyte development.....</b>	<b>17</b>
<b>1.4 Monocyte derived progeny.....</b>	<b>19</b>
1.4.1 Macrophages and Dendritic cells.....	19
1.4.2 Osteoclasts.....	23
<b>1.5 Fibrocytes.....</b>	<b>24</b>
1.5.1 Overview of Fibrocytes.....	24
1.5.2 Fibrocyte differentiation.....	28
1.5.3 Inhibition of fibrocyte generation.....	30
<b>1.6 Fibrocytes and Fibrotic diseases.....</b>	<b>32</b>
1.6.1 Fibrocytes and Th-2 cytokines.....	33
1.6.2 Treating animal models of fibrosis with SAP.....	35
1.6.3 Mechanisms by which fibrocytes populate peripheral organs.....	39
1.6.4 Fibrocytes and Human fibrosis.....	41
<b>1.7 Fibrocytes and wound healing.....</b>	<b>43</b>
1.7.1 Fibrocytes and burn injury.....	46
<b>1.8 Fibrocytes as mesenchymal stromal cells.....</b>	<b>47</b>

<b>1.9 Overview of fibroblasts.....</b>	<b>48</b>
<b>1.10 Transcriptome analysis.....</b>	<b>50</b>
<b>1.11 Justification of this project.....</b>	<b>51</b>
<b>1.12 Aims of this project.....</b>	<b>54</b>
<b>2.0 METHODS AND MATERIALS.....</b>	<b>55</b>
<b>2.1 Abbreviations.....</b>	<b>55</b>
<b>2.2 Cell preparation.....</b>	<b>58</b>
<b>2.3 Differentiating fibrocytes.....</b>	<b>58</b>
<b>2.4 Differentiating Macrophages.....</b>	<b>59</b>
<b>2.5 Viable cell counts using a haemocytometer.....</b>	<b>59</b>
<b>2.6 Cell counts using an eye piece micrometer.....</b>	<b>60</b>
<b>2.7 Separation of peripheral blood mononuclear cells.....</b>	<b>61</b>
<b>2.8 CD14<sup>+</sup> selection using Magnetic beads.....</b>	<b>61</b>
<b>2.9 Flow cytometry to check CD14<sup>+</sup> purity.....</b>	<b>62</b>
<b>2.10 Cell Surface epitope staining for flow cytometry.....</b>	<b>62</b>
<b>2.11 Growing fibroblast cell lines.....</b>	<b>64</b>
<b>2.12 Photographing fibrocytes and macrophages.....</b>	<b>66</b>
<b>2.13 Testing different detachment solutions on fibrocytes.....</b>	<b>66</b>

<b>2.14 Use of Puramatrix Hydrogel™ in tissue culture.....</b>	<b>67</b>
<b>2.15 Identification of live cells with DiOC6 using flow cytometry.....</b>	<b>68</b>
<b>2.16 Cell surface epitope staining on slides.....</b>	<b>69</b>
<b>2.17 Time-lapse photography of fibrocytes and macrophages.....</b>	<b>72</b>
<b>2.18 Cell sorting of monocyte populations to culture in different media.....</b>	<b>73</b>
<b>2.19 Microarray analysis of fibrocytes, macrophages and fibroblasts.....</b>	<b>77</b>
2.19.1 Sample collection.....	77
2.19.2 Microarray preparation.....	79
2.19.3 RNA Extraction for microarray analysis.....	79
2.19.4 Reverse Transcription PCR.....	79
2.19.5 Quantification of amount of cDNA in each sample.....	80
2.19.6 Dye Labelling .....	81
2.19.7 Probe Quantification .....	82
2.19.8 Probe hybridisation .....	83
2.19.9 Preparation of microarray slides.....	83
2.19.10 Post hybridisation washes.....	84
2.19.11 Slidescanning.....	85
2.19.12 Analysis of microarrays.....	85
<b>3.0 CULTURING AND QUANTIFYING FIBROCYTES.....</b>	<b>88</b>
<b>3.1 Generating Fibrocytes.....</b>	<b>88</b>

<b>3.2 Quantifying Fibrocytes.....</b>	<b>90</b>
<b>3.3 Optimising culture conditions of both serum-free and serum-containing fibrocytes.....</b>	<b>97</b>
<b>3.4 Harvesting Fibrocytes.....</b>	<b>102</b>
3.4.1 Testing different detachment solutions on serum-free fibrocyte cultures.....	102
3.4.2 Culturing cells on Puramatrix™ Hydrogel.....	107
3.4.3 Differentiating serum-free fibrocytes on coated plates.....	110
<b>3.5 Marker expression of serum-free and serum-containing fibrocytes.....</b>	<b>111</b>
<b>3.6 Discussion of chapter.....</b>	<b>116</b>
<b>4.0 COMPARING SERUM-FREE AND SERUM-CONTAINING FIBROCYTES.....</b>	<b>120</b>
<b>4.1 Generation of fibrocytes from PBMC and CD14<sup>+</sup> selected cells.....</b>	<b>120</b>
<b>4.2 Donor Variability.....</b>	<b>122</b>
<b>4.3 Motility of fibrocytes.....</b>	<b>124</b>
<b>4.3 Effect of serum-containing medium on serum-free derived fibrocytes.....</b>	<b>130</b>
<b>4.4 Treating serum-free derived fibrocytes with serum.....</b>	<b>136</b>
<b>4.5 Effect of serum-containing media on marker expression of serum-free fibrocytes.....</b>	<b>143</b>
<b>4.6 Determining what part of serum is causing the loss of fibrocyte morphology.....</b>	<b>147</b>

<b>4.7 Discussion of chapter.....</b>	<b>152</b>
<b>5.0 CULTURING FIBROCYTES FROM SORTED MONOCYTE POPULATIONS.....</b>	<b>160</b>
<b>5.1 Introduction.....</b>	<b>160</b>
<b>5.2 Cell sorting monocyte subsets.....</b>	<b>161</b>
<b>5.3 Differentiating fibrocytes and macrophages from sorted monocyte populations.....</b>	<b>164</b>
<b>5.4 Discussion of chapter.....</b>	<b>168</b>
<b>6.0 TRANSCRIPTOME ANALYSIS.....</b>	<b>170</b>
<b>6.1 Introduction.....</b>	<b>170</b>
<b>6.2 Transcriptome analysis.....</b>	<b>170</b>
<b>6.3 Aims of this chapter.....</b>	<b>173</b>
<b>6.4 Preparing samples for transcriptome analysis.....</b>	<b>175</b>
<b>6.5 Normalising microarray data.....</b>	<b>178</b>
<b>6.6 Relationship between experimental replicates.....</b>	<b>181</b>
<b>6.7 Analysis of all four cell populations.....</b>	<b>183</b>
6.7.1 Unsupervised clustering of all four cell populations.....	183
6.7.2 Significance analysis of all four cell populations.....	187

<b>6.8 Analysis of serum-free fibrocytes, serum-containing fibrocytes and macrophages.....</b>	<b>192</b>
6.8.1 Unsupervised analysis.....	192
6.8.2 Statistical analysis of microarrays.....	192
6.8.3 Predictive analysis of microarrays.....	195
<b>6.9 Analysis of serum-free fibrocytes versus serum-containing fibrocytes....</b>	<b>203</b>
6.9.1 SAM analysis of serum-free fibrocytes versus serum-containing fibrocytes.....	203
6.9.2 Functional annotation of genes identified by SAM analysis of serum-free versus serum-containing fibrocytes.....	204
6.9.3 PAM analysis of serum-free fibrocytes versus serum-containing fibrocytes.....	208
<b>6.10 Analysis of fibrocytes versus macrophages.....</b>	<b>213</b>
6.10.1 SAM analysis of fibrocytes versus macrophages.....	213
6.10.2 PAM analysis of fibrocytes versus macrophages.....	215
<b>6.11 Predictive analysis of fibroblasts versus fibrocytes and macrophages..</b>	<b>224</b>
<b>6.12 Discussion of chapter.....</b>	<b>235</b>
<b>7.0 FINAL DISCUSSION.....</b>	<b>245</b>
<b>8.0 REFERENCES.....</b>	<b>256</b>

## LIST OF FIGURES

<b>Figure 1.1</b> Dichotomy model of haematopoiesis.....	2
<b>Figure 1.2</b> Alternative model of haematopoiesis.....	4
<b>Figure 1.3</b> Second alternative model of haematopoiesis.....	6
<b>Figure 1.4</b> Effect of cytokines on fibrocyte differentiation.....	34
<b>Figure 1.5</b> SAP inhibits IL-4 or IL-13 induced fibrocyte differentiation.....	35
<b>Figure 2.1</b> Purity check of a CD14 <sup>+</sup> selection using MACS beads.....	64
<b>Figure 3.1</b> Culture conditions for generating fibrocytes.....	89
<b>Figure 3.2</b> Photographs of Fibrocytes.....	90
<b>Figure 3.3</b> Percentage and number of serum-free fibrocytes differentiated on two different substrates.....	92
<b>Figure 3.4</b> Determination of the ideal number of fields of view and replicate wells required for precise counting results.....	94
<b>Figure 3.5</b> Determination of the ideal number of fields of view when counting three wells to get precise counting results.....	96
<b>Figure 3.6</b> Testing precision of counting three fields of view from three replicate wells.....	97
<b>Figure 3.7</b> Number of differentiated fibrocytes for different seeding concentrations of PBMC.....	97
<b>Figure 3.8</b> Optimum time point to wash out non-adherent cells to ensure minimal monocyte loss.....	99
<b>Figure 3.9</b> Number of differentiated fibrocytes for different seeding concentrations of CD14+ selected cells.....	101
<b>Figure 3.10</b> Percentage serum-free fibrocyte detachment.....	104

<b>Figure 3.11</b> Photographs of fibrocyte cultures pre- and post treatment with commercially available detachment agents.....	105
<b>Figure 3.12</b> Photographs of fibrocyte cultures pre- and post treatment with ice cold EDTA.....	106
<b>Figure 3.13</b> A schematic of how Puramatrix™ hydrogel detaches adherent cells.....	107
<b>Figure 3.14</b> DiOC6 staining of dermal fibroblasts cultured on Puramatrix™ .....	109
<b>Figure 3.15</b> Images of stromal markers on serum-free, serum-containing fibrocytes, macrophages and a fibroblast cell line.....	112
<b>Figure 3.16</b> Images of haematopoietic markers on serum-free, serum-containing fibrocytes, macrophages and a fibroblast cell line.....	113
<b>Figure 3.17</b> Images of irrelevant matched controls on serum-free, serum-containing fibrocytes, macrophages and a fibroblast cell line.....	114
<b>Figure 4.1</b> Number of fibrocytes differentiated in serum-free and serum-containing fibrocyte media from either PBMC or CD14 <sup>+</sup> selected cells.....	121
<b>Figure 4.2</b> Donor variability of serum-free derived fibrocytes.....	123
<b>Figure 4.3</b> Donor variability of serum-containing derived fibrocytes.....	124
<b>Figure 4.4</b> Examples of motility of serum-free differentiated fibrocytes.....	126
<b>Figure 4.5</b> Examples of motility of serum-containing differentiated fibrocytes.....	127
<b>Figure 4.6</b> Examples of motility of macrophages.....	128
<b>Figure 4.7</b> Motility of serum-free, serum-containing fibrocytes and macrophages.....	129
<b>Figure 4.9</b> Effect of switching medium on fibrocytes differentiated from PBMC.....	131
<b>Figure 4.9</b> Effect of switching medium on fibrocytes differentiated from CD14 <sup>+</sup> cells .....	133
<b>Figure 4.10</b> Control experiment to ensure change in fibrocyte morphology is due to addition of serum.....	135

<b>Figure 4.11.</b> Fibrocytes differentiated in serum-free fibrocytes medium were changed to serum-containing fibrocyte medium and videoed.....	136
<b>Figure 4.12</b> Effect of FCS on serum-free fibrocytes.....	137
<b>Figure 4.13</b> Effect of heat-inactivated FCS on serum-free fibrocytes.....	138
<b>Figure 4.14</b> Effect of human serum on serum-free fibrocytes.....	139
<b>Figure 4.15</b> Effect of heat-inactivated human serum on serum-free fibrocytes.....	140
<b>Figure 4.16</b> Change in length of serum-free fibrocytes treated with serum over time.....	141
<b>Figure 4.17</b> Number of serum-free fibrocytes following a titration of serum.....	143
<b>Figure 4.18</b> Effect of changing media of serum-free generated fibrocyte to either serum-free or serum-containing fibrocyte medium.....	144
<b>Figure 4.19</b> Effect of changing media of serum-free generated fibrocyte to either serum-free or serum-containing fibrocyte medium.....	146
<b>Figure 4.20</b> Effect of heat-treated FCS on serum-free generated fibrocyte.....	148
<b>Figure 4.21</b> Effect of fractionated FCS on serum-free generated fibrocyte.....	149
<b>Figure 4.22</b> Effect of SAP and EDTA equivalents on serum-free generated fibrocyte.....	151
<b>Figure 4.23</b> Effect of SAP and EDTA equivalents on PBMC cultured in serum-free fibrocyte medium.....	152
<b>Figure 4.24</b> Schematic of how XY co-ordinate is calculated in motility analysis.....	153
<b>Figure 5.1</b> Logical gating when sorting monocyte populations from PBMC.....	162
<b>Figure 5.2</b> Purity check of sorted monocyte populations.....	163
<b>Figure 5.3</b> Photographs of differentiated fibrocytes and macrophages from sorted monocyte populations.....	165
<b>Figure 5.4</b> Number of fibrocytes and macrophages differentiated from sorted monocyte populations.....	167

<b>Figure 6.1</b> Example of real time L27 gene PCR results for twelve samples.....	178
<b>Figure 6.2</b> Example image of microarray containing 37632 spots and subarrays.....	179
<b>Figure 6.3</b> Box-plot of normalised intensities of each sample.....	181
<b>Figure 6.4</b> Correlation between experimental replicates and other samples.....	183
<b>Figure 6.5</b> Hierarchical cluster analysis tree of unsupervised data from all four cell populations.....	185
<b>Figure 6.6</b> Principle component plot of unsupervised data from all four cell populations...	187
<b>Figure 6.7</b> SAM plot of four way analysis of microarrays using a 0.1% FDR.....	188
<b>Figure 6.8</b> Hierarchical cluster analysis tree of samples based on 12602 significantly different genes from SAM analysis.....	190
<b>Figure 6.9</b> Principle component plot of samples based on 12602 significantly different genes from SAM analysis.....	191
<b>Figure 6.10</b> Unsupervised cluster analysis of serum-free fibrocyte, serum-containing fibrocyte and macrophages samples.....	193
<b>Figure 6.11</b> Cluster analysis of serum-free fibrocyte, serum-containing fibrocyte and macrophage samples based on 4563 significantly different genes from SAM analysis.....	194
<b>Figure 6.12</b> Cross validation probabilities of prediction set in PAM analysis.....	196
<b>Figure 6.13</b> Cross validation probabilities of test set in PAM analysis.....	197
<b>Figure 6.14</b> Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the 27 samples.....	201
<b>Figure 6.15</b> Cluster analysis of serum-free fibrocyte, serum-containing fibrocyte and macrophage samples based on 28 predictive genes from PAM analysis.....	202
<b>Figure 6.16</b> Cluster analysis of serum-free fibrocyte and serum-containing fibrocyte samples based on 6614 significantly different genes from SAM analysis.....	204
<b>Figure 6.17</b> Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-free and serum-containing fibrocyte samples.....	211

<b>Figure 6.18</b> Cluster analysis of serum-free and serum-containing fibrocytes samples based on 36 predictive genes from PAM analysis.....	212
<b>Figure 6.19</b> Cluster analysis of fibrocytes and macrophage samples.....	214
<b>Figure 6.20</b> Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-free fibrocytes and macrophage samples.....	217
<b>Figure 6.21a</b> Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-containing fibrocytes and macrophage samples.....	220
<b>Figure 6.21b</b> Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-containing fibrocytes and macrophage samples.....	221
<b>Figure 6.22</b> Cluster analysis of fibrocytes and macrophage samples based on predictive genes from PAM analysis.....	223
<b>Figure 6.23a</b> Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-free fibrocytes and fibroblast samples.....	226
<b>Figure 6.23b</b> Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-free fibrocytes and fibroblast samples.....	227
<b>Figure 6.24a</b> Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-containing fibrocytes and fibroblast samples.....	230
<b>Figure 6.24b</b> Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum- containing fibrocytes and fibroblast samples.....	231
<b>Figure 6.25a</b> Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the macrophage and fibroblast samples.....	233
<b>Figure 6.25b</b> Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the macrophage and fibroblast samples.....	234
<b>Figure 7.1</b> PCA of Monocytes, monocyte derived progeny and fibroblasts.....	235

## LIST OF TABLES

<b>Table 1.1</b> Hematopoietin family of cytokines.....	10
<b>Table 1.2</b> TNF family of cytokines.....	11
<b>Table 1.3</b> IL-10 family of cytokines.....	11
<b>Table 1.4</b> IL-12 family of cytokines.....	12
<b>Table 1.5</b> Unassigned cytokines.....	12
<b>Table 1.6</b> CC subfamily of chemokines.....	14
<b>Table 1.7</b> CXC subfamily of chemokines.....	15
<b>Table 1.8</b> C subfamily of chemokines.....	16
<b>Table 1.9</b> CX <sub>3</sub> C subfamily of chemokines.....	16
<b>Table 1.10</b> Markers positively expressed on fibrocytes.....	26
<b>Table 1.11</b> Markers negatively expressed on fibrocytes.....	27
<b>Table 2.1</b> List of antibodies used for flow cytometry.....	63
<b>Table 2.2</b> List of primary antibodies used in immunostaining.....	71
<b>Table 2.3</b> List of secondary antibodies used in immunostaining.....	72
<b>Table 2.4</b> List of antibodies used in cell sorting of monocyte populations.....	76
<b>Table 3.1</b> Summary of staining results on serum-free, serum-containing fibrocytes, macrophages and fibroblast cell lines.....	115
<b>Table 6.1</b> Prepared samples for transcriptome analysis.....	176
<b>Table 6.2</b> Summary of test probability results for PAM analysis.....	198

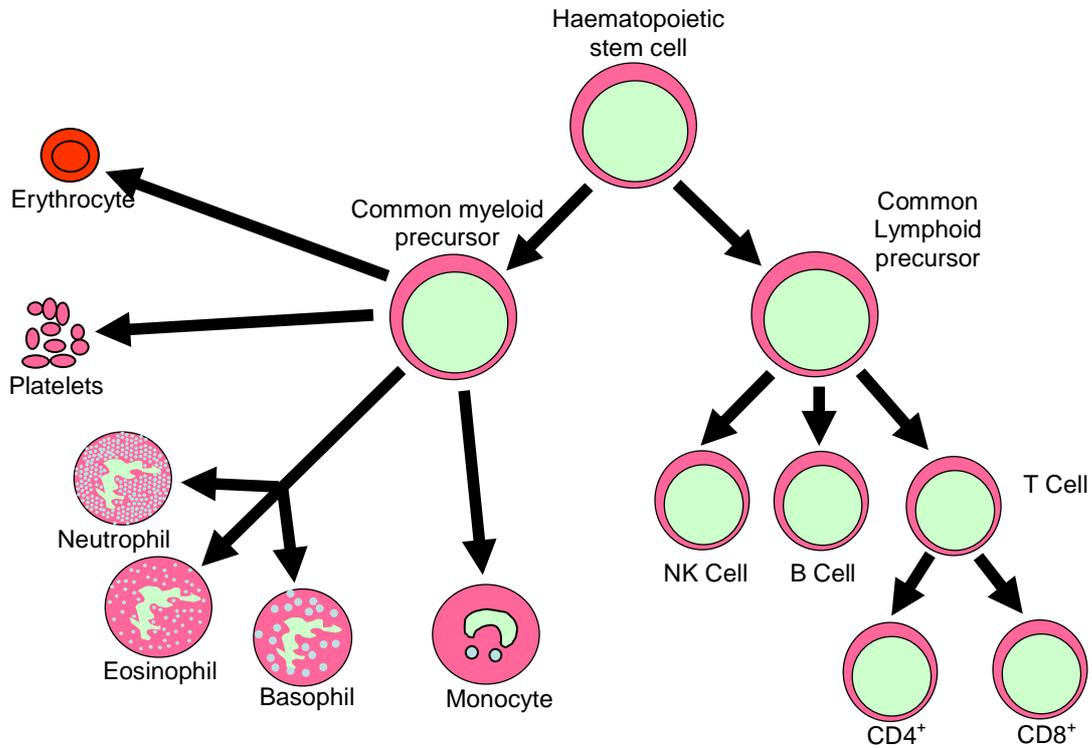
<b>Table 6.3</b> List of genes used eight times and above in the ten iterations of PAM to classify the 28 samples.....	190
<b>Table 6.4</b> Processes enriched in DAVID when looking at those genes that were higher in serum-free fibrocytes than serum-containing fibrocytes.....	206
<b>Table 6.5</b> Processes enriched in DAVID when looking at those genes that were higher in serum-containing fibrocytes than serum-free fibrocytes.....	207
<b>Table 6.6</b> Summary of test probability results for PAM analysis of serum-free and serum-containing fibrocytes.....	209
<b>Table 6.7</b> List of genes used eight times and above in the ten iterations of PAM to classify the serum-free and serum-containing fibrocyte samples.....	210
<b>Table 6.8</b> List of genes used eight times and above in the ten iterations of PAM to classify the serum-free fibrocyte and macrophage samples.....	216
<b>Table 6.9</b> List of genes used eight times and above in the ten iterations of PAM to classify the serum-containing fibrocyte and macrophage samples.....	219
<b>Table 6.10</b> List of genes used eight times and above in the ten iterations of PAM to classify the serum-free fibrocyte and fibroblast samples.....	225
<b>Table 6.11</b> List of genes used eight times and above in the ten iterations of PAM to classify the serum-containing fibrocyte and fibroblast samples.....	229
<b>Table 6.12</b> List of genes used eight times and above in the ten iterations of PAM to classify the macrophage and fibroblast samples.....	232

# 1.0 INTRODUCTION

## 1.1 Haematopoiesis

Blood cells of all lineages are generated through the process of haematopoiesis. Each adult produces approximately 200 billion erythrocytes, 100 billion leukocytes, and 100 billion platelets every day<sup>1</sup> and this can increase by more than 10 fold during a high demand for particular cells<sup>1</sup>. The correct development of the immune system from the haematopoietic stem cell is extremely important in its ability to protect the body from invasion and attack, therefore the differentiation pathways of the cells of the immune system have been researched for many years.

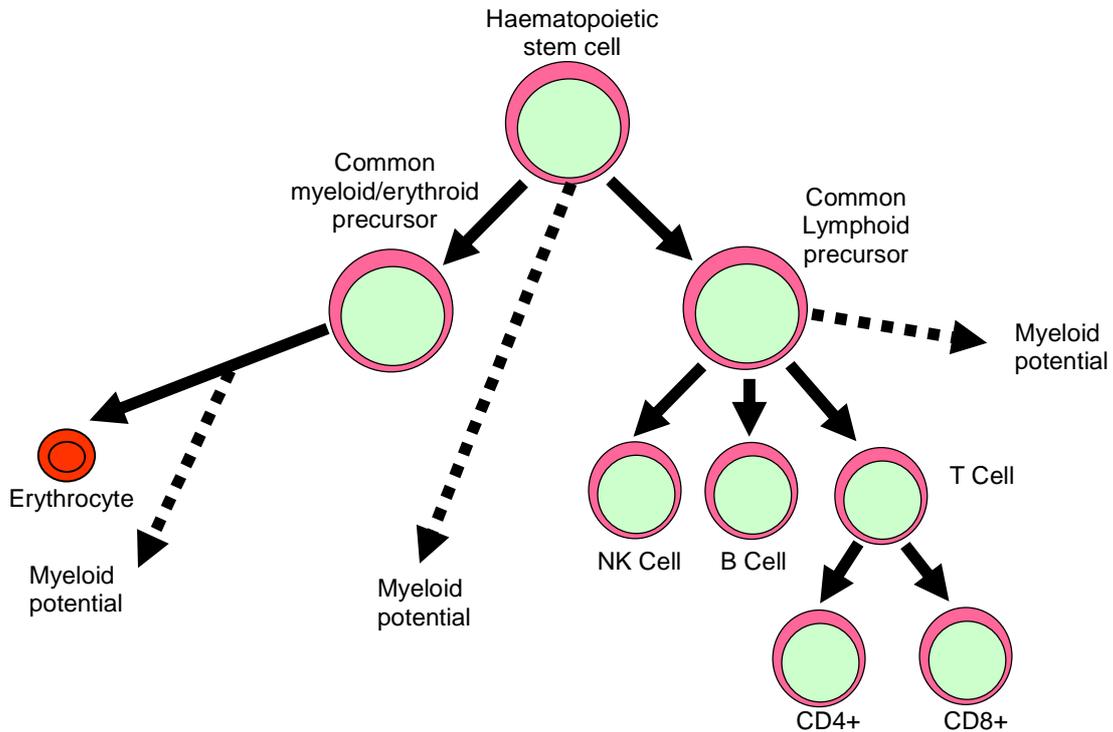
It was 30 years ago that Abramson demonstrated that myeloid and lymphoid cells are derived from the same pluripotent stem cells in mice<sup>2</sup> and the dichotomy model of differentiation developed (Figure 1.1). Over the years various studies have supported the concept that the lymphoid cells, T and B lymphocytes are closely related. Due to their ability to recognize antigen specifically, their morphology and the observation that patients with severe combined immune deficiency have neither of these cells<sup>3</sup>. Two studies in mice in the 1990's indicated that early progenitors in the adult thymus had T and B cell differentiation potential but only limited myeloid potential<sup>4;5</sup>. Until more recently comparably little work has been done specifically on the myeloid lineage, other than in the development of red blood cells (reviewed in Kaushansky *et al*<sup>1</sup>).



**Figure 1.1 Dichotomy model of haematopoiesis.** Schematic diagram of the dichotomy model of haematopoietic cell differentiation<sup>2</sup>.

In 2002 work on myeloid lineage in humans supported the dichotomy model of haematopoiesis, Manz *et al* stated that the evidence available, as well as their own published work in humans, indicated that the commitment to either the lymphoid or myeloid lineages are exclusive events<sup>6</sup>. They reported that human common myeloid progenitors are the clonogenic progenitors of the granulocyte/macrophage and megakaryocyte/erythrocyte progenitors. They also stated that none of these myeloid progenitors exhibit *in vitro* differentiation activity for B or NK cells, or express CD7 or CD10, markers of early lymphocytes<sup>6</sup>.

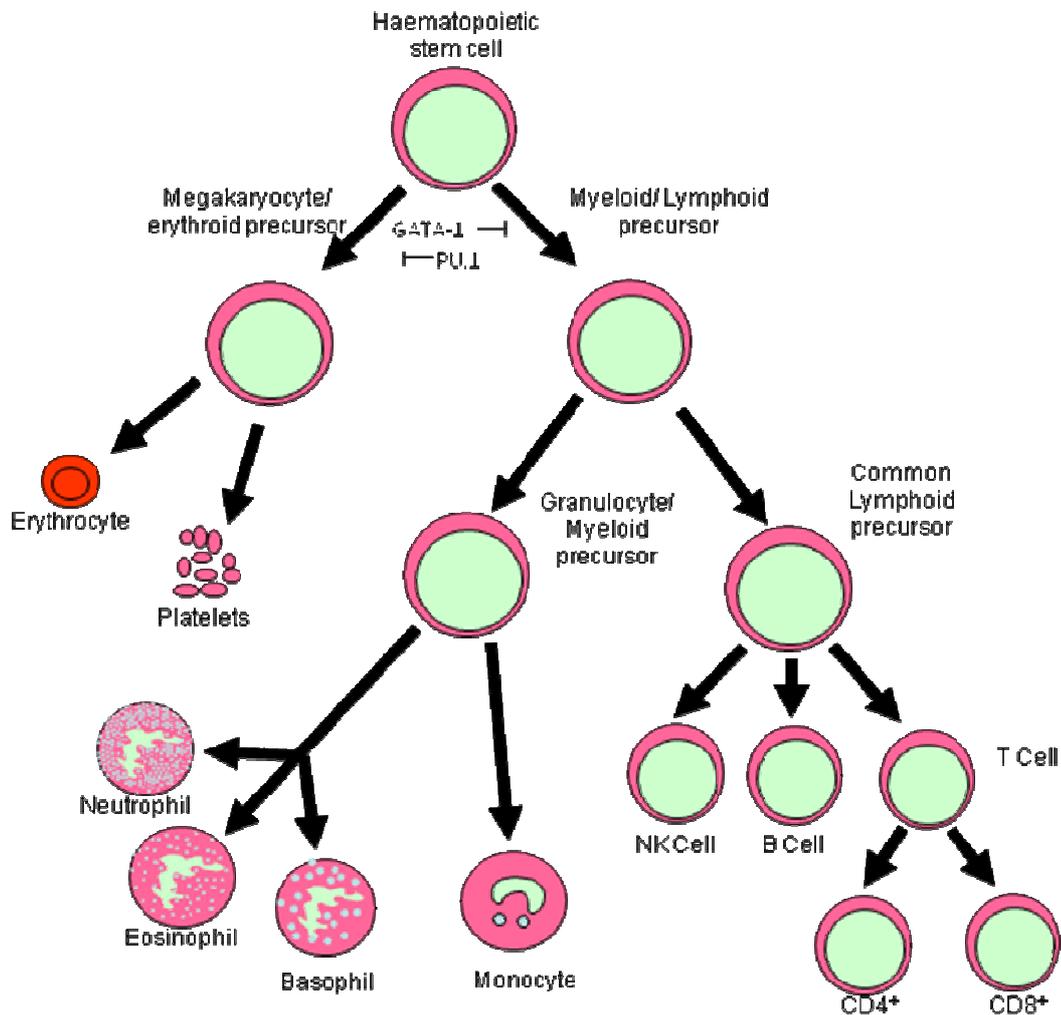
Following work investigating lineage commitment during lympho-haematopoiesis an alternative model of haematopoiesis was proposed in 2006 by Kawamoto *et al*, using a clonal assay system<sup>3,7</sup>. In this system an environment that equally supported the development of various cell lineages was developed on the cultural foetal thymus lobes from which endogenous thymocytes had been deleted. Cytokines were added to enhance the production of cells other than T cells and the single cells were cultured under high oxygen submersion conditions. The cells generated were collected and analyzed by flow cytometry to determine which cell lineages were generated in which culture. Although the results did not always reflect a complete developmental potential of the initially seeded progenitor cell, careful analysis of the frequency of progenitors in each organ and cellular subpopulations, along with the flow cytometry results, could determine the developmental potential<sup>3</sup>. From this work the group proposed a new model for haematopoiesis in which cells with myeloid potential could differentiate from many different cell types (Figure 1.2), as they found that common lymphoid progenitors still had the capacity to produce myeloid cells<sup>3</sup>.



**Figure 1.2 Alternative model of haematopoiesis.** Schematic diagram of the model of haematopoietic cell differentiation proposed by Kawamoto *et al*<sup>3,7</sup>.

However foetal thymus lobes in which thymocytes are not present is not physiologically possible and the whole experimental system was extremely artificial, therefore it is perhaps not surprising that cells with myeloid potential could differentiate from many different cell types due to the cytokine cocktail they were being given, and the lack of antagonistic signals. During haematopoiesis a cell does not just receive one set of signals sending it down a certain differentiation pathway, it can receive many conflicting signals and how the cell responds to all of these signals determines how it goes on to differentiate.

Evidence was provided in 2005 that suggested that a population of FMS-like tyrosine kinase 3 (Flt3) positive lympho-myeloid stem cells exist that lack the potential of erythro-megakaryocytic lineages, suggesting an update to the lineage map of haematopoiesis<sup>8</sup>. Flt3 is a tyrosine-kinase, a cell membrane bound enzyme that will signal into the cell when it binds with itself as a homodimer. It was shown that multipotent progenitor (MPP) cells that do not express Flt3 differentiate specifically down the erythroid/megakaryocyte pathway, whereas those which do express Flt3 have a reduced erythroid/megakaryocyte potential and give rise to both lymphoid and myeloid populations. This suggested that cells of both the innate and adaptive immune response arise from the same common progenitor<sup>8</sup>. The genetic basis of this separation, identified by Flt3 expression, has been shown previously to be due to the transcription factors PU.1 and globin transcription factor 1 (GATA-1). Transcription factors are proteins that bind to specific sequences of DNA controlling transfer, or transcription. Gene disruption studies in mice have shown the importance of GATA-1 for erythroid and megakaryocyte differentiation<sup>9</sup> and PU.1 for myeloid and lymphoid development<sup>10</sup>. PU.1 and GATA-1 were found to be functional antagonists of each other, due to the inhibition of transactivation potential, respectively, by direct physical interaction between the two proteins<sup>11-14</sup>. More recently work has shown that the reciprocal activation of GATA-1 and PU.1 primarily organises the hematopoietic lineage fate decision to form the earliest hematopoietic branch point, separating cells of erythroid/megakaryocyte potential from those of myeloid/lymphoid potential<sup>15</sup> (reviewed<sup>16</sup>).



**Figure 1.3 Second alternative model of haematopoiesis.** Schematic diagram based on the model of haematopoietic cell differentiation proposed by Laslo *et al* 2008<sup>16</sup>.

The level of the transcription factor PU.1 has been found to continue to affect the outcome of cell differentiation, as having dictated a common lymphoid/myeloid precursor the amount available determines a myeloid over lymphoid direction of differentiation. B-cell development requires the presence of a small amount of the transcription factor PU.1, however 4-5 times that concentration will drive macrophage differentiation, at which point B-cell development is

actively inhibited<sup>16;17</sup>. The molecular determinates for this graded response to PU.1 are unknown however PU.1 does not act independently, many other signals are also required for a cell to be committed to either myeloid or B-cell fates. It is believed that at the lymphoid/myeloid progenitor stage signals are already being released to prime a cell for a certain lineage, although still allowing the cell to have the potential for other pathways (reviewed<sup>16</sup>).

The transcription factor E2A is known to be important for the generation of B lymphocytes, however before determining that lineage it is thought to promote the generation of the lymphoid myeloid progenitors by blocking the expression of a critical erythroid and megakaryocytic lineage determinant. It is also thought to prime the transcription of B lymphocyte lineage specific genes<sup>16</sup>. Ikaros is another transcription factor required for B lymphocyte development, it activates the recombination activating gene (RAG) expression and IgH gene rearrangements<sup>18</sup>. Ikaros deficient mice were still able to generate lymphoid/myeloid progenitor cells, however from that point all B-cell development was blocked and there was an excessive myelopoiesis<sup>16;18</sup>. The loss in B-cell development due to the lack of Ikaros could be reversed however by the expression of the transcription factor early B-cell factor (EBF)<sup>19</sup>. It is thought that Ikaros acts on the lymphoid myeloid progenitor by not only inducing further B-cell lineage genes but also by repressing the expression of myeloid lineage ones<sup>16</sup>.

Many transcription factors are required for T lymphocyte commitment, such as PU.1, arabidopsis ME12-like protein 1 (AML1), globin transcription factor 3 (GATA-3) and E2A, however the most important factor is Notch-1. Notch-1 signalling is blocked in the bone marrow by the transcription factor leukaemia/lymphoma related factor (LRF), disruption of which leads to the development of T cell progenitors. However in the thymic cortex Notch-1 signalling promotes T lymphocyte development and has been shown to inhibit B-cell development, which may be due to Notch-1 inhibiting B-cell specific transcription factors (reviewed<sup>16</sup>). So despite B and T lymphocyte development being quite similar with the distinctive use of the RAG genes for recombination of their receptor genes, and their role in recognising antigen, their developmental pathways diverge quite early on, due to their dichotomous response to Notch signalling<sup>20</sup>.

Once the transcription factor PU.1 has ensured the myeloid/lymphoid lineage development rather than erythro/megakaryocyte<sup>10;15;21</sup>, it then goes on to be involved in the further development of the myeloid lineage<sup>17;22;23</sup>. In addition to PU.1, CCAAT enhancer-binding protein-alpha (C/EBP $\alpha$ ) is also important in specifying the granulocyte/myeloid lineage over the lymphoid<sup>24;25</sup>. The relative amounts of these two transcription factors are thought to regulate the fate of myeloid lineage cells into either monocyte or neutrophils<sup>26</sup>. High levels of PU.1 activate further transcription factors such as early growth response 1, 2 (Egr-1,2)/NGFI-A-binding protein 2 (Nab-2) which together go on to activate monocyte specific genes and repress neutrophil development. In contrast C/EBP $\alpha$  activates the transcription factor growth factor

independence 1 (Gfi-1) which has the opposite effect of repressing monocyte specific genes and activate neutrophil ones (reviewed<sup>16</sup>). In the review by Laslo *et al*<sup>16</sup> they go on to propose that the primary cell fate determinates such as PU.1 and C/EBP $\alpha$  as well as the secondary cell determinates Egr/Gfi-1 work together in feed forward loops to promote transcription of lineage specific genes, so reinforcing cell fate decisions.

## 1.2 Cross talk between cells

Both while cell differentiation is taking place and afterwards, as cells are undertaking their roles within the body, how they interact with each other and ultimately 'talk' to one another is very important. The 'cues' that one cell releases can have major effects on not only the cells around it but cells distant from it as well. One way that cells interact with one other is by the release of cytokines, these can be proteins, peptides or glycoproteins that are regulators produced widely throughout the body and act as signalling molecules, allowing cross talk and communication. They can act in an autocrine (on the cell that produced them), paracrine (on cells nearby) or endocrine (on cells distant from them) fashion and are critical to the development and functioning of both the innate and adaptive immune response. Each cytokine will bind particular cell surface receptors which will initiate a cascade of signals into the cell triggering many different responses<sup>27</sup>.

Cytokines and their receptors are grouped based on their structure. There are two main structural families, the hematopoietin family (Table 1.1), which includes many interleukins

involved in both adaptive and innate immunity, as well as growth hormones, and the tumor necrosis factor (TNF) family (Table 1.2), which also functions in both adaptive and innate immunity but includes many membrane bound members. Other families of cytokines include the interferons, the IL-10 family (Table 1.3), the IL-12 family (Table 1.4) and unassigned cytokines (Table 1.5). Originally the name interleukin was used as a standard nomenclature to identify those cytokines that were secreted by, and acted on leukocytes, however since then many more cytokines have been identified that have diverse origin, structure and effects and the interleukin nomenclature has become confusing<sup>27</sup>.

<b>Cytokine</b>	<b>Alternative name</b>	<b>Producer cells</b>	<b>Receptor's</b>
Epo	Erythropoietin	Kidney cells, hepatocytes	EpoR
IL-2	T cell growth factor	T cells	CD25( $\alpha$ ), CD122( $\beta$ ), CD132( $\gamma$ c)
IL-3	Multicolony CSF	T cells thymic epithelial cells	CD123, $\beta$ c
IL-4	BCGF-1 BSF-1	T cells, mast cells	CD124, CD132( $\gamma$ c)
IL-5	BCGF-2	T cells, mast cells	CD125, $\beta$ c
IL-6	IFN- $\beta$ <sub>2</sub> , BSF-2, BCDF	T cells, macrophages, endothelial cells	CD126, CD130
IL-7		Non- T cells	CD127, CD132( $\gamma$ c)
IL-9		T cells	IL-9R, CD132( $\gamma$ c)
IL-11	AGIF	Stromal fibroblasts	IL-11R, CD130
IL-13	P600	T cells	IL-13R, CD132( $\gamma$ c), CD24
G-CSF	Granulocyte stimulating factor	Fibroblasts and monocytes	G-CSFR
IL-15	T-cell growth factor	Many non-T cells	IL-15R, CD122 CD132( $\gamma$ c)
IL-21		T <sub>H</sub> 2 cells	IL-21R, IL-2R ( $\gamma$ c)
GM-CSF	Granulocyte-macrophage stimulating factor	Macrophages, T cells	CD116 $\beta$ c
OSM	OM, oncostatin	T cells, macrophages	CD116 $\beta$ c
LIF	Leukaemia inhibitory factor	Bone marrow stroma, fibroblasts	LIFR, CD130

**Table 1.1 Hematopoietin family of cytokines.** Sixteen members of the hematopoietin family of cytokines are listed by their name. Alternative names are also indicated, as are the cells that produce them and their receptor, where available. Table adapted from Appendix III in<sup>27</sup>.

Cytokine	Alternative name	Producers	Receptor's
TNF- $\alpha$	Cachectin	Macrophages, NK cells, T cells	p55, p75, CD120a, CD120b
TNF- $\beta$	lymphotoxin, LT, LT- $\alpha$	T cells, B cells	p55, p75, CD120a, CD120b
LT- $\beta$		T cells, B cells	LT- $\beta$ R or HVEM
CD40 ligand	CD40-L	T cells, mast cells	CD40
Fas Ligand	FasL	T cells	CD95 (Fas)
CD27 Ligand	CD27L	T cells	CD27
CD30 Ligand	CD30L	T cells	CD30
4-1BBL		T cells	4-1BB
TRAIL		T cell, monocyte	DR4, DR5, DCR1, DCR2, OPG
OPG-L	RANK-L	Osteoblasts, T cells	RANK, OPG
APRIL			TAC1, BCMA
LIGHT		Activated T cells	
TWEAK			TWEAKER
Blys	BAFF	Monocytes, dendritic cells	TAC1, BCMA

**Table 1.2 TNF family of cytokines.** Fourteen members of the TNF family of cytokines are listed by their name. Alternative names are also indicated, as are the cells that produce them and their receptor, where available. Table adapted from Appendix III in<sup>27</sup>

Cytokine	Alternative name	Producers	Receptor's
IL-10	Cytokine synthesis inhibitory factor	T cells, macrophages, EBV-transformed B cells	IL-10R $\alpha$ + IL-20R $\beta$ c
IL-19		Monocytes	IL-20R $\alpha$ + IL-20R $\beta$ c
IL-20		Monocytes	IL-20R $\alpha$ + IL-20R $\beta$ c, IL-22R $\alpha$ c + IL-20R $\beta$ c
IL-22	IL-TIF	T cells, NK cells	IL-22R $\alpha$ c + IL-10R $\beta$ c
IL-24	MDA7	Monocytes, T cells	IL-22R $\alpha$ c + IL-20R $\beta$ c, IL-20R $\alpha$ + IL-20R $\beta$ c
IL-26	AK155	T cells, NK cells	IL-20R $\alpha$ + IL-10R $\beta$ c
IL-28A, B	IFN- $\lambda$ 2,3		IL-28R $\alpha$ c + IL-10R $\beta$ c
IL-29	IFN- $\lambda$ 1		IL-28R $\alpha$ + IL-10R $\beta$ c

**Table 1.3 IL-10 family of cytokines.** Eight members of the IL-10 family of cytokines are listed by their name. Alternative names are also indicated, as are the cells that produce them and their receptor, where available. Table adapted from Appendix III in<sup>27</sup>

Cytokine	Alternative name	Producers	Receptor's
IL-12	NK-cell stimulatory factor	Macrophages, dendritic cells	IL-12R $\beta$ 1c + IL-12R $\beta$ 2
IL-23		Dendritic cells	IL-12R $\beta$ 1c + IL-23R
IL-27		Monocytes, macrophages, dendritic cells	WSX-1+CD130c

**Table 1.4 IL-12 family of cytokines.** Three members of the IL-12 family of cytokines are listed by their name. Alternative names are also indicated, as are the cells that produce them and their receptor, where available. Table adapted from Appendix III in<sup>27</sup>.

Cytokine	Alternative name	Producers	Receptor's
TGF- $\beta$		Chondrocytes, monocytes, T cells	TGF- $\beta$ R
IL-1 $\alpha$		Macrophages, epithelial cells	CD121a and CD121b
IL-1 $\beta$		Macrophages, epithelial cells	CD121a and CD121b
IL-1 RA		Monocytes, macrophages, neutrophils, hepatocytes	CD121a
MIF		T cells, pituitary cells	
IL-16		T cells, mast cells eosinophils	CD4
IL-17	mCTLA-8	CD4 memory cells	
IL-18	IGIF, interferon- $\gamma$ inducing factor	Activated macrophages and kupffer cells	IL-1R related protein
IL-25		T <sub>H</sub> 2 cells, mast cells	

**Table 1.5 Unassigned cytokines.** Nine unassigned cytokines are listed by their name. Alternative names are also indicated, as are the cells that produce them and their receptor, where available. Table adapted from Appendix III in<sup>27</sup>.

Another group of cytokines, chemokines, or chemotactic cytokines, are low molecular weight (8-10kDa) polypeptides that are involved in the activation, recruitment and retention of leukocytes in both homeostatic and inflammatory conditions<sup>28</sup>. Homeostatic chemokines are expressed constitutively within lymphoid tissue and are involved in controlling migration in normal processes of tissue maintenance or development. However inflammatory chemokines are specifically up-regulated at sites of inflammation, often stimulated by pro-inflammatory cytokines such as interleukin 1 (IL-1). These chemokines are thought to be involved in recruiting

leukocytes, such as monocytes, neutrophils and other effector cells from the peripheral blood to site of infection or tissue damage, often to initiate an immune response or to promote wound healing<sup>29;30</sup>. Homeostatic and inflammatory chemokine groups are not mutually exclusive, same chemokines can be involved in both processes.

Chemokine nomenclature is based on the locations of distinct cysteine residues that form disulphide bonds which are the key to holding their 3-dimensional shape. There are two major (CC and CXC) and two minor (C and CX<sub>3</sub>C) families based on these cysteine signature motifs, where X indicates any residue between the cysteines<sup>31</sup>.

The CC chemokine (or  $\beta$ -chemokines) subgroup has at least 27 different members reported in mammals (Table 1.6), usually containing four cysteines, although a small number have six<sup>32</sup>. This subfamily of chemokines is known to induce the migration of monocytes and other cell types such as NK cells and dendritic cells as well as inducing cellular migration by binding to, and activating, CC chemokine receptors. Ten CC chemokine receptors have been discovered to date and are called CCR1-10<sup>32</sup>.

Systematic name	Other name	Target cell	Receptor
CCL1	I-309	Neutrophil, T cells	CCR8
CCL2	MCP-1	T Cell, monocyte, basophil	CCR2
CCL3	MIP-1 $\alpha$	Monocyte/macrophages, T cell, NK cell, basophil, Immature dendritic Cell, bone marrow cell	CCR1, 5
CCL4	MIP-1 $\beta$	Monocyte/macrophages, T cell, NK cell, basophil, Immature dendritic Cell, bone marrow cell	CCR1, 5
CCL5	RANTES	Monocyte/macrophages, T cell, NK cell, basophil, dendritic cell, eosinophil	CCR1,3,5
CCL6	MRP-2	Monocyte/macrophage, neutrophil	CCR1
CCL7	MCP-3	T cell, monocyte eosinophil, basophil, dendritic cell	CCR2,3
CCL8	MCP-2	T cell, monocyte eosinophil, basophil	CCR2
CCL9/10	MIP-1 $\gamma$	Dendritic cells, osteoclasts	CCR1
CCL11	Eotaxin	Eosinophil	CCR3
CCL12	None	Eosinophil, monocyte, T cell	CCR2
CCL13	MCP-4	T cell, monocyte, eosinophil, basophil, dendritic cell	CCR2,3
CCL14	HCC-1	Monocyte	CCR1
CCL15	MIP-5/HCC-2	T cell, monocyte, neutrophil, dendritic cell	CCR1,3
CCL16	HCC-4	Monocyte	CCR1
CCL17	TARC	T cell, immature dendritic cell, NK cell	CCR4
CCL18	DC-CK1	Naive T cell, T cell	Unknown
CCL19	MIP-3 $\beta$	Naive T cell, mature dendritic cell, B cell	CCR7
CCL20	MIP-3 $\alpha$	T cell, PBMC	CCR6
CCL21	6Ckine	Naive T cell, B cell,	CCR7
CCL22	MDC	Immature dendritic cell, NK cell, T cell thymocytes	CCR4
CCL23	MPIF-2	Monocyte, T cell	Unknown
CCL24	Eotaxin-2/MPIF-2	T cell, eosinophil, basophil	CCR3
CCL25	TECK	Macrophage, thymocytes, dendritic cell	CCR9
CCL26	Eotaxin-3/MIP-4 $\alpha$	Eosinophil, basophils	CCR3
CCL27	CTACK	T cell	CCR10
CCL28	MEC	T cell, eosinophil	CCR10,3

**Table 1.6 CC subfamily of chemokines.** All 27 members of the CC subfamily of chemokines are listed by their systematic name, other names are also indicated, as are the target cells these chemokines will affect and their receptor. Table adapted from Appendix IV in<sup>27</sup>.

The CXC family is the second major chemokine subfamily, as its name suggests, the two N-terminal cysteines of CXC chemokines (or  $\alpha$ -chemokines) are separated by one amino acid, represented with an 'X'. In mammals 16 different CXC chemokines have been described, divided into two categories, those with a specific amino acid sequence (or motif) of glutamic acid-

leucine-arginine (or ELR for short) immediately before the first cysteine of the CXC motif (ELR-positive), and those without an ELR motif (ELR-negative) (Table 1.7). ELR-positive CXC chemokines specifically induce the migration of neutrophils, and interact with chemokine receptors CXCR1 and CXCR2. CXC chemokines that lack the ELR motif tend to be chemoattractant for lymphocytes. CXC chemokines bind to CXC chemokine receptors, of which seven have been discovered to date, designated CXCR1-7<sup>32</sup>.

Systematic name	Other name	ELR+/-	Target cell	Receptor
CXCL1	GRO $\alpha$	+	Neutrophil	CXCR2
CXCL2	GRO $\beta$	+	Neutrophil	CXCR2
CXCL3	GRO $\gamma$	+	Neutrophil	CXCR3
CXCL4	PF4	-	Fibroblast	CXCR3
CXCL5	ENA-78	+	Neutrophil	CXCR2
CXCL6	GCP-2	+	Neutrophil	CXCR2
CXCL7	LDGF-PBP	+	Fibroblast, neutrophil	CXCR2
CXCL8	IL-8	+	Neutrophil, basophil, T cell	CXCR1, 2
CXCL9	Mig	-	Activated T cell	CXCR3
CXCL10	IP-10	-	Activated T cell	CXCR3
CXCL11	i-TAC	-	Activated T cell	CXCR3
CXCL12	SDF-1 $\alpha/\beta$	-	CD34+ bone marrow cell, T cell, dendritic cell, B cell, activated CD4 T cell	CXCR4
CXCL13	BLC/BCA-1	-	Naive B cells, activated CD4 T cells	CXCR5
CXCL14	BRAK	+	T cell, Monocytes	Unknown
CXCL15	Lungkine	+	Neutrophils	CXCR2
CXCL16	BUNZO/STRC33	-	T cell, NK T cell	CXCR6

**Table 1.7 CXC subfamily of chemokines.** Members of the CXC subfamily of chemokines are listed by their systematic name, other names are also indicated, as are the target cells these chemokines will affect and their receptor. Table adapted from Appendix IV in<sup>27</sup>.

The third group of chemokines is known as the C chemokines (or  $\gamma$  chemokines), and is unlike all other chemokines in that it has only two cysteines; one N-terminal cysteine and one cysteine downstream. Two chemokines have been described for this subgroup and they are called XCL1 (lymphotactin- $\alpha$ ) and XCL2 (lymphotactin- $\beta$ )<sup>32</sup> (Table 1.8).

Systematic name	Other name	Target cell	Receptor
XCL1	Lymphotactin- $\alpha$	T cell, NK cell	XCR1
XCL2	Lymphotactin- $\beta$	Those expressing XCR1	XCR1

**Table 1.8 C subfamily of chemokines.** Members of the C subfamily of chemokines are listed by their systematic name, other names are also indicated, as are the target cells these chemokines will affect and their receptor. Table adapted from Appendix IV in<sup>27</sup>.

Finally, there is only 1 member to date of the fourth subgroup of chemokines, fractalkine (or CX<sub>3</sub>CL1). in which the two cysteines have three amino acids between them, termed the CX<sub>3</sub>C chemokine (or  $\delta$ -chemokine)<sup>32</sup> (Table 1.9).

Systematic name	other name	Target cell	Receptor
CX <sub>3</sub> CL1	Fractalkine	T cell, monocyte, neutrophil	CX <sub>3</sub> CR1

**Table 1.9 CX<sub>3</sub>C subfamily of chemokines.** The one member of the CX<sub>3</sub>C subfamily of chemokines is listed with its systematic name, its other name, the cells it can target and its receptor. Table adapted from Appendix IV in<sup>27</sup>.

Chemokines interact with G-protein coupled receptors (GPCR) that have seven trans-membrane spanning  $\alpha$  helix domains<sup>33-35</sup>. To date 20 chemokine receptors have been described for the 46 human chemokines, with additional complexity via the potential formation of homodimers and heterodimers<sup>36</sup>. GPCR can be split into four categories based on the chemokines they interact with, CXCR bind CXC chemokines, CCR bind CC chemokines, CX<sub>3</sub>XR1 binds the sole CX<sub>3</sub>C chemokine and XCR1 the XC chemokines. As the name suggests GPCR are linked to G-proteins

that transmit cell signals following ligand binding. These can go on to promote many signaling cascades which are involved in chemotaxis, degranulation and release of superoxide anions<sup>37</sup>.

### 1.3 Monocyte development

The development of monocytes takes place in the bone marrow, and as discussed previously involves a complex crosstalk of transcription factors to ensure the development of first the granulocyte/myeloid precursor from the myeloid/lymphoid precursor, and then monocytes from the granulocyte/myeloid precursor<sup>38</sup>. After all this co-operation and cross-talk of transcription factors a differentiated monocyte can be released from the bone marrow into the peripheral blood<sup>39</sup> by the interactions of chemokine receptors and their ligands. Monocyte emigration is highly affected by the level of inflammation within the body as well as the presence of a high fat diet<sup>40</sup>. The chemokine receptor CCR2 and its ligands CCL7 and CCL2 have been shown to be important in the emigration of monocytes from the bone marrow<sup>40</sup>.

Once in the blood monocytes circulate for a few days, before entering the tissues and giving rise to a variety of tissue resident macrophages throughout the body<sup>39</sup>, as well as specialized cells such as dendritic cells<sup>41,42</sup>, osteoclasts<sup>43,44</sup> and fibrocytes<sup>45-49</sup>. Monocytes make up 5-10% of the circulating leukocytes in the human body however they are extremely heterogeneous in their size, degree of granularity and nuclear morphology, which can sometimes lead to confusion in

their identification at the extremes of these<sup>50</sup>. These are important cells of the immune system that link inflammation and the innate part of the immune system, to the adaptive portion.

Three subsets of monocytes have been defined, based on phenotype and chemokine expression. In the 1980s various groups described the existence of two monocyte populations in humans that differed in phenotype and functions (reviewed in<sup>40</sup> and<sup>51</sup>). The dominant population consisted of larger, more active cells with both phagocytic and myeloperoxidase activity, as well as a higher super oxide release. The smaller monocytes had low peroxidase activity, but were able to release IL-1 and mediate antibody-dependent cytotoxicity<sup>40;51</sup>. Subsequently it was found that this larger population represented between 80-90% of all monocytes and could be identified by the expression of CD14 but not CD16<sup>52</sup>. They also expressed high levels of the chemokine receptor CCR2 but low levels of CX<sub>3</sub>CR1 and in response to LPS *in vitro* they released IL-10<sup>53-55</sup>. The smaller monocytes however were identified by their expression of CD16 and low levels of CD14<sup>56</sup>, expressing high levels of CX<sub>3</sub>CR1 and low levels of CCR2<sup>53;55</sup>. These cells were termed proinflammatory<sup>52;57</sup>, as in response to LPS they released TNF- $\alpha$  and have been reported as being in higher numbers in the blood of patients with acute inflammation<sup>58</sup> and infectious diseases<sup>59;60</sup>.

More recently the small population of CD16<sup>+</sup> monocytes has been suggested to consist of two distinct populations with different functions<sup>51</sup>. Monocytes which express both CD16 and CD14 have been seen to express the Fc receptors CD64 and CD32, have phagocytic activity and are

responsible for the expression of both TNF- $\alpha$  and IL-1 in response to LPS<sup>61</sup>. However those CD16<sup>+</sup> monocytes that have a very low level of CD14 are much less efficient at phagocytosis, do not express the Fc receptors or produce either TNF- $\alpha$  or IL-1 in response to LPS<sup>62</sup>.

## **1.4 Monocyte derived progeny**

### **1.4.1 Macrophages and Dendritic cells**

Two major cell types that can differentiate from monocytes are macrophages and dendritic cells (DCs), however the exact origin and lineage relationships of these cells remains poorly understood. Both macrophages and dendritic cells are involved in the scavenging of dying cells, pathogens, and molecules through phagocytosis, endocytosis and the use of pattern recognition receptors<sup>63</sup>. Both can be termed antigen presenting cells (APC) and are widely dispersed throughout the body participating in the initial capture and processing of potential antigens (innate immunity) and then in the activation of specific T and B lymphocyte effector mechanisms (adaptive immunity)<sup>63</sup>. Although these two cell types have overlapping functions within the body, they are distinct. Macrophages are tissue-resident cells that take part in the innate immune response<sup>64</sup>, whereas dendritic cells are professional antigen presenting cells that trigger and help regulate the adaptive immune response<sup>65</sup>.

Although initially it was thought that both macrophages and DCs differentiated entirely from monocytes that had circulated in the blood and then travelled into tissues it is now thought that, although this is still the case for some subtypes, it is not the case for all. Characteristics

such as half life, their replacement after bone marrow graft and whether inflammation has an effect on their differentiation or not allow macrophages and DCs to be split into three groups<sup>40</sup>. Group one includes cells which, following bone marrow transplantation, remain host derived unless the cells are specifically depleted by irradiation<sup>66;67</sup>. For example certain macrophages, such as microglia of the central nervous system, and epidermal DC's, such as Langerhans cells remain host derived, suggesting that there may be some kind of self renewal taking place which is separate from bone marrow derived cells, such as the monocyte<sup>40</sup>. In 2007 Massberg *et al* reported work on haematopoietic stem cells and progenitor cells<sup>68</sup> and showed that progenitor cells have the ability to circulate and then proliferate within extramedullary tissues so giving rise to tissue resident cells<sup>68</sup>. In the presence of Toll-like receptor (TLR) agonists the differentiation of these cells was increased, hence they can respond in both steady state and inflammatory conditions<sup>68</sup>.

The second group consist of cells such as the conventional DCs (cDCs). These can be either CD8a<sup>+</sup> or CD8a<sup>-</sup> and are located in all lymphoid organs. cDCs have a very short half life and renew in steady-state from bone marrow precursors, without a monocyte intermediate<sup>42;69-71</sup>. Finally the third group of cells is a short lived population which responds to inflammatory cues by differentiating from bone marrow derived monocytes into monocyte derived DCs and tissue resident macrophages<sup>39;40</sup>, which was initially thought to be the way that all macrophages and DCs differentiated.

Tissue resident macrophages are extremely heterogeneous, studies using monoclonal antibodies have shown that these cells express different phenotypes dependent on their location, perhaps suggesting specialisation for their particular microenvironments. For example alveolar macrophages found in the lungs express a high number of pattern recognition receptors and scavenger receptors<sup>72;73</sup> which are involved in clearing microorganisms, viruses and environmental particles; whereas macrophages isolated from the lamina propria in the gut have a high phagocytic and bactericidal activity but produce few pro-inflammatory cytokines<sup>74</sup>. Tingible body macrophages in the germinal centres of tonsils have been shown to clear apoptotic lymphocytes generated during the development of an immune response<sup>75</sup> and rarely come into contact with external pathogens. In response to inflammatory and immune stimulation additional monocytes can also be recruited in increased numbers to local sites, where local cues such as macrophage colony stimulating factor (M-CSF) cause macrophage differentiation, however they have been shown to display different phenotypes from the originally resident macrophages<sup>64</sup>.

The surface receptors seen on macrophages are extremely versatile and can be involved in regulating a range of functions including differentiation, growth and survival, adhesion, migration, phagocytosis, activation, and cytotoxicity<sup>64</sup>. These receptors also allow them to recognize a wide range of endogenous and exogenous ligands and respond appropriately, whether it be in the role of homeostasis or as host defense in innate immunity<sup>64</sup>.

Monocyte derived DCs *in vitro* exist in two functionally and phenotypically distinct states, immature and mature. Immature dendritic cells express relatively low levels of MHC class I and II proteins and co stimulatory molecules on their surface, although large amount of MHC class I and II are being made and sequestered intracellularly into lysosomes<sup>65</sup>. Immature dendritic cells are very adept at endocytosis and readily take up antigen, though are very inefficient at processing it and presenting it to T cells<sup>65</sup>. Once the dendritic cell has detected a microbial product or pro-inflammatory cytokines *in vitro* it matures, reducing its ability to take up antigen but becoming extremely efficient at presenting antigen to T cells. There is also a massive cytoplasmic reshuffle with MHC class II molecules being moved from intracellular compartments to the plasma membrane, along with an increased expression of co-stimulatory molecules for T cells<sup>65</sup>. The maturing dendritic cells can alter their chemokine receptor profile to help promote the movement of the cell from the tissue to home to the lymphoid organs, during which these cells extend long projections called dendrites, possibly to help with the interaction with T cells<sup>65</sup>. However when studied *in vivo* Nolte *et al* showed that inflammatory mediators originating from non-hematopoietic tissues are not sufficient, or necessarily required for DC activation<sup>76</sup>. They saw that radio-resistant cells throughout the body, which respond strongly to LPS by initiating local and systemic inflammatory responses, are not sufficient, or necessary to change DC localization, phenotype or T cell stimulatory function, despite the high levels of TNF and IL-1 released<sup>76</sup>.

### 1.4.2 Osteoclasts

Osteoclasts are a multinucleated cell type, formed by the fusion of mononuclear macrophages. They are the major cell type involved in re-absorption of bone and work in partnership with osteoblasts, which form bone, to regulate the size and mass of the skeleton. Unlike osteoblasts which are mesenchymally derived, osteoclasts are haematopoietic in origin<sup>77;78</sup> differentiating from monocytes<sup>43</sup>. Once monocytes have received the macrophage signal, M-CSF, binding to its receptor, colony stimulating factor 1 receptor (c-Fms), they begin to differentiate into macrophages, but this is also a signal to early osteoclast precursors for survival and proliferation<sup>77</sup>. RANK (receptor activator of nuclear factor (NF)-kappaB,) is expressed on osteoclasts and their precursors and it was found that the interaction with its ligand, RANK-L is the signal for osteoclast generation. *In-vivo* RANK-L is expressed on stromal cells, osteoblasts and activated T lymphocytes<sup>77</sup>, however *in-vitro* RANK-L can be added as a soluble factor to a cell culture in the presence of M-CSF differentiated macrophages, to see the production of osteoclasts. Once differentiated osteoclasts become polarised towards bone, developing a ruffled edge which involves the complex infolding of the plasma membrane, thought to allow the formation of microtubules that transport the acidifying vesicles to the bone surface<sup>77</sup>. Although no surface markers specifically identify osteoclasts their massive multinucleated morphology does, as does the presence of tartrate-resistant acid phosphatase or TRAP, known to be highly expressed by osteoclasts, as well as the osteoclast specific protease cathepsin K<sup>79</sup>.

Osteoclasts have been recently shown to only differentiate from the CD16<sup>-</sup> fraction of monocytes, when both the CD16<sup>+</sup> and CD16<sup>-</sup> fractions of monocytes were examined to look at their ability to differentiate into osteoclasts. The CD16<sup>-</sup> fraction formed ten times as many osteoclasts as the CD16<sup>+</sup> fraction and following various tests it was found that although the CD16<sup>+</sup> fraction of cells do respond to M-CSF and RANK-L treatment, by differentiating into osteoclasts, they do not respond to the same extent as the CD16<sup>-</sup> fraction, possibly due to the reduced level of  $\alpha\beta3$  integrin on their surface, which is vital for osteoclast differentiation and activation<sup>44</sup>. This was shown when osteoclasts could not differentiate *in vitro* from macrophages of integrin  $\beta3$  deficient mice<sup>80;81</sup> due to the lack of activation of ERK (extracellular signal-regulated kinase) and c-Fos, both of which are essential for osteoclastogenesis<sup>82;83</sup>. Therefore although osteoclasts differentiate from monocytes, they only differentiate from the larger CD14<sup>+</sup> CD16<sup>-</sup> population.

## 1.5 Fibrocytes

### 1.5.1 Overview of Fibrocytes

It was reported as long ago as 1892 that blood mononuclear cells were capable of transforming into connective tissue following studies in tadpoles. This observation was then confirmed by Maximov in 1928, however both reports were criticised as sceptics suggested that the results seen were due to contaminating fibroblasts entering the blood sample and expanding in culture (reviewed in Postlethwaite *et al* 2004<sup>84</sup>). Experimental studies in 1969 used cannulation and diffusion chamber techniques to demonstrate that collagen-producing cells were not

contaminates dislodged from the blood vessel wall, but were derived from circulating blood elements (reviewed in Postlethwaite *et al* 2004<sup>84</sup>). It was not until work undertaken during the 1990's however that the sceptics were quietened as connective tissue cells were observed to develop from blood mononuclear cells by a number of different groups<sup>85-87</sup>.

Fibroblast-like cells that have developed from peripheral blood are referred to as many different things in the literature and are all reported to have slightly different characteristics. However, most groups are in agreement that these cells require the presence of CD14<sup>+</sup> cells (monocytes), either alone or within PBMC to differentiate. Following *in vitro* culture for anywhere between 3 days and 3 weeks, depending on conditions, long thin spindle shaped cells develop that phenotypically look very much like mesenchymally-derived fibroblasts<sup>45-47;85;86</sup>.

It has been suggested that these cells are extremely important in wound healing<sup>87</sup>, in 1994 Bucala *et al* reported the presence of fibroblast-like cells, or as they referred to them 'fibrocytes' in a model of wound healing in mice<sup>87</sup>. The term fibrocyte combines the Greek 'kytos' meaning cell and 'fibro' which is from the Latin meaning fiber<sup>88</sup>. Bucala's group surgically implanted wound chambers into mice subcutaneously, 24 hours later a rapid influx of peripheral blood cells were seen and the unexpected development of a large number of adherent spindle shaped cells that resembled fibroblasts. Fibroblasts do migrate slowly from adjacent connective tissue to sites of healing however following the entry of a large number of circulating inflammatory cells into the wound site, and the presence of a large number of these fibroblast-

like cells they suggested that there was a connection between them. Bucala *et al* did some preliminary studies on these cells *in vitro* and found that they had similar markers to fibroblasts, such as collagen I and III, vimentin, fibronectin and that they were negative to non-specific esterases, found on monocytes and granulocytes<sup>87</sup>. Further studies confirmed the presence of fibroblast-like cells in cultures of peripheral blood and that, in addition to expressing the fibroblast markers mentioned above, they also expressed leukocyte associated cells surface markers such as the common leukocyte antigen CD45, the pan myeloid antigen CD13, and the haematopoietic stem cell marker CD34, listed in table 1.10<sup>87</sup>. However it was found that these cells did not express epithelial and endothelial markers, monocytes/macrophage specific markers, CD14 and CD16, or proteins produced by dendritic cells and precursors<sup>87</sup> (Table 1.1). Further work since 1994 has shown that fibrocytes can also produce both extracellular matrix (ECM) components as well as ECM-modifying enzymes<sup>45;47;87;89</sup>, primarily matrix metalloproteinase-9 (MMP-9)<sup>90</sup>.

Marker	Role	Cell subpopulations seen on
Vimentin	Intermediate Filament	Fibroblasts
Fibronectin	Pericellular Matrix	Mesenchymal cells, lymphoid cells
Collagen I	Extracellular matrix	Fibroblasts
Collagen III	Extracellular matrix	Fibroblasts
CD11b	Adhesion molecule	Monocytes, granulocytes, NK cells
CD13	Aminopeptidase N	Myeloid and dendritic cells
CD18	Integrin $\beta$ 2	Lymphoid and myeloid cells
CD34	Precursor antigen	Haematopoietic progenitors embryonic fibroblasts
CD45	Leukocytes Common Antigen	Leukocytes
CD71	Transferrin receptor	Macrophages, activated cells

**Table 1.10 Markers positively expressed on fibrocytes.** Cell surface phenotype of human peripheral blood fibrocytes, positive expression seen on fibrocytes<sup>87</sup>

Marker	Role	Cell subpopulations seen on
Esterase	Lysosomal enzyme	Monocytes and granulocytes
TCR ( $\alpha\beta$ , $\gamma\delta$ )	Antigen receptor	T cells
Cytokeratin	Matrix protein	Epithelial cells
Von Willebrand	Coagulation factor	Endothelial cells and platelets
Desmin	Intermediate filament	Muscle cells
$\alpha$ -Actin	Cytoskeletal protein	Smooth muscle cells
Laminin	Matrix protein	Blood vessels, muscle cells, nerve etc
CD3	TCR-associated antigen	T cells
CD4	Class II recognition	Helper/inducer cells
CD8	Class I recognition	Cytotoxic/suppressor T cells
CD10	Endopeptidase (CALLA)	B cell populations
CD11a	LFA-1 antigen	Lymphoid and myeloid cells
CD14	LBP receptor	Monocytes/macrophages
CD16	FcyRIII (Ig receptor)	Monocytes, granulocytes, NK cells
CD19	B-cell antigen	B cells
CD25	IL-2 receptor	Activated macrophages/T-/B- cells
CD33	Pan-myeloid antigen	Myeloid and monocytic precursors
CD38	T10 antigen	Precursor cells, activated T-/B-cells
CD44	Lymphocytes homing receptor	Leukocytes and erythrocytes
CD54	ICAM-1	Monocytes and lymphocytes
CD56	NCAM	NK and T-cell subpopulations

**Table 1.11 Markers negatively expressed on fibrocytes.** Cell surface phenotype of human peripheral blood fibrocytes, negative surface expression seen on fibrocytes<sup>87</sup>

In 1997 it was reported that fibrocytes were potent antigen presenting cells capable of priming naïve T cells<sup>91</sup>. Using monoclonal antibodies it was determined that fibrocytes expressed the full complement of surface proteins that have been shown in other cell types to be necessary for antigen presentation, and following *in vitro* functional experiments concluded that they are almost as potent as dendritic cells at presenting antigen to naïve T cells<sup>91</sup>. This data indicated that fibrocytes may play a critical role in the initiation of antigen specific immunity. This coupled with the observation that fibrocytes induce an angiogenic phenotype in cultured endothelial

cells, promoting angiogenesis *in vivo* in mice<sup>90</sup> could help to account for the persistent inflammatory state seen in some disease states.

### 1.5.2 Fibrocyte differentiation

In 1991 a group observed that neo-fibroblasts, (their term for peripheral blood derived fibroblast-like cells) that developed from monocytes *in vitro* would only do so after the loss of T lymphocytes. They suggested that T lymphocytes, stimulated by the cytokine interleukin-2 (IL 2) inhibit the transformation of monocytes into fibroblasts in culture<sup>85;86</sup>. In 2001 Bucala's group reported a differentiation pathway of fibrocytes and their potential mechanism of migrating to wound sites. They determined that a peripheral blood population consisting of predominately CD14<sup>+</sup> but not CD14<sup>-</sup> cells gives rise to fibrocytes *in vitro*. However in contrast to the observations of Labat *et al* ten years previously, Bucala's group reported that fibrocytes would only differentiate in the presence of T cells and that cell-cell contact, not just conditioned medium, was necessary for their differentiation<sup>45</sup>. Yang *et al* found that direct contact between the CD14<sup>-</sup> and CD14<sup>+</sup> fraction was not required for the differentiation of CD14<sup>+</sup> cells into fibrocytes, however CD14<sup>-</sup> conditioned medium was<sup>46</sup>. Therefore there were many discrepancies between the different groups in relation to fibrocyte differentiation.

Fibroblast-like cells had been reported to be seen in humans in 1991 by Labat *et al*<sup>85;86</sup> in different diseased states. They found monocytes in patients suffering from osteomyelosclerosis<sup>85</sup>, Engelmann's disease<sup>85</sup> and cystic fibrosis<sup>86</sup> could spontaneously

transform into neo-fibroblastic structures *in vitro*, that phenotypically looked very similar to the fibrocytes reported by Bucala *et al*<sup>87</sup>. Both osteomyelosclerosis and Engelmann's disease are characterised by excessive osteoblastic bone formation and they report that long term mononucleated blood cell cultures appeared to contain fibroblastoid cells embedded in a matrix surrounded by macrophages<sup>85</sup>. They then studied the spleens of these patients and found that after 50 days of culture large adherent macrophages gave rise to fibroblast-like cells that kept some macrophage markers for a time, such as LeuM3 and CD68, before losing them<sup>85</sup>. In the same year this group reported that monocytes isolated from blood from patients with cystic fibrosis also spontaneously transformed into neo-fibroblasts *in vitro*. In contrast to the findings of Bucala *et al* 3 years later they reported that "neo-fibroblasts" that developed from monocytes did stain positively for non specific esterase activity initially, but that this was no longer seen in more differentiated neo-fibroblasts. Immuno-fluorescence staining by this group showed that the fibroblast marker 5B5 was not only seen on their neo-fibroblasts that had differentiated from monocytes but was also seen on macrophages, indicating the potential of these macrophages to synthesize collagen. In the case of both papers they discuss the relevance of these cells in these disease states, all of which are characterised by fibrosis, in the bones of osteomyelosclerosis and Engelmann's disease; and in the pancreas, lungs and liver of cystic fibrosis patients. This group observed that these cells can develop from 'normal' patients however note that this was at a much lower rate than from their diseased patients<sup>86</sup>. This led them to suggest the hypothesis that under normal circumstances these cells are mainly resident in the bone marrow, but circulate within the blood during pathological situations<sup>85</sup>.

Both Yang and Hartlapp *et al* observed a positive correlation between the levels of serum transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and the number of fibrocytes seen in cultures of PBMC<sup>46;90</sup>. However whereas Hartlapp *et al* was using healthy controls Yang *et al* observed this in peripheral blood from burns patients. Work on the number of fibrocytes seen in burns patients concluded that the development of fibrocytes was up-regulated systemically as a higher number of cells differentiated from the peripheral blood of burns patients than from healthy controls<sup>46</sup>. As TGF- $\beta$ 1 is known to be important in tissue repair and fibrosis then its effects on fibrocytes are not surprising<sup>92</sup>.

### 1.5.3 Inhibition of fibrocyte generation

The mechanisms that inhibit the differentiation of fibrocytes from CD14<sup>+</sup> peripheral blood monocytes are of extreme importance, as unchecked fibrocyte differentiation could lead to an enhanced and sustained fibrosis at sites of inflammation. Pilling *et al* identified that serum amyloid P (SAP), found in serum, inhibits the differentiation of fibrocytes<sup>47</sup>. SAP is pentraxin protein which is produced by the liver and secreted into the blood where it circulates as a stable pentamer<sup>93;94</sup>. SAP appears to have an important role in the resolution phase of inflammation as it can cause opsonisation and engulfment of bacteria by binding to the surface sugar residues<sup>95</sup>. It can also bind free DNA and chromatin generated by apoptotic cells, preventing secondary immune responses<sup>96;97</sup>.

SAP can bind to all three classical Fc $\gamma$  receptors, Fc $\gamma$ RI (CD64), Fc $\gamma$ RII (CD32) and Fc $\gamma$ RIII (CD16), although it has a preference to Fc $\gamma$ RI and Fc $\gamma$ RII. This means that when SAP coats a bacteria it can then react with phagocytic cells, such as macrophages, causing opsonisation<sup>98;99</sup>. All three receptors also bind to the Fc portion of immunoglobulin G (IgG) and are found on the surface of a variety of haematopoietic cells. The most high affinity receptor is Fc $\gamma$ RI (CD64), it binds monomeric IgG and is found on peripheral blood monocytes<sup>100</sup>. Whereas both Fc $\gamma$ RII (CD32) and Fc $\gamma$ RIII (CD16) are low affinity receptors that will only bind aggregated IgG, Fc $\gamma$ RII is expressed on monocytes and B cells and Fc $\gamma$ RIII on natural killer cells and subpopulations of monocytes<sup>51;101;102</sup>.

The inhibition of fibrocyte differentiation by SAP was found to be due to the interaction of SAP with the Fc $\gamma$  receptors, signalling intracellularly through Syk and Sre related tyrosine kinases<sup>48</sup>. Aggregated or cross-linked IgG but not monomeric was also found to be able to inhibit fibrocyte differentiation through the same mechanism. Only the Fc portion of IgG could inhibit fibrocyte differentiation through the signalling of the Fc $\gamma$  receptors though, the F(ab')<sub>2</sub> fragments were found to have no effect<sup>48</sup>. These results suggest that monocytes can differentiate into fibrocytes in situations where SAP and aggregated IgG levels are low.

The potential importance of SAP in fibrocyte differentiation is illustrated in patients with scleroderma, a disease consisting of a pathological thickening and hardening of the skin caused by swelling and thickening of fibrous tissue. Normally men have much higher levels of SAP in

their serum than women and interestingly 80% of scleroderma patients are women. It was observed that sera from patients with scleroderma were less able to inhibit fibrocyte differentiation than that from healthy controls and that scleroderma patients also had lower levels of the SAP protein. Perhaps suggesting that changes in circulating levels of SAP may have an important role in fibrosis<sup>47</sup>.

## 1.6 Fibrocytes and Fibrotic diseases

Fibrocytes have been reported to express stromal markers such as fibronectin, collagen I and collagen III<sup>47;87</sup>, as well as extracellular matrix (ECM) components and ECM-modifying enzymes<sup>45;47;87;89</sup> therefore it is not surprising that their role in fibrotic diseases is under investigation. Fibrosis is the abnormal and excessive production of ECM proteins by fibroblasts or fibroblast-like cells, often accompanied by inflammation and so therefore can include an excessive of leukocytes, which can lead to the maintenance of high cytokine levels and the inhibition of ECM degrading enzymes. Fibrosis is also associated with repeated episodes of injury, creating positive-feedback loops, which increase ECM protein deposition and ultimately alters the tissue or organ physiologically, often inflicting functional constraints<sup>103</sup>.

Fibrotic tissue is often described as containing myofibroblasts; these are fibroblasts that have differentiated following tissue injury into contractile and secretory myofibroblasts. These myofibroblasts are important in wound healing but when contraction and ECM protein deposition becomes excessive they can severely damage tissue, such as in fibrotic diseases<sup>104</sup>.

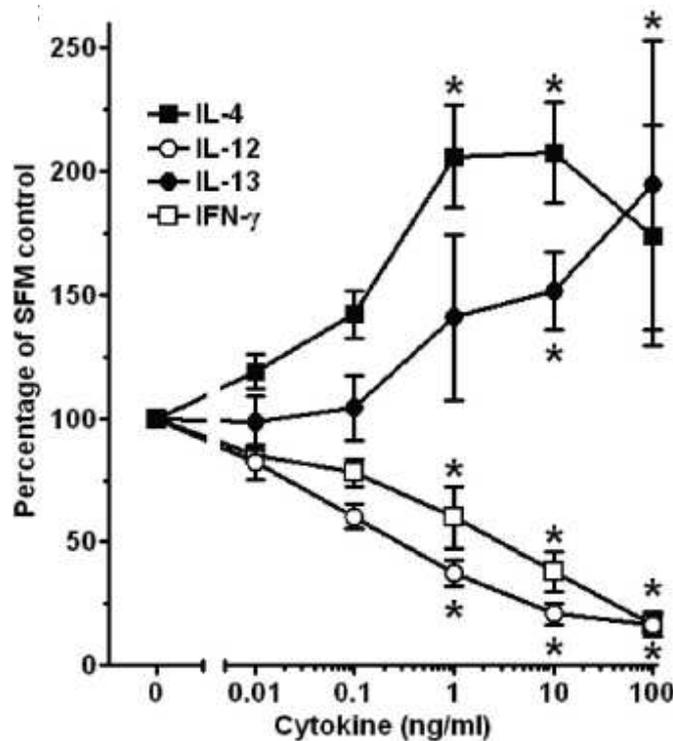
Stimulation of fibroblasts by transforming growth factor  $\beta$  (TGF- $\beta$ ) is thought to have an important role in producing these myofibroblasts<sup>105</sup> and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), a contractile protein incorporated into stress filaments, is often used as a marker. Once developed, myofibroblasts sustain a contractile force over a long period of time, which is generated by the tension exerted by stress fibres in the surrounding extracellular matrix<sup>106</sup>, contributing to the damaging effect they have on organs and tissues when there is an excessive of them in fibrosis.

### 1.6.1 Fibrocytes and Th-2 cytokines

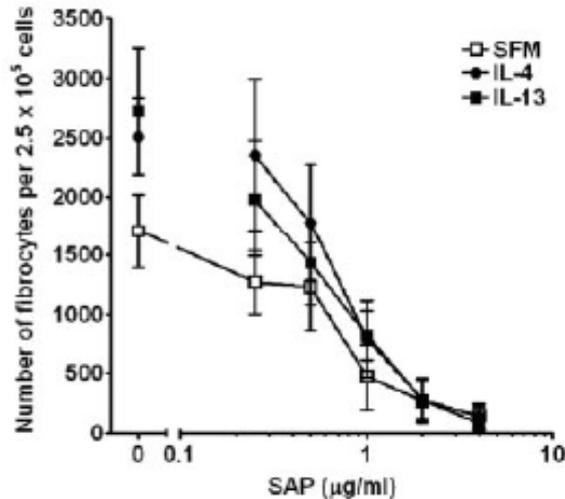
The pro-fibrotic cytokines involved in fibrosis are described as T<sub>H</sub>-2 cytokines, as they are known to be produced by the subset of CD4<sup>+</sup> T helper 2 (T<sub>H</sub>-2) cells. They include the cytokines IL-4 and IL-13 which stimulate collagen production and deposition by fibroblasts<sup>107</sup>, as well as promoting the activation and differentiation of fibroblasts into myofibroblasts<sup>108</sup>. They have also been associated with the involvement of the fibrotic inflammatory environment in a number of disease states, such as asthma, cardiac fibrosis, hepatic fibrosis, early rheumatoid arthritis, scleroderma and various lung fibrosing pathologies<sup>107;109-112</sup>.

As both fibrocytes and T<sub>H</sub>-2 cytokines are associated with fibrosis<sup>107;109;111</sup> the relationship between them was investigated by Shao *et al* in 2008 and found that the T<sub>H</sub>-2 cytokines IL-4 and IL-13 interacted directly with monocytes to promote fibrocyte differentiation<sup>103</sup>. Whereas the T<sub>H</sub>-1 cytokines, known to regulate T<sub>H</sub>-2 cytokines by exerting reciprocal inhibitory effects, such

as IL-12 and interferon- $\gamma$  (IFN- $\gamma$ ) acted in the opposite direction and prevented differentiation of fibrocytes<sup>103</sup> (Figure 1.4). The T<sub>H</sub>-1 and T<sub>H</sub>-2 cytokines were found to be able to counteract each other's effects, showing no change in the rate of fibrocyte differentiation compared to a media control. The addition of SAP however inhibited the effect of the T<sub>H</sub>-2 cytokines<sup>103</sup> (Figure 1.5).



**Figure 1.4 Effect of cytokines on fibrocyte differentiation.** Part of figure 1 from<sup>32</sup>. PBMCs at  $2.5 \times 10^5$  cells per ml were cultured in serum-free fibrocyte media for 5 days with the indicated concentrations of cytokines. Cells were then air-dried, fixed and stained and fibrocytes enumerated by morphology. As the number of fibrocytes differentiating from PBMCs varies between individuals, the effect of IL-4, IL-12, IL-13 and IFN- $\gamma$  on the differentiation of fibrocytes from different donors was analysed. For each donor, counts were normalized to the no-cytokine control to obtain a percent of control. Results are expressed as the mean  $\pm$  SEM of percent of control (n=6 donors). Statistical significance in comparison with the no-cytokine control was determined by ANOVA; \*,  $P < 0.05$ <sup>32</sup>.



**Figure 1.5 SAP inhibits IL-4 or IL-13 induced fibrocyte differentiation.** Part of figure 8 from<sup>32</sup>. PBMCs were cultured in serum-free fibrocyte media for 5 days with increasing concentrations of SAP in the presence or absence of 3ng/ml IL-4 or IL-13. Compared with PBMCs cultured with increasing concentrations of SAP alone, at SAP concentrations of 1µg/ml, IL-4 or IL-13 had no effect on the number of fibrocytes. Results are expressed as the mean  $\pm$ SEM of the number of fibrocytes per  $2.5 \times 10^5$  cells ( $n=6$  separate donors)<sup>32</sup>.

This group went on to suggest that in many chronic inflammatory conditions the presence of both the  $T_H$ -2 cytokines, IL-4 and IL-13 and the  $T_H$ -1 cytokines, IL-12 and IFN- $\gamma$  may promote fibrocyte differentiation, depending on the ratio between them possibly leading to fibrosis. However the presence of SAP in an inflammatory site may prevent this, although currently little is known about the activity or quantity of SAP in an inflammatory site it is promising as a potential therapy for fibrosis<sup>103</sup>.

## 1.6. 2 Treating animal models of fibrosis with SAP

As the plasma protein SAP has been suggested to have potential as a therapy for fibrosis animal models of fibrotic disease have been treated with it<sup>111-113</sup>. In a mouse model of ischemic

cardiomyopathy the presence of fibroblasts and fibrosis was reported to be markedly reduced following treatment with SAP<sup>111</sup>. What they termed 'blood-borne fibroblast precursors', expressing both collagen I and CD45, were found to be recruited to the heart, by CCL2, when a myocardial infarction was induced. This murine model of closed-chest myocardial infarction and reperfusion allowed for the examination of chemokine induction after the dissipation of surgical trauma<sup>114</sup>. When SAP was administered to mice at the time of myocardial infarction induction these 'blood-borne fibroblast precursors' were not recruited and fibrosis was markedly reduced, although CCL2 was still released and inflammation still occurred<sup>111</sup>. Further work found that the mechanism that SAP uses to reduce this fibrosis is again due to its interaction with FCγ receptors<sup>113</sup>.

As well as *in vivo* mouse work this group also looked at the migratory capacity of fibrocytes in the presence of SAP. Previously they have shown that monocytes are attracted to the site of injury by MCP-1 (CCL2). They set up an *in-vitro* trans-endothelial migration study using transwells with a layer of human umbilical vein endothelial cells (HUVECs) on the filter. PBMC were plated out for 3-4 days before being allowed to migrate through the HUVECs in response MCP-1, following this fibrocytes differentiated in the bottom chamber within a few days. Interestingly if SAP was added to the top of the transwell at the beginning monocytes migrated but fibrocyte differentiation did not occur in the bottom, however if SAP was put in the bottom of the transwell fibrocyte differentiation was seen following monocyte migration<sup>113</sup>. This implied that although preventing fibrocyte differentiation SAP does not prevent migration of the

monocytes. However, either something to do with the act of migration, or even developmental status of cells meant that the addition of SAP later, i.e. in the bottom of the transwell does not have the same effect<sup>113</sup>. These observations provide further evidence of the importance of fibrocytes in fibrosis and this paper goes on to suggest that monocytes are required to migrate through endothelial cells to be able to mature into fibrocytes rapidly<sup>113</sup>.

Fibrocytes have also been reported to be the precursors for bronchial myofibroblasts in asthma<sup>115</sup>. Fibroblast-like cells, having a similar surface antigen expression profile as reported by Bucala *et al* in fibrocytes, were detected in the bronchial mucosa of patients with allergic asthma. Their numbers were seen to increase during an asthmatic reaction induced by allergen exposure, however there was no direct evidence that these cells seen in the bronchial mucosa were recruited from the peripheral blood<sup>115</sup>. In 2004 however, Phillips *et al* published that a population of CD45<sup>+</sup> collagen I<sup>+</sup> fibrocytes migrate in response to CXCL12 and traffic to the lungs in a murine model of bleomycin-induced pulmonary fibrosis<sup>116</sup>. They also saw that the increasing level of collagen deposition in the lungs correlated with the recruitment of these cells and therefore hypothesised that circulating fibrocytes contribute to the pathogenesis of pulmonary fibrosis<sup>116</sup>.

Bleomycin was actually developed as a chemotherapeutic drug for testicular cancer, however a side effect of it in ~10% of patients is lung fibrosis<sup>117</sup>. The treatment of bleomycin in rodents has become the most widely used model of lung fibrosis<sup>118</sup>. It causes an accumulation of leukocytes,

especially macrophages, producing inflammation, and activates fibroblasts and fibroblast-like cells to produce collagen, events similar to those seen in human lung fibrosis<sup>118-120</sup>.

In 2007 Pilling *et al* reported that bleomycin treated rats and mice treated with SAP had a decreased level of lung fibrosis compared to the untreated<sup>112</sup>. In rats the inflammation was reduced with less leukocytes, fibrocytes and activated fibrocytes and peripheral blood oxygen content was maintained<sup>112</sup>. The ability of SAP to reduce bleomycin-induced pulmonary fibrosis suggests that fibrosis is maintained by fibrocytes, as their differentiation has been shown to be inhibited by the presence of SAP<sup>47</sup>. However it may also be acting by reducing the numbers of mature fibrocytes as well as their precursors. The noted reduction in leukocyte recruitment following SAP treatment may also be linked to an inhibition of fibrocytes as they are known to release cytokines that promote leukocyte migration<sup>89</sup>, less fibrocytes, means less recruitment. Of course the effect of SAP could be completely unrelated to fibrocytes, SAP binds apoptotic material, the presence of it may have caused a rapid removal of the apoptotic material by the resident macrophages, so reducing leukocyte recruitment and reducing fibrosis<sup>112;121</sup>. As the literature does suggest the role of fibrocytes in pulmonary fibrosis<sup>115;116;122</sup>, and SAP is known to inhibit fibrocytes differentiation both *in vitro*<sup>47</sup> and *in vivo*<sup>111</sup> it has reasonable to assume that SAP is reducing fibrosis by acting on fibrocytes in this model rather than the rapid removal of apoptotic material, although this may also be taking place at the same time.

### 1.6.3 Mechanisms by which fibrocytes populate peripheral organs

Since 2004 when Philips *et al* published that fibrocytes migrated through the peripheral blood in response to CXCL12<sup>116</sup> a number of groups have shown, in mouse models, that this is not the primary mechanism that fibrocytes populate the peripheral organs<sup>49;111;123</sup>. The *in vivo* differentiation of fibrocytes, from circulating precursors, occurs mainly at the tissue sites and not in the peripheral blood. As discussed already Haudek *et al* saw a reduction in fibrocyte number and reduced fibrosis in a mouse model of ischemic cardiomyopathy when SAP was given as a treatment and suggested that SAP was interfering with the development of fibrocytes from the monocyte precursors taken up during the inflammatory response<sup>111</sup>, (as is seen *in vitro*<sup>47</sup>), rather than affecting fibrocyte recruitment to the site.

Frid *et al* used neonatal animal models of hypoxia induced pulmonary vascular remodelling to look at the role of fibrocytes in chronic pulmonary hypertension<sup>123</sup>. Circulating monocytes of chronically hypoxic rats were selectively labelled *in vivo* and were later identified in the remodelled layer of the pulmonary arteries expressing the marker CD45 and producing collagen, suggesting these cells differentiated in the layer from the monocyte precursors taken up from the circulation<sup>123</sup>, rather than themselves being recruited from the circulation.

Thirdly Varoce *et al* looked at which circulating cells were involved in the vascular remodelling in an ovine model of carotid artery intimal hyperplasia, induced by vascular graft implantation. Circulating leukocytes were labelled and a subpopulation of them infiltrated the intima *in vivo*

to then go on to take on a fibrocyte phenotype, of CD45<sup>+</sup> and Vimentin<sup>+49</sup>. Again suggesting fibrocyte differentiation was at the site of injury rather than these cells being recruited.

In a pulmonary model of fibrosis, induced by intratracheal instillation of fluorescein isothiocyanate (FITC), minced lung explants and bronchoalveolar lavage (BAL) cultures saw the emergence of fibrocytes after 10 to 14 days in a serum-free culture medium<sup>124</sup>, however whether these are due to differentiation or were already present is unknown. However these fibrocytes were lost when the same experiment was performed on CCR2<sup>-/-</sup> mice. CCR2<sup>-/-</sup> have previously been shown to be protected from fibrosis induced by FITC injury, despite inflammatory cells still being recruited<sup>125</sup>. Bone marrow from CCR2<sup>+/+</sup> mice transplanted into lethally irradiated CCR2<sup>-/-</sup> mice restored the level of fibrocytes seen within the lung explants and BAL cultures. These results suggest a pivotal role of CCR2 in accumulation of fibrocytes in sites of fibrosis<sup>124</sup>. The observations can be transferred to humans as well as the recruitment of monocytes is also dependent on the chemokine CCR2<sup>40</sup>.

Continuing work by Moore et al has reported that one of CCR2s ligands, CCL12, has an important role in fibrosis in regards to fibrocytes<sup>126</sup>. Neutralising CCL12 by antibody treatment protected mice from FITC induced pulmonary fibrosis, whereas neutralising the other CCR2 ligands such as CCL2 and CCL7 was less effective. They suggest therefore that CCL12 is the ligand involved in fibrocyte accumulation at sites of fibrosis<sup>126</sup>. Interestingly the human homolog for

murine CCL12 is actually human CCL2, which has been shown to be important in human systemic sclerosis<sup>127</sup>, cystic fibrosis<sup>128</sup> and cardiac fibrosis<sup>129</sup>.

In a mouse model of renal fibrosis, where unilateral ureteral obstruction was performed, a considerable number of fibrocytes expressing both CD45 and collagen I were seen in the interstitium, these fibrocytes were also positive for CCR7<sup>130</sup>. The blockade of CCR7's ligand CCL21 by anti-CCL21 antibodies reduced renal fibrosis, which was confirmed in CCR7<sup>-/-</sup> mice<sup>130</sup>.

Although evidence now suggests that fibrocytes differentiate at the site of inflammation<sup>49;111;123</sup> in the tissue, rather than be traffic there from the circulatory system, Phillips *et al's* observation of the importance of the interaction of the chemokine CXCL12 and its receptor CXCR4 in the role of fibrocytes in bleomycin induced model of lung fibrosis is extremely important. One of the subpopulations of monocytes thought to be able to differentiate into fibrocytes, identified by CD14<sup>+</sup> CD16<sup>-</sup>, expresses both CXCR4 and CCR7<sup>50</sup>, both of which have both been implicated in the accumulation of fibrocytes in mouse models of fibrotic diseases<sup>116;130</sup> and so may be involved in their recruitment into sites which then go on to include fibrocytes. .

#### **1.6.4 Fibrocytes and Human fibrosis**

In the last decade the role of fibrocytes in fibrosis has been heavily reported on. Fibrocytes have been discussed as being involved in the pathogenesis of human lung fibrosis<sup>115;116;124;126;131;132</sup>, as well as renal fibrosis<sup>130;133</sup> and liver fibrosis<sup>134;135</sup>. They have also been suggested as having a

role in the fibrosis seen in systemic sclerosis<sup>136</sup> and in the fibrocellular membranes seen in proliferative vitreoretinopathy in the eyes<sup>137</sup>.

In 2007 Mehrad *et al* reported on fibrocytes in human fibrotic interstitial lung disease<sup>131</sup>. The role of CXCL12 expression on fibrocytes associated with mouse lung fibrosis was suggested by Phillips *et al*<sup>116</sup> in 2004 and Mehrad took this further by looking at the level of the CXCL12 chemokine in lung tissue from patients suffering from fibrotic usual interstitial pneumonia (UIP) or non-specific interstitial pneumonia (NSIP). They found that it significantly higher in UIP and NSIP patients than healthy controls. They described circulating fibrocytes as Collagen I<sup>+</sup>, CD45<sup>+</sup> cells in peripheral blood and found that UIP and NSIP had significantly more of these than healthy controls as well as significantly more circulatory CXCL12<sup>131</sup>. They concluded from this the importance of fibrocytes in human lung fibrosis and suggested the possibility that the mechanism surrounding this chemokine and fibrocytes could be used in some way as a therapeutic target for treatment of these diseases.

There is a growing literature linking fibrocytes, or haematopoietic derived fibroblast-like cells, to fibrosis, and what was once a field dominated by the mesenchymal derived fibroblasts is accepting that the role of inflammatory cells and fibrocytes may be much more important than first thought. In fact a systemic fibrotic disease characterised by the thickening or hardening of the skin of the extremities in people with significant renal insufficiency is thought to be mediated by fibrocytes<sup>138</sup>. Nephrogenic systemic fibrosis or NFS was first reported in 1997,

although is only ever seen in patients who suffer from renal dysfunction<sup>139</sup>, it is not related to the severity of the renal dysfunction, whether the patient has received a transplant or been on dialysis; and neither the cause or duration of the underlying renal disease is directly related to the severity of NFS<sup>138</sup>. They define the fibrocytes in this work by a spindle shaped morphology and the co-expression of CD34 and procollagen I. Skin biopsies from patients suffering from NFS contained cells of this description<sup>140</sup> which have not previously been described in the skin<sup>141</sup>. Other factors suggest a systemic factor being the cause, such as the symmetry of the lesions, the rapid development of the lesions, indicating recruitment from the circulatory system rather than mitosis; the presence of the fibrosis in otherwise unaffected organs and an active case of NSF resembles a wound healing site, with resolved cases of NSF being indistinguishable from a healed wound<sup>138;140</sup>. The cause of NSF is still unclear and further investigation into what links renal dysfunction to the onset of this disease is required, however the fact that it appears to be a systemic disease due to the role of fibrocyte; and the subsequent treatments found to help have implications in the treatment of all fibrotic diseases<sup>138</sup>.

## **1.7 Fibrocytes and wound healing**

Wounds result from an abrupt, physical disruption of the normal architecture of tissue; they can be due to trauma, burns or inflammatory processes and require immediate attention by the body's immune system to prevent infection and tissue invasion; and to restore normal tissue integrity. Inflammatory cells and stromal cells such as myofibroblasts and fibrocytes are needed

to ensure correct wound healing; it is when this act of wound healing becomes excessive that fibrosis occurs.

When fibrocytes were termed by Bucala *et al* in 1994 it was whilst looking at a wound healing model in mice, as they wanted to study the acute cellular responses of wound repair<sup>87</sup>. The mouse model they used involved wound chambers, short lengths of sponge filled, silastic tubing, implanted surgically in the subcutaneous tissues of a mouse's back. Following implantation wound fluid was aspirated periodically and a massive influx of inflammatory cells was seen within 24hrs, consisting of neutrophils, monocytes and lymphocyte populations. When these chambers were eventually removed, and looked at under light microscopy they observed a large number of adherent, spindle shaped cells that were fibroblast-like. Although it was possible that it could be infiltrating fibroblasts from the surrounding area they considered it too much of a coincidence that a large number of peripheral blood cells had entered the wound chamber immediately before the emergence of these cells spindle shaped cells<sup>87</sup>. They went on to do *in-vitro* analysis of cultured peripheral blood cells identified CD45<sup>+</sup> collagen I<sup>+</sup> cells that they termed fibrocytes.

More recently the role fibrocytes in wound healing has been researched in other mouse models and has provided evidence that fibrocytes contribute to the myofibroblast population seen in wounded skin; and that they originate from the bone marrow<sup>142;143</sup>. Myofibroblasts are vital in the healing process and secrete extra cellular matrix proteins involved in tissue repair as well as

$\alpha$ -SMA, a contractile protein. In 2005 Mori *et al* identified a substantial number of the  $\alpha$ -SMA positive cells, in the skin of wounded mice, that also expressed the surface markers CD13 and CD45, suggesting they had differentiated from a haematopoietic origin and not from mesenchymal-derived tissue fibroblasts<sup>142</sup>. This group also isolated cells of a fibrocyte phenotype at different time points from the wounded tissue and found that these cells became  $\alpha$ -SMA between 4 and 7 days post-wounding, indicating fibrocytes were differentiating into myofibroblasts at the tissue site. When looking at this differentiation *in vitro* this group observed that myofibroblast differentiation from fibrocytes was accelerated by the addition of TGF- $\beta_1$ <sup>142</sup>, which is known to be involved in the differentiation of fibroblasts into myofibroblasts<sup>105</sup>.

Due to the observed role of fibrocytes in sites of wound healing<sup>45;46;87;89;124;144</sup> conditions of poor wound healing have been investigated to determine whether an inability to support fibrocyte differentiation or the inhibition of fibrocyte differentiation was responsible. Diabetic patients have poor wound healing abilities therefore PBMC were cultured with increasing amounts of glucose and 10 $\mu$ g/ml of insulin or increasing amounts of insulin, in a serum-free environment. In the absence of either glucose or insulin it was observed that all the cells died, and no fibrocytes were seen. At low levels of glucose (0.004-0.025g/L) fibrocyte differentiation was poor but occurred, at intermediate levels (0.1-2g/L) the same level of fibrocyte differentiation was seen as previously observed<sup>47</sup>; and with high levels of glucose (greater than 8g/L) an increased number of fibrocytes were seen. The effect of glucose on fibrocytes was further increased with

the addition of pyruvate and increasing levels of insulin<sup>138</sup>. Therefore whether the body can produce fibrocytes and to what extent could be highly important in their wound healing capacity.

### 1.7.1 Fibrocytes and burn injury

When an individual suffers from extensive burns hypertrophic scars and keloids represent two different forms of aberrant wound healing that are often seen<sup>145</sup>. Collagen fibrils arranged in a nodular structure are seen in hypertrophic scars, and keloids contain thick collagen fibres, made up of numerous fibrils packed together<sup>145</sup>. An inflammatory infiltrate is seen in both forms of scarring, however is more pronounced in the hypertrophic form and is composed of T cells, monocytes and dendritic-like cells. The nodules of the hypertrophic scars also have enhanced expression of TGF- $\beta_1$  as well as  $\alpha$ -SMA positive myofibroblasts<sup>145</sup>.

In 2002 Yang *et al* reported that burns patients were more efficient at producing fibrocytes than healthy controls<sup>46</sup>, they also found a positive correlation between levels of serum TGF- $\beta_1$  and the percentage fibrocytes developed in those patients<sup>46</sup>. This group later went on to identify spindle shaped cells co-expressing leukocyte specific protein-1 (LSP-1; known to be on fibrocytes<sup>144</sup>) and pro-collagen I in post burn hypertrophic scars suggesting that fibrocytes are involved post-burn hypertrophic scars.

In 2006 Wang *et al* looked at the effect fibrocytes have on fibroblasts in burns patients<sup>146</sup>. They reported that conditioned medium from fibrocytes could up-regulate the production of collagen from fibroblasts, induce proliferation and cause differentiation from dermal fibroblasts to myofibroblasts. They also report that the degree in which the fibrocyte can do this is directly correlated to the size of the patient's burn. However they do not see this ability in fibrocytes from healthy controls<sup>146</sup>.

## 1.8 Fibrocytes as mesenchymal stromal cells

There is some literature which refers to cells with fibroblast-like morphology that have been derived from CD14<sup>+</sup> monocytes and have the capacity to differentiate into several mesenchymal type cell lineages. Kuwama *et al* reported these cells in 2003 and referred to them as 'monocyte derived mesenchymal progenitors' (MOMPs)<sup>147</sup>. They cultured CD14<sup>+</sup> cells from peripheral blood for 7-10 days then collected these cells for use in various assays where they reportedly differentiated osteoblasts, skeletal myoblasts, chondrocytes and adipocytes by varying the type of growth medium<sup>147</sup>. They verified these cell types by genes and protein expressions<sup>147</sup>. In the same year Zhao *et al* reported a subset of cells that act as a pluripotent stem cells derived from peripheral blood monocytes<sup>148</sup>. Phenotypically these cells represented fibroblasts but express the haematopoietic markers CD14, CD34 and CD45. By the addition of various cytokines and growth factors they induced various cells types to differentiate from these cells *in vitro*, T lymphocytes, epithelial, neuronal, endothelial and hepatocytes. They verified these cell types by immunostaining and, in the case of the T lymphocytes, a cytotoxicity assay<sup>148</sup>.

Although the 'stem' cells described above appear to have the same expression profile and morphological characteristics as fibrocytes the literature as a whole does not agree that tissue-resident mesenchymal stem cell/multipotent mesenchymal stromal cells and fibrocytes are the same cell<sup>149;150</sup>. Although there is a lot of confusion in the literature over the nature of these tissue-resident mesenchymal stem cell/multipotent mesenchymal stromal cells, as the terminology to describe them has not been standardised, it is agreed they are not the same as fibrocytes. Two of the defining surface markers of fibrocytes are the expression of CD34 and CD45<sup>47;87</sup>, as these markers depict a haematopoietic origin and separates them from fibroblasts. One of the criteria's for these tissue-resident mesenchymal stem cell/multipotent mesenchymal stromal cells which is agreed on is that they do not express CD34 or CD45<sup>145;149;150</sup>. In culture they also have different morphological characteristics, although both spindle shaped and fibroblast-like fibrocytes are described as having a slightly plump body, with two elongated thin ends, whereas the tissue-resident mesenchymal stem cell/multipotent mesenchymal stromal cells are described as much more stretched out in shape, pale in colour and do not have sharp ends.

## 1.9 Overview of fibroblasts

Fibroblasts are a type of stromal cell that are found ubiquitously throughout the body, providing mechanical strength to tissues by supporting the extra cellular matrix<sup>151</sup>. They are identified by their morphology, ability to adhere to plastic, production of extra cellular matrix and lack of

epithelial, vascular and leukocyte lineage markers<sup>152</sup>. Although initially thought to have a mainly structural role throughout the body, in the synthesis and remodelling of extra cellular matrix, they have more recently been reported as being involved in maintaining the homeostasis of adjacent cell types, such as epithelial and endothelial cells, by both producing and responding to growth factors<sup>153</sup>. Fibroblasts have also found to produce cytokines and chemokines, playing a key role in the development of an immune response<sup>152</sup>; as well as being implicated in the development and duration of an inflammatory response, and the resolution<sup>154;155</sup>. Fibroblasts were thought to be the same cell throughout the body, however they have been found to be phenotypically different from one tissue type to another, and it has been suggested that they are not a homogeneous population even within single tissues, but exist as subsets of cells, in a similar way that macrophages and dendritic cells do<sup>156</sup>. In 2002 Chang *et al* reported that when analysing the transcriptome of human fibroblasts from different anatomical locations within the body samples from the same anatomical location clustered more closely together, in hierarchical clustering analysis, than samples from the same individual, suggesting that fibroblasts from different locations in the body should be considered distinct differentiated cell types<sup>157</sup>.

Fibroblasts are believed to originate from three distinct origins, primary mesenchyme, local epithelial-mesenchymal transition (EMT) and bone marrow derived precursors<sup>84</sup>. Whilst mesenchymal cells are believed to be the major contributor of tissue fibroblasts the EMT hypothesis is described as a way that mesenchymal derived epithelial cells are reshaped into

fibroblasts following epithelial stress, such as inflammation or tissue injury<sup>152</sup>. It has also been suggested that bone marrow derived circulating precursors can develop into fibroblasts, namely the fibrocyte<sup>84;111;116;158;159</sup>, however whether fibrocytes become fibroblasts, or are just similar morphologically is still an issue for debate.

## 1.10 Transcriptome analysis

With the exception of certain cells of haematopoietic origin, every cell in the body has exactly the same DNA within its nucleus. The factors that make cells develop down different differentiation paths is the way that this DNA is transcribed and translated into proteins; so a cell can be described as the sum of all the genes it expresses, which is collectively termed the transcriptome. Which mRNA species are transcribed from the DNA is modulated by DNA-binding regulatory proteins, for example the mRNA transcribed to cause a neuronal cell to develop will be very different to that transcribed in the development of, for example, a hepatocyte.

Transcriptome analysis or gene expression profiling has been applied to various malignant and immune disorders such as leukaemia, lymphoma, systemic lupus erythematosus and rheumatoid arthritis, as well as many others<sup>160-164</sup>. This approach has helped to identify important diagnostic and prognostic markers as well as potential therapeutic targets. Gene expression profiling has also been used to look at the relationships between subsets of peripheral blood cells to establish, or confirm potential lineages<sup>165-169</sup>. Work reported in 2005 by two separate groups looked at different subsets of T lymphocytes, naïve, central and effector

memory; and both came to very similar conclusions of their lineage relationships by using cluster analysis<sup>165;166</sup>.

## 1.11 Justification of this project

Much of the early literature on fibrocytes refers to these cells as circulating cells of the peripheral blood<sup>45;46;87-91;115;116;142;170</sup>, however in the methods section of these reports they culture PBMC in medium, wash off the non-adherent cells after 1 or 2 days then wait for 7-10 days to see fibrocytes. As fibrocytes have been reported to differentiate from CD14<sup>+</sup> cells<sup>45;47-49;85;86</sup> it is more logical that, like some subtypes of macrophages for example, these cells differentiate from monocytes at the sites they are needed. This has been supported by work in mouse models of fibrosis<sup>49;111;123</sup>, which were discussed earlier.

There is also a large discrepancy running through all the fibrocyte literature, in some reports *in vitro* derived fibrocytes are generated in a serum-free media and in others a serum-containing media. Although marker expression between the cells generated in the two media types are the same<sup>47;87;145</sup> there are some differences seen. Serum-free derived fibrocytes express high levels of prolyl 4-hydroxylase, the enzyme required for the synthesis of new collagen<sup>48</sup>, whereas those generated in serum-containing media are reported as having low levels of prolyl 4-hydroxylase activity in comparison to mature fibroblasts<sup>146</sup>. Also SAP, a plasma protein found in serum, inhibits fibrocyte differentiation in serum-free media *in vitro*<sup>47</sup> and *in vivo*<sup>111</sup>. SAP is present in the serum of the serum-containing fibrocyte media, although fibrocytes in this media take

longer to differentiate than serum-free fibrocytes they do differentiate, therefore the SAP is having no or very little effect on their differentiation. Therefore the question arises, are these fibrocytes seen to differentiate in serum-free and serum-containing media the same cell? Or are they two different, although clearly related cell types, despite having the same surface marker express profile and morphology.

A large portion of the literature describes fibrocytes as differentiating from monocytes. Monocytes found in peripheral blood are actually made up of 3 subpopulations, identified in humans by their expression of the surface markers CD14 and CD16. The subpopulation  $CD14^+CD16^-$  constitutes approximately 80-90% of monocytes with the remaining 10-20% made up of  $CD14^+CD16^+$  and  $CD14^{lo}CD16^+$  populations. Another monocyte derived cell type, osteoclasts, has been reported to only differentiate from the  $CD16^-$  fraction of monocytes<sup>44</sup> ( $CD14^+CD16^-$ ) and these observations lead us to suggest that the differences seen between the two types of fibrocytes in the literature may be due to them differentiating from different subpopulations of monocytes, therefore the differential potential of different subsets of monocytes into fibrocytes could be very interesting.

To define the connection between these two types of fibrocytes seen in the literature understanding what defines a fibrocyte is vital. As the identity of a cell can be defined as the sum of the genes it expresses then comparing the transcriptome of these cells to each other by transcriptome analysis may provide the clearest answer to this question. Transcriptome analysis

also allows fibrocytes to be compared to other monocyte-derived progeny, such as macrophages, and mesenchymal derived fibroblasts, allowing for speculation on the lineage relationships between both the types of fibrocytes, and these other cell types.

As both serum-free and serum-containing derived fibrocytes are morphologically and phenotypically very similar, if not almost identical, then if they are different cell types based on functional characteristics and transcriptome analysis their roles within the body will still be highly related. Due to the surface marker expression of these cells their roles in wound healing will both be important, however as they prefer different environments they could potentially be involved at different time points of the healing process. We could hypothesis that the serum-containing derived fibrocytes are involved at the beginning of inflammatory response, helping to initiate the wound healing response and the serum-free fibrocytes near the end when the environment becomes more suitable for them.

## 1.12 Aims of this project

- To determine the ideal procedure for quantifying fibrocyte differentiation.
- To optimise culture conditions for differentiating fibrocytes in both serum-free and serum-containing fibrocyte medium, and determine whether that the cells used in this thesis have the same marker expression as those described in the literature.
- To determine which monocyte subpopulation/s fibrocytes differentiate from and whether serum-free and serum-containing fibrocytes differentiate from different subpopulations.
- To compare serum-free and serum-containing generated fibrocytes by both *in vitro* and transcriptional protocols to determine if they are the same cell generated by two different methods or two different, although related, cell types that potentially could be involved in different time points of a wound healing response.
- To compare serum-free and serum-containing fibrocytes with *in vitro* derived donor matched macrophages and fibroblast cell lines to determine potential lineage relationships.

## 2.0 METHODS AND MATERIALS

### 2.1 Abbreviations

avi	Audio video interleave
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
cDNA	Complementary de-oxy ribonucleic acid
CO <sub>2</sub>	Carbon dioxide
DC	Dendritic cell
DiOC6	Dihexaoxacarbocyanine iodide
DMEM	Dulbecco's modified essential medium
DNA	De-oxy ribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleotide triphosphate
dTTP	Deoxythymidine triphosphate
DTT	Dithio threitol
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorter
FCS	Foetal calf serum

GAL	Generic array logic
GEPAS	Gene expression pattern analysis suite
GPS	L-glutamine, benzylpenicillin, streptomycin
HI-FCS	heat-inactivated foetal calf serum
IL	Interleukin
IMDM	Iscove's modified Dulbecco's Medium
ITS+3	insulin, transferrin and selenite
MACS	Magnet assisted cell selection
NEAA	Non-essential amino acids
P.A.L.M.	Photoactivated localization microscope
PAM	Prediction analysis of microarrays
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI 1640	Roswell Park Memorial Institute medium 1640
SAM	statistical analysis of microarrays
SAP	Serum amyloid P
SOP	Sodium Pyruvate

WinMDI      Windows Multiple Document Interface Flow Cytometry Application program

w/v          Weight/volume

## 2.2 Cell preparation

Fibrocyte cell culture was carried out using one of two medium recipes. Serum-free fibrocyte medium consisted of Rosswell Park Memorial Institute medium 1640 (RPMI 1640; Sigma), supplemented with 1% GPS (1.64mM L-glutamine, 40U/ml benzylpenicillin, 0.4mg/ml streptomycin; Sigma), 1% HEPES buffer (Sigma), 1% ITS+3 liquid media supplement (containing insulin, transferrin, sodium selenite, linoleic acid, oleic acid and bovine serum albumin; Sigma) 1% non-essential amino acids (NEAA; Sigma) and 1% sodium pyruvate (SOP; Sigma)<sup>47</sup>. The serum-containing fibrocyte medium consisted of Dulbecco's modified eagle medium (DMEM; Sigma) supplemented with 1% GPS and 20% foetal calf serum (FCS; Biosera)<sup>87</sup>. Macrophage medium consisted of Iscove's modified Dulbecco's medium (IMDM; Sigma) 1% GPS, 10% human serum (Biosera) and 50ng/ml macrophage-colony stimulating factor (M-CSF; Peprotech). Non-sterile solutions were filtered through 0.22µm filters and the majority of tissue culture plastics, tubes and filters were purchased from Sarstedt Ltd. 24-well plates used were purchased from BD biosciences, as were the glass chamber slides.

## 2.3 Differentiating fibrocytes

To differentiate fibrocytes from PBMC or CD14<sup>+</sup> selected cells they were resuspended at the required density in either serum-free or serum-containing fibrocyte medium and plated out into either 8-well glass chamber slides (500µl), 24-well (2mls) or 96-well (200µl) plastic plates. Differentiation took approximately 3-7 days in the serum-free fibrocyte medium or 10-14 days

in the serum-containing fibrocyte medium. At day 4 the non-adherent cells were washed out of PBMC cultures and the medium replaced.

## **2.4 Differentiating Macrophages**

To differentiate macrophages from PBMC they were resuspended at  $1 \times 10^6$  cell/ml, the required density in macrophage medium, and plated out into either 8 well glass chamber slides (500 $\mu$ l), 24 well (2mls) or 96 well (200 $\mu$ l) plastic plates and macrophages left to differentiate. At day 4 the non-adherent cells were washed out and the media replaced. Differentiation took approximately 7 days.

## **2.5 Viable cell counts using a haemocytometer**

Cell suspensions were diluted 1:1 with 0.4% trypan blue (Sigma) and incubated for 1 minute at room temperature. 8 $\mu$ l was then inserted beneath a coverslip on an improved Neubauer counting chamber (haemocytometer) (Weber Scientific, UK Materials), the number of cells were counted within a 5x5 square of the counting chamber and multiplied by two for the trypan blue dilution, then by  $10^4$  to provide an estimate of cells per ml. Multiple squares were counted to provide an average count. Dead cells were stained blue by the trypan blue and therefore excluded from the count.

## 2.6 Cell counts using an eye piece micrometer

An eye piece micrometer (Olympus) consisting of a grid, 10mm by 10mm, with 1mm graduations, was used to count fibrocytes and macrophages on the microscope (Olympus CX2). When counting cell cultures at least three separate fields of view were counted. To calibrate the eyepiece micrometer a stage micrometer was purchased, 1mm long with 0.01mm divisions (Pyser-SGI Limited). Once the eye piece micrometer had been calibrated the total number of cells in each population could be estimated from the total number of cells counted. For each objective on the microscope the multiplication factor required to estimate the total number of fibrocytes present was calculated.

At 10x magnification the area of 100 squares of the micrometer was  $1.1025\text{mm}^2$ , the area of a glass well was  $70\text{mm}^2$  therefore  $70/1.1025 = 63.492$ , the factor by which the average cell number from the 3 fields of view was multiplied by to estimate the total cell number. For 20x magnification the area of 100 squares of the micrometer was  $0.2756\text{mm}^2$ , the area of a glass well was  $70\text{mm}^2$  therefore  $70/0.2756 = 253.97$ .

All graphs to do with fibrocyte counting data throughout the thesis were made using GraphPad Prism version 3.00 software. When statistical analysis was performed on fibrocyte counting data the analysis package GraphPad Prism, version 3.00, was also used.

## 2.7 Separation of peripheral blood mononuclear cells

Peripheral blood was donated by healthy controls, following informed consent, heparinised and diluted 1:1 with RPMI 1% GPS, 1% HEPES. Peripheral blood mononuclear cells (PBMC) were then separated from the blood using ficoll density centrifugation (Ficoll-Paque™ Plus, Amersham Biosciences). 15ml of diluted peripheral blood was layered over 8mls of ficoll and centrifuged at 225g for 30 minutes at 20°C. The PBMC were then removed using a Pasteur pipette and washed five times in RPMI 1% GPS, 1% HEPES, centrifuging at 300g for 8mins. The heparin used during sample collection was preservative free and obtained from CD Pharmaceutical Ltd. All cell culture was performed in category 2 tissue culture flow cabinets.

## 2.8 CD14<sup>+</sup> selection using Magnetic beads

PBMC were isolated as specified above and suspended at 80µl per 10<sup>7</sup> cells in MACS buffer (phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA; Sigma)) and 2mM EDTA, (pH 7.2, Sigma). 20µl of CD14<sup>+</sup> MACS beads (Milteny Biotec) per 10<sup>7</sup> PBMC were added and left at 4°C for 15 minutes. Unattached beads were washed away by the addition of 10mls of MACS buffer and centrifugation at 300g for 8 minutes, twice. After choosing the relevant separation column, dependent on cell number, this was primed with MACS buffer on a magnet before the cell suspension added to the top in a volume dependent on the size of the column. The column was then washed three times with MACS buffer and the CD14<sup>-</sup> cells collected. The column was removed from the magnet and washed again with MACS buffer to

collect the CD14<sup>+</sup> fraction of cells. The CD14<sup>+</sup> cells were counted using a haemocytometer and spun down to be resuspended in the required medium. A sample of cells was removed to be stained with antibodies and so that the purity of the CD14<sup>+</sup> selection could be checked using flow cytometry.

## **2.9 Flow cytometry to check CD14<sup>+</sup> purity**

Analysis of the purity of CD14<sup>+</sup> cell selection of PBMC was carried out on a Beckman Coulter Epics XL bench-top flow cytometer (Beckman Coulter). The cytometer calibration was standardised using Flow-set fluorospheres (Beckman Coulter) and data was analysed using the Windows Multiple Document Interface Flow Cytometry Application program (WinMDI Version 2.8). For analysis, a live gate was set around viable cells based on their forward scatter versus side scatter profile.

## **2.10 Cell Surface epitope staining for flow cytometry**

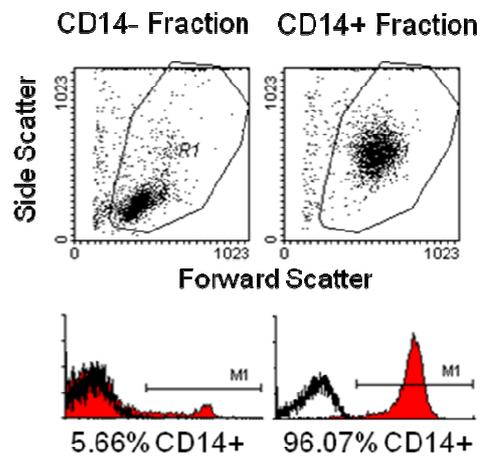
To check the purity of the CD14<sup>+</sup> microbead selection staining was performed on  $1 \times 10^5$  cells, or as many as could be spared from the CD14<sup>+</sup> and CD14<sup>-</sup> cell fractions. Each sample was put in a 96-well flexi plate and spun at 4°C for 5min at 300g before being resuspended in 50µl of an antibody mix made up using PBS 2% BSA. Protein level matched irrelevant isotype control samples were also prepared. Samples were left for 20 minutes at 4°C before 200µl of PBS 2% BSA was added and the plate centrifuged at 300g for 5min at 4°C and resuspended in 100µl PBS

2% BSA. Antibody stained samples were stored at 4°C until analysis on the flow cytometer. List of antibodies used is shown in table 2.1.

Antibody Name	Conjugated to	Dilution	Manufacturer
Anti-human CD14	FITC	1/50	Immunotools
Anti-mouse IgG1	FITC	1/50	Immunotools

**Table 2.1 List of antibodies used for flow cytometry.** Antibodies used to test the purity of a CD14<sup>+</sup> cell selection using magnetic microbeads.

When determining the purity of the CD14<sup>+</sup> population a live cell gate was drawn on a dot plot of forward scatter versus side scatter and the percentage of cells CD14<sup>+</sup> determined. The CD14<sup>+</sup> cell fraction indicates the purity of the selected sample. Figure 2.1 is an example of the analysis of a purity check with the CD14<sup>+</sup> cell fraction being 96% CD14<sup>+</sup>, and the CD14<sup>-</sup> cell fraction being 5.66% CD14<sup>+</sup>. Therefore, in the case of this selection the purification was quite high and some CD14<sup>+</sup> cells have not been selected by the beads.



**Figure 2.1 Purity check of a CD14<sup>+</sup> selection using MACS beads.** The live cells are gated on using forward versus side scatter and the percentage of cells that are CD14<sup>+</sup> looked at using single colour histograms. The isotype control is indicated with the open histograms and the CD14<sup>+</sup> staining by the closed red histograms.

## 2.11 Growing Fibroblast cell lines

The fibroblast cell lines used were previously set up within the group by Dr Andrew Filer and originated from samples of tissue removed from an affected rheumatoid arthritis joint with informed consent from the patient. Complete fibroblast medium was prepared, RPMI 1640, supplemented with 1% GPS, 10% FCS, 1% NEAA and 1% SOP. Aliquots of frozen fibroblasts were bought up from liquid nitrogen storage at -150°C and defrosted quickly by placing in a 37°C water bath, defrosted cells were then added to 24mls of warmed complete fibroblast medium and centrifuged at 300g for 8 minutes. Cells were washed twice more before being counted, on a haemocytometer, using trypan blue exclusion dye and, depending on the number of viable cells, resuspended in an appropriate volume of complete fibroblast medium before being put into a suitable vessel. The cells were plated out at high densities to ensure that the fibroblasts

could to support each other as they went through the lag phase of growth from storage in liquid nitrogen. The cells were left to adhere overnight and the medium refreshed the following day to remove dead cells.

When feeding the fibroblast cultures the conditioned medium was collected and 66% of the final volume of medium replaced with fresh complete fibroblast medium. The collected medium was centrifuged at 300g for 5 minutes to remove cell debris and added back to the cell cultures as conditioned fibroblast medium to make the volume up to 100%.

When fibroblast cultures were 100% confluent, or the cells required for use in experiments, the cells were removed from the plate with 2x trypsin EDTA (Sigma) prepared from 10x stock solution using sterile PBS. The conditioned fibroblast medium was removed from the cells, and once centrifuged mixed with fresh complete fibroblast medium in a ratio of 1:2. The cells were washed in sterile PBS, as the FCS in the medium would inactivate the trypsin EDTA, enough 2x trypsin EDTA was added to cover the monolayer of cells and they were incubated at 37°C for 5 minutes or less. Detachment of the cells was checked microscopically and complete fibroblast medium added to the cultures to inactivate the trypsin EDTA. The cells were centrifuged at 300g for 8 minutes and the supernatant discarded twice before being resuspended in an appropriate volume of 33% fibroblast conditioned medium and 66% complete fibroblast medium and transferred to an appropriate vessel for use.

## **2.12 Photographing fibrocytes and macrophages**

Cultures of differentiated fibrocytes were photographed on day 11, and differentiated macrophages on day 7. The microscope used was a Zeiss Axiovert 200 with a Hamamatsu digital camera (C4742-95), the images were captured by Simple PCI version 5.0 computer software.

## **2.13 Testing different detachment solutions on fibrocytes**

Fibrocytes were differentiated in 24-well plates from PBMC in serum-free fibrocyte medium and the efficiency of various detachment agents tested. In preparation the fibrocyte medium was removed and every well washed in PBS. Photographs of each well were taken pre-treatment and the number of fibrocytes calculated by taking the mean fibrocyte number, based on three fields of view of each well. The PBS was removed and the various detachment agents added and incubated at either 37°C or 4°C, dependent on treatment. After 40 minutes of treatment, with the wells being checked approximately every 10 minutes, 1ml of RPMI/ 1%GPS/ 1% HEPES was added to each well to deactivate the agents. The detachment agent plus media were removed and replaced with PBS to allow post-treatment photographs to be taken and the number of fibrocyte to be again counted using an eye piece graticle.

Trypsin EDTA was prepared as in protocol in section 2.11 however was used at both 2x and 10x (stock) concentration. The detachment agent Accutase (PAA) was also used on a separate well,

500µl was added and the well incubated at 37°C for 40 minutes. Finally three concentrations of EDTA were tested, 0.5M, 0.05M and 0.005M EDTA (Sigma). The EDTA was diluted in PBS to the required concentration and 500µl added of each concentration to three separate wells. The wells were treated and incubated at 4°C for 40 minutes. The percentage fibrocytes detached was calculated by dividing the number of fibrocytes pre-treatment by the number of fibrocytes in the same well post-treatment, and multiplying by one hundred.

## **2.14 Use of Puramatrix Hydrogel™ in tissue culture**

Puramatrix™ hydrogel consists of standard amino acids (1% w/v) and 99% water and under physiological conditions the peptide component self assembles into a 3D hydrogel with a fibrous structure. To create the 3D hydrogel the stock Puramatrix™ was sonicated for 30 minutes and diluted with sterile water to the concentrations required. When using it for the first time a titration of concentrations was recommended to determine which was the optimal for the cells that were being cultured. Initially a titration between 1 and 0% w/v (1, 0.75, 0.5, 0.25 and 0% w/v) was used, but in later experiments it was determined that these concentrations were too high so the titration was scaled down to between 0.25 and 0% w/v (0.25, 0.2, 0.15, 0.1, 0.05 and 0% w/v). The Puramatrix™ hydrogel was set up in 24 well plates, 250µl of each concentration was added to a different well and 500µl RPMI 1%GPS 1%HEPES carefully layered over the top. The plate was then kept at room temperature for an hour and the medium carefully replaced with fresh. Every 30 minutes for 90 minutes the medium was replaced and the plate kept at room temperature. The purpose of layering the medium over the gel was to exchange the water

used to make up the gel with the medium by osmosis, therefore the more washes the gel had the less water remained within the makeup of it. Fibroblasts were used to test the system and after 90 minutes were layered over the gel in 1.5mls of the appropriate medium and left to culture at 37°C with 5% CO<sub>2</sub>. Two control wells were included, one where the well went through the same procedure as those with hydrogel in i.e. 0% w/v, and one where fibroblasts were just plated out following no treatment.

To dissolve the gel and so remove the adherent cells the media was removed from the cells and Dispase (BD Bioscience) added, 10 units/per cm<sup>2</sup>, which, in the case of a 24-well plate was 200µl. The dispase was left to work for 2hrs at 37°C then the whole mixture pipetted to disperse the cell suspension and break up any remaining hydrogel. The action of the dispase was stopped by chelation of Ca<sup>++</sup> and Mg<sup>++</sup> using 5-10mM EDTA and the cell suspension washed several times at low speed before analysis. Dispase was also used on the well that contained no hydrogel to detach the fibroblasts.

### **2.15 Identification of live cells with DiOC6 using flow cytometry**

Staining was performed on fibroblasts detached by dispase when testing the Puramatrix™ hydrogel. Fibroblasts recently detached from the hydrogel or control wells were transferred to a flexi plate and spun for 3 minutes at 4°C at 300g before the supernatant was flicked off and 100µl PBS 2% BSA added. 100µl of DiOC6 was also added at a working concentration of 2nM. The cells were incubated in the DiOC6 for 15 minutes at 37°C before the wells were filled with

PBS 2% BSA and the flexi plate spun for 3 minutes at 4°C at 300g. The supernatant was then flicked off and the cells resuspended and washed again for 3 minutes at 4°C at 300g, before being finally resuspended in 100µl of PBS 2% BSA. Prior to running on the flow cytometer the stained cells were added to 300µl PBS. Analysis of fibroblasts detached by dispase when testing the Puramatrix™ hydrogel was carried out on a Beckman Coulter Epics XL bench-top flow cytometer (Beckman Coulter). The cytometer calibration was standardised using Flow-set fluorospheres (Beckman Coulter) and data was analysed using the WinMDI Version 2.8 package. DiOC6 was identified by the 488 laser on the flow cytometer; it is a mitochondrial membrane dye which will only bind to live cells with intact mitochondrial membrane. Therefore when analysing this data live cells were gated on using DiOC6 positivity.

## **2.16 Cell surface epitope staining on slides**

Cultures of fibroblasts, serum-free fibrocytes, serum-containing fibrocytes and macrophages were set up in 8-well chamber slides, 12 wells for each cell type. Fibrocytes were taken at day 11, macrophages at day 7 and fibroblasts at the point they were approximately 60% confluent. For staining of these cultures all medium was removed from each chamber slide and, using the tool provided, the top chambers removed, to leave just the glass slide with the adherent cells attached. The slide was then washed three times in PBS for 5 minutes before being left to dry and fixed in -20°C acetone (Sigma) for 15 minutes. At this point slides were stored at -20°C until staining.

Slides were removed from freezer and allowed to warm up to room temperature. Using a hydrophobic pen the wells were drawn around to prevent any cross contamination of antibodies. The cells were rehydrated in PBS/2% BSA for 5 minutes and blocking was performed with 10% serum (type dependent on secondary antibody to be used on that well) to prevent non-specific staining of the secondary antibody, and left for 20 minutes. The serum was removed and the slides gently washed in PBS with a stirring bar. Once excess liquid had been removed 50µl of the primary antibody mixes were added to the centre of each well, diluted in PBS/2% BSA. The slides were left overnight in a humidified incubator. The following day the antibodies were removed and the slides washed twice in PBS with a gentle stirring bar. Again, once excess liquid had been removed, the secondary antibody mix was added, diluted in PBS/2% BSA, and left for one hour in a humidified incubator. After an hour the secondary antibodies were removed and the slides washed twice more, for 10 minutes, in PBS with a stirring bar. The outside of the wells was dried as much as possible and the slides dipped in the nuclear counter-stain Hoechst (20µg/ml; Bis-benzimid H33258 Fluorochrome) for two minutes and rinsed once more in PBS to remove any remaining counter-stain. The slides were dried and to retard fading slides were mounted in 2.4% 1,4-Diazabicyclo[2.2.2]octane (DABCO; Aldrich) in glycerol (Fisher Scientific) pH 8.6. The slides were then stored at -20°C until analysed.

The antibody stained slides were analysed on the Zeiss Axiovert 100M confocal microscope, and images collected using the Zeiss laser scanning microscope LSM 5.10, version 3.2 SP2, computer program. A well stained with an irrelevant matched isotype control was completed for every

antibody type and when analysing the slides the irrelevant's were used to set the photo-multipliers, so that the irrelevant appeared negative. Using these settings any staining seen with the matched antibodies was specific for that antibody and not unspecific. A list of the primary antibodies used is shown in table 2.2, and the secondary antibodies in table 2.3.

Antibody	Amount of antibody added	Dilution used	Manufacturer	Blocking serum	Secondary antibody used
Rabbit $\alpha$ human collagen I	50ng	1/100	Rockland (600-401-103-0.1)	Donkey	Donkey $\alpha$ rabbit IgG Cy5
Rabbit $\alpha$ human collagen III	200ng	1/25	Rockland (600-401-105-0.1)	Donkey	Donkey $\alpha$ rabbit IgG Cy5
Rabbit $\alpha$ human fibronectin		1/100	Sigma (F3648)	Donkey	Donkey $\alpha$ rabbit IgG Cy5
Rabbit Immunoglobulin fraction	200ng	1/4000	Dako (X0903)	Donkey	Donkey $\alpha$ rabbit IgG Cy5
Mouse $\alpha$ human CD68 (IgG2b)	1 $\mu$ g	1/25	BD Pharmingen (556059)	Goat	Goat $\alpha$ mouse IgG2b Alexa Fluor 633
Negative Control IgG2b	1 $\mu$ g	1/10	Dako (X0944)	Goat	Goat $\alpha$ mouse IgG2b Alexa Fluor 633
Mouse $\alpha$ human CD13 (IgG1)	2 $\mu$ g	1/25	Immunotools (21270131)	Goat	Goat $\alpha$ mouse IgG1 Alexa Fluor 633
Mouse $\alpha$ human CD3 (IgG1)	750ng	1/20	Dako (M0835)	Goat	Goat $\alpha$ mouse IgG1 Alexa Fluor 633
Mouse $\alpha$ human CD45 (IgG1)	2 $\mu$ g	1/25	Immunotools (21270451)	Goat	Goat $\alpha$ mouse IgG1 Alexa Fluor 633
Negative Control IgG1	2 $\mu$ g	1/5	Dako (X0931)	Goat	Goat $\alpha$ mouse IgG1 Alexa Fluor 633
Mouse $\alpha$ human Vimentin (IgG1)	2 $\mu$ g	1/100	Dako (M725)	Goat	Goat $\alpha$ mouse IgG1 F(ab) FITC
Negative Control IgG1	2 $\mu$ g	1/5	Dako (X0931)	Goat	Goat $\alpha$ mouse IgG1 F(ab) FITC

**Table 2.2 List of primary antibodies used in immunostaining.** The primary antibodies used in immunostaining are listed, along with the amount used to stain, the dilution the antibody was used at, its manufacture, which serum was used to block and which conjugated secondary antibody was used.

Secondary antibodies	Dilution used	Manufacturer
Donkey $\alpha$ rabbit IgG Cy5	1/125	Jackson (711176152)
Goat $\alpha$ mouse IgG2b Alexa Flur 633	1/100	Molecular probes A-(21146)
Goat $\alpha$ mouse IgG1 Alexa Flur 633	1/100	Molecular probes A-(21126)
Goat $\alpha$ mouse IgG1 F(ab) FITC	1/100	Southern Biotec (1032-02)

**Table 2.3 List of secondary antibodies used in immunostaining.** The secondary antibodies used in immunostaining are listed, along with the dilution the antibody was used at and its manufacture.

## 2.17 Time-lapse photography of fibrocytes and macrophages

Serum-free fibrocytes, differentiated from PBMC, and serum-containing fibrocytes, differentiated from CD14<sup>+</sup> selected cells were cultured in 8-well class chamber slides and used at day 11. The microscope used for time-lapse photography was the Zeiss Axiovert 200 with a Hamamatsu digital camera (C4742-95). The chamber slides were placed in a temporary incubator which attaches to a heated stage that fitted to the microscope. The CO<sub>2</sub> supply was set at 5% CO<sub>2</sub>, and the heated stage set at 37°C. To get the clearest time-lapse images the lid of the cultures was removed from the chamber slides, to prevent condensation, and sterile mineral oil layered over the media (all done in category 2 tissue culture hoods), which prevented evaporation. The computer program Simple PCI, version 5.0, controlled the microscope, and was set to take photographs of the culture every five minutes for at least 6 hours. The images were saved in a datafile database which could then be used to create avi video files. The video files were set to run at 15 frames/second and each frame had a time stamp added to it, indicating the time from start.

The videos were then analysed on Image pro-Plus, version 4.5, where various readings could be taken. The package analysed the video frame by frame, and individual cells could be drawn around and the centre of the shape found. The centre of the shape was depicted as an XY coordinate of the frame, this cell was then drawn around in the next frame and the new XY coordinate recorded, using Pythagoras' theorem the distance the cell had moved over time could be calculated. This process was repeated every three frames (so 15 minute intervals on the video) and using this, the average speed of each cell calculated. Ten cells were analysed for each video so that the average cell speed in each video could be found. As well as the speed of movement other readings of each cell could also be taken, once the cell has been drawn around the analysis package also provided the length and area of the shape. The length of the shape reading was used when looking at the effect serum had on serum-free fibrocytes.

## **2.18 Cell sorting of monocyte populations to culture in different media**

Fluorescence-activated cell sorting (FACS) was performed using a Mo-Flo MultiLaser flow cytometer (Dako Cytomation) operated by Roger Bird. Cell sorting involved labelling cells with antibodies conjugated to different fluorochromes. Labelled cells can then be ran through a cell sorter and, as a single cell stream, pass through the beam of a blue argon laser, the laser excites the attached fluorochromes and the fluorochromes emits light within specific wave lengths, dependent on the fluorochrome, detectors identify cells expressing these fluorochromes by the wavelength of the emitted light. Based on the combinations of fluorochromes they have bound to their surface by antibodies cells can be identified and subsequently collected. On this

particular machine up to four different populations, expressing different combinations of fluorochromes, could be collected at one time, and any cells not expressing these combinations of fluorochromes were discarded.

To prepare cells for cell sorting approximately  $100 \times 10^6$  PBMC were isolated from 100mls of peripheral blood. Three wells of a 96-well plate were set up at  $1 \times 10^6$  cells/ml in serum-free, serum-containing fibrocyte medium and macrophage medium as a PBMC control for culturing later. Of the remaining cells one aliquot of  $2 \times 10^6$  cells and four aliquots of  $1 \times 10^6$  cells were taken and stored at  $4^\circ\text{C}$  in 1.5ml microfuge tubes. Finally the remaining cells were labelled with antibodies against the surface markers CD14, CD16 and CD56, for antibody information see table 2.4. The antibody cocktails were made up in PBS/2%BSA. The large sample of cells was spun down at 300g for 8mins and resuspended in 1ml of antibody cocktail in a 1.5ml microfuge tube. The aliquot of  $2 \times 10^6$  PBMC was resuspended in 800 $\mu\text{l}$  PBS/2% BSA and stored on ice as an unlabelled cell control. One of the smaller aliquots of  $1 \times 10^6$  cells was labelled with matched irrelevant controls for each antibody, and the other three set up to compensate the cell sorter, all in 1.5ml microfuge tubes. Although the large population of cells were labelled with CD14/CD16/CD56 all three compensation tubes were made up using CD14 conjugated antibodies. Labelling with this marker provided a relatively large, bright population that gave plenty of events to compensate. All antibody/cell combinations were left for 30 minutes before an excess of PBS/2%BSA was added to fill the tube. The tubes were spun down 500g for 5 minutes, then resuspended in PBS/2% BSA and spun again. The irrelevant and compensation

tubes were resuspended in 100µl PBS/2% BSA and filtered through separate 50µm filters (cup-type, DakoCytomation) that had been pre-wet with 100µl of PBS/2% BSA. 200µl of unlabelled cells were also added to each of these filters to have labelled and unlabelled monocytes in the same sample. The filters were then washed twice with 100µl PBS/2% BSA to give a final volume of approximately 600µl. The cells labelled with all three markers were resuspended in 1ml PBS/2%BSA, and also filtered through a 50µm filter. The filter had been pre-wet with 200µl of PBS/2%BSA and following the cells was washed 3 times with 200µl PBS/2% BSA to give a final volume of 1.8mls. All cells were filtered into polypropylene tubes with lids (Becton Dickinson) and stored on ice until sorting.

The computer program used to control the sorting was Summit v4.3 from Dako. When sorting the PBMC a forward/side scatter gate was drawn around monocytes, to exclude nonviable cells and lymphocytes; using a dot-plot of CD14 verses CD56 the CD56<sup>-</sup> fraction of cells was also gated to exclude neutrophils. Both the forward/side scatter gate and CD56<sup>-</sup> gate were applied to a dot-plot of CD14 verses CD16 and the populations CD14<sup>+</sup>CD16<sup>-</sup>CD56<sup>-</sup>, CD14<sup>+</sup>CD16<sup>+</sup> CD56<sup>-</sup> and CD14<sup>lo</sup> CD16<sup>+</sup> CD56<sup>-</sup> were gated on so sorting of these populations could be performed.

Tube	Antibodies	Manufacturer	Dilution
Irrelevant	Mouse IgG1 Control-FITC	Immunotools (21335013)	1/100
	Mouse IgG1 Control-PE	Immunotools (21335014)	1/100
	Mouse IgG2a Control-PE-Cy5	Immunotools (21335025)	1/100
FITC compensation	Mouse $\alpha$ human CD14-FITC	Immunotools (21270143)	1/20
PE Compensation	Mouse $\alpha$ human CD14-PE	Immunotools (21270144)	1/200
PE-Cy5 Compensation	Mouse $\alpha$ human CD14-PE-Cy5	Immunotools (21330145)	1/100
Cells to be sorted	Mouse $\alpha$ human CD16-FITC (IgG1)	Immunotools (21279163)	1/100
	Mouse $\alpha$ human CD56-PE (IgG1)	Dako (R7251)	1/50
	Mouse $\alpha$ human CD14-PE-Cy5 (IgG2a)	Immunotools (21330145)	1/100

**Table 2.4 List of antibodies used in cell sorting of monocyte populations.** For cell sorting of monocyte populations the tubes listed above were prepared with the indicated antibodies. The dilution the antibodies were used at and the manufacturer of the antibody is also listed. The fluorochromes the antibodies are conjugated to are also shown, fluorescein isothiocyanate (FITC), phycoerythrin (PE) and phycoerythrin linked to Cy5 (PE-Cy5).

The purity of the sorted populations was checked by running a small number of sorted cells back through the machine. Depending on the number of cells collected in each population, the cells were plated out at  $2 \times 10^5$  cells/ml in serum-free fibrocyte, serum-containing fibrocyte and macrophage medium. At day 7 the number of macrophages in the macrophage medium was counted, based on a fried egg morphology. At day 11 the number of fibrocytes were counted in serum-free, serum-containing and macrophage medium.

## **2.19 Microarray analysis of fibrocytes, macrophages and fibroblasts**

### **2.19.1 Sample collection**

A large number of serum-free, serum-containing fibrocytes, macrophages and fibroblasts were required for transcriptome analysis using microarrays. To collect serum-free fibrocytes approximately fifty million PBMC were isolated, as previously described, and plated out in 24 well plates at  $1 \times 10^6$  cell/ml, 2ml per well, in serum-free fibrocyte media. At day 4 the non-adherent cells were washed out and the media replaced. To collect serum-containing fibrocytes approximately eighty million PBMC were isolated, and the CD14<sup>+</sup> cells selected using magnetic bead selection, these were then plated out in serum-containing fibrocyte medium at  $2 \times 10^5$  cell/ml in 24 well plates, 2ml per well. At day eleven fibrocytes differentiated in both media types were harvested. The media was washed out of each well and replaced with 1ml sterile PBS. The number of wells set up was divided into three equal sets and the number of fibrocytes calculated from three wells of each set. The PBS in each well was then removed and 700 $\mu$ l of lysis buffer (RNeasy mini kit, Qiagen) used to lyse the cells in the wells in each set. For each fibrocyte culture three aliquots of lysed cells were produced and stored at -70°C for RNA extraction later.

To collect macrophages for the transcriptome analysis approximately fifty million PBMC were isolated and plated out in 24-well plates at  $1 \times 10^6$  cell/ml, 2ml per well, in macrophage media. At day 4 the non-adherent cells were washed out and the media replaced; and at day seven the macrophages were harvested. As with the fibrocytes, on the day of harvesting the media was

washed out of each well and replaced with 1ml sterile PBS. The number of wells set up was divided into three equal sets and the number of macrophages calculated from three wells of each set. The PBS in each well was removed and 700µl of lysis buffer (RNeasy mini kit, Qiagen) used to lyse the cells in the wells in each set. For each macrophage culture there were three aliquots of lysed cells produced and stored at -70°C for RNA extraction later.

To collect fibroblasts for transcriptome analysis a 75mm<sup>2</sup> culture flask of fibroblasts was allowed to become 100% confluent, the cells detached using trypsin EDTA, as described earlier, and counted using a haemocytometer. The harvested fibroblasts were split into three equal aliquots, spun down and resuspended in 350µl lysis buffer (RNeasy mini kit, Qiagen), then stored at -70°C for RNA extraction later.

As well as three aliquots of each culture giving technical replication, three separate cultures were set up for each cell type, giving biological replication. The biological replicates of serum-free, serum-containing fibrocytes and macrophages were donor matched, so the same three donors were used for each *in vitro* derived cell. The fibroblasts used were established cell lines, so three different cell lines were used, but not donor matched to the *in-vitro* derived cells. Overall 36 samples of lysed cells were prepared, nine for each cell type, consisting of 3 biological replicates of 3 technical replicates.

### **2.19.2 Microarray preparation**

The microarrays used were custom printed spotted microarrays, containing oligos supplied by Operon. The 70-mer oligos consisted of 37,000 genes from the Human Genome Array Ready Oligo set (AROS) version 4.0 and, until use, were stored in a lyophilized form at  $-80^{\circ}\text{C}$ . Prior to use they were resuspended at a concentration of  $40\mu\text{M}$  in 3x SSC (for recipe of SSC see section 2.19.8) and left to resuspend overnight at  $4^{\circ}\text{C}$ . The resuspended oligos were then spotted onto Ultra-GAPS coated slides following the protocol supplied using a Microgrid TAS II arrayer (BioRobotics) at 70% humidity. After spotting the arrays were stabilised and immobilised as per their instructions using a UV cross-linker. The arrays were then stored in a desiccated chamber at  $-80^{\circ}\text{C}$ . The microarrays were prepared by Liverpool Microarray Facility, Professor Andy Cossins.

### **2.19.3 RNA Extraction for microarray analysis**

Cell sample aliquots were defrosted at room temperature and the RNA extracted using the RNeasy mini kit from Qiagen as per manufacturer's instructions. Following extraction the RNA was treated to remove any contaminating DNA (DNase I kit, Ambion). The RNA was then stored at  $-20^{\circ}\text{C}$  for future use.

### **2.19.4 Reverse Transcription PCR**

For each sample two reverse transcription reactions were set up, consisting of  $16\mu\text{l}$  RNA and  $1\mu\text{l}$  oligo(dT)<sub>12-18</sub> primer (Invitrogen). Each reaction was heated to  $65^{\circ}\text{C}$  for 10 minutes before being cooled to  $4^{\circ}\text{C}$  for the next reagents to be added. A further reaction mix was prepared for each

sample, 8 $\mu$ l of first strand buffer, 4 $\mu$ l 0.1M DTT and 2 $\mu$ l Superscript II (200U/ $\mu$ l) (all from Superscript II RNase H Reverse Transcriptase kit, Invitrogen). Plus 2 $\mu$ l dATP (10mM), 2 $\mu$ l dCTP (10mM), 2 $\mu$ l dGTP (10mM) and 2 $\mu$ l dTTP (10mM) dNTPs (made up from 100mM PCR Grade dNTP Set; Invitrogen), and 1 $\mu$ l de-ionised water, the samples were then kept at 50°C for 2 hours. The newly formed cDNA was purified using the Nucleospin extraction kit (Macherey-nagel), as per manufacturer's instructions, and the eluted cDNA stored at -20°C.

#### **2.19.5 Quantification of amount of cDNA in each sample**

To ensure that approximately the same amount of cDNA was hybridised to each microarray slide from each sample a quantitative real-time L27 gene PCR reaction was prepared. By calculating from the initial cell number lysed each real-time PCR reaction was prepared using the equivalent of 1000 cells worth of cDNA for each sample, and each reaction was set up in triplicate. The human reference sample (see next section for review) was used as a positive control for the reaction but also set up as a standard curve. The stock solution of the human reference sample was 106ng/ $\mu$ l, dilutions of 1, 1/2, 1/20, 1/50 and 1/100 were prepared and also run in triplicate. Each reaction mix consisted of 8.5 $\mu$ l of template, with de-ionised water used to make up the volume if required, 0.5 $\mu$ l of each of the forward, reverse primers and labelled probe, and 10 $\mu$ l of the real-time PCR mastermix (Applied Biosystem Taqman 2x PCR mastermix; Roche). Reactions were made up in a 96-well plate (Applied Biosystems), with six wells prepared as negative controls. The plate was loaded into the Stratagene MX3000P real-time PCR machine, the program the samples were tested on was 50°C for 2 minutes, 95°C for 10

minutes, then 40 cycles of 95°C for 15 sec and 60°C for 1 minute. Following the reactions the results were analysed using the MxPro, Mx3005P version 3.20 software and using the relative amounts of samples compared to the standard curve readings calculated how much cDNA each sample contained.

The primer and probe set for human L27 was designed using Primer Express version 1.0 (ABI).

The primers and probe sequences were as follows –

L27 sense: GCAAGAAGAAGATCGCCAAG

L27 antisense: AAACCGCAGTTTCTGGAAGA

TaqMan probe for L27: CCCTTGGACAAAACACTGTCGT (VIC labelled).

The TaqMan probe for L27 was VIC labelled and specificity for the target genes was checked by a BLAST search against Genbank sequences.

### **2.19.6 Dye Labelling**

Before hybridising the isolated cDNA to the microarray slides it first needed to be labelled with Cy3-dCTP. To do this 1µg of cDNA, calculated for each sample from the L27 quantitative real-time PCR and 20µl of random primer mix (Bioprime Labelling Kit, Invitrogen) were made up to 43µl with deionised H<sub>2</sub>O. The human reference sample (Universal reference RNA; Stratgene) was also labelled as a positive control of both hybridisation, and of the slides by acting as an internal control in the microarray analysis, to show any inter-slide variation. The human reference sample was composed of the total RNA from ten different human cell lines, including adenocarcinoma (mammary gland), hepatoblastoma (liver), adenocarcinoma (cervix), histiocytic

lymphoma (macrophage; histocyte), embryonal carcinoma (testis), lymphoblastic leukaemia (T lymphoblast), glioblastoma (brain) and plasmacytoma (myeloma; B lymphocyte). It was produced in RNA form and was reverse transcribed as described above then stored in aliquots at -20°C at a stock concentration of 106ng/μl. For every cell sample to be labelled with Cy3-dCTP a reaction mix was also prepared for a reference sample to be labelled with Cy5-dCTP, using 530ng of cDNA. Each reference/cell sample mix was heated to 95°C for 5 minutes before being cooled to 4°C. The following reagents were then added to each reaction, 1.2μl dATP (10mM), 0.72μl dCTP (10mM), 1.2μl dGTP (10mM), 1.2μl dTTP (10mM) (all made up from 100mM dNTP Set PCR Grade, Invitrogen), 1μl 40U/μl Klenow enzyme (Bioprime Labelling Kit, Invitrogen), 1μl of either Cy3-dCTP (cell samples) or Cy5-dCTP (reference sample) dye (Amersham Biosciences), made up to 7μl with deionised H<sub>2</sub>O. The reaction was kept at 37°C for 8 hours and the labelled cDNA purified with the nucleospin extraction kit and eluted in 70μl of elution buffer.

### 2.19.7 Probe Quantification

The labelled cDNA was measured for pmols of labelled product by use of a spectrophotometer (Pharmacia Biotech Ultrospec 2100 pro) at 550nm (Cy3-dCTP) or 650nm (Cy5-dCTP). The concentration of probe was then calculated using the equation below

$$\frac{\text{Absorbance}}{0.15(\text{Cy3}) \text{ or } 0.25 (\text{Cy5})}$$

The labelled cDNA was then stored in the absence of light at 4°C for hybridisation to be performed later on the same day.

Aliquots of 100pmols of labelled cDNA for cell and reference samples were concentrated together using microcon concentration columns (Millipore) as per manufacturer's instructions, eluting into 30µl H<sub>2</sub>O.

### **2.19.8 Probe hybridisation**

Each probe (reference and cell sample together) was prepared for hybridisation by being denatured in a hybridisation solution consisting of 20µl formamide (Sigma), 20µl 20x SSC, 0.4µl 20% SDS, 5µg PolyA DNA (Invitrogen), 8µg Cot1 DNA (Invitrogen) made up to 50µl with H<sub>2</sub>O for 5 minutes at 95°C, then kept at 4°C until use.

- *20x SSC- Dissolve 175.3g sodium chloride (NaCl; Fisher scientific), 88.2g sodium citrate (Fisher scientific) in 800mL water, pH adjusted to 7.0 with 10N sodium hydroxide (NaOH) and volume adjusted to 1L with water.*
- *10% SDS (Sodium dodecyl sulphate)- 100g electrophoresis grade SDS in 900ml water, heat to 68°C to assist in dissolution, pH adjusted to 7.2 by adding concentrated hydrochloric acid (HCL) and volume adjusted to 1L.*

### **2.19.9 Preparation of microarray slides**

Before hybridisation microarray slides had to be treated with both pre-soak solution and pre-hybridisation solution. Pre-soak solution (Pronto!<sup>TM</sup>, microarray hybridisation kit; Corning) was prepared as per manufacturer's instructions and the required number of slides incubated at

42°C for 20 minutes. Slides were washed three times in water and placed in pre-hybridisation solution at 42°C for 2 hours. Pre-hybridisation solution was made up of 12.5ml formamide (Sigma), 12.5ml 20x SSC, 0.25ml 20% SDS and 50µl 100mg/µl BSA (Sigma), made up to 50ml with water.

After 2 hours the slides were washed in water and spun dry at 1500rpm for 15 minutes in a centrifuge at room temperature. Once dry the slides were added to a hybridisation chambers (Corning) and the preparation instructions for the chamber followed. The labelled probe mix was then carefully applied to the surface of the slide and a Lifter Slip (VWR) slotted on the top. The hybridisation chamber was sealed, wrapped in a damp paper towel and tinfoil and left for 16-20 hours in a hybridisation oven at 42°C.

#### **2.19.10 Post hybridisation washes**

Slides were removed from the hybridisation chambers and the cover-slips removed by being immersed in 2x SSC, 0.1% SDS at 42°C. The slides were washed using a AdvaWash machine, version 3.4 from Advantix. The machine used 3 buffers, buffer 1, kept at 42°C, 2 x SSC + 0.1% SDS; buffer 2, at room temperature, 0.2 x SSC and buffer 3, at room temperature, 0.05 x SSC. The machine washed the slides for 10 minutes in wash buffer 1, 10 minutes in wash buffer 2, 15 minutes in wash buffer 3, and two lots of wash buffer 3 for 4 minutes. Finally the slides were spun dry at 1500rpm for 15 minutes in a centrifuge at room temperature.

### **2.19.11 Slide scanning**

Microarray slides were scanned using a Scanarray GX+ scanner and analysed using Scanarray express version 3.03.0002 using variable PMT voltages. A prepared GAL file was used to annotate the slide images and the intensity data was extracted and exported as gpr files using the same software. These were uploaded into the Gene Expression Pattern Analysis Suite (GEPAS) (Bioinformatics Unit CNIO; <http://gepas.bioinfo.cnio.es>) software for normalization, which was performed using print-tip loess normalisation with background subtraction, initially within each slide and subsequently between slides.

### **2.19.12 Analysis of microarrays**

For microarray analysis, normalised data was exported as a tab delimited file to the software application TIGR Multiexperiment Viewer (TMEV), version 2.0 (free for Academic Institutions at <http://www.tigr.org/tdb/euk>)<sup>171</sup> and this was used to perform exploratory analysis (e.g. hierarchical clustering, and Principal component analysis (PCA)) as well as to identify differentially expressed genes (statistical analysis of microarrays (SAM)).

Hierarchical cluster analysis of the samples was performed using Euclidean distance clustering. Sample classes, such as different cell types were colour coded on the dendrogram tree in order to make easier the biological interpretation. Principle component analysis was performed as an alternative data exploration technique on the samples using a incomplete approximation procedure.

Significant analysis of microarrays was performed on the normalised data using either a multiclass analysis (three way) or a two class unpaired analysis. These analyses were performed, using 100 permutations of the data original data to compute the FDR. More permutations would have been preferable but computer limitations meant 100 was the maximum possible. The false discovery rate used to define a differentially expressed gene may differ between analysis and it is indicated in the specific results chapters. However in most cases a 0.1% FDR was chosen. The genes found to be significantly differentially expressed were then set as the data source and hierarchical and principle component cluster analyses performed.

Normalised data was exported to Prediction Analysis of Microarray (PAM), version 2.1 for identifying gene subsets predictive of different cell identities. This program is implemented as a plug-in within Microsoft Excel, files were set up with gene name and ID in the first two columns. A random selection of six samples of each cell type were used as a training set whereas three samples were used as the test set. Ten different iterations of PAM were performed with different random selection of training and test sets.

PAM was performed setting the shrinkage threshold to allow maximum predictivity on the training set with a model size of at least 100 genes. Using these genes the cell type of the remaining samples (prediction set) were predicted. The top 100 genes in each of the ten gene lists were ranked by the number of times they appeared (i.e. used as a predictive gene); and those genes which had been used 8 times and above selected. The normalised data for each of

these genes was then extracted to be represented in a graph, or loaded into TMEV for hierarchical and principle component cluster analysis.

To determine any potential biological meaning behind these gene lists the database for annotation, visualization and integrated discovery (DAVID), was used. This is a web-based tool ([www.david.abcc.ncifcrf.gov](http://www.david.abcc.ncifcrf.gov) <http://www.david.abcc.ncifcrf.gov>) that maps the genes contained in a given gene list to biological annotation databases such as GO. It then it identifies functional pathways which are over-represented in the input gene list<sup>172</sup>. It is extremely useful to formulate hypothesis on the role of these genes in biological systems.

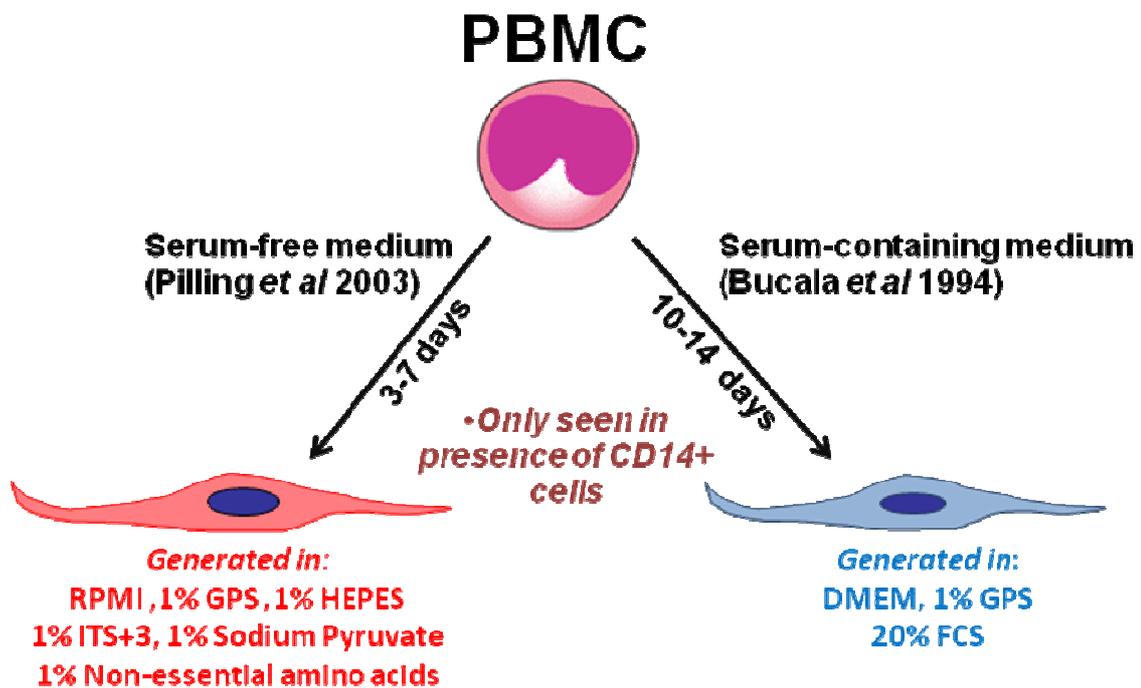
The ideal gene number to analyse with DAVID is between 200-2000 genes<sup>172</sup> however the gene list we wanted to use was far too large for this so to reduce this a three-fold difference cut off was introduced, only those genes whose ratio to reference was three fold different or more between the two cell populations were used. The list of genes which remained was split into two, based on which cell type had the highest gene expression, using the mean  $\log_2$  ratio reading across the samples for each population. The two lists were inputted separately into DAVID and a cut off of an enrichment score of greater than 2 and a FDR of less than 5 was used when looking at the biological processes provided.

## 3.0 CULTURING AND QUANTIFYING FIBROCYTES

### 3.1 Generating Fibrocytes

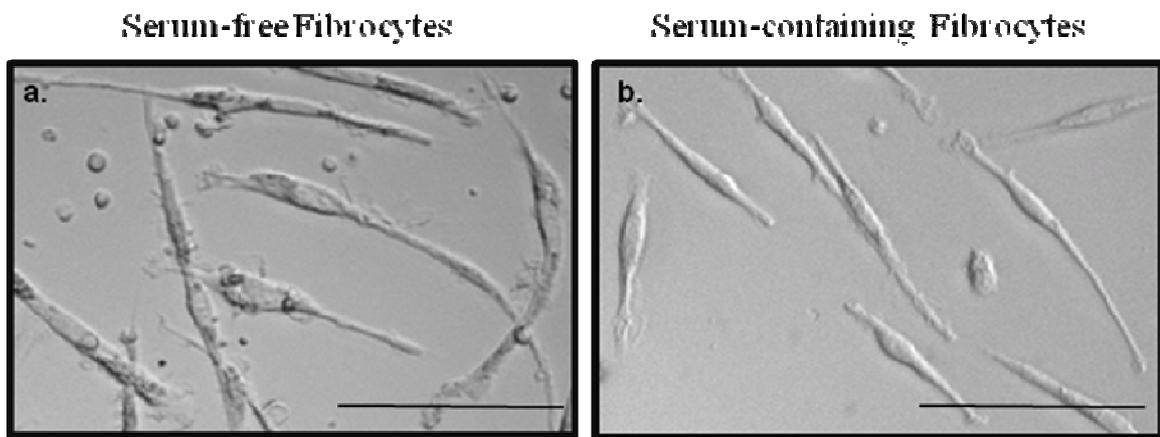
As discussed in the introduction fibrocytes have been shown to differentiate from the CD14<sup>+</sup> monocyte population within peripheral blood<sup>45-47</sup> *in vitro*, however there are two methods used to generate these, in both the presence<sup>45;87</sup> and absence<sup>47</sup> of serum (Figure 3.1). There are also discrepancies within the literature on the optimum conditions for culture, therefore for the purpose of this thesis this chapter is to address the ideal culture conditions for generating fibrocytes, to allow for the questions raised in the overall aims to be answered.

More specifically the aims of this chapter are to determine the most precise method to quantify fibrocyte differentiation and to determine the optimum culture conditions for both serum-free and serum-containing fibrocytes. To discuss various potential methods of isolating a pure sample of fibrocytes; and to determine that the cells being differentiated have the same expression profile as those described in the literature.



**Figure 3.1 Culture conditions for generating fibrocytes.** A schematic to represent the culture conditions described in the literature for generating fibrocytes in both serum-free and serum-containing fibrocyte medium<sup>47;87</sup>.

Using the serum-containing fibrocyte medium, according to the literature, fibrocytes differentiate *in vitro* after 10-12 days<sup>45;87;89-91</sup>. However in the case of the serum-free fibrocyte medium fibrocytes are seen to differentiate after just 3-5 days; this was shown to be due to the lack of serum amyloid P (SAP) which is found in serum<sup>47;48</sup>. For the purpose of this report the medium used by Pilling *et al* will be referred to as serum-free fibrocyte medium and the medium used by Bucala *et al* serum-containing fibrocyte medium. To demonstrate the fibroblast like morphology of fibrocytes figure 3.2 shows an example of fibrocytes differentiated in either serum-free (a) or serum-containing (b) fibrocyte medium photographed at day 12 of culture.

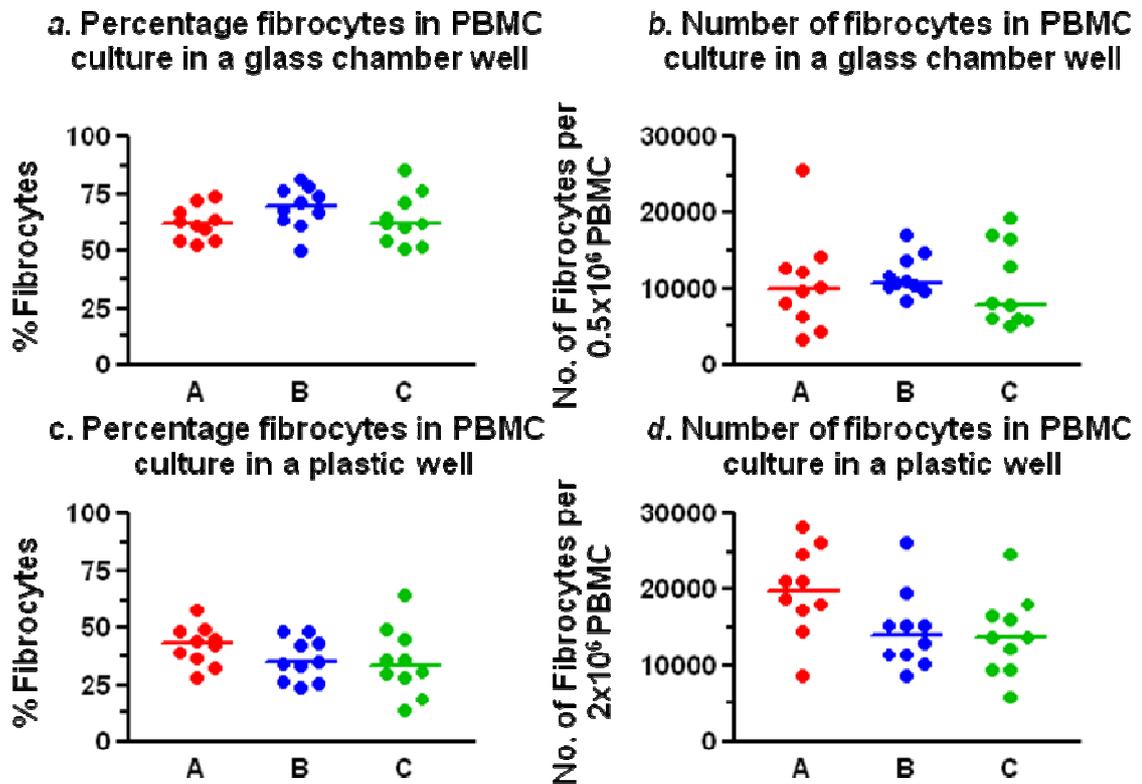


**Figure 3.2 Photographs of Fibrocytes.** Fibrocytes differentiated in serum-free fibrocyte medium from isolated PBMC plated out at  $1 \times 10^6$  cell/ml (a). Fibrocytes differentiated in serum-containing fibrocyte medium from positively selected CD14<sup>+</sup> cells, plated out at  $2 \times 10^5$  cell/ml (b). Both were photographed on day 12 of culture. Scale bar indicates 100 $\mu$ m.

### 3.2 Quantifying Fibrocytes

Whilst doing preliminary work for this thesis it was found that serum-free derived fibrocytes were extremely adherent, which will be discussed later in this chapter, however to be able to establish the best conditions for *in vitro* fibrocyte generation a criteria for counting differentiated fibrocytes *in situ* had to be developed. The best phenotype for the identification of fibrocytes is their spindle shaped, fibroblast like morphology (Figure 3.2). Initially fibrocyte cultures were photographed periodically, using a Zeiss inverted microscope, and the number of cells in the photographs counted using the two categories, fibrocyte or non-fibrocyte. From this data the percentage of differentiated fibrocytes could be calculated. Subsequently an eye piece micrometer was purchased to allow for differentiated cells to be counted *in situ* on the microscope, without the need for photographs. When using the eye piece micrometer three

random fields of view were counted for each culture condition and the mean percentage and number of fibrocytes calculated for each well. Fibrocyte differentiation is extremely heterogeneous and when counting using the eye piece micrometer it became clear that just counting three random fields of view from one well was not sufficient to get an accurate picture of what was happening in the cultures. On one day the three fields of view randomly chosen may have a very low percentage/number of fibrocytes and on the next day the three fields of view, randomly chosen, could contain a high number of fibrocytes, making it appear that there has been a large increase over night, or vice versa. Therefore an experiment was performed to look at how many fields of view in how many replicate wells needed to be counted to get a precise fibrocyte count. Three wells of a glass 8-well chamber slide and a plastic 24-well plate were seeded with PBMC in serum-free fibrocyte medium at  $1 \times 10^6$  cells/ml and the non-adherent cells washed out at day four. On day eleven ten fields of view were counted in each well and the percentage and number of serum-free fibrocytes calculated (Figure 3.3).



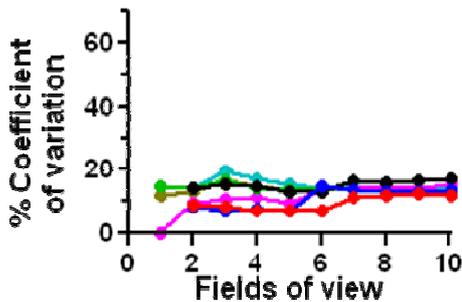
**Figure 3.3 Percentage and number of serum-free fibrocytes differentiated on two different substrates.** Fibrocytes were differentiated in serum-free fibrocyte medium from PBMC on a glass 8 well chamber slide and plastic 24 well plate. Using the eye piece micrometer cells were counted in ten fields of view of 3 replicate wells (A, B and C) for both substrates. The percentage fibrocytes (a.) and number of fibrocytes (b.) in PBMC culture of glass chamber slide with the mean shown. The percentage fibrocytes (c.) and number of fibrocytes (d.) in PBMC culture of plastic plate with the mean shown.

The percentage fibrocytes seen in the 3 wells of the glass chamber slide ranged between 50 and 85% fibrocytes, however the number of fibrocytes seen had a much larger range, which may have been due to the heterogenic way these cells differentiate, with some fields of view having many more fibrocytes than others. The percentage fibrocytes seen in the plastic plate was lower than that seen in the glass chamber slide, which may indicate the cells substrate preferences of

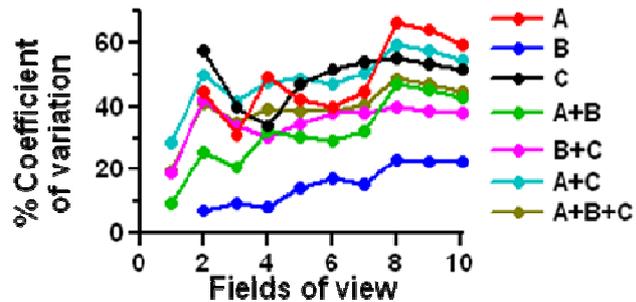
glass over plastic. However, as for the glass chamber slides, the range in the number of fibrocytes calculated in the plastic wells was much higher than the range of percentage fibrocytes, again probably due to the heterogeneity of the culture.

Using the fibrocyte percentage and number data calculated from the ten fields of view the percentage coefficient of variation was chosen to determine the level of variation seen within the different fields of view. This helped to determine the number of fields of view and replicate wells that need to be counted to get precise counting results. The percentage coefficient of variation was calculated looking at single wells (A, B or C), two wells (A+B, A+C or B+C) or all three wells (A+B+C), with an increasing number of fields of view included. These analyses were all performed for percentage and number of fibrocytes in glass chamber slides and plastic plates (Figure 3.4).

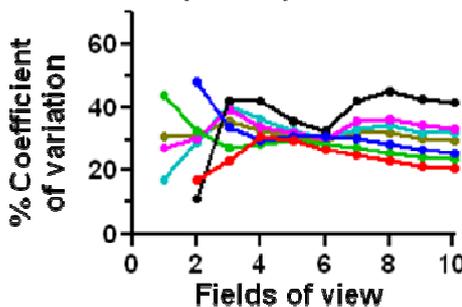
a. Percentage coefficient of variation of % fibrocytes in PBMC culture of a glass chamber slide



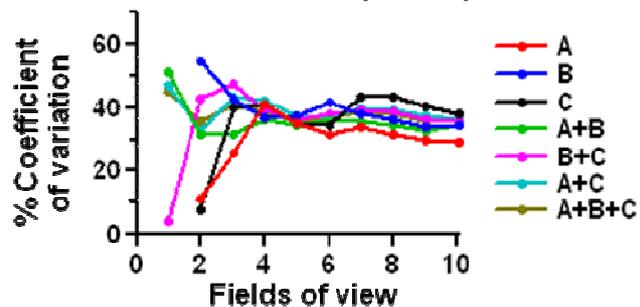
b. Percentage coefficient of variation of number of fibrocytes in PBMC culture of a glass chamber slide



c. Percentage coefficient of variation of % fibrocytes in PBMC culture of a plastic plate



d. Percentage coefficient of variation of number of fibrocytes in PBMC culture of a plastic plate

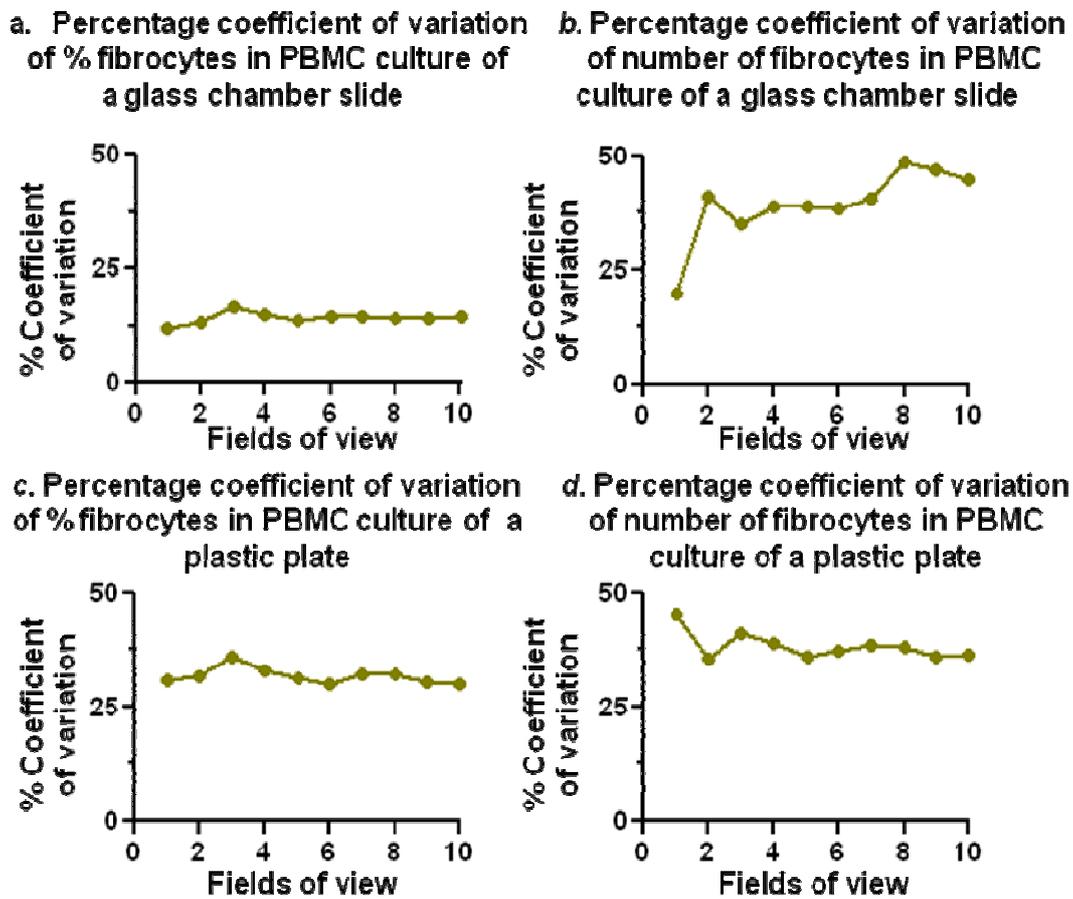


**Figure 3.4 Determination of the ideal number of fields of view and replicate wells required for precise counting results.** Percentage coefficient of variation of an increasing number of fields of view and increasing number of wells for fibrocytes differentiated in serum-free fibrocyte medium from PBMC. Using the eye piece micrometer cells were counted in ten fields of view of 3 replicate wells (A, B and C) for both substrates and the data analysed as above. The percentage coefficient of variation of percentage fibrocytes (a.) and number of fibrocytes (b.) differentiated from PBMC in glass chamber slide. The percentage coefficient of variation of percentage fibrocytes (c.) and number of fibrocytes (d.) differentiated from PBMC in plastic plate.

Due to the heterogeneity of the cultures the variation between the different fields of view and wells was always going to be high, in some cases the percentage coefficient of variation was in the 60's (Figure 3.4). However the purpose of these experiments was to calculate at what point

the percentage coefficient became stable and no longer changed so that no matter how many fields of view or wells were counted it never actually got any lower, and therefore indicated the level of variation within the cultures, not of the counting.

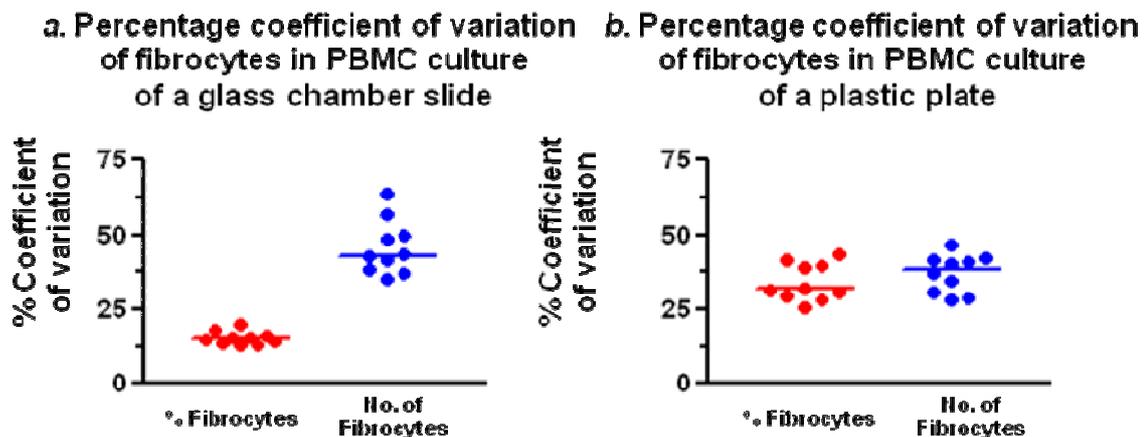
When just looking at the percentage coefficient of variation of increasing fields of view in three wells (Figure 3.5) three fields of view appeared to be the point where the variation became stable, more than three fields of view did not lower the level of variation seen and three fields of view for three wells for each condition is technically feasible. Therefore counting three fields of view from three replicate wells was decided to be the most precise way to quantify fibrocyte cultures.



**Figure 3.5 Determination of the ideal number of fields of view when counting three wells to get precise counting results.** Percentage coefficient of variation of an increasing number of fields of view counted in three wells of fibrocytes differentiated in serum-free fibrocyte medium from PBMC. Using the eye piece micrometer cells were counted in up to ten fields of view of 3 different wells (A, B and C) for both substrates and the data analysed as above. The percentage coefficient of variation of percentage fibrocytes (**a.**) and number of fibrocytes (**b.**) differentiated from PBMC in glass chamber slide. The percentage coefficient of variation of percentage fibrocytes (**c.**) and number of fibrocytes (**d.**) differentiated from PBMC in plastic plate.

To test that three fields of view from three wells was a suitable method ten permutations of three fields of view from the ten counted for each well were chosen by using a random number generator. These were put together with three random fields of view from the other two wells

and the percentage coefficient of variation calculated for both the percentage and the number of fibrocytes seen. Figure 3.6 illustrates the range of the percentage coefficients of variation seen for these ten permutations. Although the fibrocyte percentage and number percentage coefficient of variations are very different on the glass chamber slide, the 10 permutations cluster closely together indicating that although the degree of variation is high within the plate, counting three fields of view of three wells is enough to ensure the counts are precise.

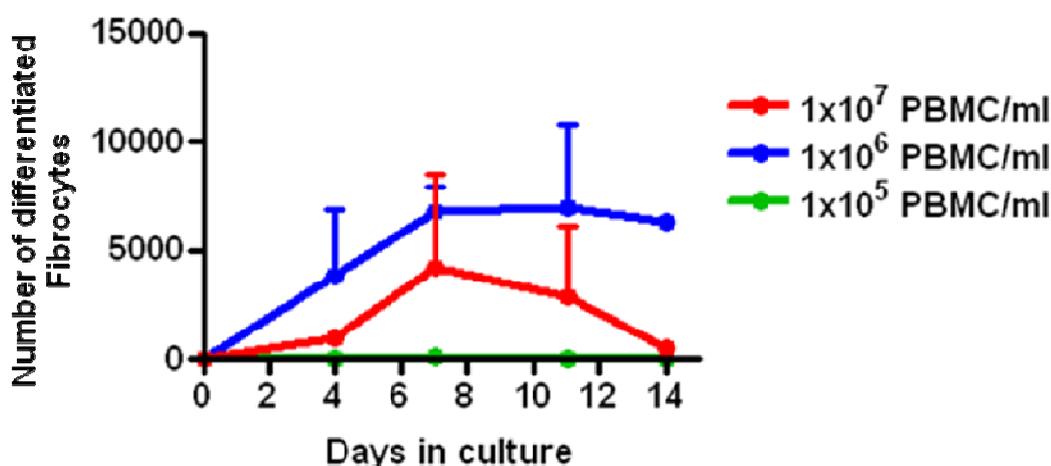


**Figure 3.6** Testing precision of counting three fields of view from three replicate wells. Percentage coefficient of variation of fibrocytes differentiated in serum-free medium from PBMC for ten replicates of 3 randomly chosen fields of view from 3 wells. For (a.) glass wells and (b.) plastic wells.

### 3.3 Optimising culture conditions of both serum-free and serum-containing fibrocytes

As with many other cultured cells the concentration at which PBMC are plated out can influence the percentage, and number of fibrocytes seen to differentiate, due to cell density effects<sup>171;172</sup>.

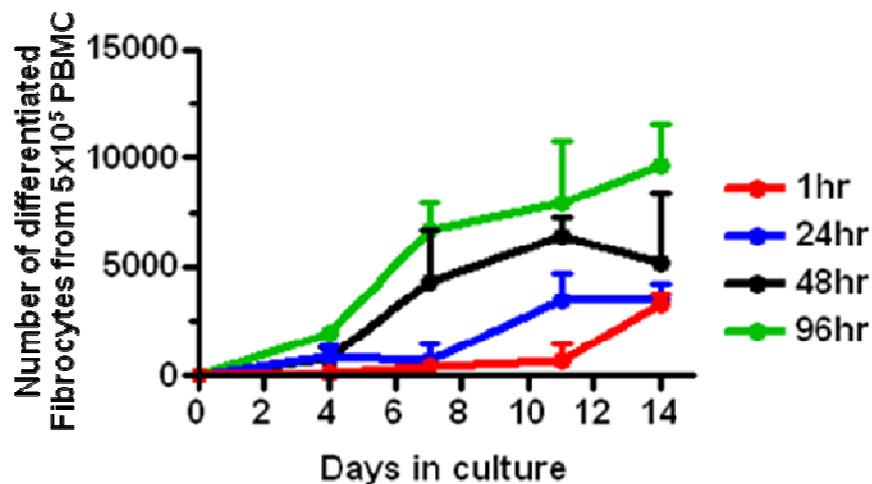
To determine the optimal seeding density for the generation of serum-free derived fibrocytes, PBMC were isolated and seeded at  $1 \times 10^7$ / $1 \times 10^6$ / $1 \times 10^5$  cells per ml into glass chamber slides in serum-free fibrocyte medium (Figure 3.7). The number of fibrocytes was calculated on days 4, 7, 11 and 14.



**Figure 3.7** Number of differentiated fibrocytes for different seeding concentrations of PBMC. PBMC were plated out at the indicated concentrations in glass 8 well chamber slides in serum-free fibrocyte medium. At days 4, 7, 11 and 14 the number of fibrocytes was calculated. Error bars indicate standard deviation about the mean of the three fields of view counted for each condition from one experiment. Experiment representative of  $n=3$ .

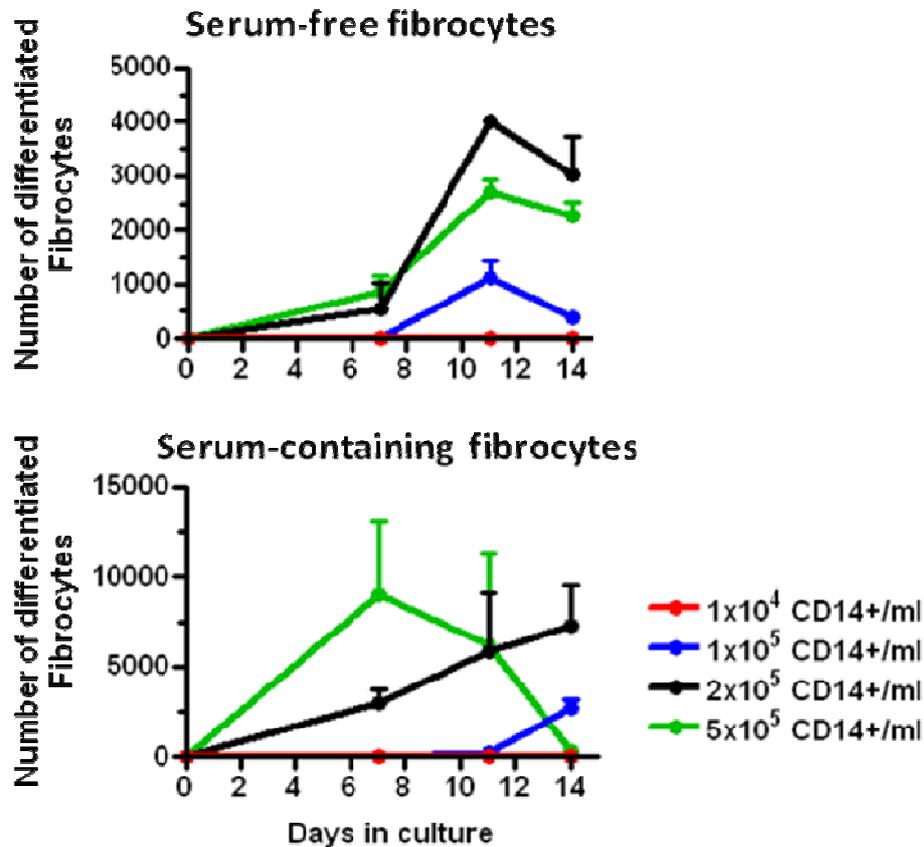
The ideal seeding concentration was found to be  $1 \times 10^6$  cells/ml as it gave the most serum-free fibrocytes at every time point counted. Whenever PBMC were plated out in serum-containing fibrocyte medium however very few fibrocytes differentiated in our hands, therefore it was decided that the majority of work on them would be performed on cells that had differentiated from  $CD14^+$  selected cells.

When counting the number of fibrocytes in culture, following set up from PBMC, it was difficult to see the differentiating adherent cells underneath the non-adherent cells, therefore the non-adherent cells were removed. To ensure these were being washed out at the ideal time point a time course was performed. It was important to ensure that the non-adherent cells were not being washed out at a time point at which the monocytes were still adhering, to ensure minimal loss in monocyte number and maximum fibrocyte differentiation. Non-adherent cells were washed out at 1hr, 24hr, 48hr and 96hrs after PBMC were plated out in serum-free fibrocyte medium at  $1 \times 10^6$  cells/ml. The number of fibrocytes were calculated on days 4, 7, 11 and 14 and at every time point the number of fibrocytes were counted the optimum wash out time was 96hrs (Figure 3.8).



**Figure 3.8 Optimum time point to wash out non-adherent cells to ensure minimal monocyte loss.** PBMC were plated out at  $1 \times 10^6$  cell/ml in glass chamber slides in serum-free fibrocyte medium. At 1hr, 24hr, 48hr and 96hr time points the non-adherent cells were washed out and at days 4, 7, 11 and 14 the number of fibrocytes was calculated. Error bars indicate standard deviation about the mean of the three fields of view counted for each condition from one experiment. Experiment is representative of  $n=3$ .

Fibrocytes have been reported to differentiate from CD14<sup>+</sup> monocytes<sup>45;47-49;85;86</sup> and as usable numbers of serum-containing fibrocytes repeatedly failed to differentiate from PBMC the optimum seeding concentration for CD14<sup>+</sup> selected cells in each media type was also determined. CD14<sup>+</sup> cells were selected using magnetic bead selection, (described in the methods section, 2.8) and only purities of 95% and above were used. To ensure the correct seeding density of the CD14<sup>+</sup> selected cells a titration was set up including  $1 \times 10^4$ / $1 \times 10^5$ / $2 \times 10^5$ / $5 \times 10^5$  cells per ml plated into glass chamber slides in both serum-free and serum-containing fibrocyte media (Figure 3.9). The number of fibrocytes were calculated at days 7, 11 and 14 for each condition.



**Figure 3.9** Number of differentiated fibrocytes for different seeding concentrations of CD14<sup>+</sup> selected cells. CD14<sup>+</sup> selected cells were plated out at the indicated concentrations in glass 8 well chamber slides in serum-free and serum-containing fibrocyte medium. At days 7, 11 and 14 the number of fibrocytes was calculated. Error bars indicate standard deviation about the mean of the three fields of view counted for each condition. Experiment is representative of n=3.

In both media types the optimum seeding concentration was  $2 \times 10^5$  cells/ml, although in the serum-containing fibrocyte medium, at the earlier time points,  $5 \times 10^5$  cells/ml appeared to be better, however this dropped off very quickly, whereas the number of fibrocytes in the lower concentration continued to increase. Therefore  $2 \times 10^5$  cells/ml was chosen as the optimum cell concentration for plating out the CD14<sup>+</sup> selected cells in both media.

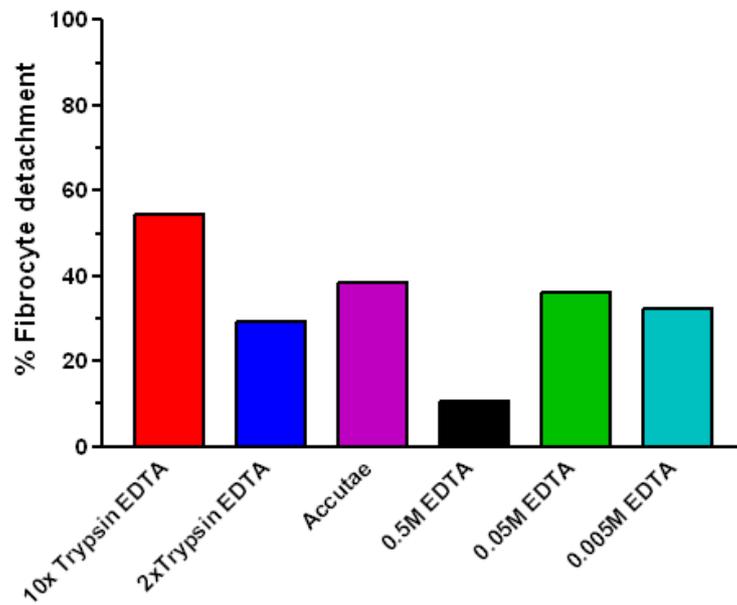
## 3.4 Harvesting Fibrocytes

### 3.4.1 Testing different detachment solutions on serum-free fibrocyte cultures

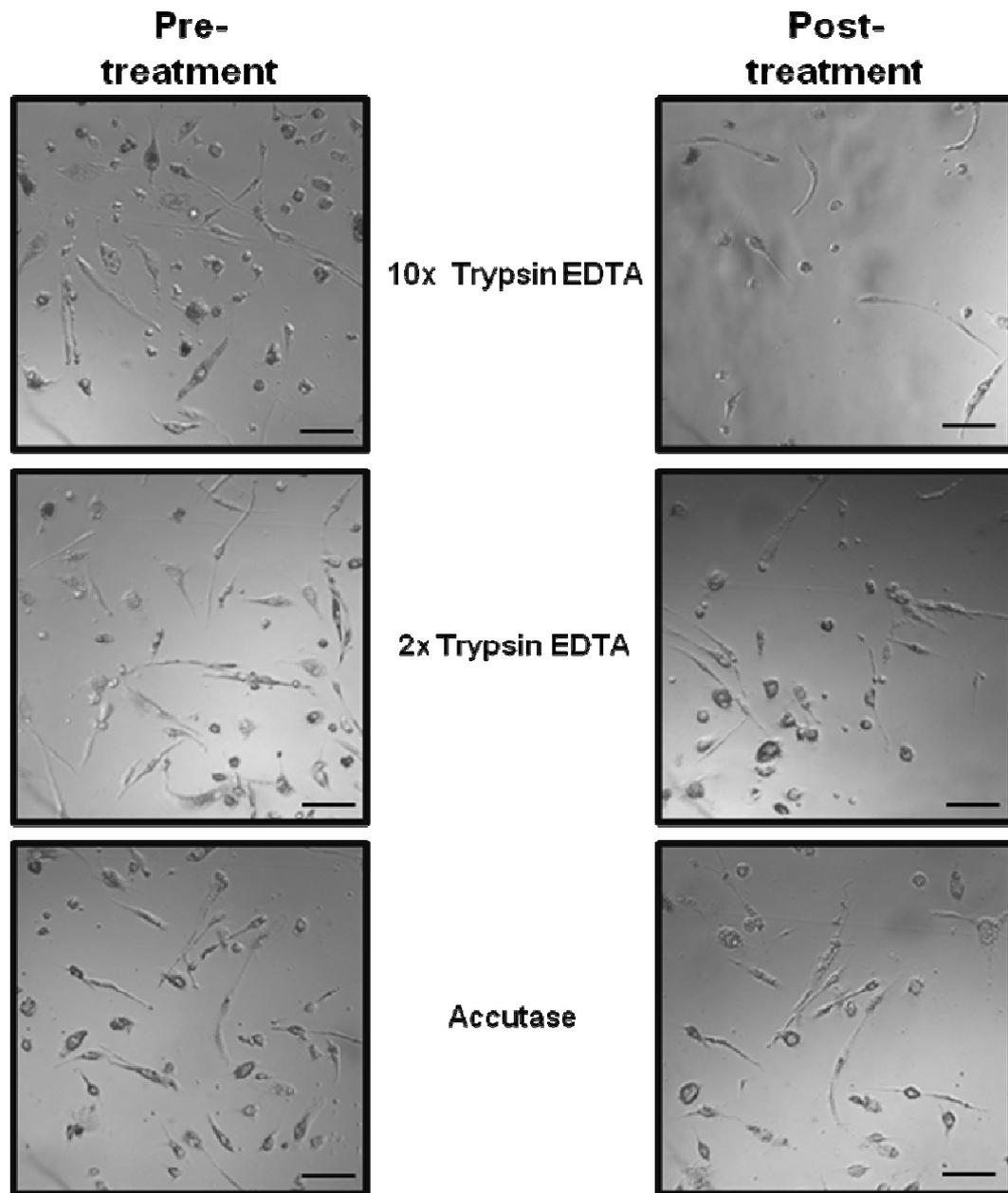
Although it has been reported that fibrocytes will detach from plastic following treatment with ice cold 0.05M EDTA<sup>46;87;90;91</sup> preliminary experiments in our group did not find this to be the case. Fibrocyte cell culture conditions do not produce a homogeneous population therefore being able to detach the cells from the plastic to subsequently analyse the effect of treatments, or to sort them to gain a pure cell population was required. Consequently an experiment was designed to investigate the effect of different detachment solutions on fibrocyte cultures. The different detachment solutions tested included, 0.005M EDTA, 0.05M EDTA, 0.5M EDTA (Sigma, at 4°C), Accutase (PAA) and Trypsin EDTA (Sigma). Manufactures' protocols were followed for Trypsin EDTA (2x) and Accutase. However a higher concentration of Trypsin EDTA was also used (10x).

PBMC were set up in serum-free fibrocyte media and on day 11, prior to treatment, each culture was counted to determine the number of differentiated fibrocytes and the cultures photographed. Following forty minutes of treatment all wells had some detached cells in suspension, however it was impossible to tell what they were, and both fibrocytes and non-fibrocytes still remained attached to the bottom of the well. To deactivate each detachment agent 1ml of RPMI/1%GPS/1% HEPES was added to each well, the wells then washed with PBS, and the number of attached fibrocytes counted again before cultures re-photographed.

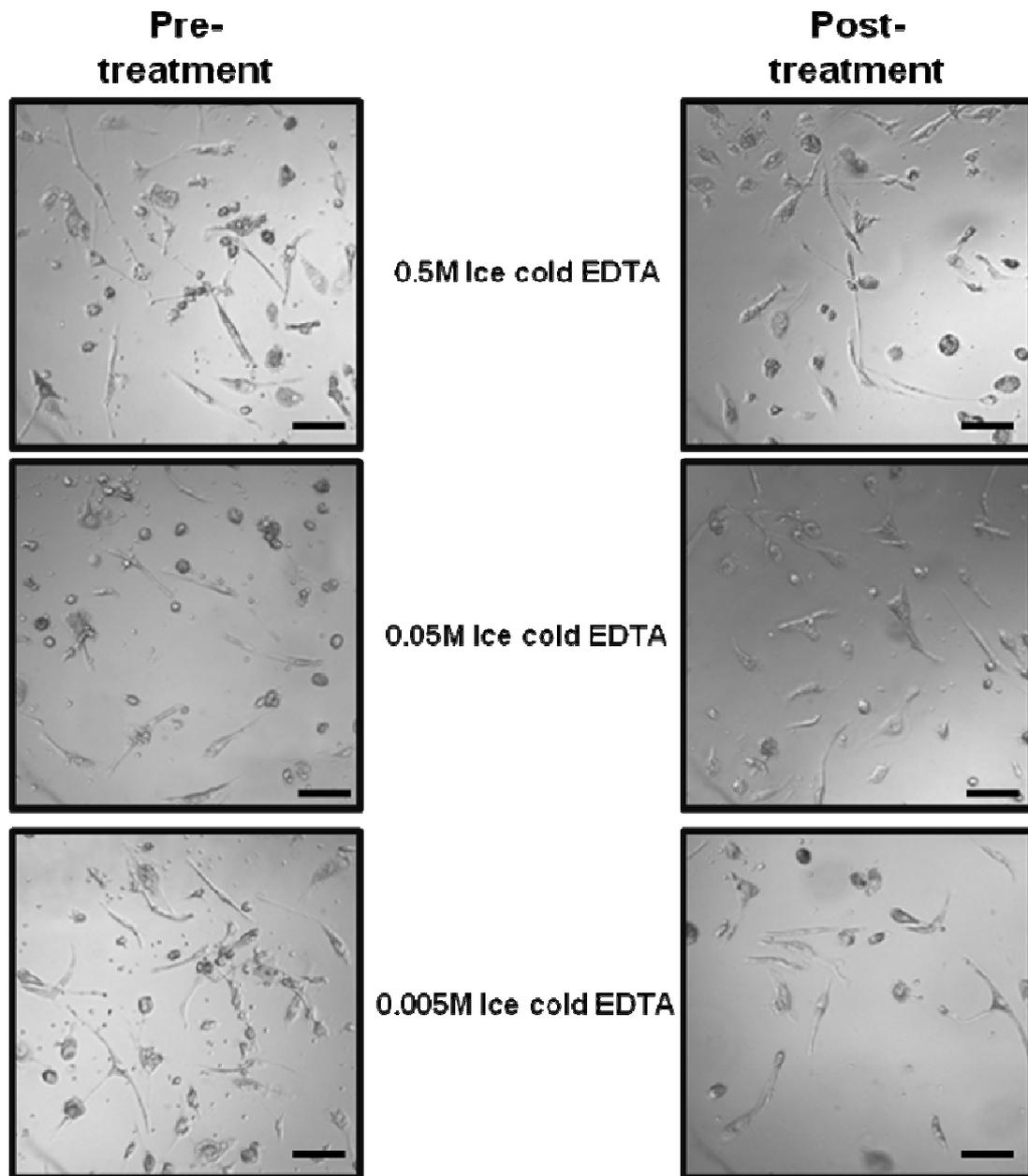
Figure 3.10 shows the results determined by counting the number of fibrocytes before and after treatment. The number of fibrocytes that detached is represented as a percentage of the number of fibrocytes pre-treatment. With every treatment less than 60% of the serum-free fibrocytes seen in culture detached following 40 minutes of treatment. The detachment agent which worked the most efficiently was 10x Trypsin EDTA, however it still only detached 55% of the fibrocytes, the remaining 45% that remained could represent a distinct population of fibrocytes which are more adherent, therefore a representative population of fibrocytes could not be collected. The other detachment agents detached less than 40% of the serum-free fibrocytes (Figure 3.10), this is also illustrated in the photographs in figures 3.11 and 3.12, pre and post treatment. Although there are perhaps less non-fibrocytes following treatment there are still fibrocytes clearly in the post treatment photographs, indicating that none of these detachment populations are suitable.



**Figure 3.10 Percentage of serum-free fibrocyte detachment** The number of serum-free fibrocytes was determined pre and post treatment. The number of fibrocytes detached by treatment is shown as a percentage of the number of cells counted pre-treatment. Data representative of n=2 experiments.



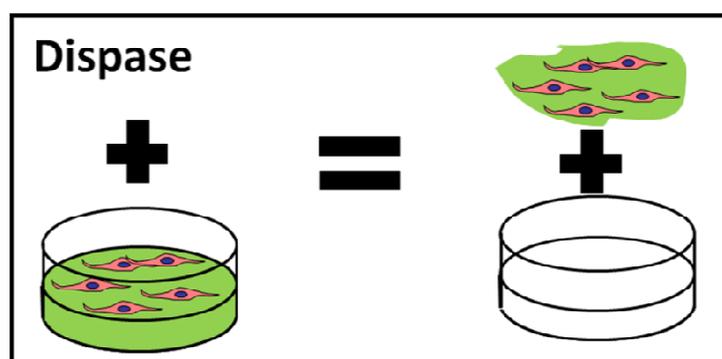
**Figure 3.11 Photographs of fibrocyte cultures pre and post treatment with commercially available detachment agents.** At day 11 three cultures of serum-free derived fibrocytes were photographed before treatment with either Trypsin EDTA (Sigma- concentrations 10x and 2x) or Accutase (PAA) and after 40 minutes of treatment. Scale bar represents 100 $\mu$ m.



**Figure 3.12** Photographs of fibrocyte cultures pre- and post treatment with ice cold EDTA. At day 11 three cultures of serum-free derived fibrocytes were photographed before treatment with either 0.5/0.05/0.005M EDTA (Sigma- at 4°C) and after 40 minutes of treatment. Scale bar represents 100µm.

### 3.4.2 Culturing cells on Puramatrix™ Hydrogel

Differentiating fibrocytes on Puramatrix™ Hydrogel from BD™ Biosciences was another option for obtaining a pure culture of fibrocytes in suspension. This hydrogel was a synthetic matrix designed to create defined three dimensional micro-environments for cell culture experiments. The peptide hydrogel consisted of standard amino acids (1% w/v) and 99% water and under physiological conditions the peptide components self assembled into a 3D hydrogel with a fibrous structure. The theory behind using this gel was that once fibrocytes had differentiated on the gel it could then be dissolved with dispase, a detachment agent able to dissolve the Puramatrix™ gel, allowing the collection and sorting of the detached fibrocytes into a pure culture.



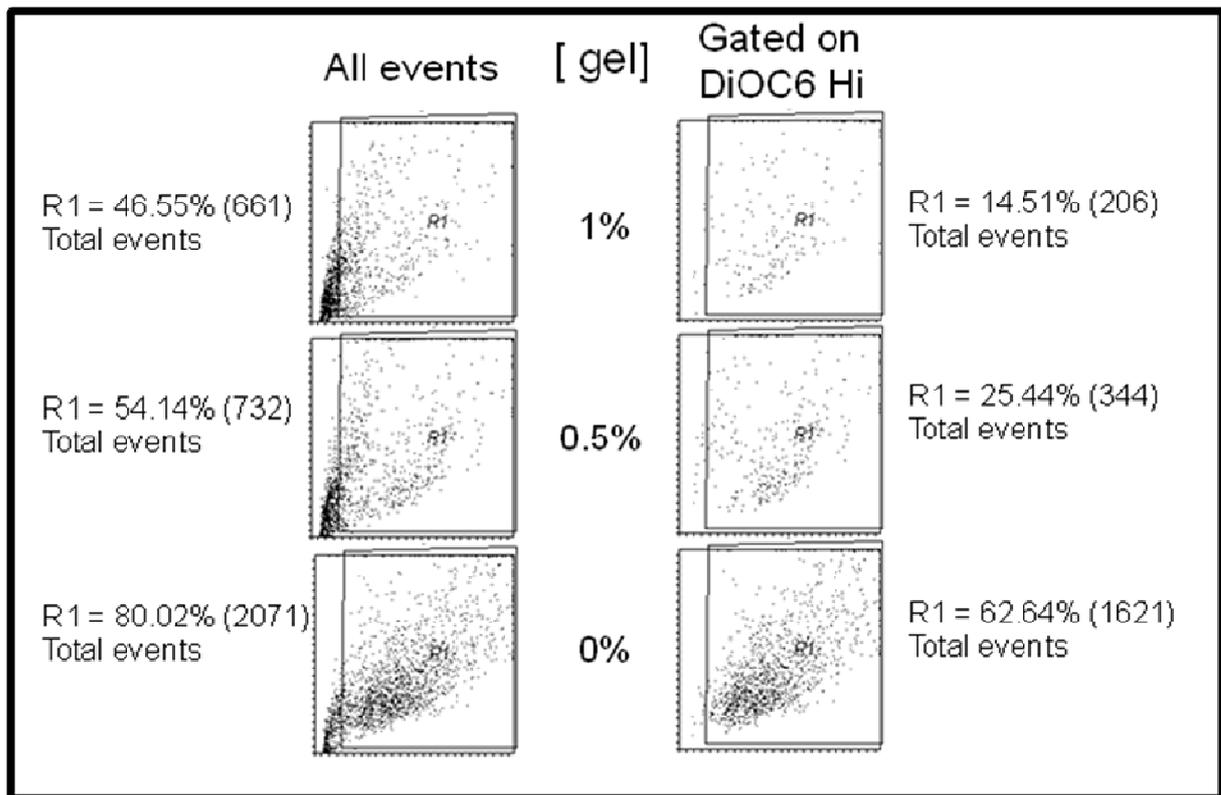
**Figure 3.13** A schematic of how Puramatrix™ hydrogel detaches adherent cells. After making the hydrogel the cells would be differentiated on it, then the Puramatrix™ hydrogel dissolved, using dispase, and in the process detaching the cells, so allowing collection of normally adherent cells for cell sorting into a pure culture.

Initially the optimal percentage of amino acids (gel concentration) needed to be determined as the manufacturers advised that different cell types required slightly different percentage w/v's. For ease of tissue culture dermal fibroblasts were used when optimising the Puramatrix™ gel. A

titration of gel concentrations was performed in a 24-well plate, with final concentrations of 1, 0.75, 0.5, 0.25 and 0% w/v, made up using the stock hydrogel and water. As a control one well had no hydrogel added, just water. Then over a series of washes the water content of the gel was replaced by fibroblast medium and the gel allowed to solidify.  $2 \times 10^4$  dermal fibroblasts were seeded into each well, as well as a sixth untouched well. After 7 days the cells were observed under the microscope and dispase used to dissolve the gel and detach the cells. To identify live cells anything detached by the dispase was stained with DiOC6, a lipophilic dye selective for polarised mitochondria and therefore will only stain live cells.

When looking at the cells on the Puramatrix<sup>TM</sup>, very few fibroblasts had proliferated and some had not even stretched out into their distinctive morphological characteristic. However the two wells that contained no hydrogel had a confluent culture of fibroblasts all stretched out. This suggested that every concentration of hydrogel that was tested was unsuitable for fibroblast culture and therefore unlikely to be suitable for fibrocyte culture. Figure 3.14 confirmed this, the DiOC6 labelled detached cells were ran on the flow cytometer in a fibroblast specific program. When just looking at the forward scatter versus side scatter plots of the samples obtained from 1, 0.5 and 0% gel (w/v) a lot more debris could be seen down the left hand side, i.e. debris and dead cells in the samples that were cultured on Puramatrix<sup>TM</sup> gel (1 and 0.5%) compared to the no hydrogel well (0%), there were also a lot fewer events in the fibroblast gate (R1). When the DiOC6<sup>hi</sup> samples were back gated onto the forward scatter versus side scatter plots the actual percentage of live cells was again much less in the samples that had been

cultured on Puramatrix™ gel compared to the 0% control, confirming that these concentrations of Puramatrix™ gel are not suitable for fibroblast cultures.



**Figure 3.14 DiOC6 staining of dermal fibroblasts cultured on Puramatrix™.** Identification of live cells by DiOC6 staining of detached dermal fibroblasts cultured on Puramatrix™ gel at different concentrations. Dermal fibroblasts were plated out at  $2 \times 10^4$  on increasing concentrations of Puramatrix™ hydrogel before being detached using dispase treatment. The detached cell samples were labelled with DiOC6 and looked at on the flow cytometer to determine the proportion of live fibroblasts versus total cells detached.

This experiment was then repeated using much lower concentrations of Puramatrix™ hydrogel, 0.25, 0.20, 0.15, 0.10, 0.05 and 0% w/v. Those fibroblasts on the lower concentration hydrogel were seen to proliferate and spread out, however they did not appear to proliferate as well as

the 0% controls. At this point we attempted to differentiate serum-free fibrocytes on the hydrogel. They were also seen to differentiate at the lower concentrations of gel, however when dispase was used to dissolve the matrix and detach the cells they did not detach, suggesting that they were able to differentiate at the lower concentrations of hydrogel because they went through the hydrogel and actually attached to the plastic of the well, at which point dispase could not detach them. Therefore although suitable for fibroblast culture Puramatrix™ was found to be unsuitable for the isolation of differentiating fibrocytes.

### **3.4.3 Differentiating serum-free fibrocytes on coated plates**

Various options for improving differentiation efficiency and detachment of serum-free fibrocytes were suggested by different company representatives, including various coatings and temperature sensitive plates.

Plates with various coatings were trialled to see if differentiation efficiency improved and/or if detachment efficiency increased. Serum-free fibrocytes did differentiate on plates coated with collagen I, collagen IV, laminin, poly-D-lysine and fibronectin however there were a lot less differentiated cells than uncoated plastic plates or glass slides (data not shown), therefore the use of these plates was not pursued.

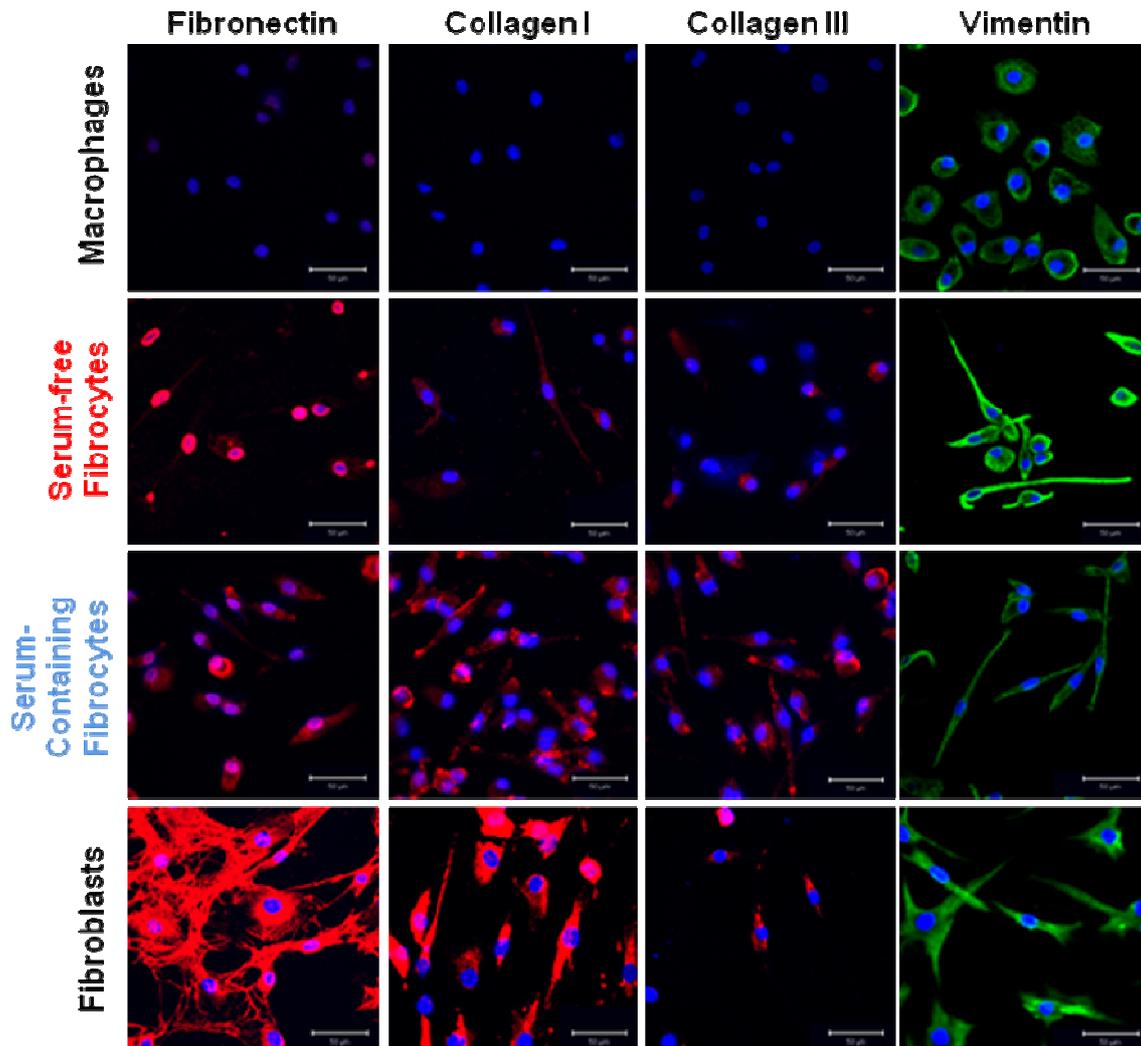
Temperature sensitive plates were also trialled, these were plates which were coated with a substrate that was solid at 37°C but when removed from the incubator for any length of time at

20°C the substrate would detach, allowing for collection of adherent cells. In theory the idea was good however the serum-free fibrocytes would not differentiate on the temperature sensitive substrate (Data not shown).

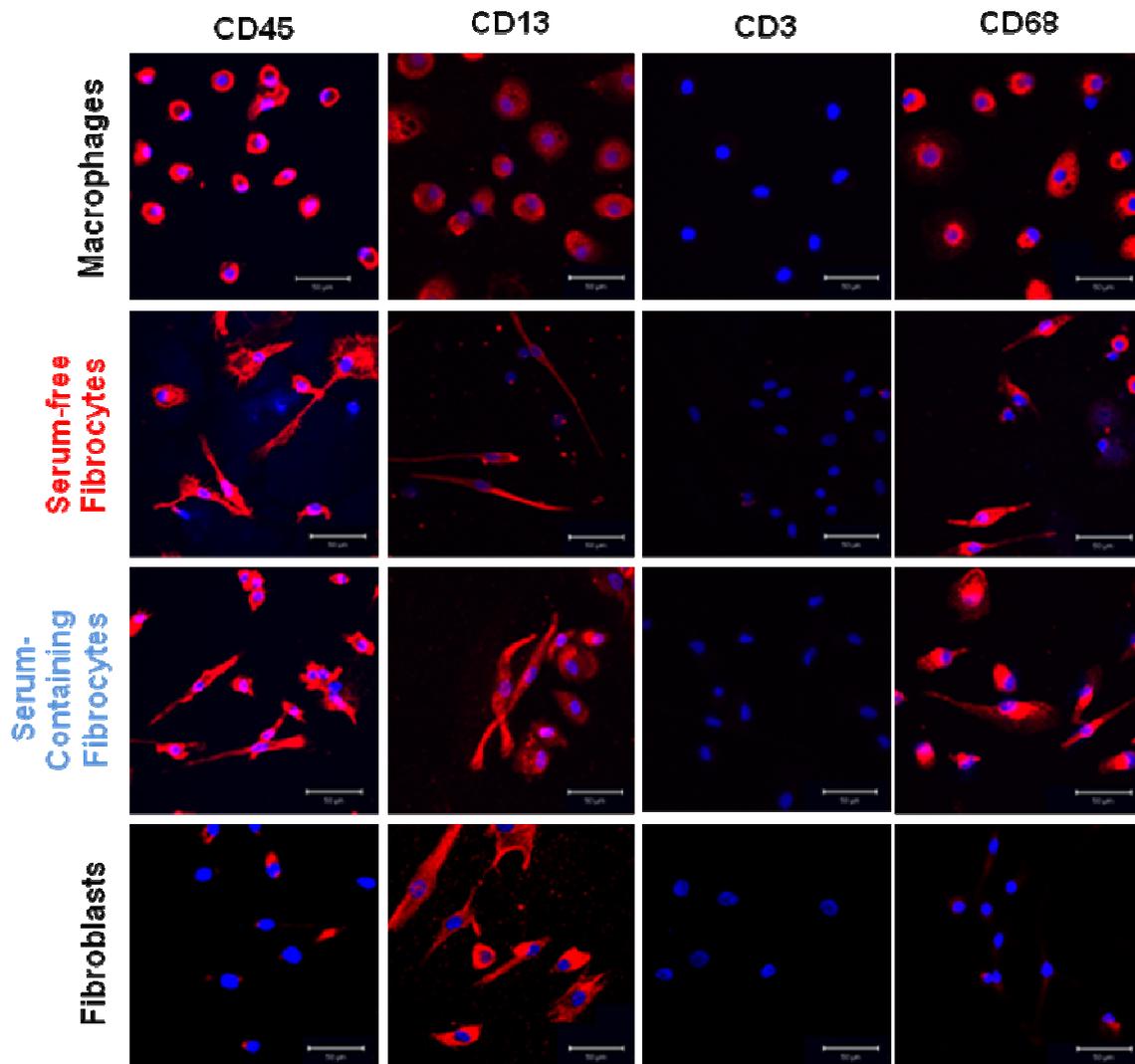
### **3.5 Marker expression of serum-free and serum-containing fibrocytes**

To ensure that the cells being used to answer questions in this thesis express the same markers as those described within the literature both serum-free and serum-containing fibrocyte cultures were antibody stained for markers of which expression is reported. Fibrocytes are known to express both stromal and haematopoietic markers<sup>47;87</sup>; the stromal markers looked at here were fibronectin, collagen I, collagen III and vimentin (Figure 3.15) and the haematopoietic markers CD45, CD13 and CD68 (Figure 3.16). As positive and negative controls for both sets of markers donor matched macrophages and a fibroblast cell line were also stained. CD3 was used as a negative marker for all cell types. Matched irrelevant controls are shown in figure 3.17.

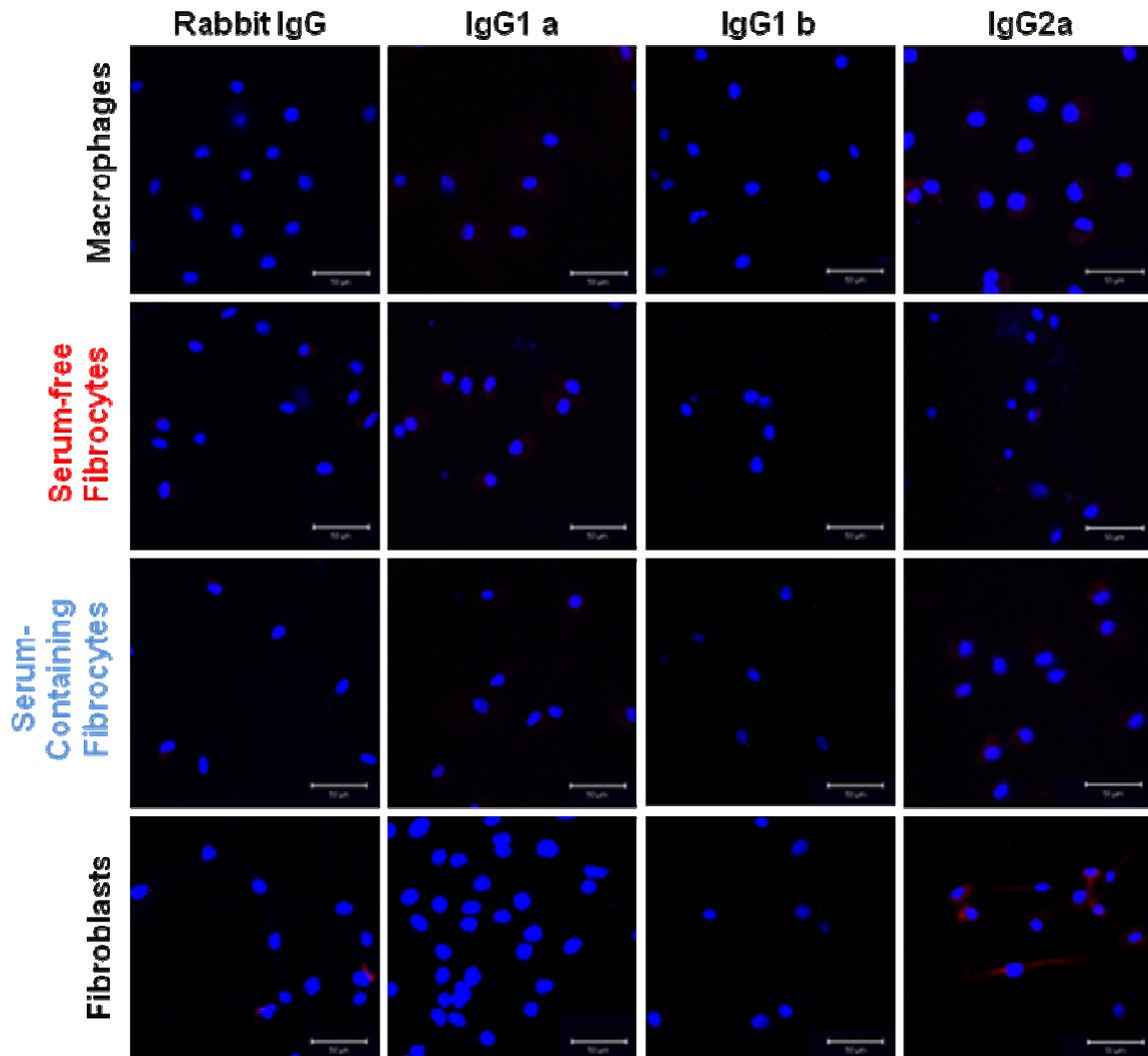
Serum-free fibrocytes were differentiated from PBMC and serum-containing fibrocytes differentiated from CD14<sup>+</sup> selected cells on glass chamber slides. At day 11, in both cases, the cultures were fixed for staining. Macrophages were differentiated from PBMC in specific macrophage media (see materials and methods) on glass chamber slides and fixed for staining on day 7. Fibroblast cell lines were plated out on glass chamber slides at either passage 4 or 5 and fixed 48hrs later, allowing for a suitable confluency.



**Figure 3.15** Images of stromal markers on serum-free, serum-containing fibrocytes, macrophages and a fibroblast cell line. Serum-free fibrocytes were differentiated from PBMC and serum-containing fibrocytes differentiated from CD14<sup>+</sup> selected cells on glass chamber slides, at day 11 fixed for staining. Macrophages were differentiated from PBMC on glass chamber slides and fixed for staining on day 7. Fibroblast cell lines were plated out on glass chamber slides at approximately passage 4 and fixed 48hrs later, allowing for a suitable confluency. Matched irrelevant control for Fibronectin, Collagen I and Collagen III is rabbit IgG and matched irrelevant control for vimentin is IgG1 b (Figure 3.17). Images shown represent one donor from  $n = 3$  for *in vitro* derived cells and one cell line from  $n = 3$  for fibroblast cell lines. Scale bar indicates 50µm.



**Figure 3.16** Images of haematopoietic markers on serum-free, serum-containing fibrocytes, macrophages and a fibroblast cell line. Serum-free fibrocytes were differentiated from PBMC and serum-containing fibrocytes differentiated from CD14<sup>+</sup> selected cells on glass chamber slides, at day 11 fixed for staining. Macrophages were differentiated from PBMC on glass chamber slides and fixed for staining on day 7. Fibroblast cell lines were plated out on glass chamber slides at approximately passage 4 and fixed 48hrs later, allowing for a suitable confluency. Matched irrelevant control for CD45, CD13 and CD3 is IgG1 a and matched irrelevant control for CD68 is IgG2b (Figure 3.17). Images shown represent one donor from n = 3 for *in vitro* derived cells and one cell line from n = 3 for fibroblast cell lines. Scale bar indicates 50µm.



**Figure 3.17** Images of irrelevant matched controls on serum-free, serum-containing fibrocytes, macrophages and a fibroblast cell line. Serum-free fibrocytes were differentiated from PBMC and serum-containing fibrocytes differentiated from CD14<sup>+</sup> selected cells on glass chamber slides, at day 11 fixed for staining. Macrophages were differentiated from PBMC on glass chamber slides and fixed for staining on day 7. Fibroblast cell lines were plated out on glass chamber slides at approximately passage 4 and fixed 48hrs later, allowing for a suitable confluency. Images shown represent one donor from n = 3 for *in vitro* derived cells and one cell line from n = 3 for fibroblast cell lines. Scale bar indicates 50μm

Stromal	Marker	Macrophages	Serum-free fibrocytes	Serum-containing fibrocytes	Fibroblasts
	Fibronectin	-	+	+	++
	Collagen I	-	+	+	++
	Collagen III	-	+	+	+
	Vimentin	++	++	++	++
Haematopoietic	Marker	Macrophages	Serum-free fibrocytes	Serum-containing fibrocytes	Fibroblasts
	CD45	+	+	+	+/-
	CD13	+	+	+	+
	CD68	+	+	+	-
	CD3	-	-	-	-

**Table 3.1 Summary of staining results on serum-free, serum-containing fibrocytes, macrophages and fibroblast cell lines.** Summary of figures 3.15 and 3.16. The symbol + indicates positive staining and – negative staining.

A summary of the staining results is shown in table 3.1. The staining indicated that serum-free and serum-containing fibrocytes have the same marker expression profile, positive for both stromal and haematopoietic markers, matching what is reported in the literature<sup>47,87</sup>.

Interestingly the fibronectin staining in both types of fibrocytes was much more peri-nuclear than in the fibroblasts, where it was seen along the whole length of the cell.

### 3.6 Discussion of chapter

Fibrocytes can be generated by two different protocols, in the absence<sup>47</sup> or presence<sup>87</sup> of serum, data in this chapter shows that both culture medium generate cells of a fibroblast-like morphology with the same marker expression profile as described in the literature<sup>47;87</sup> (Figures 3.2, 3.15-3.17 and Table 3.1). To quantify fibrocyte differentiation in culture, counting the number of differentiated cells in 3 fields of view and across 3 separate wells of a culture plate was found to be the most precise way of getting quantifiable results, due to the heterogeneous nature of fibrocyte differentiation (Figure 3.3 – 3.6). In 2003 Pilling *et al* enumerated fibrocyte differentiation by identifying them by their elongated morphology and the presence of an oval nucleus in 5 different fields of view of a 96-well plate<sup>47</sup>. In 2006 and 2008 the same group used the same method but with duplicate wells rather than single wells, with each culture being counted by at least two independent observers<sup>48;103</sup>. Therefore using cell counts based on cell morphology is a protocol deemed suitable for fibrocyte quantification within the literature and although multiple observers were not used here multiple wells were to try and provide precision.

The optimum culture conditions for differentiating fibrocytes vary within the literature, with reports using a starting concentration of PBMC that varies between  $2.5 \times 10^5$  cells/ml<sup>47;48;103</sup>,

$1 \times 10^6$  cells/ml<sup>46;89;91</sup>,  $2 \times 10^6$  cells/ml<sup>146</sup> and  $1 \times 10^7$  cells/ml<sup>90</sup>. Some reports do not wash out the non-adherent cells at all<sup>47;48;103;116</sup>, whereas others wash them out at 24hrs<sup>87;89-91</sup>, 48hrs<sup>45</sup> or 72hrs<sup>46;146</sup>. Therefore it was vital for the optimal culture conditions for this laboratory to be determined. For serum-free fibrocytes these were found to be at a concentration of  $1 \times 10^6$  PBMC/ml of media in either plastic or glass wells (Figure 3.7). To enable quantifiable counting the ideal time to wash off non-adherent cells was also found to be at day 4 or 96hrs from the time the cultures were set up (Figure 3.8). This was seen at every time point following set up, suggesting that until 96hrs either the differentiating monocytes were not quite adherent enough to cope with the washing or, and more likely, something within the non-adherent fraction of PBMC, or released by it, is required to get optimum fibrocyte differentiation. Various factors have been discussed as being important for fibrocyte differentiation, the pro-fibrotic cytokines IL4 and IL13 have been shown to promote fibrocyte differentiation<sup>103</sup>. Previous to them the presence of T cells was shown to be important for their differentiation *in vitro*. However it is still unclear whether direct cell-cell contact between T cells and fibrocyte precursors is required or whether it is just soluble factors released by these cells<sup>45;46</sup>. Also this effect of T cells on fibrocyte differentiation is still seen in the presence of blocking anti-cytokine antibodies for the profibrotic cytokines IL4 and IL13<sup>138</sup>, suggesting there is another factor, or factors involved.

The number of differentiated serum-containing fibrocytes from PBMC cultures was found to be extremely low in our hands, therefore the optimum cell density for both types of fibrocytes was

established from CD14<sup>+</sup> selected cells and found to be  $2 \times 10^5$  CD14<sup>+</sup> cells/ml media for both serum-free and serum-containing generated fibrocytes (Figure 3.9).

Neither serum-free nor serum-containing fibrocytes differentiate as pure populations, there were always cells without a spindle shaped morphology present in the cultures, therefore attempts were made to detach serum-free derived fibrocytes so that a pure population could be obtained. A pure sample of each population would allow for more precise comparisons between them to be made as there would be no question of non-fibrocytes biasing the results. A detached pure population would also allow for techniques such as flow cytometry to be used. Within the literature ice-cold 0.05% EDTA diluted in PBS was found to be sufficient to detach fibrocytes<sup>45;46;89;91;146;173</sup>, however this was not the case for cells differentiated in our hands as neither 0.5M, 0.05M or 0.005M were able to detach the serum-free derived fibrocytes (Figures 3.10 and 3.12). Although one report does detach fibrocytes using the commercial product Accutase<sup>116</sup>, we unfortunately found no commercially available detachment agents able to do so; Trypsin EDTA, Accutase and Dispase were all tested (Figures 3.10 and 3.11 ). We also found that the efficiency of serum-free fibrocyte differentiation was not improved by using plates coated with collagen I, collagen IV, laminin, poly-D-lysine or fibronectin, despite fibronectin coated plates being used in the literature<sup>89;91</sup>. Fibrocytes were found to not differentiate on Puramatrix<sup>TM</sup> hydrogel or temperature sensitive plates. No protocols tested enabled a pure fibrocyte culture to be isolated, therefore the effects of any treatment on fibrocyte

differentiation were quantified by counting the number of differentiated fibrocytes in culture based on morphology.

The cases in the literature where fibrocytes were detached using either EDTA or Accutase had differentiated their fibrocytes in serum-containing conditions<sup>45;46;89;91;116;144</sup>. Fibrocytes differentiated in serum-free conditions were not detached but quantified *in situ* by counting differentiated fibrocytes<sup>47;48;103</sup>. In this thesis the detachment agents were being tested on the serum-free derived fibrocytes, therefore our inability to detach them is not unseen within the literature. Although the literature suggests that detachment agents may have worked on the serum-containing fibrocytes this was not pursued as a detached population of fibrocytes derived under both methods were required for comparison, if a representative population of serum-free fibrocytes could not be detached there was no requirement for serum-containing derived fibrocytes to be detached.

The experiments within this chapter determined the optimum culture conditions for our laboratory to differentiate serum-free and serum-containing derived fibrocytes and methods of quantification. All further experiments within this thesis are based on these.

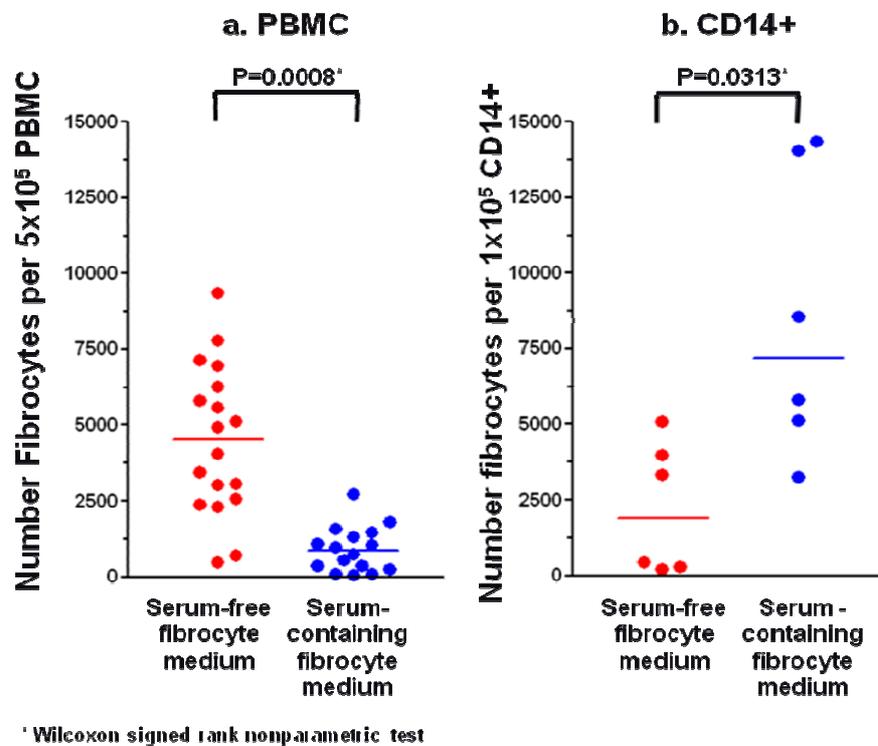
## 4.0 COMPARING SERUM-FREE AND SERUM-CONTAINING FIBROCYTES

### 4.1 Generation of fibrocytes from PBMC and CD14<sup>+</sup> selected cells

Both serum-free and serum-containing fibrocytes are reviewed in the literature as being the same cell type, due to their similar morphology and expression profile in immunostaining<sup>47;87</sup>, however there are some differences also mentioned. Serum-containing fibrocytes take 10-14 days to appear *in vitro* whereas serum-free are seen after just 3-5 days<sup>47;87</sup>. Also, serum-free fibrocyte differentiation is inhibited by the presence of the plasma protein SAP found in serum<sup>47</sup>, whereas serum-containing fibrocytes, as the name suggests, are differentiated in the presence of serum<sup>87</sup>. It has also been observed that serum-free fibrocytes express high levels of prolyl 4-hydroxylase, the enzyme required for the synthesis of new collagen<sup>48</sup>, whereas those generated in serum-containing media show low levels of prolyl 4-hydroxylase activity<sup>145;146</sup>, however both of these observations have been made separately and have not been directly compared.

The aim of this chapter is to compare the generation and motility of serum-free and serum-containing fibrocytes and to look at the effect serum has on serum-free derived fibrocytes. Due to our own observations, and the differences seen between the two types of fibrocytes described in the literature, the percentage and number of fibrocytes generated in both media

types were investigated in both PBMC and CD14<sup>+</sup> selected cultures. PBMC were isolated and plated out at  $1 \times 10^6$  cells/ml in both serum-free and serum-containing fibrocyte media and on day 4 (96 hours), the non-adherent cells washed out and the media replaced. CD14<sup>+</sup> cells were positively selected using magnetic beads selection (see materials and methods) from PBMC and plated out at  $2 \times 10^5$  cells/ml in both serum-free and serum-containing fibrocyte media. The number of fibrocytes were calculated at day 11 from triplicate wells (Figure 4.1).

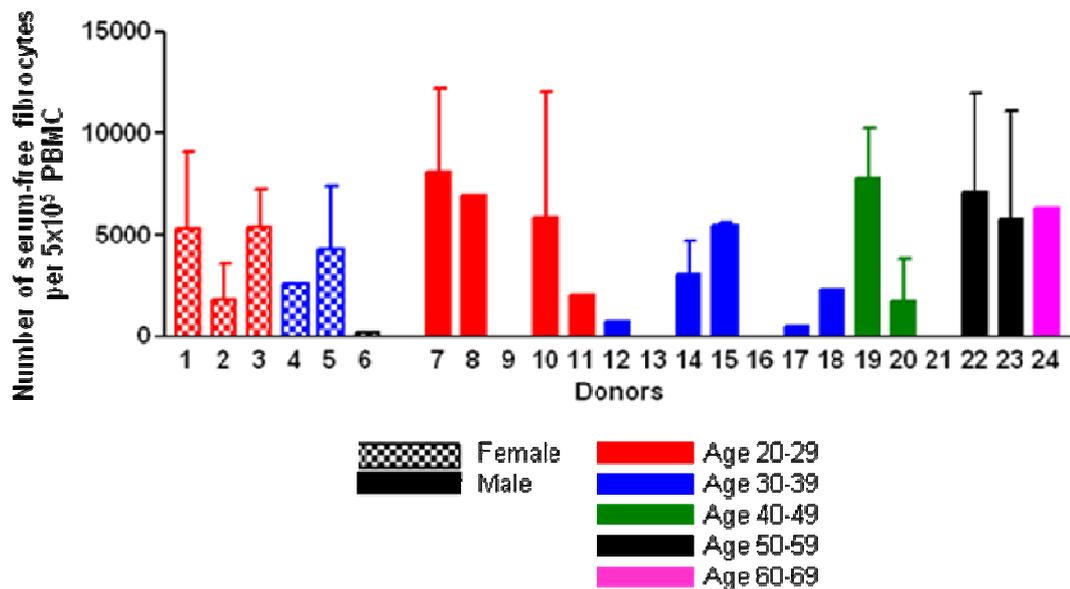


**Figure 4.1** Number of fibrocytes differentiated in serum-free and serum-containing fibrocyte media from either PBMC or CD14<sup>+</sup> selected cells. **a.** The number of fibrocytes differentiated in serum-free and serum-containing fibrocyte medium from isolated PBMC, plated out at  $1 \times 10^6$  cell/ml and counted at day 11, mean is indicated (n = 16). **b.** The number of fibrocytes differentiated in serum-free and serum-containing fibrocyte medium from positively selected CD14<sup>+</sup> cells, plated out at  $2 \times 10^5$  cell/ml and counted on day 11, mean is indicated (n = 6). Wilcoxon signed rank nonparametric test of significance performed on each.

When using the Wilcoxon signed rank nonparametric statistical test to compare the number of fibrocytes seen to differentiate in the two different media types the results were significantly different for fibrocytes differentiated from both PBMC and CD14<sup>+</sup> selected cells. Significantly more fibrocytes were seen to differentiate in serum-free fibrocyte media compared to serum-containing media when differentiated from PBMC. However in contrast significantly more fibrocytes differentiated in serum-containing fibrocyte media compared to serum-free when generated from CD14<sup>+</sup> selected cells. This suggests that fibrocytes differentiated in the two types of media are not the same cell type, as they are acting differently under the same conditions.

## **4.2 Donor Variability**

In the course of working on fibrocytes it has become obvious that there is a large donor to donor variation on the number of fibrocytes that will differentiate, which has been observed in both serum-free and serum-containing fibrocyte medium. PBMC isolated from some donors have the capacity to produce a high number of differentiated fibrocytes, whereas other donors produce a very low number. The donor variability of fibrocytes differentiated from PBMC in serum-free fibrocyte media is illustrated in figure 4.2.

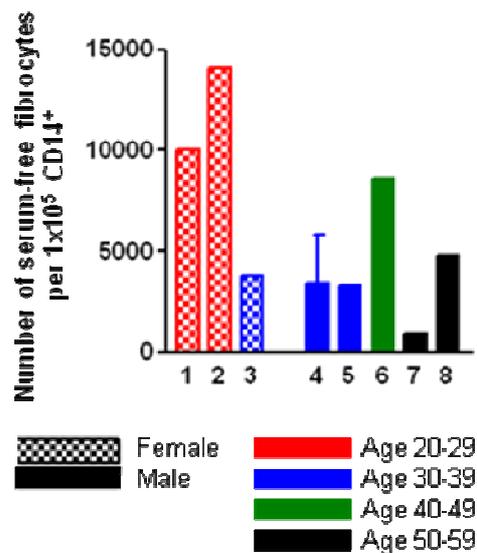


**Figure 4.2 Donor variability of serum-free derived fibrocytes.** The number of fibrocytes differentiated in serum-free fibrocyte medium from isolated PBMC plated out at  $1 \times 10^6$  cell/ml and counted at day 11 for 24 donors. If an error bar is present it represents the mean and standard deviation for more than 1 culture from that donor. No error bar indicates  $n = 1$  for that donor. Hashed bars indicate females and solid bars male; colour represents age range of donor.

Cultures were set up at  $1 \times 10^6$  cells/ml in serum-free fibrocyte media and the number of fibrocytes calculated at day 11. Hashed bars depict females, and solid bars males; the colours indicate different age ranges. If an error bar is shown the bar on the graph represents the mean and standard deviation for multiple readings for that donor. No error bar indicates the results of an  $n=1$ . A large variability in the number of fibrocytes differentiating is seen within healthy donors that does not seem to correspond to age or sex.

Figure 4.3 shows the donor variability for fibrocytes differentiated in serum-containing fibrocyte medium from  $CD14^+$  selected cells. As with the donor variability seen in serum-free derived

fibrocytes there is a large variation in the number of fibrocytes which will differentiate from different donors. This variability does not appear to be related to age or sex of the donor and no other quantified features within the donors appeared to account for it.

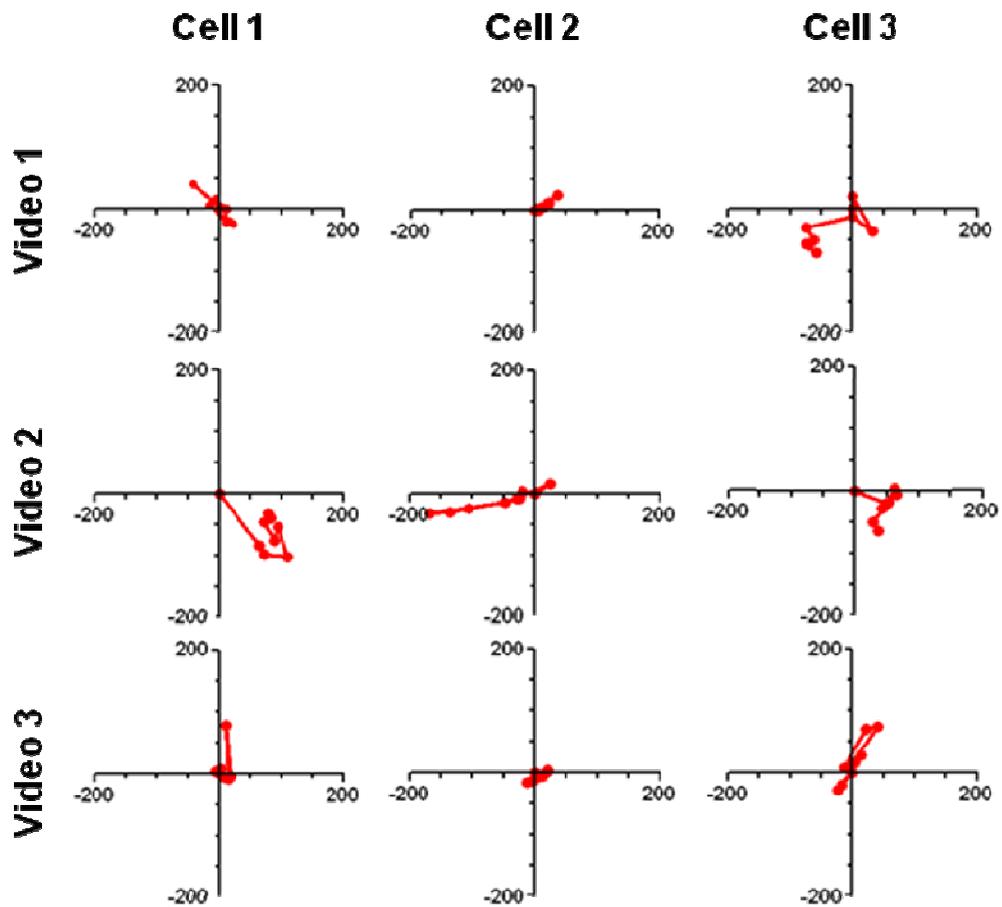


**Figure 4.3 Donor variability of serum-containing derived fibrocytes.** The number of fibrocytes differentiated in serum-containing fibrocyte medium from positively selected CD14 cells plated out at  $2 \times 10^5$  cell/ml and counted at day 11 for 8 donors. If an error bar is present represents mean and standard deviation for more than 1 culture from that donor. No error bar indicates  $n=1$  for that donor. Hashed bars indicate females and solid bars male; colour represents age range of donor.

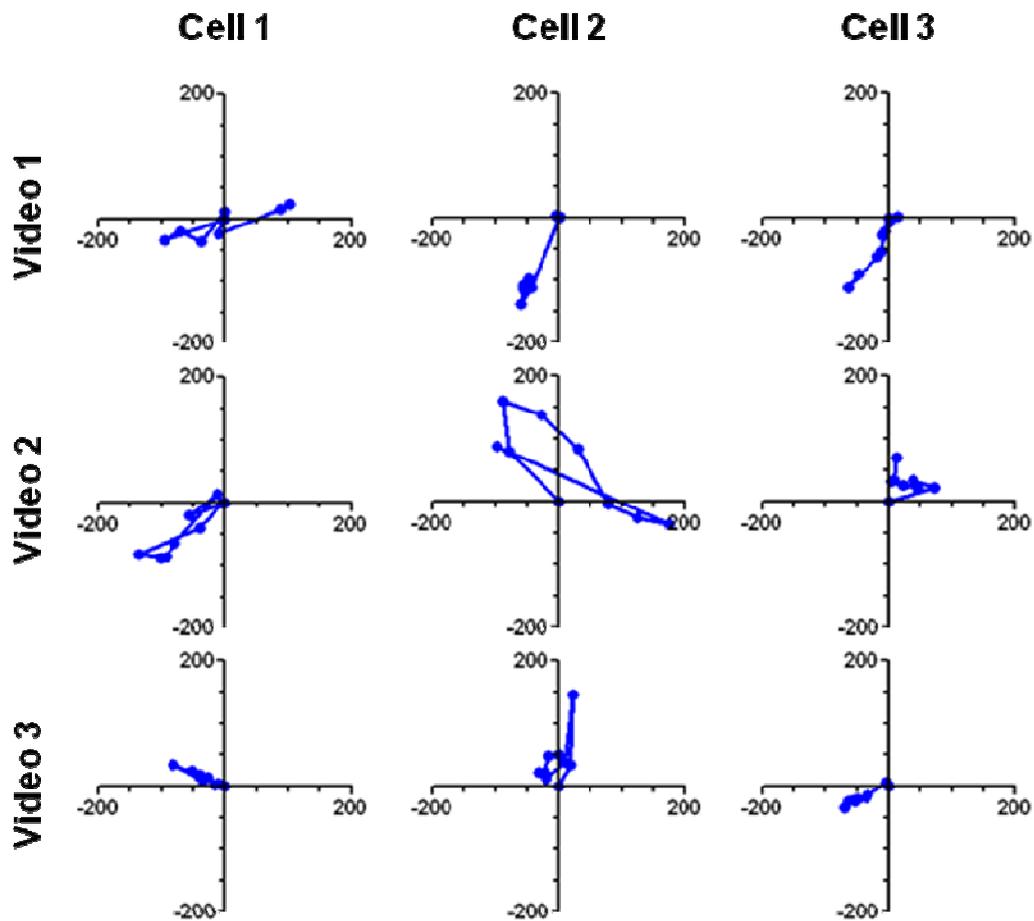
### 4.3 Motility of fibrocytes

Observations made when looking at serum-free and serum-containing fibrocytes by time-lapse photography suggested that the serum-containing fibrocytes were more motile than the serum-free fibrocytes. To analyse this three cultures of serum-free fibrocytes, differentiated from PBMC, and three cultures of serum-containing fibrocytes, differentiated from CD14<sup>+</sup> selected

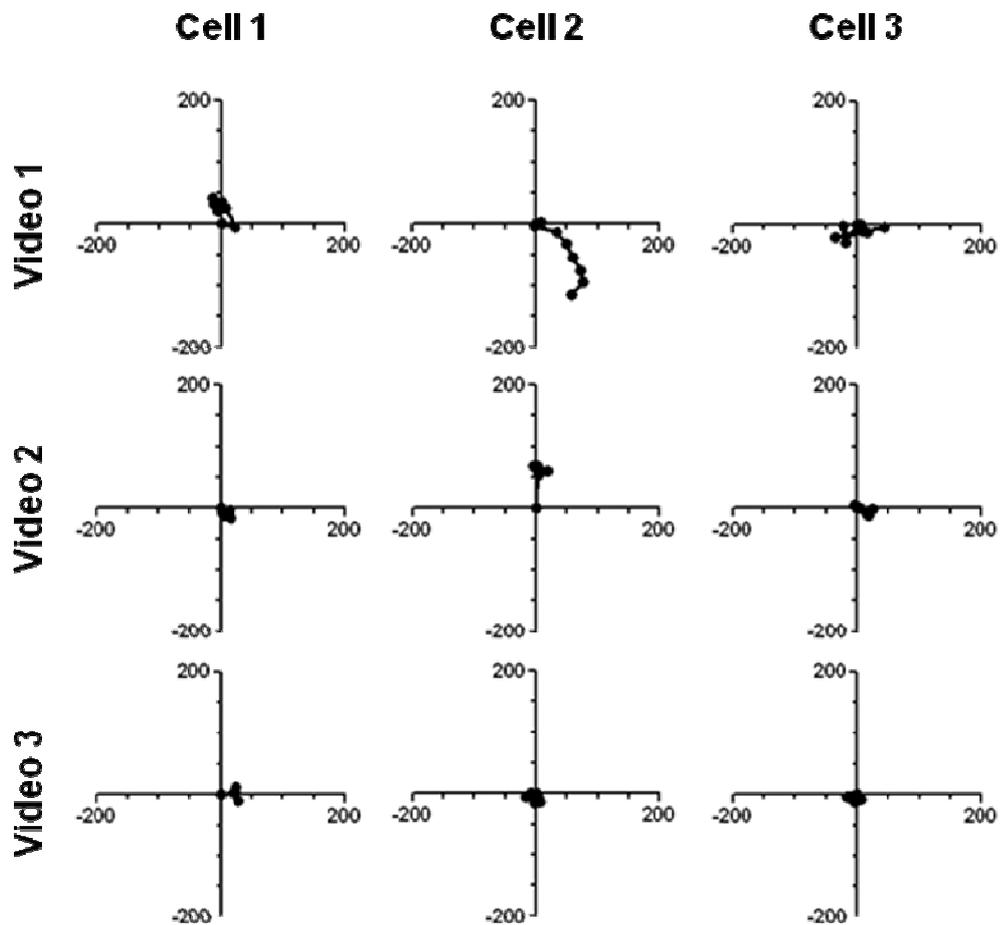
cells, were prepared and photographs taken of the cultures every five minutes for six hours, the resulting photographs were used to produce time-lapse videos. Three cultures of macrophages were also prepared and documented in the same way. A video analysis package was used to calculate the speed of 10 cells from each video in  $\mu\text{m}/\text{hour}$ . To do this the XY position of each of 10 cells in the frame were recorded every three frames (every 15 minutes), and their movement was calculated from their change in location. Examples of the movement of three of the ten cell analysed in each of the serum-free fibrocyte cultures are shown in figure 4.4, and serum-containing in figure 4.5. The movement in  $\mu\text{m}$  from their position at time 0hr is shown for 6 hours. Examples of the movement of three of the ten cell analysed in each of the macrophages cultures are shown in figure 4.6.



**Figure 4.4 Examples of motility of serum-free differentiated fibrocytes.** Three time-lapse photography videos, each of 6 hours in length involving photographs taken every 5 minutes were made of three separate serum-free fibrocyte cultures. Videos were made at day 11 after culturing PBMC in serum-free fibrocyte medium, on glass chamber slides, at  $1 \times 10^6$  cells/ml, non-adherent cells removed at 96 hours. The movement over 6 hours is shown in  $\mu\text{m}$  from their position at time 0hr for three of the ten cells analysed in each video. For videos see supplementary figures S1-S3.



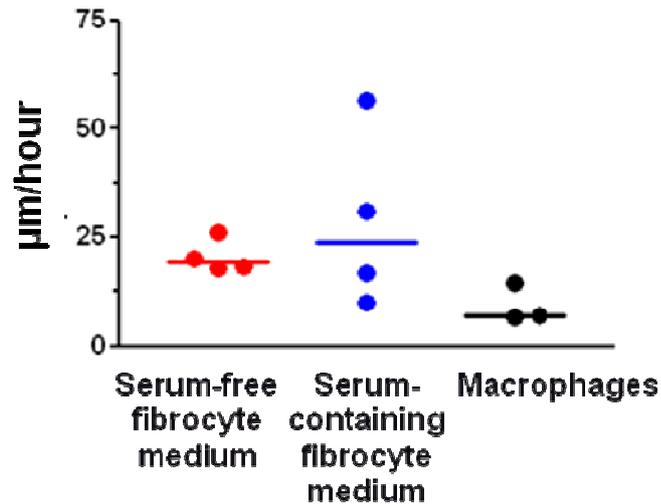
**Figure 4.5 Examples of motility of serum-containing differentiated fibrocytes.** Three time-lapse photography videos, each of 6 hours in length involving photographs taken every 5 minutes were made of three separate serum-containing fibrocyte cultures. Videos were made at day 11 after culturing CD14<sup>+</sup> selected cells, on glass chamber slides, in serum-containing fibrocyte medium at  $2 \times 10^5$  cells/ml. The movement over 6 hours is shown in  $\mu\text{m}$  from their position at time 0hr for three of the ten cells analysed in each video. For videos see supplementary figures S4-S6.



**Figure 4.6 Examples of motility of macrophages.** Three time-lapse photography videos, each of 6 hours in length involving photographs taken every 5 minutes were made of three separate macrophage cultures. Videos were made at day seven after culturing PBMC in macrophage medium on glass chamber slides, at  $1 \times 10^6$  cells/ml, non-adherent cells removed at 96 hours. The movement over 6 hours is shown in  $\mu\text{m}$  from their position at time 0hr for three of the ten cells analysed in each video. For videos see supplementary figures S7-S9.

To calculate the level of motility of each time-lapse video the average speed of the 10 cells was calculated. Despite the initial suggestions that serum-containing fibrocytes were more motile than the serum-free fibrocytes figure 4.7 shows this is not the case. There is more variation in the level of motility seen in the serum-containing fibrocyte cultures, which may be why the

initial suggestion was made, however the mean speed is no different to that of the serum-free fibrocytes. Both types of fibrocytes were more motile than the macrophages however, although not significantly.



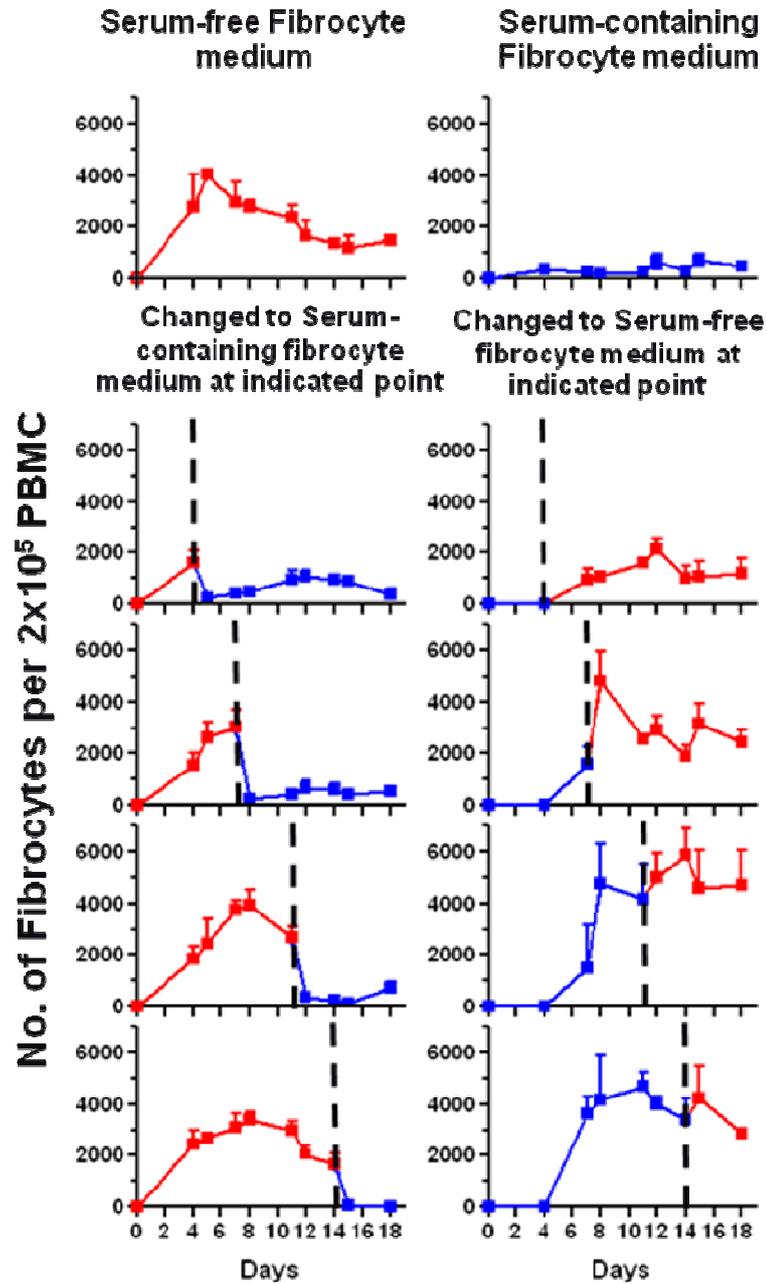
**Figure 4.7 Motility of serum-free, serum-containing fibrocytes and macrophages.** Time-lapse photography videos, each of 6 hours in length involving photographs taken every 5 minutes were made of separate cultures of each of serum-free, serum-containing fibrocytes and macrophages, cultured on glass chamber slides. Videos were made at day seven for the macrophages and day 11 for the fibrocytes. The graph represents the average speed of 10 cells from each video.

For videos see supplementary figures S1-S3 for serum-free fibrocytes, S4-S6 for serum-containing fibrocytes and S7-S9 for macrophages, all found on the disc accompanied with this thesis.

### **4.3 Effect of serum-containing medium on serum-free derived fibrocytes**

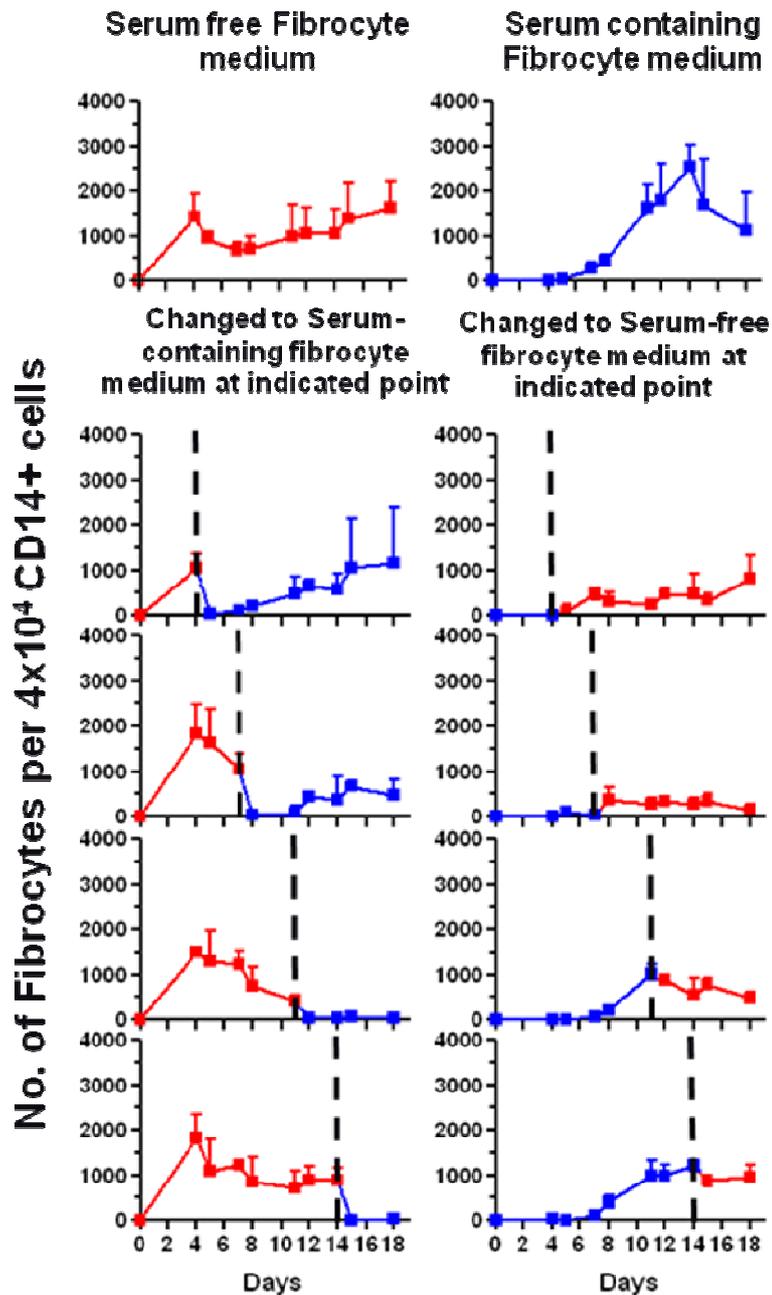
The main difference in the generation of serum-free and serum-containing fibrocytes is the presence of serum in the media. If, as the literature suggests, these are the same cell type, just generated by different protocols then once differentiation has occurred the serum-free fibrocytes should survive in a serum-containing media and the serum-containing fibrocytes in a serum-free media.

To investigate this serum-free fibrocytes and serum-containing fibrocytes were differentiated from PBMC in 96-well plates. Enough wells were set up for five conditions with three replicate wells of each to ensure as precise a fibrocyte count as possible for both media types. At day 4 all wells had their non-adherent cells washed out and the media replaced. At days 4, 7, 11 and 14 the average number of fibrocytes in every set of triplicate wells were calculated, one set then had its media changed, either serum-free to serum-containing medium or serum-containing to serum-free medium. At days 5, 8, 12, 15 and 18 the average number of fibrocytes in every set of triplicate wells was again calculated to see any overnight changes from the change in media. One set remained in the initial medium throughout to act as a control.



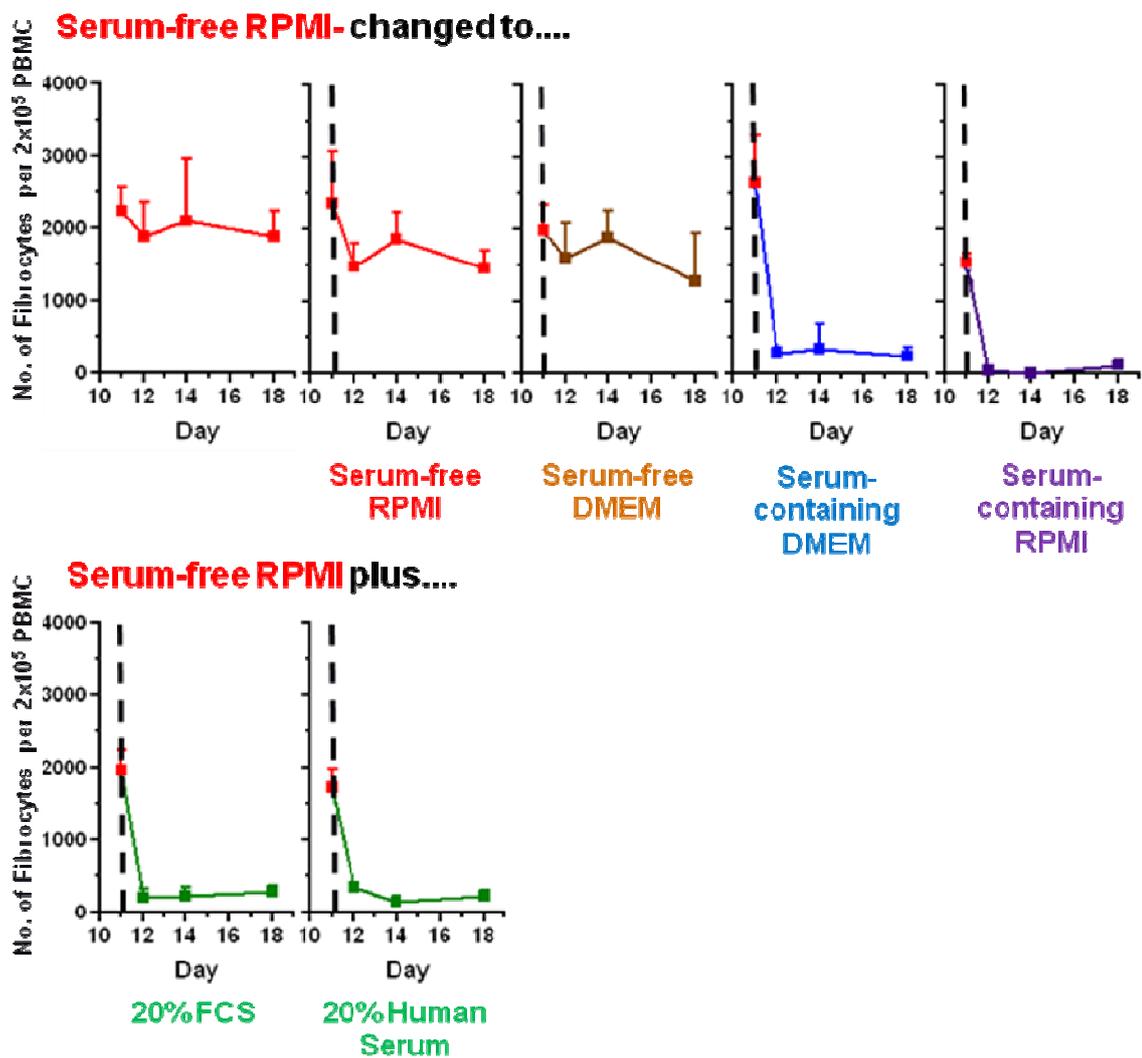
**Figure 4.8** Effect of switching medium on fibrocytes differentiated from PBMC. PBMC were set up in either serum-free or serum-containing fibrocyte media, at  $1 \times 10^6$  cell/ml. At day 4 the non-adherent cells were washed out. On the day indicated (dashed line) the media was replaced with the opposite and the number of fibrocytes counted on days 4, 5, 7, 8, 11, 12, 14, 15 and 18. Cultures in serum-free fibrocyte media are indicated in red and serum-containing in blue. Mean and standard deviation shown for three wells of each condition (representative of  $n=3$  experiments).

The left hand side of figure 4.8 indicates that whichever time point serum-free fibrocyte media was switched to a serum-containing media the number of fibrocytes dropped to almost zero within 24 hours and did not recover for the duration of the experiment. However in the reverse, switching cultures in serum-containing fibrocyte media into serum-free media the same effect was not seen, the serum-containing fibrocytes appeared to be completely unaffected by the change of media. These effects were also observed when the cultures were set up from CD14<sup>+</sup> selected cells in both media types, figure 4.9.



**Figure 4.9** Effect of switching medium on fibrocytes differentiated from CD14<sup>+</sup> cells. CD14<sup>+</sup> selected cells were set up in either serum-free or serum-containing fibrocyte media, at  $2 \times 10^5$  cell/ml. On the day indicated (dashed line) the media was replaced with the opposite and the number of fibrocytes counted on days 4, 5, 7, 8, 11, 12, 14, 15 and 18. Cultures in serum-free fibrocyte media are indicated in red and serum-containing in blue. Mean and standard deviation shown for three wells of each condition (representative of  $n=3$  experiments).

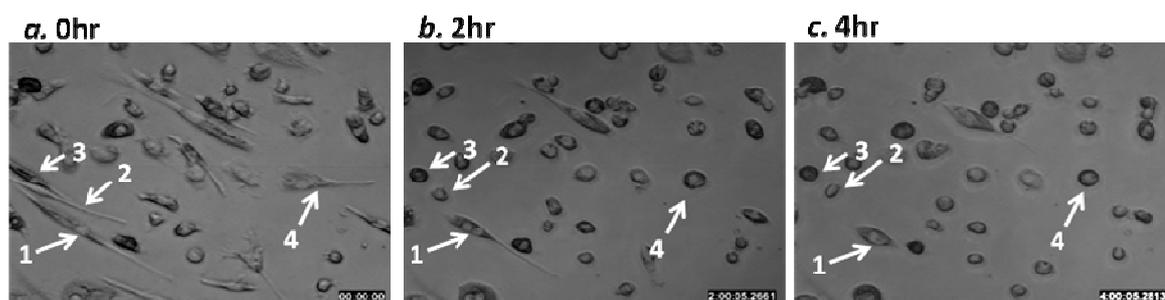
As well as the lack of serum, serum-free and serum-containing medium also differ in the types of media they are made up in, RPMI 1640 in the case of serum-free medium and DMEM in the case of serum-containing medium. To establish if it is the type of media, or the presence of serum in the serum-containing fibrocyte media that is causing this loss of fibrocyte morphology in serum-free derived fibrocytes various combinations of the two medium were made up. A serum-free media was made using DMEM as a base, and a serum-containing fibrocyte medium using RPMI 1640 as a base. PBMC were plated out at  $1 \times 10^6$  cells/ml in serum-free fibrocyte media and the non-adherent cells washed out at day 4. At day 11 one set of three wells were left untouched, one changed to serum-free fibrocyte media and one to serum-free DMEM media, all of which were counted on days 11, 12, 14 and 18 and the number of fibrocytes remained steady, with the change in media having little or no effect. However those wells which were switched into either serum-containing fibrocyte media or serum-containing RPMI 1640 saw a loss in fibrocyte morphology within 24 hours which did not recover for the duration of the experiment (Figure 4.10). This suggested that it is the addition of serum to serum-free cultures and not, either the effect of changing the media, or the base the media is made up in (RPMI 1640 or DMEM) which is causing this loss in fibrocyte morphology. In the same experimental setting FCS and human serum were added to different serum-free fibrocyte cultures and, again, the number of fibrocytes dropped within 24 hours and did not recover for the duration of the experiment either, validating our observation that the loss of cells with a fibrocyte morphology is due to the presence of serum (figure 4.10).



**Figure 4.10 Control experiment to ensure change in fibrocyte morphology is due to addition of serum.** PBMC were cultured for 11 days in serum-free fibrocyte medium then treated with various control conditions. The number of fibrocytes were counted before and after treatment. Mean and standard deviation shown for three wells of each condition (representative of  $n=3$ ).

To observe the effect serum-containing fibrocyte media has on serum-free fibrocytes a culture of serum-free fibrocytes had its media changed to serum-containing media immediately before being videoed using time lapse photography, with photographs taken every five minutes for 4

hours (supplementary figure S10). Figure 4.11 illustrates, using video stills, that the loss in fibrocyte morphology following treatment with serum-containing fibrocyte medium occurs within 4 hours, and that this loss is due to the cells rolling up.

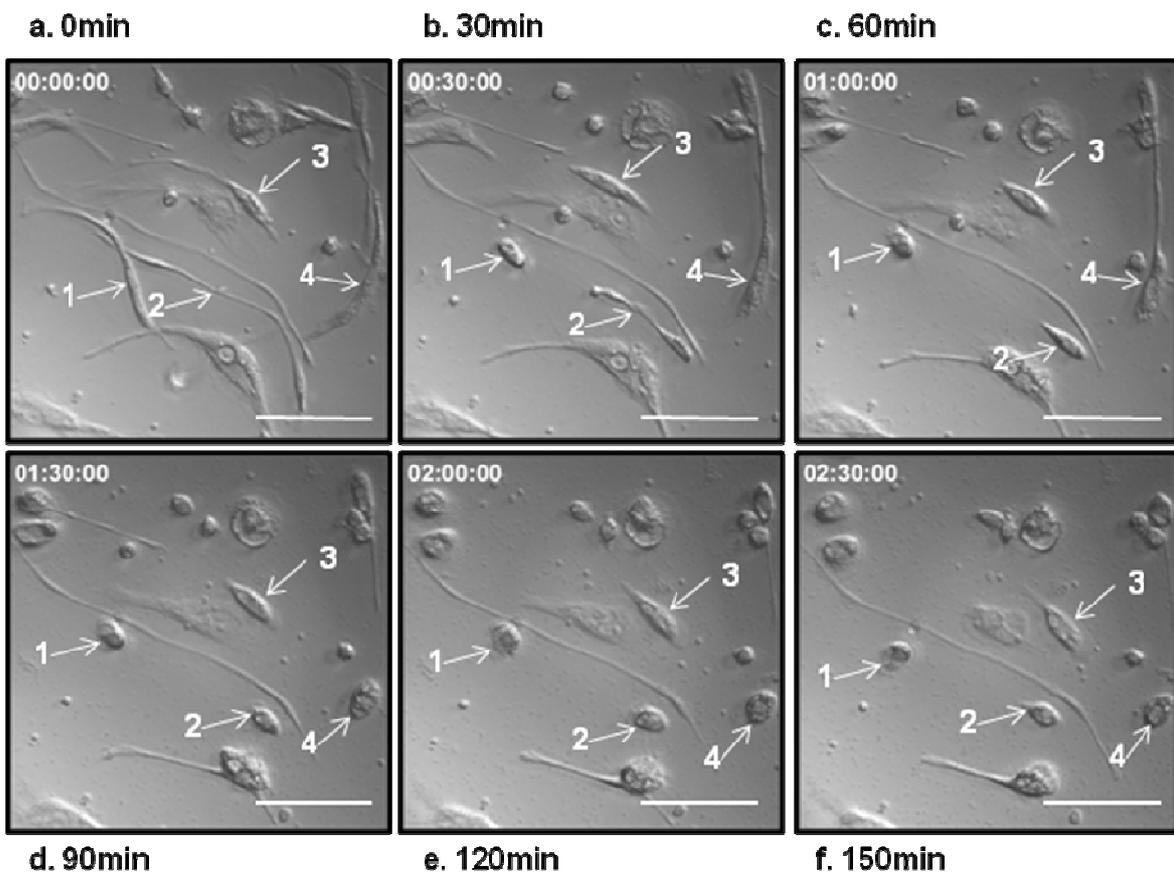


**Figure 4.11. Fibrocytes differentiated in serum-free fibrocytes medium were changed to serum-containing fibrocyte medium and videoed. *a.* Still of video at 0hr. *b.* 2hr. *c.* 4hrs with four cells highlighted. Following the change in medium all the cells have shrunk by 2hrs and completely rolled up by 4hrs (representative of n=3). See supplementary figure S10 for video.**

#### 4.4 Treating serum-free derived fibrocytes with serum

As both FCS and human serum appeared to have the same effect on the number of serum-free derived fibrocytes in culture following serum-containing media treatment this was also looked at using time lapse photography. PBMC were plated out in serum-free fibrocyte media and non-adherent cells washed out at day 4. At day 11 serum was added straight to the cultures, for a final concentration of 20%, as used in the serum-containing fibrocyte media. FCS and human serum were tested, both untreated and heat inactivated (56°C for 30 minutes). The effect of the serum was observed using time lapse microscopy and the serum added immediately before photographs began to be taken. Photographs were taken every five minutes for two and half hours and in all conditions FCS, human serum, untreated or heat inactivated, a loss of fibrocyte

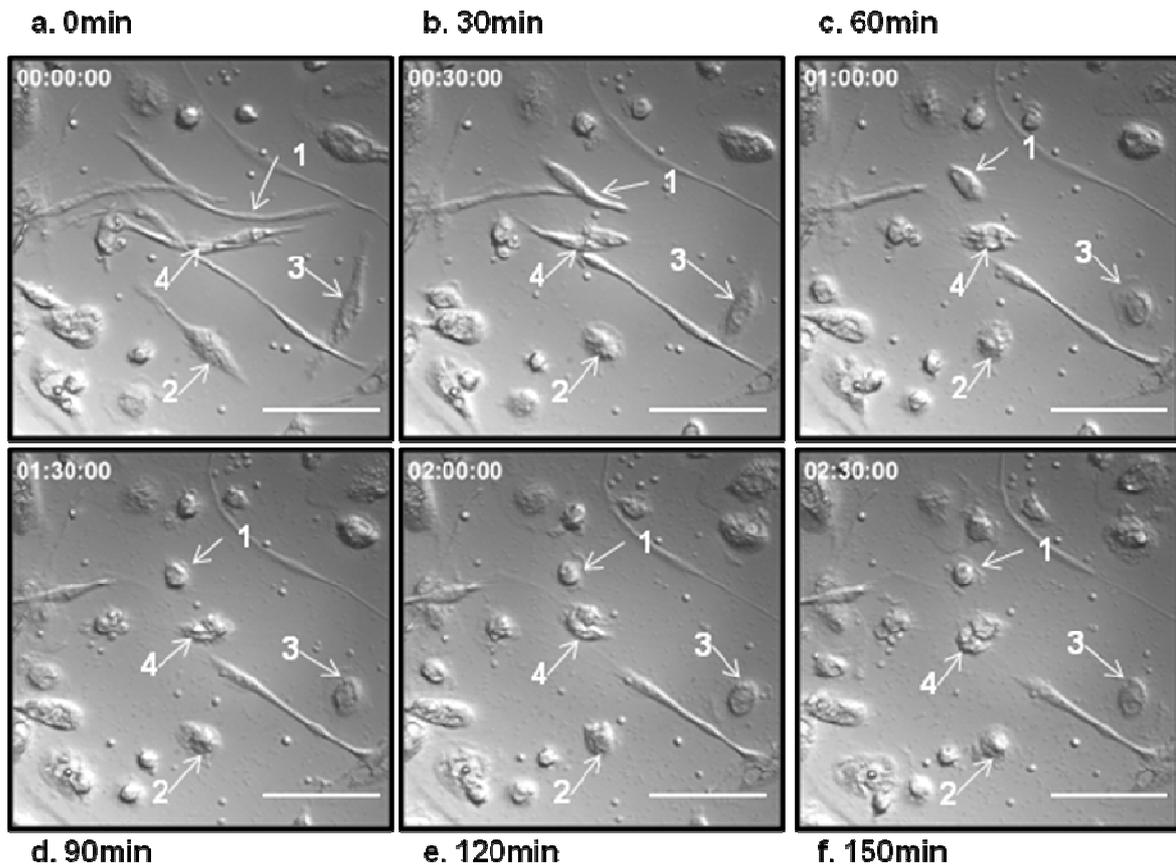
morphology was seen within the two and half hour time frame. Figure 4.12 shows stills from a time-lapse video, every 30 minutes for a serum-free fibrocyte culture treated with FCS at time 0 hours. Four cells are indicated and by two and half hours (150 minutes) all have rounded up. See supplementary figure S11 for video.



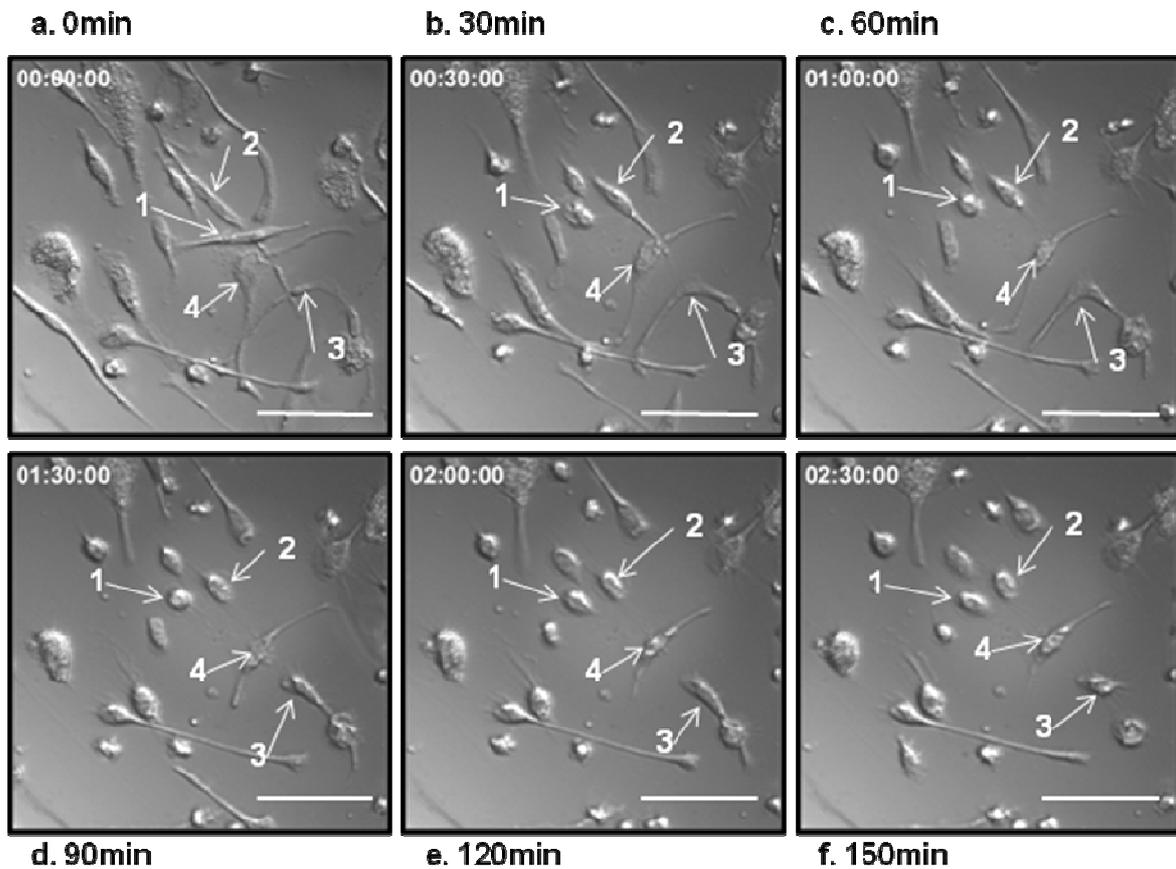
**Figure 4.12 Effect of FCS on serum-free fibrocytes.** PBMC cultured in serum-free fibrocyte media treated with FCS, for a final concentration of 20%. Photographs taken every 5 minutes for 2½ hours. Stills shown for every 30 minutes and the same four cells indicated in every still (n=1). Scale bar indicates 100µm. See supplementary figure S11 for video.

Figures 4.13, 4.14 and 4.15 show stills of serum-free fibrocytes treated with heat-inactivated FCS, human serum and heat-inactivated human serum respectively. The cells were treated

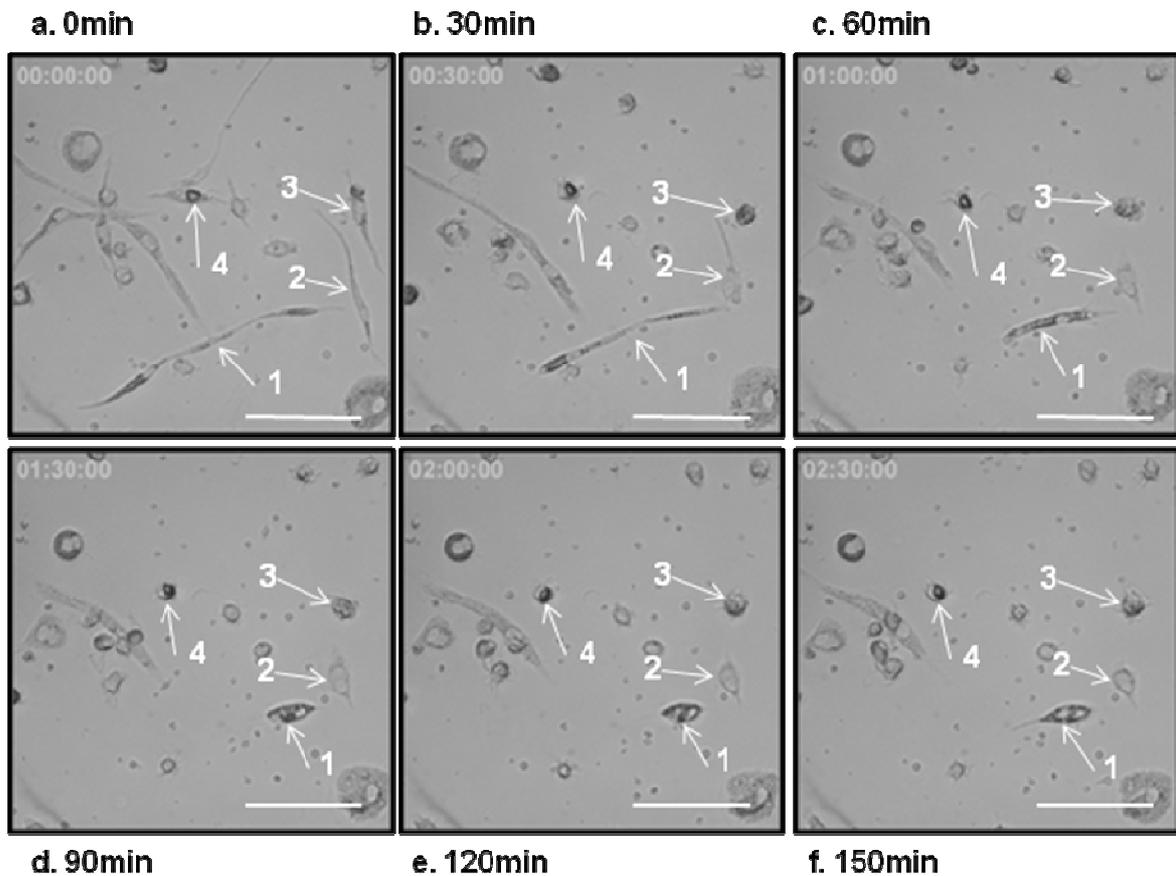
immediately before photographs were taken and four cells followed throughout the two and half hour time frames.



**Figure 4.13 Effect of heat-inactivated FCS on serum-free fibrocytes.** PBMC cultured in serum-free fibrocyte media treated with heat-inactivated FCS, for a final concentration of 20%. Photographs taken every 5 minutes for 2½ hours. Stills shown for every 30 minutes and the same four cells indicated in every still (n=1). Scale bar indicates 100µm. See supplementary figure S12 for video.



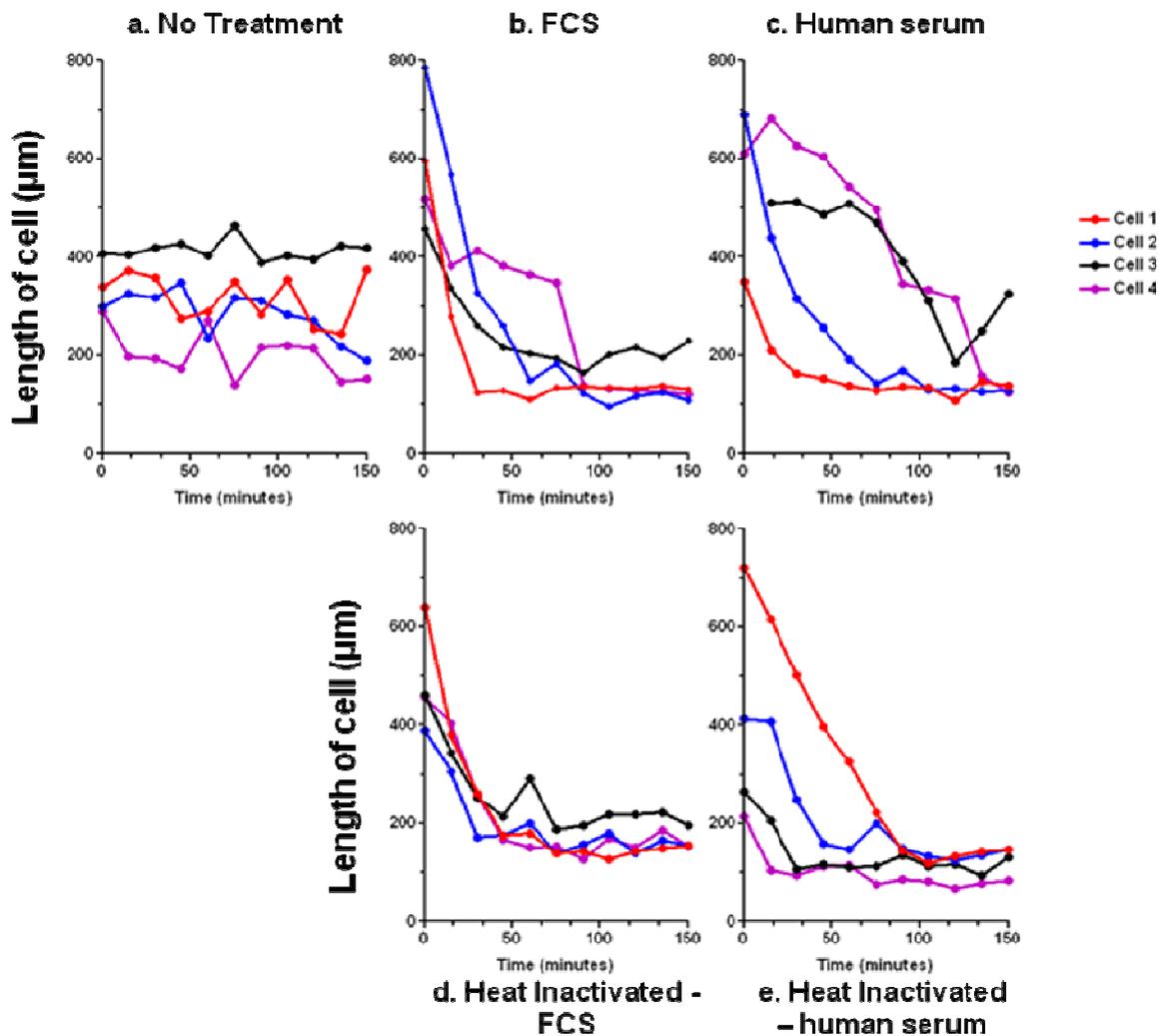
**Figure 4.14 Effect of human serum on serum-free fibrocytes.** PBMC cultured in serum-free fibrocyte media treated with human serum, for a final concentration of 20%. Photographs taken every 5 minutes for 2½ hours. Stills shown for every 30 minutes and the same four cells indicated in every still (n=1). Scale bar indicates 100µm. See supplementary figure S13 for video.



**Figure 4.15 Effect of heat-inactivated human serum on serum-free fibrocytes.** PBMC cultured in serum-free fibrocyte media treated with heat-inactivated human serum, for a final concentration of 20%. Photographs taken every 5 minutes for 2½ hours. Stills shown for every 30 minutes and the same four cells indicated in every still (n=1). Scale bar indicates 100µm. See supplementary figure S14 for video.

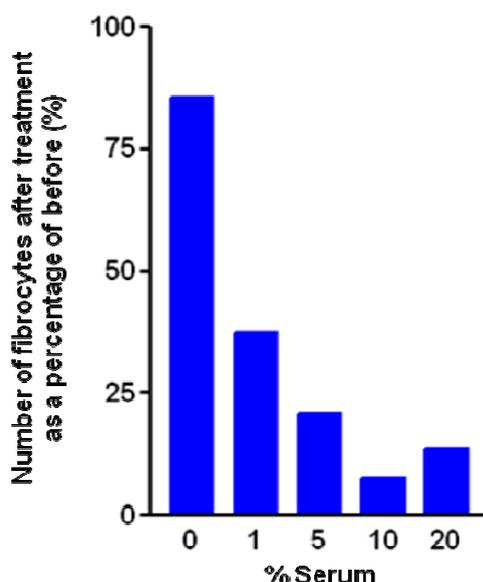
The length of the four cells indicated in figures 4.12 - 4.15 were measured, using the Image pro-plus analysis package, every three frames (15 minutes). The lengths of four cells in an untreated serum-free fibrocyte control video were also measured. Figure 4.16 shows the length of each of these cells over time following treatment with serum at day 11. As illustrated by figures 4.12 - 4.15, figure 4.16 shows that all cells had rolled up within 150 minutes (two and half hours) by

the fact that their lengths reduced quickly following treatment. Whereas the length of the cells in the control video, with no treatment, changed very little over time.



**Figure 4.16** Change in length of serum-free fibrocytes treated with serum over time. PBMC cultured in serum-free fibrocyte media either (a) untreated or treated with (b) FCS, (c) human serum, (d) heat-inactivated FCS or (f) heat-inactivated human serum, for a final concentration of 20% (n=1). Photographs taken every 5 minutes for 2½ hours. Length of four cells recorded every 15 minutes for 150 minutes for each treatment.

The effect that serum has on fibrocyte morphology can also be titrated out, in figure 4.17 the number of fibrocytes seen following treatment with FCS as a percentage of the number of fibrocytes before treatment is shown. Serum free fibrocytes were differentiated from PBMC, non-adherent cells washed out at day 4 and the number of cells calculated on day 11 for each set of three wells. FCS was then added to four of the five sets of wells for final concentrations of 1%, 5%, 10% and 20%. Twenty four hours later the number of fibrocytes were counted again and the percentage difference calculated. Although any serum caused a loss in fibrocyte morphology we can see the effect increases with the amount of serum added until approximately 10%. Therefore whatever protein constituent of serum is having an effect has so gradually, and perhaps needs a certain level to cause a complete loss in fibrocyte morphology.

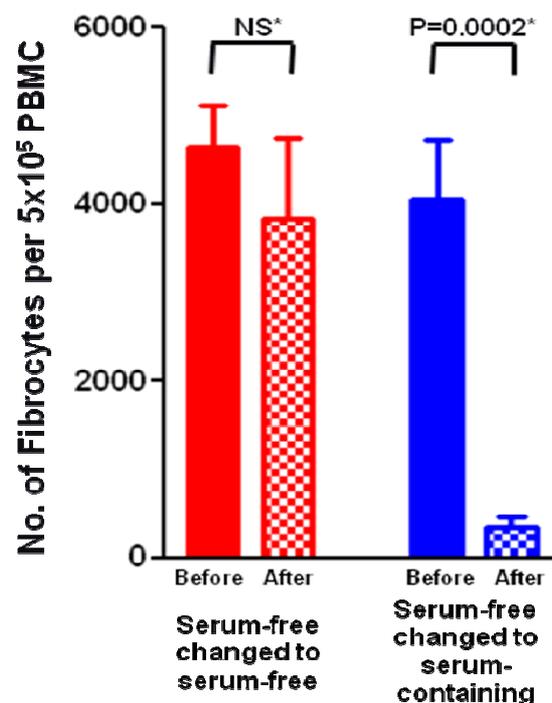


**Figure 4.17 Number of serum-free fibrocytes following a titration of serum.** PBMC cultured in serum-free fibrocyte media and at day 4 non-adherent cells washed out. At day 11 all sets of three wells were counted. FCS was then added for final concentrations of 1, 5, 10 or 20%, three wells per condition. 24 hours later number of fibrocyte counted again and percentage difference compared to before treatment calculated,  $n = 1$ .

#### 4.5 Effect of serum-containing media on marker expression of serum-free fibrocytes

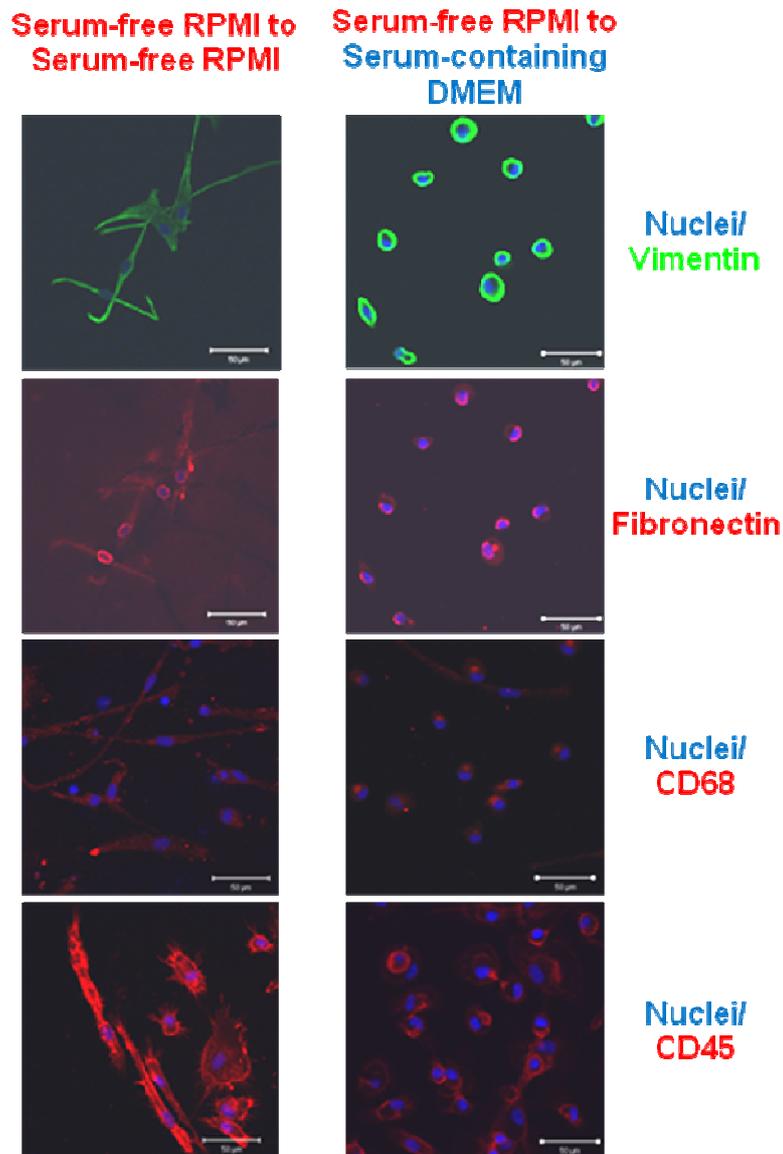
We have previously demonstrated that the loss of fibrocyte morphology following the treatment of serum-free fibrocytes with serum is due to them rolling up, however the cells do not appear to detach and die as an increase in debris within the culture was not observed, neither was a reduction in the number of attached cells, just the number of spindle shaped ones. Therefore the question arises of whether the rolled up cells remain fibrocytes, expressing both haematopoietic and stromal markers, or whether they are de-differentiating when rolling up. To address this serum-free fibrocytes were cultured from PBMC in eight wells of two 8 well

chamber slides, the non-adherent cells washed off at day 4 and at day 11 all the wells of one chamber slide had their medium changed to serum-free fibrocyte media and the other to serum-containing fibrocyte medium. Figure 4.18 shows the average number of fibrocyte per well, for the eight wells which had their media changed from serum-free medium to either serum-free or serum-containing, before and after treatment.



**Figure 4.18 Effect of changing media of serum-free generated fibrocyte to either serum-free or serum-containing fibrocyte medium.** PBMC were cultured in two 8 well chamber slides in serum-free fibrocyte media. At day 11 the number of fibrocytes were calculated in every well and the mean fibrocyte number calculated for each chamber slide. All wells of one chamber slide had their media changed to serum-free fibrocyte medium and all wells of the other chamber slide to serum-containing fibrocyte medium. 24 hours later the mean number of fibrocytes per well on each slide was calculated again. Mean and standard deviation of the number of fibrocytes in all eight wells for each treatment shown. \*Mann-Whitney non-parametric significance test performed on data from each chamber slide.

Those wells which had their media changed from serum-free to serum-free media had approximately the same number of fibrocytes in 24 hours later. However those wells which had their media changed from serum-free to serum-containing media had significantly less fibrocytes following treatment, using the Mann-Whitney non-parametric test of significance. At day 12 all media was removed from all wells and the cultures washed and fixed for immunostaining. Each condition was stained for the markers vimentin, fibronectin, CD68, CD45 and matched irrelevant controls as described in methods and materials. Figure 4.19 demonstrates that, when looking at just these markers, fibrocytes which have rolled up have the same expression profile as spindle shaped fibrocytes.



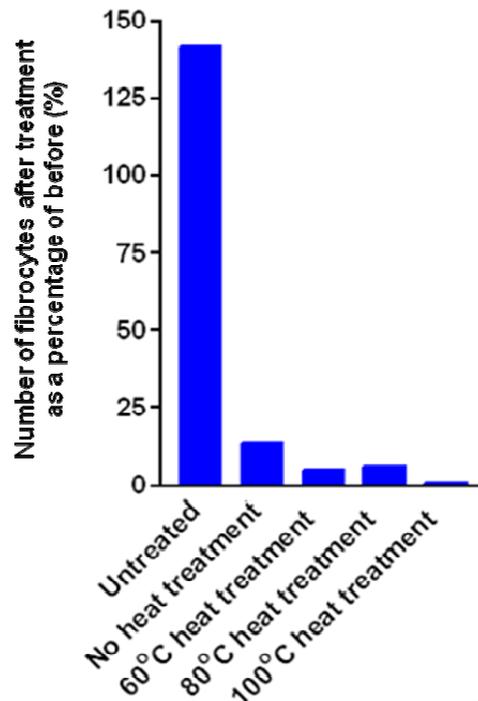
**Figure 4.19** Effect of changing media of serum-free generated fibrocyte to either serum-free or serum-containing fibrocyte medium. PBMC were cultured in two 8 well chamber slides in serum-free fibrocyte media. At day 11 all wells of one chamber slide had their media changed to serum-free fibrocyte medium and all wells of the other to serum-containing fibrocyte medium. 24 hours later the cultures were washed and fixed for staining. The markers vimentin, fibronectin, CD68 and CD45 were all looked at and all seen to be expressed in both conditions. Nuclei are shown in blue, vimentin staining in green and all the rest in red. Scale bar indicates 50µm.

Fibrocytes which have rolled up morphologically look very similar to macrophages and most importantly figure 4.19 shows that these cells still express fibronectin, which macrophages do not express (Figure 3.15). Further work needs to be done, specifically looking at the expression profile of fibrocytes which have rolled at a longer time point than 24 hours to see if they keep this expression profile or lose it, however current observations suggest that although rolled up serum-free fibrocyte treated with serum are still fibrocytes, at least for the first 24 hours.

#### **4.6 Determining what part of serum is causing the loss of fibrocyte morphology**

As discussed in the introduction the plasma protein SAP inhibits the differentiation of fibrocytes in a serum-free environment<sup>47</sup>, due to it binding to the Fcγ receptors<sup>48</sup>. We can hypothesise therefore that the factor in serum which is causing fibrocytes to roll up following treatment with serum is also SAP. SAP is an extremely stable protein known to be able to withstand high temperatures; and is a very large at 235kDa<sup>174</sup>. Some preliminary experiments have been performed to look into this hypothesis. Serum-free fibrocytes were differentiated from PBMC, at day 4 the non-adherent cells washed out and at day 11 the number of fibrocytes calculated for each set of three wells. A set of three wells were either left untreated or were treated with FCS, either normal or heat-treated for 1 hour at 60°C, 80°C or 100°C. The number of fibrocytes were counted 24 hours later and all FCS treatment, whether heat-treated or not caused a loss in

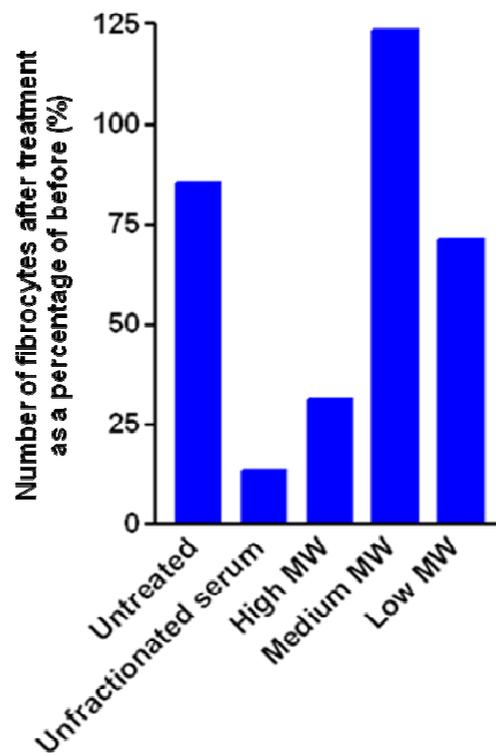
fibrocyte morphology (Figure 4.20), suggesting that the constituent of protein causing this effect on serum-free fibrocytes is extremely heat stable.



**Figure 4.20 Effect of heat-treated FCS on serum-free generated fibrocyte.** PBMC were cultured in serum-free fibrocyte media, at day 4 non-adherent cells were washed out. At day 11 all sets of three wells were counted and then each set either left untreated or treated with FCS which had either not been heat treated or had been treated to 60°C, 80°C and 100°C for one hour. 24 hours later the number of fibrocyte were counted again and the percentage difference compared to before treatment calculated, n=1.

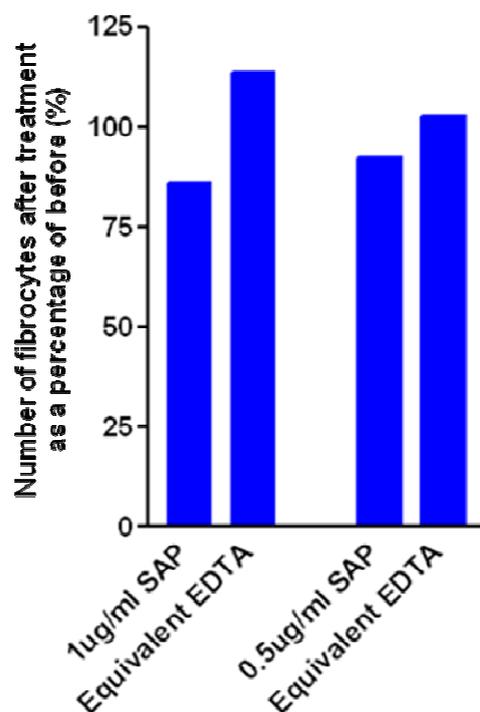
To establish the approximate size of the protein having the effect we have observed on serum-free fibrocytes FCS was fractionated using various filters. A high molecular weight aliquot was obtained containing proteins of > 100,000Da (portion we would expect SAP to be in). A medium weight aliquot of < 100,000Da, > 50,000Da and a low weight aliquot of < 50,000Da were also tested. Serum-free fibrocytes were differentiated from PBMC, at day 4 the non-adherent cells

washed out and at day 11 the number of fibrocytes calculated for each set of three wells. A set of triplicate wells were either left untreated, treated with un-fractionated serum or treated with the different molecular weight aliquots of serum listed above. As figure 4.21 shows only the un-fractionated serum and high molecular weight aliquot caused a loss of cells with fibrocyte morphology, suggesting it is a high molecular weight molecule/particle which is causing this loss in fibrocyte morphology.



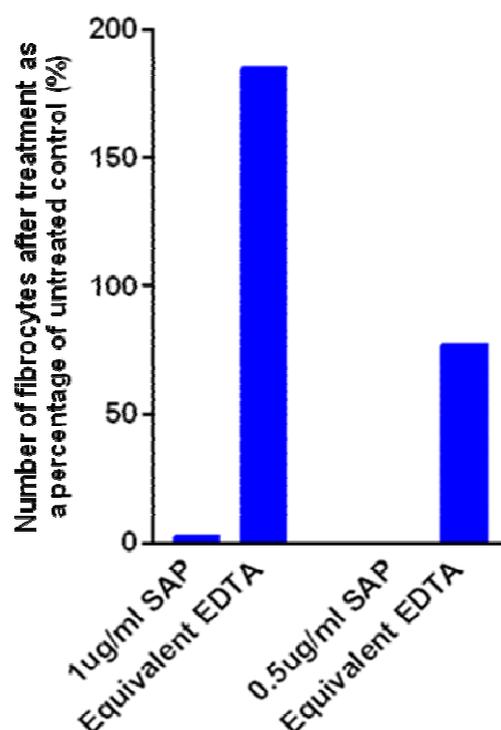
**Figure 4.21 Effect of fractionated FCS on serum-free generated fibrocyte.** PBMC were cultured in serum-free fibrocyte media, at day 4 non-adherent cells were washed out. At day 11 all sets of three wells were counted and then each set either left untreated, treated with un-fractionated FCS or treated with three aliquots of fractionated FCS, > 100,000Da, < 100,000Da but > 50,000Da or < 50,000Da. 24 hours later the number of fibrocyte were counted again and the percentage difference compared to before treatment calculated, n=1.

However when serum-free fibrocytes were treated with commercially available SAP at either 1 $\mu$ g/ml or 0.5 $\mu$ g/ml on day 11 a loss of fibrocyte morphology was not seen (Figure 4.22), despite fibrocyte differentiation being inhibited by the SAP when added at day 0 (Figure 4.23). PBMC were cultured in serum-free fibrocyte media, and at day 4 the non-adherent cells washed out. The commercial SAP is stored in EDTA therefore as a control separate serum-free fibrocyte cultures were also treated with equivalent amounts of EDTA to ensure it was not having an effect. The number of serum-free fibrocytes after treatment with either 1 $\mu$ g/ml or 0.5 $\mu$ g/ml SAP or EDTA controls, as a percentage of before treatment, show that neither the SAP nor EDTA had any effect on fibrocyte morphology as all were over 85%.



**Figure 4.22 Effect of SAP and EDTA equivalents on serum-free generated fibrocyte.** PBMC were cultured in serum-free fibrocyte media, at day 4 non-adherent cells were washed out. At day 11 all sets of three wells were counted and then either treated with SAP at 1µg/ml or 0.5µg/ml, or EDTA controls. 24 hours later the number of fibrocyte were counted again and the percentage difference compared to before treatment calculated, n = 1.

However, in the same experiment a control was set up in which either 1µg/ml or 0.5µg/ml of SAP, or equivalent amounts of EDTA, were added to serum-free fibrocyte cultures at day 0. These showed the SAP inhibiting fibrocyte differentiation, whereas the EDTA did not, when looking at the number of fibrocytes in a treated well as day 11 as a percentage of the number of fibrocytes in an untreated well at day 11 (Figure 4.23). Suggesting that the commercial SAP is active but cannot cause a loss of fibrocyte morphology if given once fibrocytes have differentiated. However further work is required as these are all only n=1 experiments.



**Figure 4.23 Effect of SAP and EDTA equivalents on PBMC cultured in serum-free fibrocyte medium.** PBMC were plated out in serum-free fibrocyte media, 3 wells were treated with either SAP at 1µg/ml or 0.5µg/ml, EDTA controls or left untreated. At day 4 the non-adherent cells were washed out and at day 11 the number of fibrocytes in each set of three wells counted. The percentage difference between the treated and untreated wells were calculated, n=1.

## 4.7 Discussion of chapter

Although it is not mentioned within the literature this chapter demonstrates that the ability of different 'normal' donors to produce both serum-free and serum-containing fibrocytes is highly variable. PBMC and CD14<sup>+</sup> cell samples from some donors produced many fibrocytes however others produce very few; this is seen consistently throughout the donors used in this thesis and does not appear to be influenced by age, or sex of the donor, although no statistical analysis of

this has been completed (Figure 4.2, 4.3). There may be other factors that are affecting these donors that have not been measured in this thesis however. For example one donor, who was noted as not producing many fibrocytes *in vitro*, is known to suffer very badly with hay fever, which may in turn be affecting the monocytes ability to differentiate into fibrocytes. However this is all speculation, and the extent of the allergen responses of other donors whose peripheral blood was unable to differentiate fibrocytes was not determined.

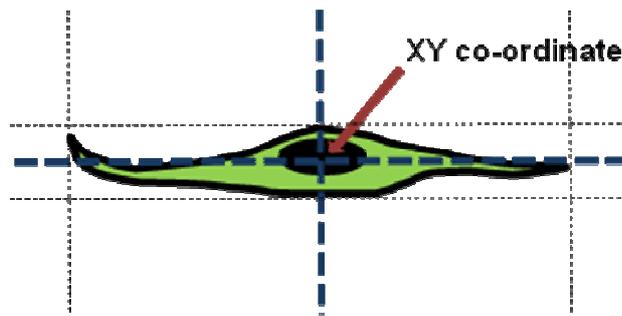
Serum-free and serum-containing derived fibrocytes are described as being the same cell type. In this chapter significantly more serum-free derived fibrocytes were generated from PBMC than serum-containing; whereas significantly more serum-containing derived fibrocytes were generated than serum-free from CD14<sup>+</sup> selected cells (Figure 4.1) when cell numbers were counted based on morphology. We could speculate that following CD14<sup>+</sup> selection something is lacking in the serum-free culture, leading to less cell differentiation; this observation is supported by the literature as both T cells and the cytokines IL-4 and IL-13 have been reported as being important in fibrocyte differentiation<sup>45;46;103</sup>. In contrast more fibrocytes differentiated in serum-containing fibrocyte media following CD14<sup>+</sup> selection compared to PBMC could suggest that something within the PBMC is inhibiting fibrocyte differentiation, and once removed many more fibrocytes are allowed to differentiate. This is supported by reports published by Labet *et al* in 1991 that suggested that T cells prevent fibrocyte differentiation, in contrast to the reports mentioned previously<sup>85;86</sup>. Of course another possibility is that the

positive selection for CD14<sup>+</sup> cells is having a signalling effect on the precursor cells, causing this increased fibrocyte differentiation in serum-containing fibrocyte media.

The monocyte population CD14<sup>+</sup>CD16<sup>-</sup> is reported to release IL10 due to the interaction of the receptor CD14 with its ligand LPS *in vitro*<sup>55</sup>. The release of IL10 is reported as down regulating T<sub>H</sub>1 specific cytokines<sup>175</sup>, which have been shown to inhibit fibrocyte differentiation,<sup>103</sup> therefore perhaps the interaction of the CD14 microbeads with the CD14 receptor is signalling to produce this chain of events that promotes fibrocyte differentiation. However we only see this in the case of serum-containing derived fibrocytes, not the serum-free, which may indicate other factors, not present in the CD14<sup>+</sup> fraction of PBMC are required to get the same level of serum-free derived fibrocyte differentiation.

When looking at the motility of fibrocytes analysis has shown that neither is more motile than the other, although both slightly more than macrophages (Figure 4.7). Compared to other haematopoietic cell populations however all three cell types tested moved very little with the mean speed being less than 25µm/hour (figure 4.7). The speed of un-treated neutrophils is reported as being between  $17 \pm 11 \mu\text{m}/\text{minute}$ <sup>176</sup> (1020 µm/hour) and  $2000 \pm 310 \mu\text{m}/\text{hour}$ <sup>177</sup>. The speed of un-treated granulocytes at  $13.3 \pm 2.8 \mu\text{m}/\text{minute}$  (816 µm/hour)<sup>178</sup>, and un-treated monocytes at  $1.8 \pm 0.3\mu\text{m}/\text{minute}$  (108 µm/hour)<sup>178</sup>. In this thesis the distance moved by these cells is calculated by their change in XY position between frames of the time lapse

video. To calculate the XY position the outer edge of the cell is drawn around and the central XY point of the shape set as the position of the cell, figure 4.24.



**Figure 4.24 Schematic of how XY co-ordinate is calculated in motility analysis.** The cell being analysed is drawn around in each frame and the XY point calculated as the centre of the shape drawn.

This works very well on round cells, however fibrocytes are spindle shaped, so a shuffle of their cytoplasm and nucleus would register as a movement even when the actual position of the cell has not changed. Also, as the length of fibrocytes can be anywhere between 300 and 800 $\mu\text{m}$  (based on own calculations see figure 4.17), an average movement of 25 $\mu\text{m}/\text{hour}$  is barely moving at all. Consequently the technique used here to measure movement is not ideal for use on cells of this shape and a more tailored way of measuring fibrocyte motility would be required to continue this work. A wound healing assay for example may be better, in which a 'wound' is made in a cell monolayer and a time lapse photography used to follow the process of repairing the 'wound'. Having the stimulus of a wound to heal may cause the fibrocytes to be more active, so move more and any differences between them could potentially be identified. Following the correct environmental cues, the migration of these cells could be much more

rapid as they have been reported to respond to the chemokines CCL2 and CCL12 in mouse models of lung fibrosis<sup>124;126</sup>, and CCL21 in a mouse model of renal fibrosis<sup>130</sup>.

Of course it is completely plausible that fibrocytes are really not very motile at all under any circumstances, if they are differentiating from monocytes in the tissue, at the site they are required, as is suggested<sup>49;111;123</sup>, then they don't need to be able to move very far, other than around the site itself. Plus their long thin shape is not advantages for movement either. However if they remain a type of fibrocyte even when rolled up, expressing both haematopoietic and stromal markers, as figure 4.9 suggests, they may be more motile in that state, however further work into whether the expression of fibrocyte markers is retained over time once cells have lost their 'fibroblast-like' morphology is required.

From looking at the time lapse videos it does appear that serum-containing fibrocytes are searching the area around them more, for example putting out more projections and perhaps looking for stimulus to move, however the technique used here to test their motility cannot quantify this as the main body of the cell doesn't tend to move.

When serum-free derived fibrocytes, generated from either PBMC or CD14<sup>+</sup> selected cells, were treated with a serum-containing fibrocyte media they were observed to roll up within four hours and not return to their original shape, at least not within two weeks (length of time observations were made) (Figure 4.8, 4.9, 4.11). This was found to be due to the presence of the

serum in the media and not the media itself (RPMI 1640 verses DMEM), or the act of changing the media (Figure 4.10). When serum was added directly to a serum-free fibrocyte culture the same effect was seen whether the serum was human or bovine in origin, untreated or heat inactivated, suggesting that the constituent of serum having the observed effect is a heat stable, conserved protein or particle (Figures 4.10, 4.12-4.16). Although treatment with serum caused serum-free fibrocytes to roll up and lose their spindle shaped morphology they continued to express both hematopoietic and stromal markers, suggesting they were actually still fibrocytes (Figure 4.19) as other monocyte derived progeny, such as macrophages, did not express these stromal markers. It has been suggested that the presence of IgG molecules and other serum proteins, such as SAP, during the initial immune response would prevent fibrocyte outgrowth, however during the resolution phase, when these molecules are cleared from the site the lower levels of these complexes would allow for the differentiation of fibrocytes and so aid in tissue regeneration and wound healing<sup>138</sup>. This would clearly fit with what happens to the serum-free fibrocytes, and why, in the presence of serum molecules they roll up, and perhaps even become dormant until the environmental cues are present for them to aid in active wound healing.

Fibrocytes which were differentiated in a serum-containing fibrocyte media have been reported as expressing antigen presenting cell markers such as HLA-DR and co-stimulatory molecules, and were able to stimulate T cell responses equivalent to those stimulated by dendritic cells<sup>91</sup>. The fact that we have found that serum-free and serum-containing fibrocytes react differently to serum may suggest that serum-containing derived fibrocytes are involved in the initial immune

response and the initiation of the wound healing process, along with other functions. The serum-free fibrocytes would appear as a second wave to continue with the process and wound healing and tissue regeneration as the environment changes. If the differentiation of serum-containing derived fibrocytes is affected by the interaction of the receptor CD14 with its ligand LPS, it could support their presence quite early on at a site of inflammation, when LPS is possibly present.

The observations made during the motility studies that serum-containing fibrocytes appear to 'search' the area, putting out more projections than the serum-free fibrocytes would also fit with this hypothesis. If these cells are involved to some extent in other processes of an inflammatory site, such as T cell stimulation they would perhaps need to be more aware of their surrounding area. Whereas if the serum-free fibrocytes are appearing when the environment is much calmer they perhaps don't need quite so vigilant to change.

Preliminary experiments suggest that the constituent of serum causing this loss of fibrocyte morphology is very heat stable (Figure 4.20), and greater than 100,000DA in size (Figure 4.21), the same characteristics as SAP. However commercially available SAP does not appear to have the same effect on serum-free fibrocytes as serum does. When commercial SAP was given to differentiated fibrocytes at day 11 it did not cause them to roll up (Figure 4.22), as the serum had, however when given at day 0 it did prevent fibrocyte differentiation in the first place (Figure 4.23). A similar effect was seen in *in vitro* mouse studies by Haudek *et al* in 2008<sup>113</sup>

whereby the addition of SAP, once fibrocyte differentiation had taken place, had no effect on the cells. These are still preliminary experiments and would need to be repeated using both commercially available SAP and donor derived SAP. As well as SAP depleted serum to confirm that it isn't actually another constituent of serum which is causing this loss in fibrocyte morphology, rather than SAP. However the current data suggest that although SAP can inhibit fibrocyte differentiation, it may not be responsible for the change in morphology seen when the cell is a fully formed fibrocyte.

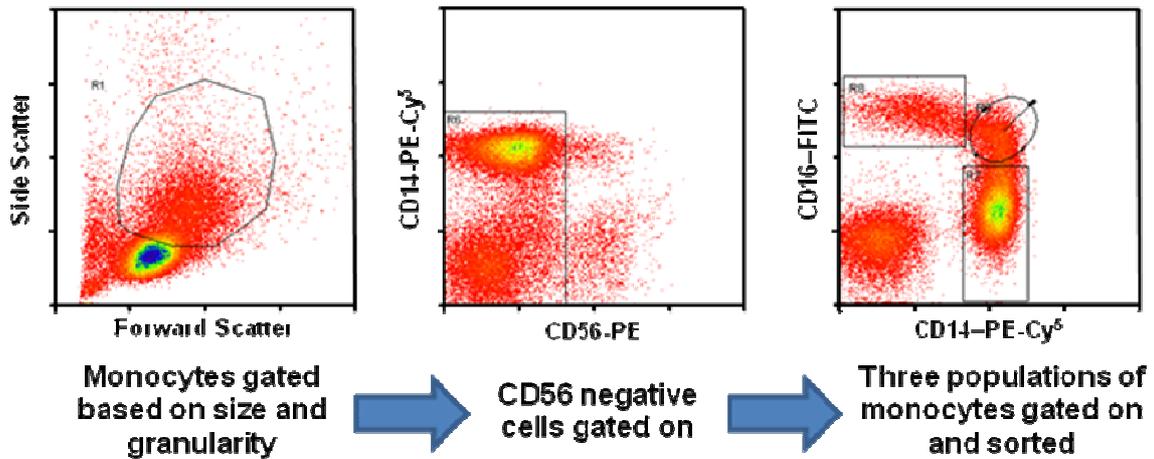
## 5.0 CULTURING FIBROCYTES FROM SORTED MONOCYTE POPULATIONS

### 5.1 Introduction

Monocytes are a population of circulating leukocytes important in the innate immune system involved in linking inflammation and the innate portion of the immune system to the adaptive part. As discussed in the introduction, three subsets of monocytes have been defined, based on phenotype and chemokine expression. The majority of human monocytes, 80-90%, are identified as being CD14<sup>+</sup> CD16<sup>-52</sup>, however two smaller populations of human monocytes have also been reported as CD16<sup>+</sup> CD14<sup>lo</sup> and CD16<sup>+</sup> CD14<sup>+</sup> <sup>51</sup>. These three populations of monocytes are thought to have different roles within the immune system<sup>40;51;53-55</sup> and it has been suggested that some of these monocyte populations are more adept than others at differentiating into different progeny. Studies into the differentiation of osteoclasts from monocytes have found that the CD16<sup>-</sup> fraction of monocytes formed ten times as many osteoclasts as the CD16<sup>+</sup> fraction<sup>44</sup>. Fibrocytes are reported as differentiating from the CD14<sup>+</sup> fraction of PBMC<sup>45-49</sup> and previous results reported in this thesis suggest that monocyte derived serum-free and serum-containing fibrocytes are different cell types. Therefore whether they differentiate from the same, or different monocyte subsets could be informative on further analysing the differences seen between them. The aims of this chapter are to determine which population of monocytes both types of fibrocytes differentiate from.

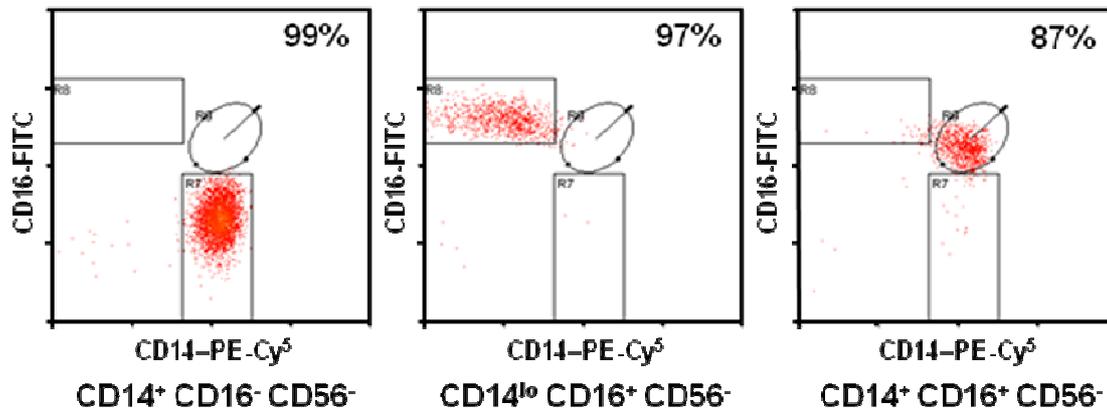
## 5.2 Cell sorting monocyte subsets

Monocyte populations were isolated using fluorescence-activated cell sorting, as discussed in the materials and methods. Briefly PBMC were isolated and labelled with antibodies against human CD14, CD16 and CD56 surface markers. These cells were then put through a cell sorter and, using logical gating, the three monocyte populations identified by surface marker expression. Figure 5.1 illustrates the logical gating used when cell sorting monocyte subsets. Firstly, using a forward verses side scatter plot, monocytes were identified by their size and granularity and selected. A plot showing the expression of CD14 and/or CD56 on the selected cells was then used to isolate only the CD56<sup>-</sup> fraction of these cells, CD56 is expressed on natural killer cells (NK cells), which can be a similar size and granularity to monocytes, by gating out any CD56<sup>+</sup> cells any of these cells included in the forward/side scatter gate are now excluded. Finally, by using both of these gates, three populations of monocytes expressing combinations of both CD14 and CD16 could be identified.



**Figure 5.1 Logical gating when sorting monocyte populations from PBMC.** To sort monocyte populations PBMC were antibody stained with the surface markers CD14, CD16 and CD56, these cells were then put through a cell sorter. To identify the required cell populations firstly monocytes were gated on using forward and side scatter properties. The CD56/CD14 expression of cells within this gate were then looked at and a second gate made around the CD56 negative population. The expression of CD14/CD16 cells were looked on cells which fell within both of these gates. Gates were drawn around the three populations of monocytes, CD14<sup>+</sup>CD16<sup>-</sup>, CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>lo</sup>CD16<sup>+</sup>, and cells expressing each of these sets of markers sorted into separate populations.

Having identified the three populations of monocytes these were sorted into separate populations and the purity of each population checked. Figure 5.2 illustrates the purity of an example sort, here the sample of the larger population of monocytes, CD14<sup>+</sup> CD16<sup>-</sup>, was 99% pure, with the other two populations, CD14<sup>lo</sup> CD16<sup>+</sup> and CD14<sup>+</sup> CD16<sup>+</sup>, 97% and 87% pure respectively.



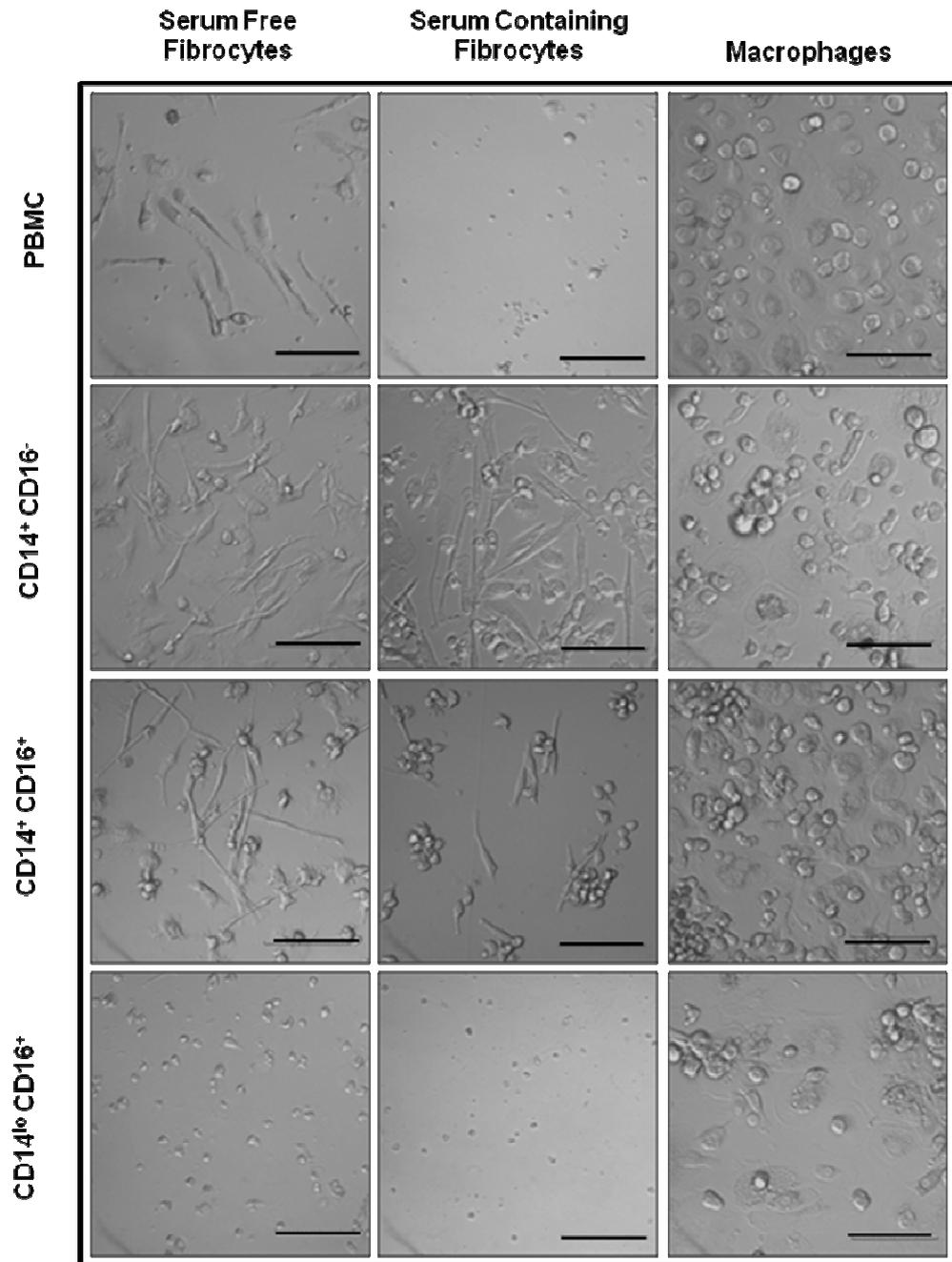
**Figure 5.2 Purity check of sorted monocyte populations.** Once the three populations of monocytes have been sorted, CD14<sup>+</sup>CD16<sup>-</sup>, CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>lo</sup>CD16<sup>+</sup>, a small number of the sorted cells were run back through the machine to check the purity. In the case of the sort shown in this figure the CD14<sup>+</sup>CD16<sup>-</sup> population was 99% pure, the CD14<sup>lo</sup>CD16<sup>+</sup> population 97% pure and the CD14<sup>+</sup>CD16<sup>+</sup> population 87% pure. An example of  $n = 17$ .

For this work seventeen separate sorts were performed, with fifteen different donors, the purity of the CD14<sup>+</sup>CD16<sup>-</sup> populations were  $\geq 98\%$ , the CD14<sup>lo</sup>CD16<sup>+</sup>  $\geq 85\%$  and the CD14<sup>+</sup>CD16<sup>+</sup>  $\geq 80\%$ . The lower purities for the two smaller populations were due to the proportion of PBMC they actually constituted. Total monocytes consist of between 5 and 10% PBMC<sup>50</sup>, however the large majority are the CD14<sup>+</sup>CD16<sup>-</sup> population. For the sort shown in figures 5.1 and 5.2 the percentage of CD14<sup>+</sup>CD16<sup>-</sup> cells of the total events was 4.5%, and for both CD14<sup>lo</sup>CD16<sup>+</sup> and CD14<sup>+</sup>CD16<sup>+</sup> populations it was 0.9%. Sorting cells of such small proportions of the total population can make it very difficult to get high purities. However, although all CD14<sup>lo</sup>CD16<sup>+</sup> populations were at least 85% pure the mean purity across the seventeen sorts was actually 93%; and the mean purity for the CD14<sup>+</sup>CD16<sup>+</sup> population 89%.

### **5.3 Differentiating fibrocytes and macrophages from sorted monocyte populations**

Once the three populations of monocytes had been sorted as many conditions as possible were set up to differentiate fibrocytes or macrophages. Ideally three wells for each population were set up in a 96 well flat bottomed plate in either serum-free, serum-containing fibrocyte medium or macrophage media. However this was not always possible due to cell number constraints so although cells were always set up at  $2 \times 10^5$  cells/ml, determined as the ideal cell concentration using CD14<sup>+</sup> selected cells (Figure 3.9), not all conditions were always set up.

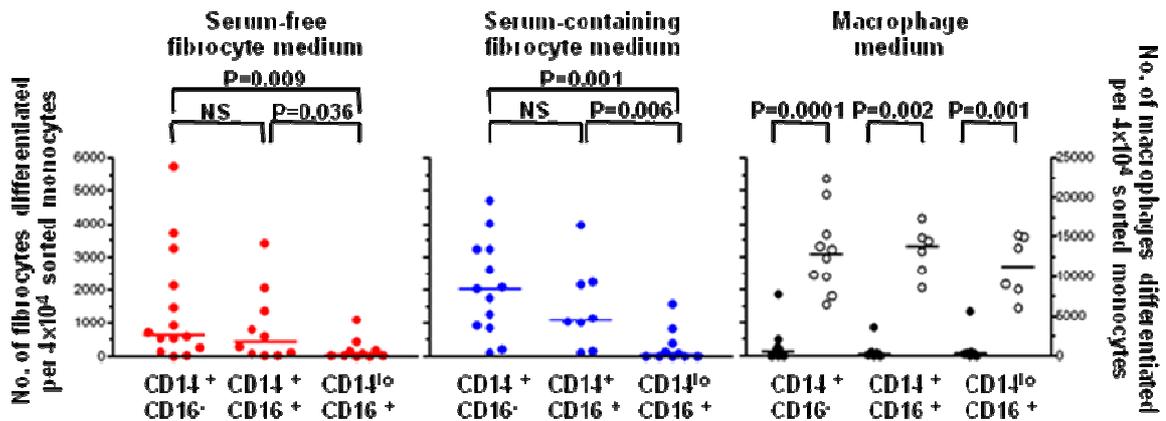
As a control PBMC, taken before sorting, were also set up in each media type at  $1 \times 10^6$  cells/ml and at day 4 the non-adherent cells washed out of these cultures. Figure 5.3 shows photographs of each culture condition at day 11 for an example sort to demonstrate the differentiation of fibrocytes and macrophages from each population.



**Figure 5.3 Photographs of differentiated fibrocytes and macrophages from sorted monocyte populations.** Once the three populations of monocytes have been sorted, CD14<sup>+</sup>CD16<sup>-</sup>, CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>lo</sup>CD16<sup>+</sup>, they were plated out at  $2 \times 10^5$  cell/ml in either serum-free, serum-containing fibrocyte media or macrophage media. As a control matched PBMC were also plated out and the non-adherent cells washed out at day 4. At day 11 an example of each culture was photographed. Scale bar indicated 100 $\mu$ m. Example of n = 17 shown.

Figure 5.3 demonstrates that each population of monocytes, as well as PBMC differentiated into macrophages, identified by their fried egg-like appearance. However the monocyte population  $CD14^{lo}CD16^{+}$  did not differentiate into fibrocytes in either the serum-free or serum-containing fibrocyte medium, whereas both  $CD14^{+}CD16^{-}$  and  $CD14^{+}CD16^{+}$  populations did. For this particular donor serum-containing fibrocytes were also not seen to differentiate from PBMC, as discussed previously in chapter 4.

As well as photographs at day 11 the number of fibrocytes or macrophages in each culture condition were calculated, based on cell morphology and as an average of triplicate wells, the results are seen in figure 5.4.



**Figure 5.4 Number of fibrocytes and macrophages differentiated from sorted monocyte populations.** Once the three populations of monocytes have been sorted, CD14<sup>+</sup>CD16<sup>-</sup>, CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>lo</sup>CD16<sup>+</sup>, they were plated out at 2x10<sup>5</sup>cell/ml in either serum-free, serum-containing fibrocyte media or macrophage media. At day 11 the number of fibrocytes and macrophages were counted based on morphology. Closed circles indicates fibrocytes, open circles macrophages (at least n=6 for each condition). Mann-Whitney, non-parametric, paired statistical test used.

In figure 5.4 cells of a fibrocyte morphology are shown with closed circles and cells of a macrophage morphology shown as open circles. Cells differentiated in serum-free fibrocyte media are shown in red, serum-containing in blue and macrophage media in black. The observation that neither serum-free, or serum-containing fibrocytes differentiate from the CD14<sup>lo</sup>CD16<sup>+</sup> population of monocytes made when looking at the photographs in figure 5.3 were confirmed in the counting data from each culture. By using the Mann-Whitney non-parametric paired test for significance significantly more fibrocytes were seen to differentiate in both serum-free and serum-containing fibrocyte medium from both the CD14<sup>+</sup>CD16<sup>-</sup> and CD14<sup>+</sup>CD16<sup>+</sup> populations of monocytes, compared to the CD14<sup>lo</sup>CD16<sup>+</sup> population. Despite all three populations giving comparable numbers of differentiated macrophages. Also to note is that very

few fibrocytes were seen to differentiate in macrophage medium, significantly less than the number of macrophages from each population.

## 5.4 Discussion of chapter

Three populations of monocyte can be purified from PBMC based on size, granularity, and the surface markers CD56, CD14 and CD16. Once sorted these monocyte populations were plated out in serum-free, serum-containing fibrocyte medium and macrophage medium, and at day 11 the number of differentiated cells calculated. Significantly more fibrocytes were seen to differentiate from both the CD14<sup>+</sup>CD16<sup>-</sup> and CD14<sup>+</sup>CD16<sup>+</sup> populations in both fibrocyte medium compared to the CD14<sup>lo</sup>CD16<sup>+</sup> population, despite all three populations being able to differentiate into macrophages (Figure 5.4), illustrated in figure 5.3. Although serum-free and serum-containing fibrocytes appear to be different cell types, based on the data discussed in chapter 4, they both differentiate from the same two subsets of monocytes (CD14<sup>+</sup>CD16<sup>-</sup>, CD14<sup>+</sup>CD16<sup>+</sup>), and interestingly neither from the third (CD14<sup>lo</sup>CD16<sup>+</sup>).

Although the origin of fibrocyte differentiation has not been examined in the literature in the same way as it is here, the population of monocytes that fibrocytes differentiate from is discussed as being the CD14<sup>+</sup>CD16<sup>-</sup> fraction<sup>47;48;145</sup>. Although a number of papers have shown that CD14<sup>+</sup> cells are required for fibrocyte differentiation<sup>45;46;48</sup> they do not necessarily show that fibrocytes do not differentiate from CD16<sup>+</sup> cells which also express CD14, or to the extent that CD14<sup>+</sup> expression is required, as the third monocyte population looked at in this chapter

actually has a low expression of CD14 rather than none at all. The population of monocytes that differentiates into fibrocytes is known to express the FC $\gamma$  receptors FC $\gamma$ R1(CD64) and FC $\gamma$ RII (CD32), as the interaction of these receptors with a ligand inhibits fibrocyte differentiation<sup>48</sup>, both the CD14<sup>+</sup>CD16<sup>-</sup> and CD14<sup>+</sup>CD16<sup>+</sup> populations have been described as expressing these receptors<sup>61</sup>. The data presented in this thesis supports these findings by showing that it is the level of CD14 that is the defining marker of fibrocyte precursors and that CD16 does not affect this process.

As osteoclasts have also been described as differentiating from a particular subset of monocytes (CD16<sup>-</sup>)<sup>44</sup> these data suggests that the differentiation potential of monocytes is different for the different subsets. Although all three subsets were able to differentiate into macrophages it may be that as the monocytes role within the immune system itself changes as does its ability to differentiate into different progeny, this has previously been suggested to explain the heterogeneity seen in monocyte populations. As the monocytes develop and mature in the blood they can be recruited to the tissue at various points in this maturation and the point they leave the blood and enter the tissues may actually define their ability to differentiate into different progeny<sup>179;180</sup>.

## 6.0 TRANSCRIPTOME ANALYSIS

### 6.1 Introduction

As discussed previously fibrocytes are haematopoietic cells with a spindle shaped, fibroblast-like morphology<sup>45;47;48;87;90;91;103</sup>. Two methods of differentiating these cells are both widely used within the literature, either in the absence<sup>47;48;103</sup> or presence<sup>45;87;90;91</sup> of serum. Data in this thesis so far suggests that, although very similar, fibrocytes differentiated by these two methods may represent different forms of the same cell type. To look at this in more detail the mRNA profiles, or transcriptomes, for both serum-free and serum-containing fibrocytes were examined, comparing them to each other as well as another monocyte derived progeny, macrophages and *in vitro* derived fibroblast cell lines.

### 6.2 Transcriptome analysis

Transcriptome analysis is a form of analysis used to compare data at the population rather than the individual cell level. The majority of cells within the body start out with the same DNA, (exceptions being sperm and eggs and certain cells of the immune system) however it is the way this DNA is translated, and then transcribed into mRNA which determines the final differentiation state of a cell. Cells that receive the same biological cues will transcribe a similar set of genes and ultimately become very similar cells. The total gene set that these cells transcribe is called their transcriptome, therefore cells of the same population will have very

similar transcriptomes; and by comparing the transcriptomes of different cell populations important differences between them can be determined. Transcriptome analysis can also be used as a tool in research into diseases, with the potential to suggest therapeutic targets and diagnostic indicators. Work done by Ramilo *et al* in 2007 suggested that microarray analyses of patients peripheral blood leukocytes might assist in the differential diagnosis of infectious diseases<sup>160</sup>, as they found that leukocytes fighting diseases such as influenza A virus, gram-negative (*Escherichia coli*) or gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*) bacteria had gene expression profiles which could be used to identify the infectious agent, therefore assisting in diagnosis<sup>160</sup>. Classification of certain types of cancer has also been improved by including transcriptome analysis as a tool. When looking at human acute leukaemia transcriptome analysis was found to easily discriminate between acute myeloid (AML) and acute lymphoblastic leukaemia (ALL)<sup>161</sup>, and work on large B-cell lymphoma (DLBCL), a clinically heterogeneous subtype of non-Hodgkin's lymphoma, showed that by using transcriptome analysis previously undetected and clinically significant subtypes of cancer could be identified<sup>162</sup>. In 2009 transcriptome analysis of adrenocortical carcinomas identified differentially expressed genes associated with malignancy, survival and hormonal activity that will go on to be investigated as possible drug targets/diagnostic or prognostic markers<sup>181</sup>.

DNA-binding regulatory proteins modulate which areas, or genes of the DNA are transcribed, or not, as they can act as both activators and repressors. There are only a finite number of these proteins that act all over the DNA, regulating the expression of a much larger number of genes,

allowing the cell to modify which genes are transcribed in response to exogenous stimuli. When the human and chimpanzee genomes were compared they were found to share 98.7% identity in genomic DNA sequences, however the two organisms are functionally diverse, illustrating how the way the DNA is translated has such a large effect on the final differentiation of individual cells and ultimately the development of an organism<sup>182</sup>. However, changes in the way an identical set of genes is regulated during differentiation will also have an effect on the total mRNA transcribed from the genome. These changes can be measured by microarray analysis and may help in determining lineage relationships between cell populations. By comparing the transcriptomes of fibrocytes to other monocyte derived progeny, such as macrophages, whether fibrocytes are a unique cell type, or just elongated macrophages, (which has also been discussed within the literature<sup>85;86</sup>) can be determined. By looking at the relationships of these two types of fibrocytes (serum-free and serum-containing) to macrophages, the question of whether there are two cell types or just one can also be examined. In some cases the literature suggests that fibrocytes are a possible precursor for fibroblasts<sup>124;145;183</sup>, this can be addressed by comparing these transcriptomes and looking at the lineage relationships between them.

Transcriptome analysis of peripheral blood monocytes, and tissue resident macrophages and DCs from the lungs of mice has been performed to look at the emigration and pulmonary differentiation of monocytes under non-inflammatory conditions<sup>184</sup>. They were able to identify differentially activated genetic programs in the circulating monocytes and their lung progeny. They found that lung DCs activated a diverse set of gene families, but largely preserved their

ability to emigrate, exhibiting a profile with high expression levels of integrin and chemokine/chemokine receptors. However, the macrophages down-regulated the same set of genes, but strongly up-regulated a distinct set of matrix metalloproteinases, potentially involved in tissue invasion and remodelling<sup>184</sup>. This work demonstrates that despite differentiating from the same population the pulmonary DCs and macrophages are transcriptionally distinct under non-inflammatory conditions, and the differences between these cell types can be identified by using this tool.

### 6.3 Aims of this chapter

The aims of this transcriptome analysis study are to determine the transcriptional relationship of both serum-free and serum-containing fibrocytes to each other, and to *in vitro* derived, donor matched macrophages; and fibroblast cell lines. However the comparisons which are being performed will also have great importance in the levels of discrimination seen and this must be considered when interpreting the results.

During the course of this analysis clustering of samples, based on their transcriptional characteristics will be used to look at the lineage relationships between the samples. The hierarchical cluster analysis uses Euclidean linkage to cluster samples based on their transcriptome, and illustrates their relationships in the form of a tree. Principal component

analysis also uses Euclidean linkage to cluster, however to illustrate the clusters it uses a three dimensional plot.

As well as clustering based on the entire transcriptome a statistical analysis of microarrays (SAM) determines those genes that are significantly different between the populations . The list of significantly different genes can then be used to cluster the samples, and can potentially provide more accurate clustering. However the more genes found to be significantly different by SAM analysis does not necessarily mean the more variation there is between cell populations. Distantly related cells may be distinguishable by a small number of genes with large consistent differences, however cells that are more closely related may only be distinguishable by subtle small changes seen within large number of genes.

Predictive analysis of microarrays (PAM) looks at the differences between cell populations, this is an alternative method of analysis to SAM and is a statistical technique for class prediction from gene expression data, using nearest shrunken centroids. It was described by Tibshirani *et al* in 2002 and is used to establish those genes that are the most reliable classifiers of two or more groups<sup>185</sup>. It uses part of the data (prediction set) to predict a list of genes that characterise those samples and then tests these predictive genes on the remaining samples (test set), to see if by just using those genes the sample type can be determined. The gene lists provided by both SAM and PAM analysis can be used to indicate potential gene populations, or individual genes, that are important in separating out the cell populations.

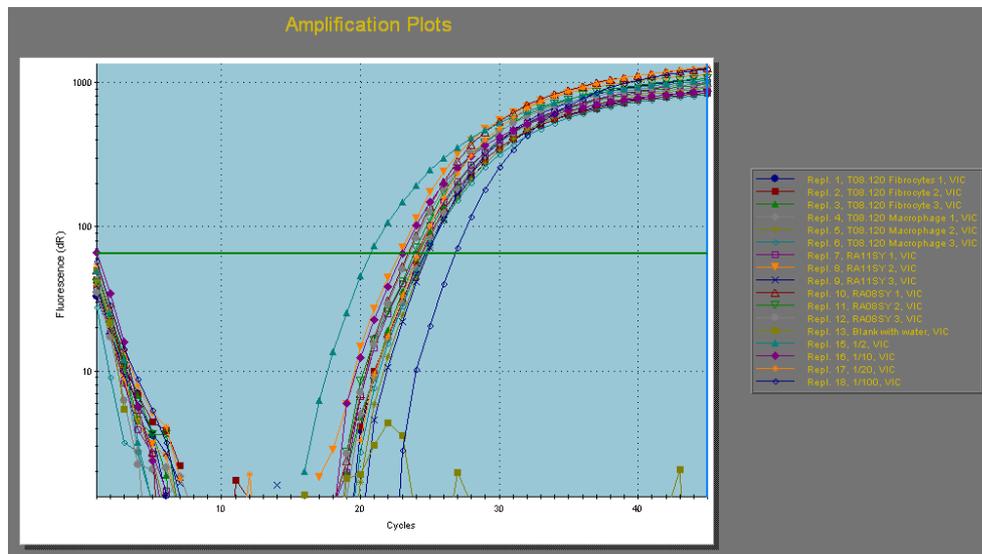
## 6.4 Preparing samples for transcriptome analysis

As discussed in the materials and methods cultures of serum-free, serum-containing fibrocytes, macrophages and fibroblast cell lines were prepared. As neither fibrocytes nor macrophages differentiate as homogeneous populations, and are extremely adherent, these cells were differentiated and the cultures then lysed in the well. Both the number of fibrocytes and macrophages were counted to calculate the purity based on cell morphology. The same three donors were used for all of the *in vitro* derived cells, therefore providing three biological replicates. For each cell type from each donor three experimental replicates were prepared, in which cells had been set up to differentiate from the same donor on the same day, however from that point onwards were treated as three separate replicates. Three different fibroblast cell lines were used, derived from three different donors, although not donor matched to the macrophages and fibrocytes. For each cell line three aliquots were taken at passage 4 to be treated as three technical replicates for each of the biological replicates. Each prepared sample, the donor it originated from, the purity and calculated cell number are indicated in table 6.1.

Cell sample	Donor	Purity (%)	Cell number
T08.21 Serum free fibrocytes 1 (T08.21 S.F.F. 1)	Donor 1	58.8	1.37x10 <sup>5</sup>
T08.21 Serum free fibrocytes 2 (T08.21 S.F.F. 2)	Donor 1	61.3	1.55x10 <sup>5</sup>
T08.21 Serum free fibrocytes 3 (T08.21 S.F.F. 3)	Donor 1	53.1	1.12x10 <sup>5</sup>
T08.21 Macrophages 1 (T08.21 Mac 1)	Donor 1	80.2	9.63x10 <sup>5</sup>
T08.21 Macrophages 2 (T08.21 Mac 2)	Donor 1	80.7	7.07x10 <sup>5</sup>
T08.21 Macrophages 3 (T08.21 Mac 3)	Donor 1	88.4	8.03x10 <sup>5</sup>
T08.281 Serum containing fibrocytes 1 (T08.281 S.C.F. 1)	Donor 1	43.9	2x10 <sup>5</sup>
T08.281 Serum containing fibrocytes 2 (T08.281 S.C.F. 2)	Donor 1	45	2x10 <sup>5</sup>
T08.281 Serum containing fibrocytes 3 (T08.281 S.C.F. 3)	Donor 1	42.2	2x10 <sup>5</sup>
T08.120 Serum free fibrocytes 1 (T08.120 S.F.F. 1)	Donor 2	50.2	0.8x10 <sup>5</sup>
T08.120 Serum free fibrocytes 2 (T08.120 S.F.F. 2)	Donor 2	53.3	1.0x10 <sup>5</sup>
T08.120 Serum free fibrocytes 3 (T08.120 S.F.F. 3)	Donor 2	47.9	0.9x10 <sup>5</sup>
T08.120 Macrophages 1 (T08.120 Mac 1)	Donor 2	74.2	1.9x10 <sup>6</sup>
T08.120 Macrophages 2 (T08.120 Mac 2)	Donor 2	79.2	1.7x10 <sup>6</sup>
T08.120 Macrophages 3 (T08.120 Mac 3)	Donor 2	71.2	1.4x10 <sup>6</sup>
T08.285 Serum containing fibrocytes 1 (T08.285 S.C.F. 1)	Donor 2	29.6	6x10 <sup>4</sup>
T08.285 Serum containing fibrocytes 2 (T08.285 S.C.F. 2)	Donor 2	29.2	6x10 <sup>4</sup>
T08.285 Serum containing fibrocytes 3 (T08.285 S.C.F. 3)	Donor 2	29.6	6x10 <sup>4</sup>
T08.135 Serum free fibrocytes 1 (T08.135 S.F.F. 1)	Donor 3	40.1	1.21x10 <sup>5</sup>
T08.135 Serum free fibrocytes 2 (T08.135 S.F.F. 2)	Donor 3	47.8	1.7x10 <sup>5</sup>
T08.135 Serum free fibrocytes 3 (T08.135 S.F.F. 3)	Donor 3	44.9	1.26x10 <sup>5</sup>
T08.143 Macrophages 1 (T08.143 Mac 1)	Donor 3	82.8	5.69x10 <sup>5</sup>
T08.143 Macrophages 2 (T08.143 Mac 2)	Donor 3	86.6	6.68x10 <sup>5</sup>
T08.143 Macrophages 3 (T08.143 Mac 3)	Donor 3	85.9	5.93x10 <sup>5</sup>
T08.255 Serum containing fibrocytes 1 (T08.255 S.C.F. 1)	Donor 3	8.7	3.8x10 <sup>4</sup>
T08.255 Serum containing fibrocytes 2 (T08.255 S.C.F. 2)	Donor 3	8	3.1x10 <sup>4</sup>
T08.255 Serum containing fibrocytes 3 (T08.255 S.C.F. 3)	Donor 3	8.4	3.5x10 <sup>4</sup>
RASY19 1	Line 1	100	2x10 <sup>5</sup>
RASY19 2	Line 1	100	2x10 <sup>5</sup>
RASY19 3	Line 1	100	2x10 <sup>5</sup>
RASY11 1	Line 2	100	6.7x10 <sup>5</sup>
RASY11 2	Line 2	100	6.7x10 <sup>5</sup>
RASY11 3	Line 2	100	6.7x10 <sup>5</sup>
RASY08 1	Line 3	100	2.9x10 <sup>5</sup>
RASY08 2	Line 3	100	2.9x10 <sup>5</sup>
RASY08 3	Line 3	100	2.9x10 <sup>5</sup>

**Table 6.1 Prepared samples for transcriptome analysis.** Four different cell types were prepared for transcriptome analysis, serum-free fibrocytes, serum-containing fibrocytes, macrophages and fibroblasts. The serum-free, serum-containing fibrocytes and macrophages were differentiated from three matched donors and lysed in the well. For each cell type for each donor three technical replicates were used. Three fibroblast cell lines were used, lysed at the same passage, with three technical replicates prepared for each one. The purity and cell number for each sample is shown, for the fibrocytes and macrophages this is based on cell morphology.

The mRNA was isolated from these samples and reverse transcribed into cDNA. To ensure approximately the same amount of cDNA from each sample was used on each of the microarrays, a real time L27 gene PCR was performed to determine the amount of cDNA in each sample. Based on the cell number calculations approximately 1000 cells worth of cDNA was put into each reaction, and each reaction was performed in triplicate. Not all samples could be tested at once, however every set of reactions included a titration of the human reference sample, where known amounts of cDNA were added, and a reaction blank. Figure 6.1 shows an example plot from one of the real time PCR for twelve samples, the mean of the triplicates for each sample are shown. All samples came up after 17 cycles, blanks either never came up at all or in the case of one set of reactions came up at cycle 39 (not shown here). On the graph the green line indicates the point in which the Ct readings of those samples were taken, during the log phase of the reaction. As the amount of cDNA put into the reference sample reaction was known, the amount of cDNA for each sample could be calculated from it, taking into account the dilution of the samples themselves as well. For each sample 1 $\mu$ g of cDNA was then labelled with fluorescent probe, mixed with labelled reference sample and hybridised to the microarrays. For a more in depth protocol see the materials and methods section.

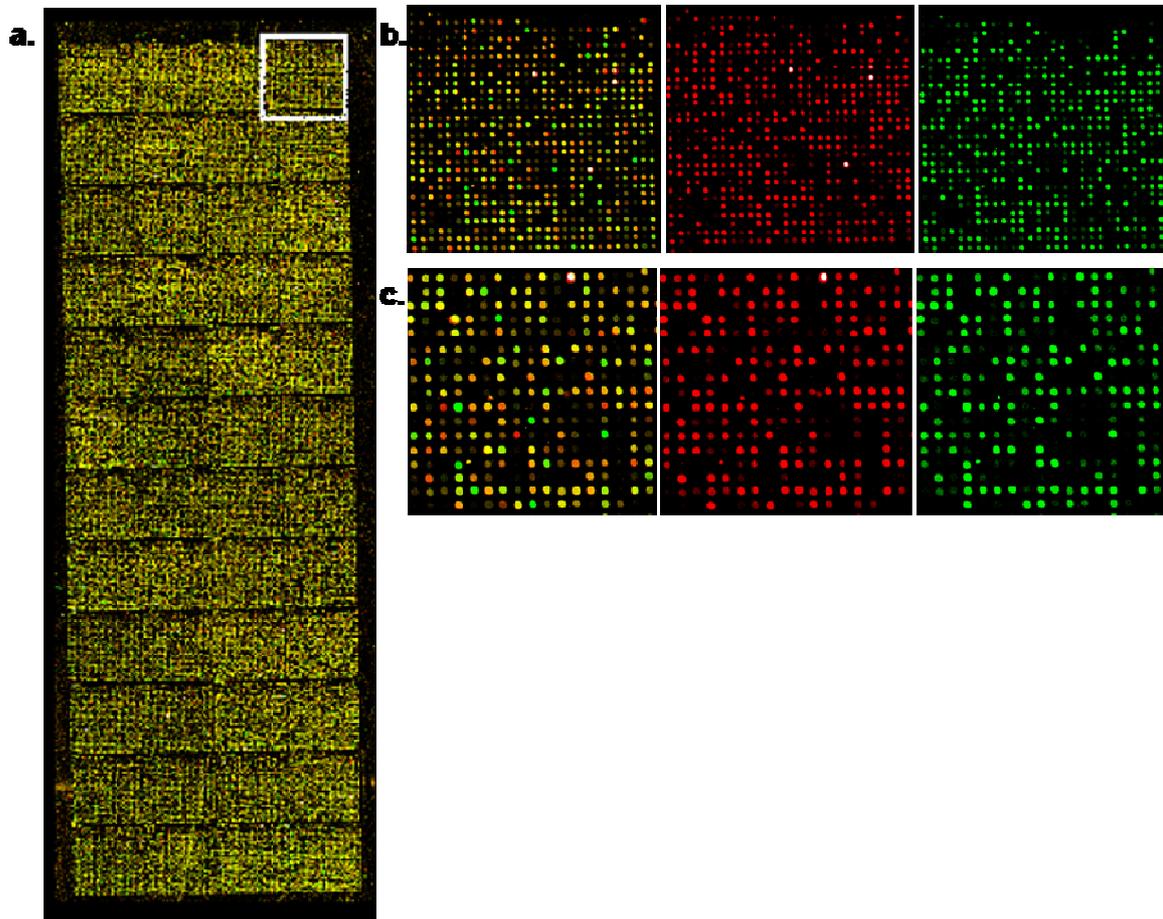


**Figure 6.1 Example of real time L27 gene PCR results for twelve samples.** Approximately 1000 cells worth of cDNA was loaded into triplicate reactions for each sample. Four batches were run as not all sample could be done at once, an example of one batch is shown. In each batch a titration of the human reference sample was included, and a known amount of cDNA used, as well as an experimental blank. Figure 6.1 show the mean of the triplicates for each sample. Green line represents the point at which the Ct readings for each sample, and the reference samples were taken.

## 6.5 Normalising microarray data

Once microarrays had been prepared with material hybridised to the slides they were scanned to measure the amount of fluorescence of the two labelled probes, the reference and cell samples for each of the 37632 spots on the array. The 37632 spots on each microarray are separated into 48 sub arrays, each containing 784 spots, see figure 6.2 (a). The reference sample reading is represented in red and the cell sample in green, when both shown, as in figure 6.2 (a), they appear yellow. However the two colours can be seen separately, figure 6.2 (b) shows the sub array identified by a white box in (a), with both the reference and cell samples shown.

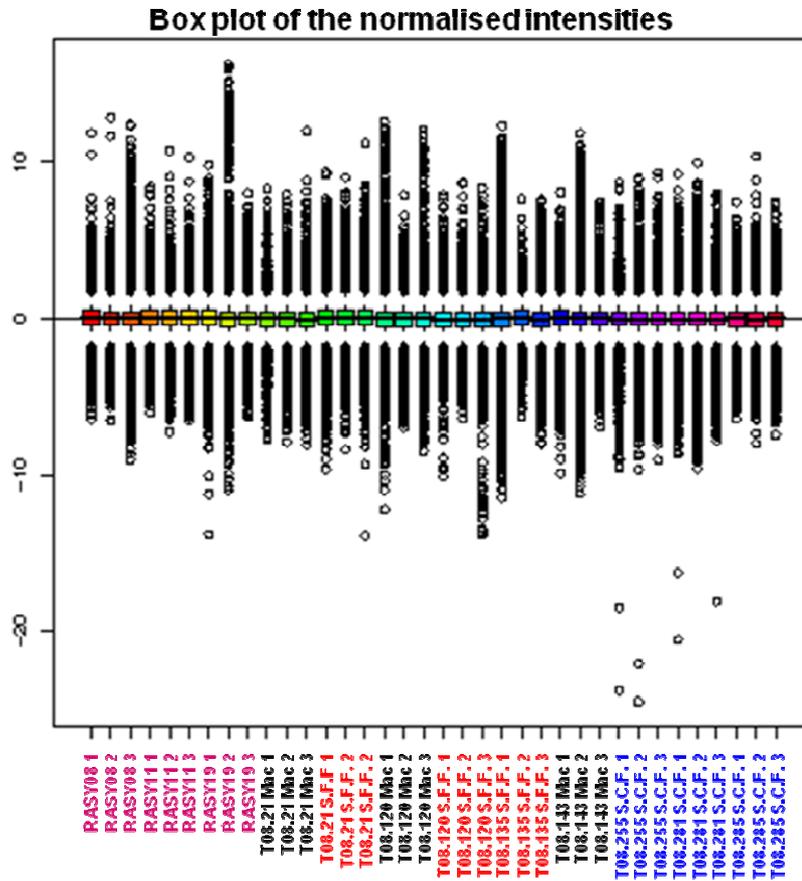
Figure 6.2 (c) also shows a smaller area of the sub array to illustrate the consistency of individual spots.



**Figure 6.2 Example image of microarray containing 37632 spots and subarrays.** A cell sample was labelled with fluorescent dye (Cy3), mixed with fluorescently labelled (Cy5) reference sample and hybridised to a microarray slide containing 37632 spots. **a.** The slide was scanned using a slide scanner and depicted as an image of spots, consisting of 48 sub arrays each containing 784 spots. **b.** Three images of the subarray highlighted in **a.** are shown. The image of just the Cy5 labelled reference sample (red), the Cy3 labelled cell sample (green) and the two images merged. **c.** Higher magnification of a section of the sub-array in **a.** with the same three images shown.

Once the slide images had been annotated with the prepared generic array logic file (GAL) the intensity data of each colour was exported and normalised, (materials and methods). By using a

reference sample as well as the test sample on each slide any inter-slide variation could be removed during normalisation, as well as controlling for any variation in the amount of sample cDNA. Figure 6.4 shows box plots of the normalised intensities of each sample. Following normalisation a reading for each gene on the array was given in the form of a ratio between the level of the gene within the test sample and the level of that same gene within the reference sample. The level of the gene in the reference sample should be very similar across all the arrays therefore the ratio readings are suitable to be used to compare across the samples.

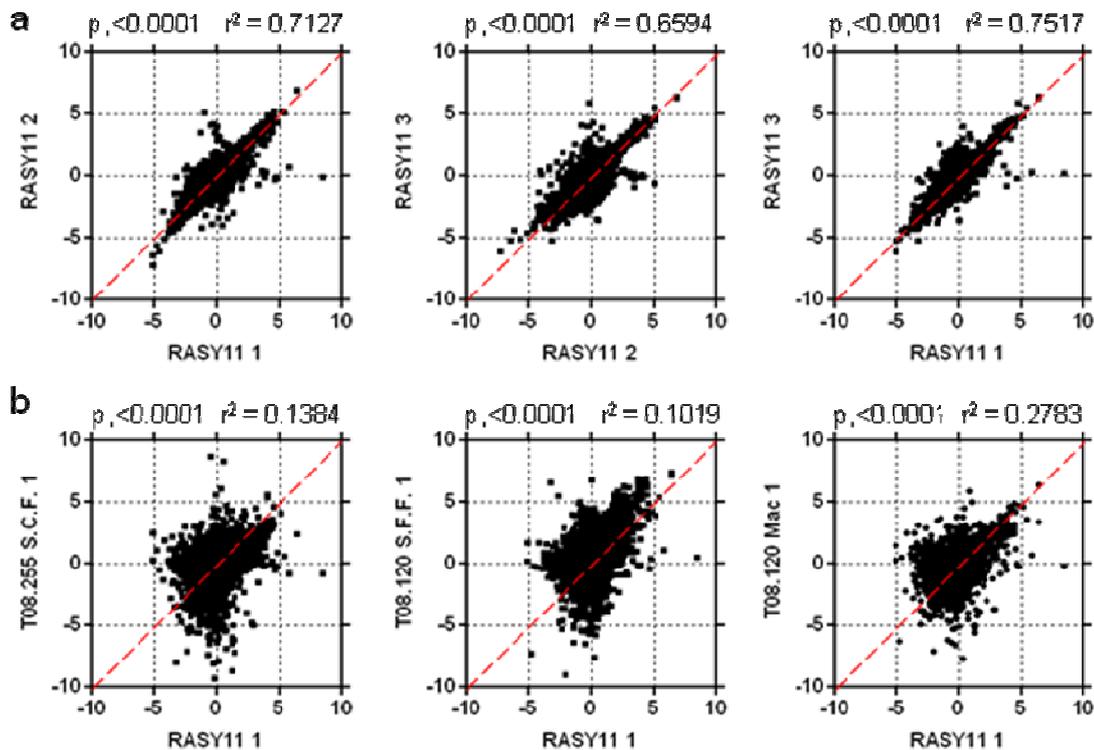


**Figure 6.3** Box-plot of normalised intensities of each sample. Samples were loaded into Gene Expression Pattern Analysis Suite (GEPAS) (Bioinformatics Unit CNIO; <http://gepas.bioinfo.cnio.es>) software for normalization, which was performed using print-tip loess normalisation with background subtraction, initially within each slide and subsequently between slides.

## 6.6 Relationship between experimental replicates

As mentioned previously all experimental replicates were treated as separate samples from the earliest possible stage of the preparation process. Following normalisation of the microarray results the relationship between the three experimental replicates for the fibroblasts cell line

RASY11 were examined. Figure 6.4 (a) shows the  $\log_2$  ratio readings for 10000 genes of each RASY11 sample plotted against each other, and a positive correlation is seen between them all (10000 genes used due to limitations in software; software used was Prism version 3.0). Correlation was calculated using the Pearson correlation statistical test and the coefficient of determination, or  $r^2$  (shown with each plot). The  $r^2$  value represents the fraction of the variance which is shared between the two samples, perfect positive correlation would be represented as  $r^2=1$ , no correlation as  $r^2=0$  and perfect negative correlation as  $r^2=-1$ . As the  $r^2$  readings seen when looking at the RASY11 cell line range between 0.65 and 0.75 we can be confident that there is a large amount of positive correlation between them. To show this is due to the samples being experimental replicates and not to do with either the reference sample, or processing, the correlation of RASY11 1 sample with an example of each of the other three cell populations being investigated was also examined. By just looking at the plots alone (figure 6.4 (b)) less correlation is seen, the  $r^2$  readings support this as all are below 0.28, indicating there is some slight correlation, due to the fact that all these samples originate from humans, however the correlation is much less than that seen between the experimental replicates of RASY11. In all cases  $P < 0.0001$ , indicating that the  $r^2$  values seen are significantly relevant. We can therefore be confident in our experimental replication.



**Figure 6.4 Correlation between experimental replicates and other samples. a.** To examine the experimental replication between the three RASY11 samples the same 10000 genes from each sample were plotted against each of the other two replicates and the level of correlation between them examined. The coefficient of determination, or r squared ( $r^2$ ) value is shown for each combination and a high level of positive correlation was seen. **b.** To determine that the level of correlation seen in a. was not related to either the presence of the reference sample, or the processing of the samples the RASY11 1 sample was compared to T08.255 serum-containing fibrocyte 1 (T08.255 S.C.F. 1), T08.120 serum-free fibrocytes 1 (T08.120 S.F.F.1) and T08.120 macrophages 1 (T08.120 Mac 1). Some positive correlation was seen but not to the same level as seen between the experimental replicates of RASY11.

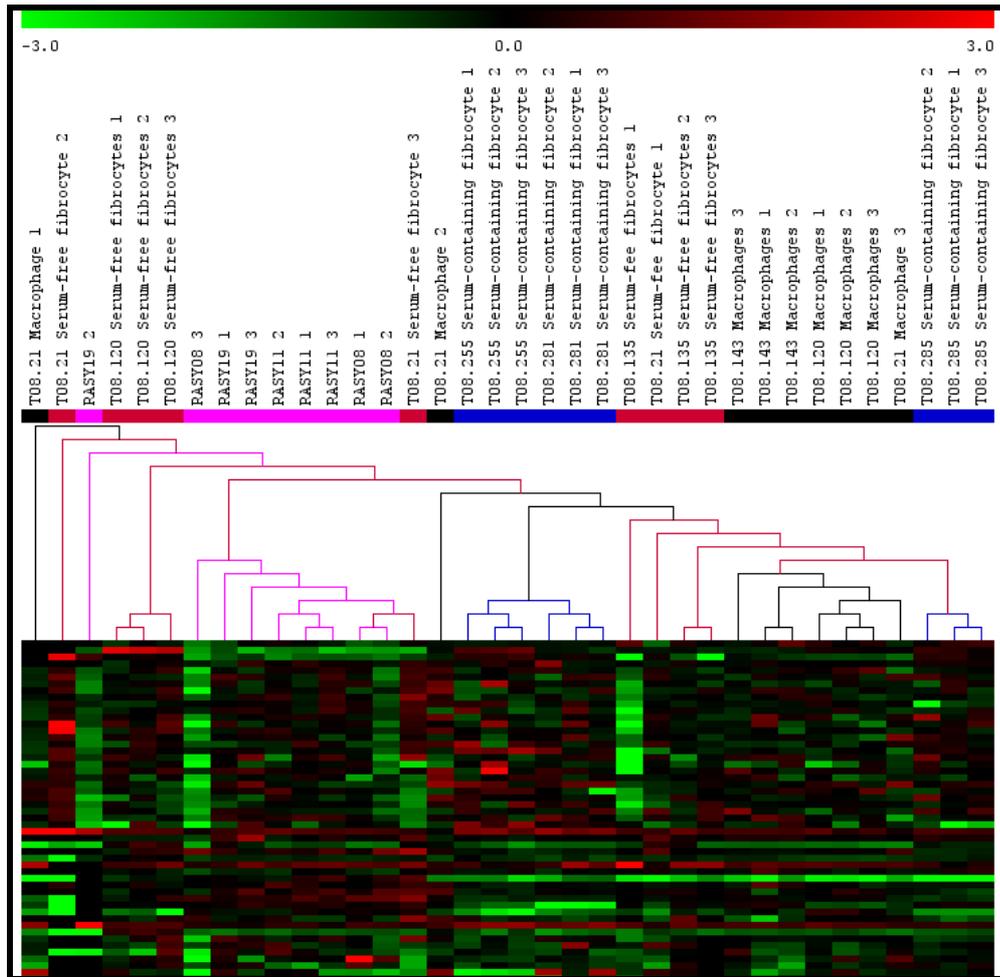
## 6.7 Analysis of all four cell populations

### 6.7.1 Unsupervised clustering of all four cell populations

Once normalised the data from all four cell populations (36 samples) was exported to the TMEV software package where, without any statistical analysis, a hierarchical cluster analysis was

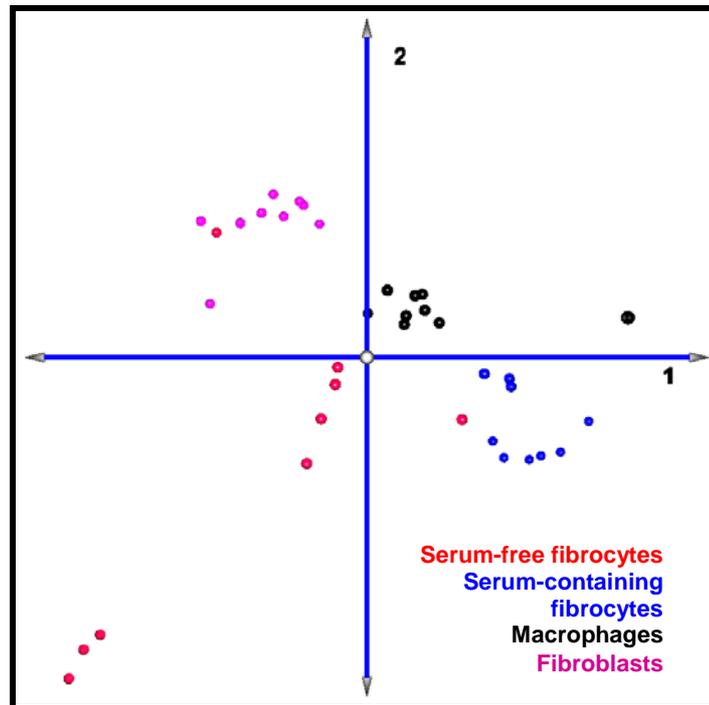
performed, using the information from 37632 genes (appendix 1). Figure 6.5 shows the very top of the hierarchical cluster figure, including the clustering tree and the information on 50 genes, although all 37632 genes were used to determine the clustering tree. Due to the amount of data involved it is impossible to show the entire figure however an example of a complete hierarchical clustering figure can be seen in figure 6.8. At the top of figure 6.5 is the clustering tree, samples that have very similar transcriptomes will cluster closer together than samples that have more diverse transcriptomes. Just because a sample is next to another though does not mean they are closely related, it is important to look at the length of the branches between samples, the smaller the distance the more closely related the samples and vice versa. Samples and branches are colour coded and these colours will remain throughout all the analyses, serum-free fibrocytes represented in red, serum-containing fibrocytes in blue, macrophages in black and fibroblasts in pink. Below the hierarchical tree information on 50 genes is shown, in no particular order, carrying on from the clustering tree a column of blocks indicates the results from one sample, labelled at the top. One row of blocks indicates a particular gene across all samples. Each block is coloured based on the  $\log_2$  ratio reading of the normalised reference sample divided by cell sample intensity reading. The more negative a  $\log_2$  ratio, the greener it will appear, and the more positive the redder it will appear. A gene that is more highly expressed in the cell sample compared to the reference sample will be negative and so green, and those expressed to a lower extent in the sample compared to the reference will be positive and so red. Those genes that have a very similar expression level in the cell sample and reference are shown in black. As the colour in the HCL represents the ratio of the cell sample to

reference of a gene, two samples that are completely different could still be represented as different shades of the same colour, as, even when different they may still both have either more or less than the reference sample.



**Figure 6.5 Hierarchical cluster analysis tree of unsupervised data from all four cell populations.** Hierarchical cluster analysis was performed on the normalised data for all thirty six samples across all four cell populations. The hierarchical cluster analysis tree is shown and information on 50 genes, although all 37632 genes were used to determine the tree. Samples are colour coded and labelled at the top, serum-free fibrocytes in red, serum-containing fibrocytes in blue, macrophages in black and fibroblasts in pink. Samples are clustered based on their similarities with the length of the branches being important rather than their proximity of the samples to each other.

Despite being completely unsupervised a great deal of clustering can be seen within this hierarchical cluster analysis. All but one of the fibroblasts samples cluster closely together, as do the majority of the macrophage samples. There are however a few outlying samples that have their own branches and don't seem to cluster with any other samples in particular (T08.21 Macrophage 1, T08.21 Serum-free fibrocyte 2 and RASY19 2). Figure 6.6 shows the same data that has been used to form this hierarchical clustering tree in the form of a Principal component plot. Principal component plots show the data along the three axes that show the largest amount of variation within the data. Principal component 1, (x axis), shows the largest variation, Principal component 2, (y axis), the second largest amount of variation and Principal component 3, (z axis- not currently seen), shows the third. As you can see from figure 6.6, other than a few samples, all four populations cluster separately from one another. There is a serum-free fibrocyte (red) sample with the serum-containing fibrocytes (blue) and one with the fibroblasts (pink). However on the whole they separate well, suggesting that the transcriptomes of all four populations are different.

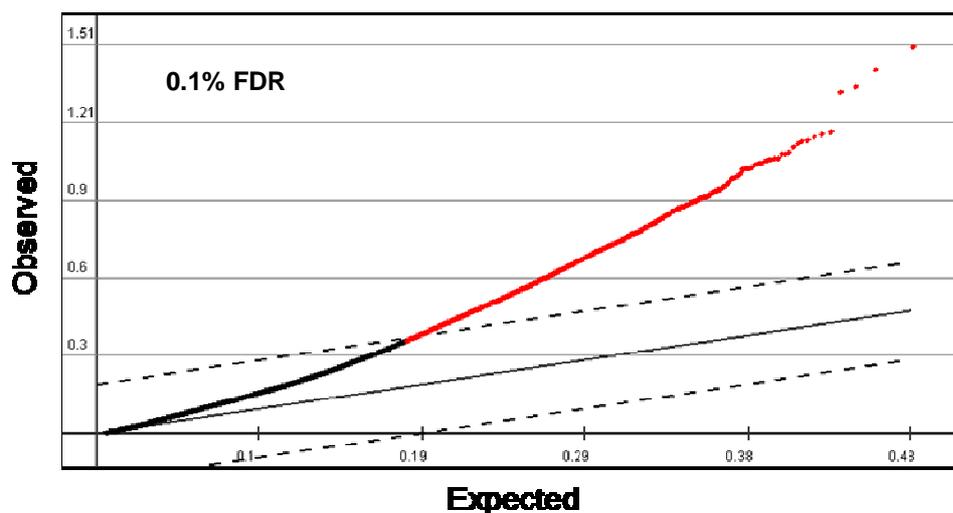


**Figure 6.6 Principal component plot of unsupervised data from all four cell populations.** Principal component analysis was performed on the normalised data for all thirty six samples across all four cell populations. The Principal component plot plots the data along the three axes that show the largest amount of variation between the examples, (although only the first two are seen in this figure).

### 6.7.2 Significance analysis of all four cell populations

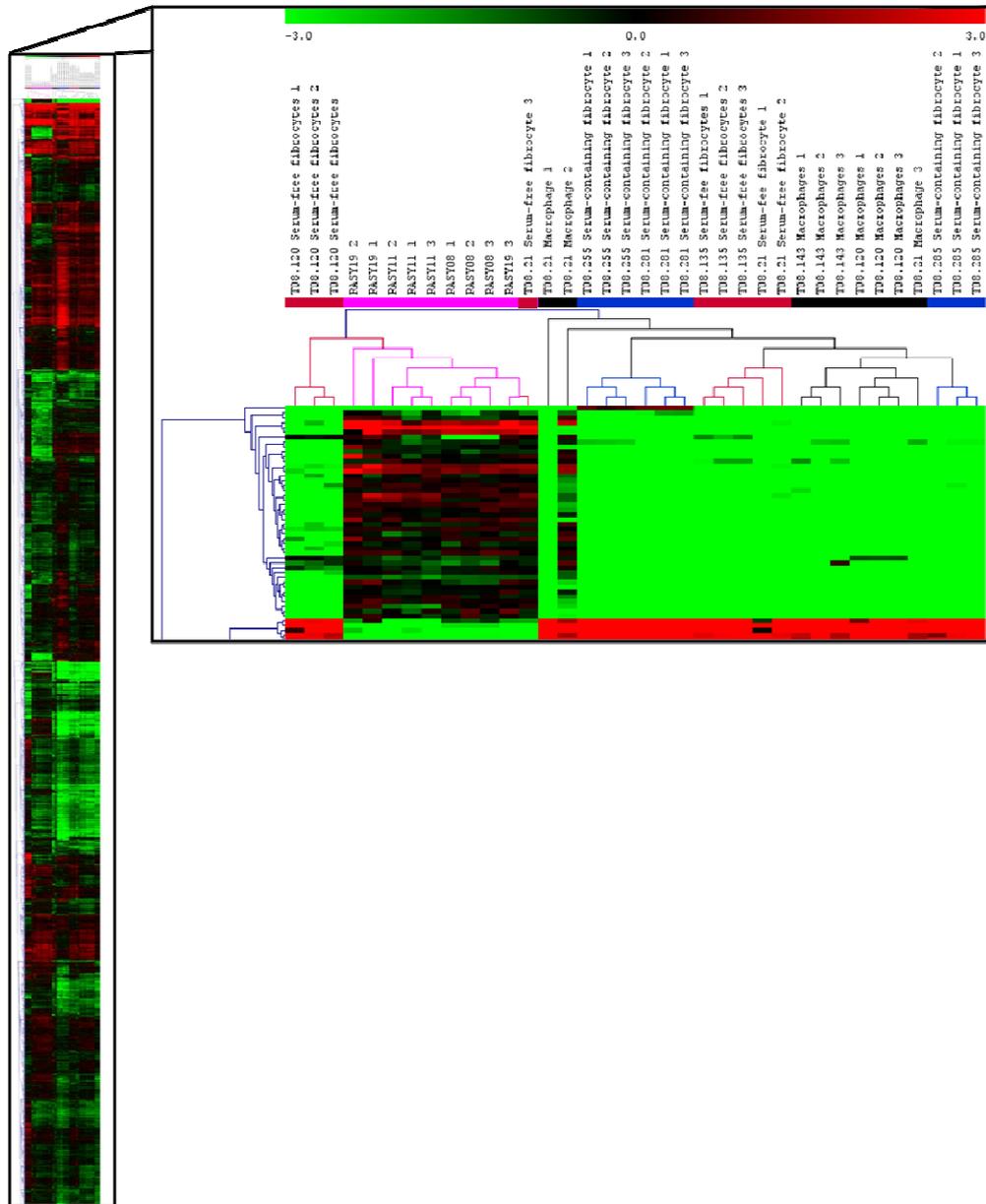
A SAM analysis of all four cell populations was performed using the TMEV software and those genes significantly different between them all identified. Figure 6.7 shows the SAM plot used, the x-axis represents the expected reading if there is no difference between the samples, and the y-axis the observed. All information is then plotted, the more divergent the plotted line is from the gray diagonal line the more significantly different genes there are. The number of significantly different genes is also dependent on the false discovery rate (FDR) used in the analysis. The FDR estimates how many genes SAM is wrongly identifying as significantly

different, the lower the FDR the better. For example in this four way analysis an FDR of 1% gave 18508 genes as being significantly different (approximately 50% of the number of genes put into the analysis), and in doing so suggests that 185 genes are being identified as significantly different when they are not, or are falsely discovered. However if an FDR of 0.1% is applied 12602 (33% of initial gene number) significantly different genes are identified with only 13 genes being falsely discovered. In the case of this analysis an FDR which is any lower than 0.1%, gives 0 genes as falsely discovered, which is not significantly relevant, so cannot be used, therefore an FDR of 0.1% was applied (Figure 6.7, appendix 2).

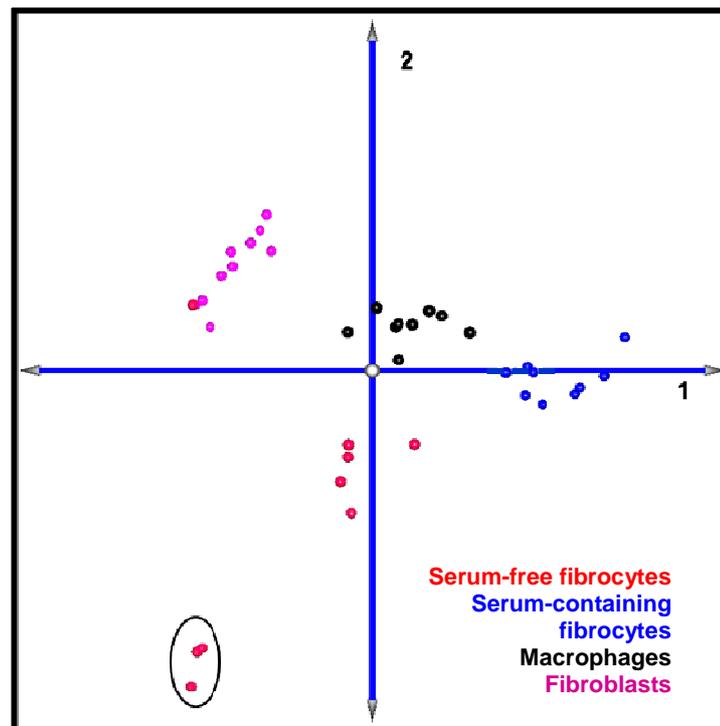


**Figure 6.7 SAM plot of four way analysis of microarrays using a 0.1% FDR.** Identification of significantly different genes between all four populations was performed using SAM, the x axis represents the expected reading if there is no difference between the samples and the y axis the observed. When plotted the more divergent the data is from the grey line the more significant genes there are. The number of significant genes is determined by using a false discover rate (FDR) cut off, depicted by the dashed lines.

Once 12602 genes had been identified as being significantly different between all four cell populations hierarchical clustering analysis was performed using them (Figure 6.8). The full hierarchical clustering figure shown in figure 6.8 allows for gene clusters and for the global separations of samples to be identified, however the most interesting part is the hierarchical tree at the top, which has been highlighted. This figure shows a definite separation of the fibroblast cell lines from the *in vitro* derived populations, however clustering of the different *in vitro* derived cells is also seen. One of the serum-free fibrocyte samples (T08.21 serum-free fibrocyte 3) is clustering strongly with the fibroblasts, despite the other technical replicates for that donor clustering nicely with other serum-free fibrocyte samples. Also observed, when looking at the hierarchical tree branches, is that three replicates of one particular donor of the serum-free fibrocytes (T08.120) appear to be more closely related to the fibroblasts than the other *in vitro* derived cells. However when the Principal component analysis for this list of 12602 genes is examined (figure 6.9) the three T08.120 serum-free fibrocyte samples cluster completely separately from anything else (identified by the ellipse), so rather than being like fibroblasts, these samples just don't cluster with any other samples at all. Interestingly all the macrophages samples and all the serum-containing fibrocyte samples form two separate clusters.



**Figure 6.8 Hierarchical cluster analysis tree of samples based on 12602 significantly different genes from SAM analysis.** Hierarchical cluster analysis was performed on the data for the 12602 genes identified as being significantly different by SAM analysis. The full hierarchical cluster analysis is shown on the left with the hierarchical cluster analysis tree and information on 50 genes magnified on the right to allow branch information to be seen. Samples are colour coded and labelled at the top, serum-free fibrocytes in red, serum-containing fibrocytes in blue, macrophages in black and fibroblasts in pink. Samples are clustered based on their similarities with the length of the branches being important rather than their proximity of samples to each other.



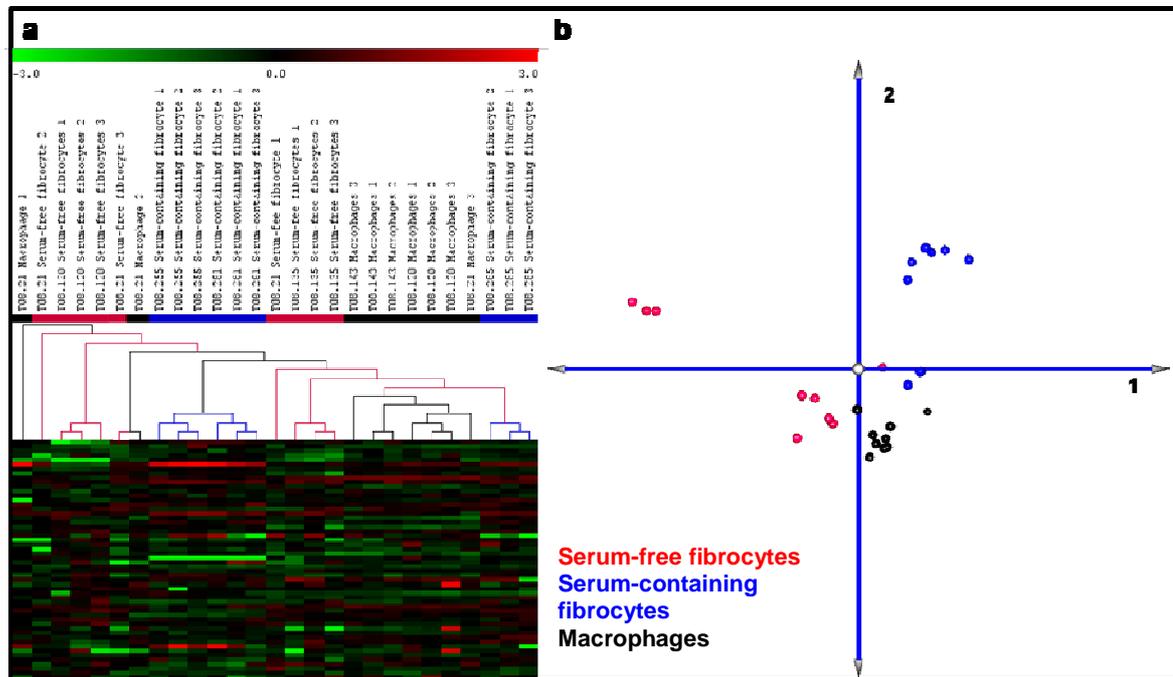
**Figure 6.9 Principal component plot of samples based on 12602 significantly different genes from SAM analysis.** Principal component analysis was performed on the data for the 12602 genes identified as being significantly different by SAM analysis. The T08.120 serum-free fibrocytes are identified by an ellipse in the bottom right hand section of the figure.

Despite clustering of the different populations of the *in vitro* derived cells (serum-free fibrocytes, serum-containing fibrocytes and macrophages) being seen, further investigation of the significantly different genes identified show that they are mainly due to the differences between the fibroblasts and everything else; and are not suitable for identifying more subtle similarities and differences between the two types of fibrocytes and macrophages. To be able to identify these differences between the monocyte derived cells the decision was made to exclude the fibroblast cell lines from the following analyses.

## **6.8 Analysis of serum-free fibrocytes, serum-containing fibrocytes and macrophages**

### **6.8.1 Unsupervised analysis**

The spot intensity data from the microarrays was re-normalised with the fibroblast samples removed, the  $\log_2$  ratio data for the remaining 27 samples (appendix 3) was then used to perform both unsupervised hierarchical clustering and Principal component analysis. Figure 6.10 (a) shows the hierarchical clustering tree and the  $\log_2$  ratio data on 50 genes, and figure 6.10 (b) the Principal component analysis. Again, despite no supervision a large degree of clustering is seen within the three cell populations, with the macrophage samples clustering the most tightly. Importantly however is the observation that serum-free and serum-containing fibrocytes clearly do not cluster together. The serum-free fibrocytes from donor T08.120 can be seen clustering quite separately from all other samples, however when looking at the Principal component analysis these samples are closest to the other serum-free fibrocytes rather than either of the other two cell populations.

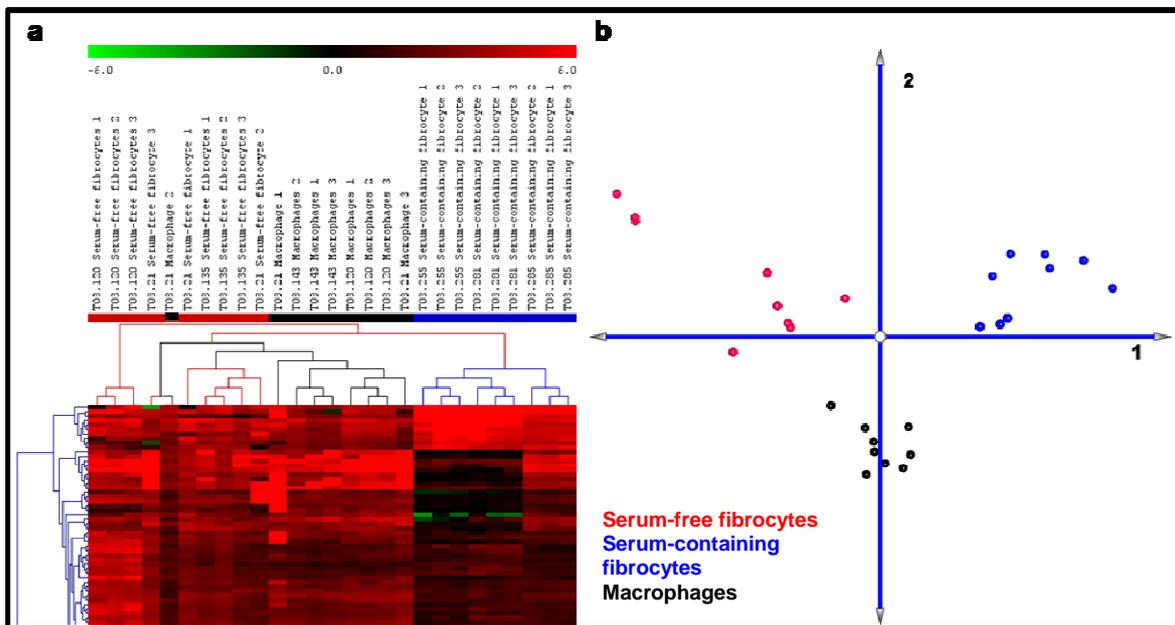


**Figure 6.10 Unsupervised cluster analysis of serum-free fibrocyte, serum-containing fibrocyte and macrophages samples.** **a.** Hierarchical cluster analysis was performed on the normalised data for the twenty seven samples across the three cell populations. The hierarchical cluster analysis tree is shown and information on 50 genes, although all 37632 genes were used to determine the tree. Samples are clustered based on their similarities, with the length of the branches between them being important rather than their proximity of the samples to each other. **b.** Principal component analysis was performed on the same data.

### 6.8.2 Statistical analysis of microarrays

SAM was performed on the normalised  $\log_2$  ratio data of the two types of fibrocytes and macrophages at an FDR of 0.01% and 4563 significantly different genes were identified (12% of the initial gene number; appendix 4). Hierarchical cluster and Principal component analyses were then performed on these genes (Figure 6.11). The hierarchical cluster analysis tree shows, again, that the three T08.120 serum-free fibrocytes cluster separately from all the other samples. The remaining serum-free fibrocyte and macrophage samples are on a separate

branch to the serum-containing fibrocytes, however, other than one macrophage sample, they do separate into two clusters. Interesting to note in context to the aims of this chapter are that serum-free and serum-containing fibrocytes are not clustering together in any way. In fact by Principal component analysis all three populations cluster completely separately.

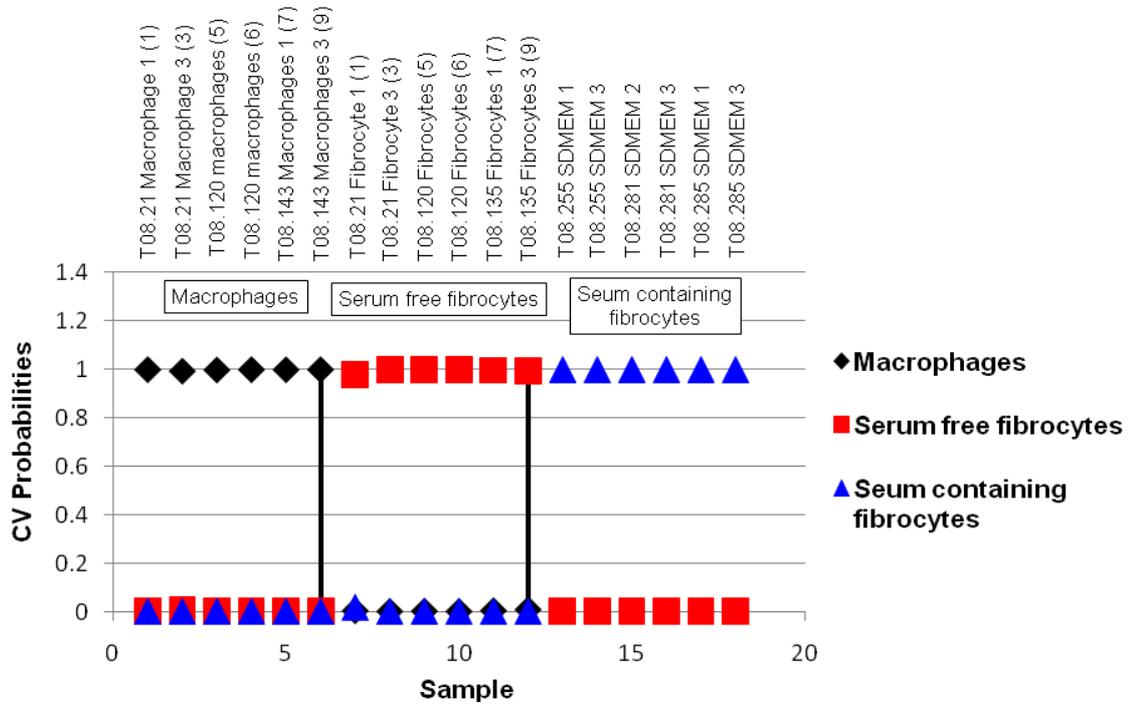


**Figure 6.11 Cluster analysis of serum-free fibrocyte, serum-containing fibrocyte and macrophage samples based on 4563 significantly different genes from SAM analysis.** Samples are colour coded, serum-free fibrocytes in red, serum-containing fibrocytes in blue and macrophages in black. **a.** Hierarchical cluster analysis was performed on the 4563 significantly different genes found between the three cell populations. The hierarchical cluster analysis tree and  $\log_2$  ratio information on 50 genes are shown, although all 4563 genes were used to determine the tree. Samples are clustered based on their similarities, with the length of the branches between them being important, rather than their proximity of the samples to each other. **b.** Principal component analysis was performed on the same data set.

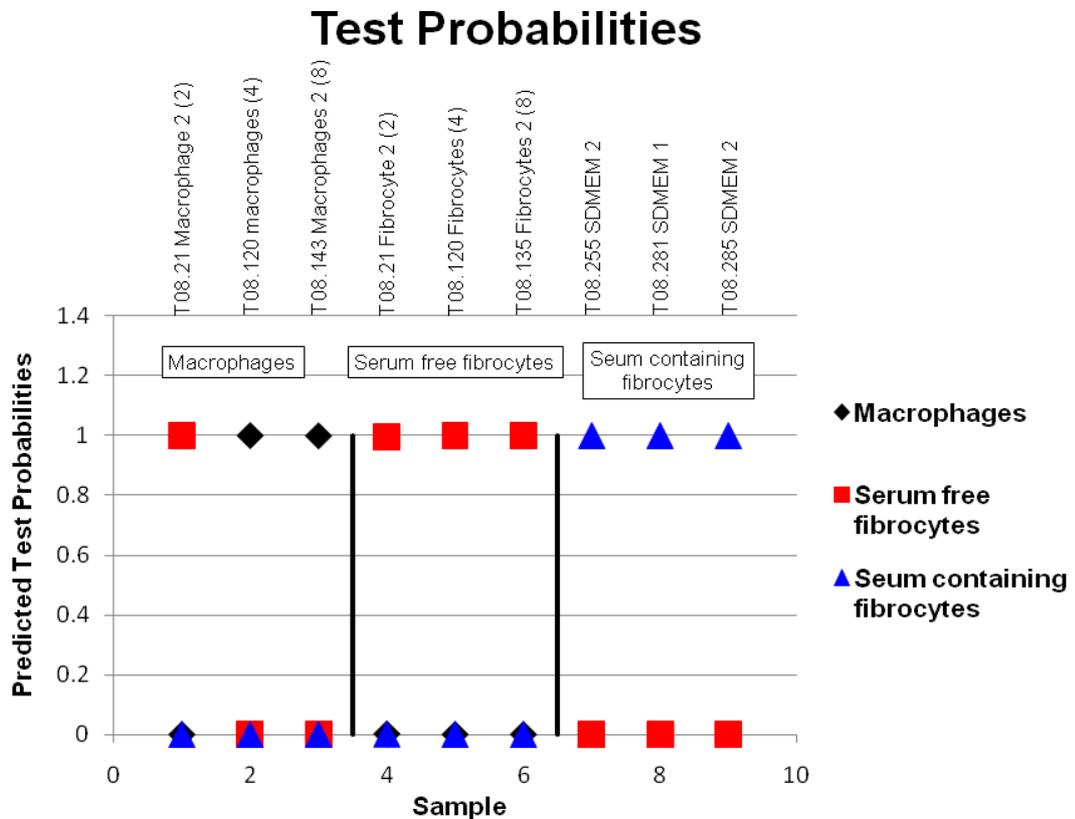
### 6.8.3 Predictive analysis of microarrays

PAM was performed on the normalised data for the three monocyte derived cell populations, figures 6.12 and 6.13 are plots from one example of a PAM. The program uses the prediction set of samples to come up with a list of genes, ranked in order of importance, which it can use to identify those samples into the correct cell populations. Figure 6.12 shows how well it does this by plotting the probability that each sample is each of the three cell populations and all 18 samples are correctly identified. However as it used this set of samples to determine the gene list the more important test is how well they classify the nine samples in the test set. Figure 6.13 shows the test probabilities for the test set and all but one of the samples is correctly classified. The one macrophages sample that it failed to classify correctly is the same one that did not cluster with all the other macrophage samples in the hierarchical cluster analyses in figures 6.10 and 6.11.

### Cross-validated Probabilities



**Figure 6.12 Cross validation probabilities of prediction set in PAM analysis.** Using the predictive set of samples a set of genes was determined that can be used to predict the population type of samples. Using this set the probability that a sample is one of the three possible populations is shown on the plot, with the actual population type shown above.



**Figure 6.13 Cross validation probabilities of test set in PAM analysis.** Using the predictive set of samples in a PAM analysis a set of genes was determined that can be used to predict the population type of samples. To test the ability of this predictive set a set of samples (test set) not involved in determining the predictive set of genes, were used. The probability that a sample is one of the three possible populations is shown on the plot, with the actual population type shown above.

Ten iterations of PAM were performed on the twenty seven samples, which consisted of nine samples of each of the three cell populations. In each iteration six samples of each population were put in the prediction set and three into the test set. The genes provided as good predictors of cell types in these ten different analyses were ranked by the number of times they appeared, and those genes which had been used in at least eight out of the ten iterations selected.

For the ten different iterations of PAM the ability of the predictive genes to identify the test set samples is summarised in table 6.2. In four out of the ten PAM analyses the gene list that had been determined misclassified a macrophage sample in the test set as a serum-free fibrocyte, every time it was the sample T08.21 Macrophage 2. Also on one occasion a serum-free fibrocyte sample was misclassified as a macrophage but at no point were any serum-containing fibrocyte samples misclassified.

		Predicted Population		
		Macrophage	Serum-free fibrocyte	Serum-containing fibrocyte
True Population	Macrophage	26/30	4/30	
	Serum-free fibrocyte	1/30	29/30	
	Serum-containing fibrocyte			30/30

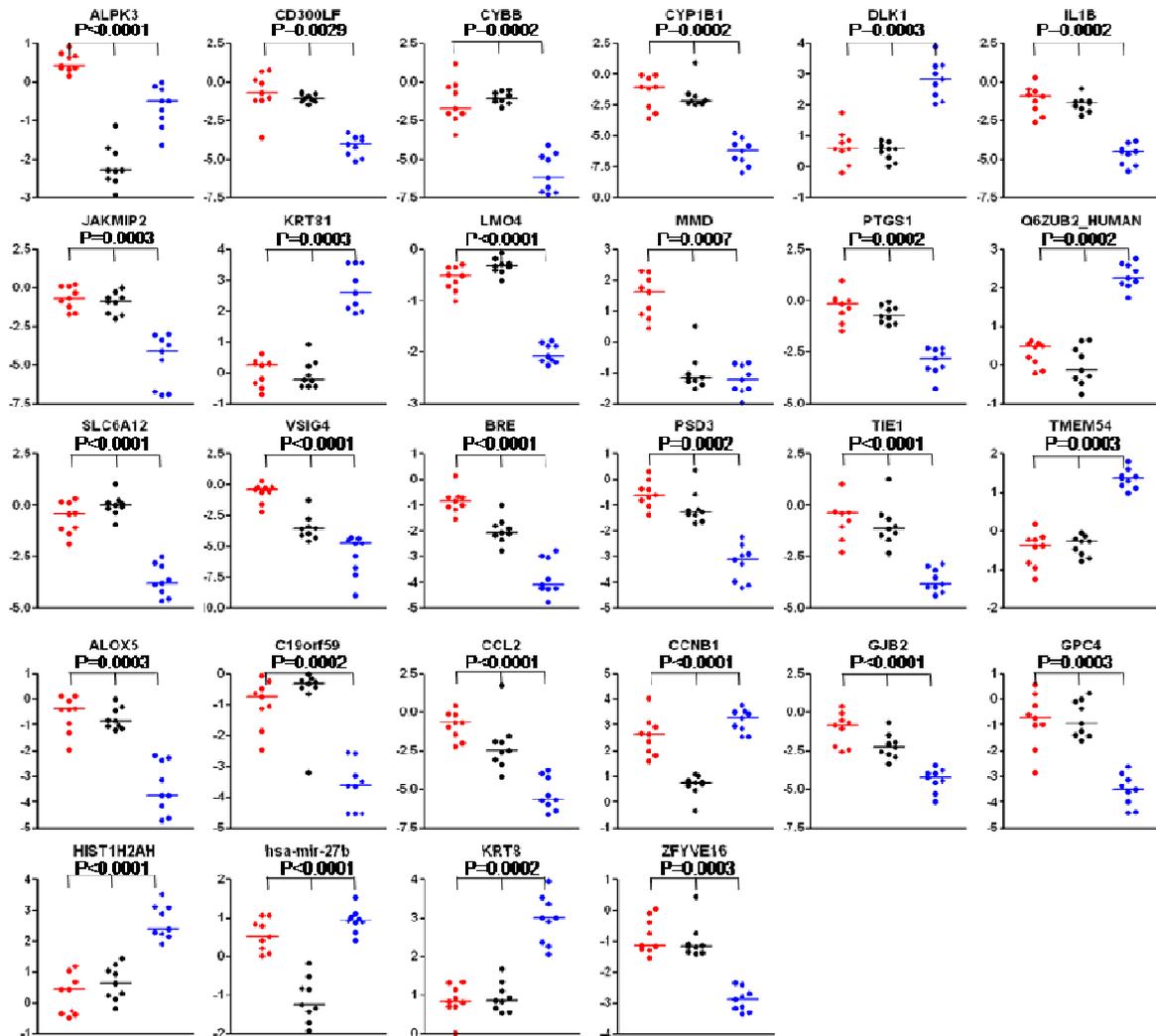
**Table 6.2 Summary of test probability results for PAM analysis.** Using the predictive set of samples in a PAM analysis a set of genes was determined that can be used to predict the population type of samples. To test the ability of this predictive set a set of samples (test set) not involved in determining the predictive set of genes, were used. This was performed on ten different iterations of the PAM analysis and the results tabulated here. The population predicted by the gene lists is shown across the top and the true population down the side.

The genes that PAM used eight times and above to classify these samples in the ten different iterations of PAM are listed in table 6.3, and the log(2) ratio readings for each sample in figure 6.14. Figure 6.14 also shows the results of a Friedman non-parametric statistical test for each gene, all are significantly different with at least  $P < 0.05$ . By looking at the log(2) ratio data the

cell type most different, so probably causing that gene to be predictive can also be identified, in most cases it was the serum-containing fibrocytes. As this is ratio data, compared to the reference sample, it is important to remember that the more negative a reading the higher the expression level of that gene in those samples is, therefore the cell type with the lowest ratio reading has the highest expression. Also to note is that all 28 genes listed as being highly predictive of these three cell populations are also present in the gene list of significantly different genes produced by SAM.

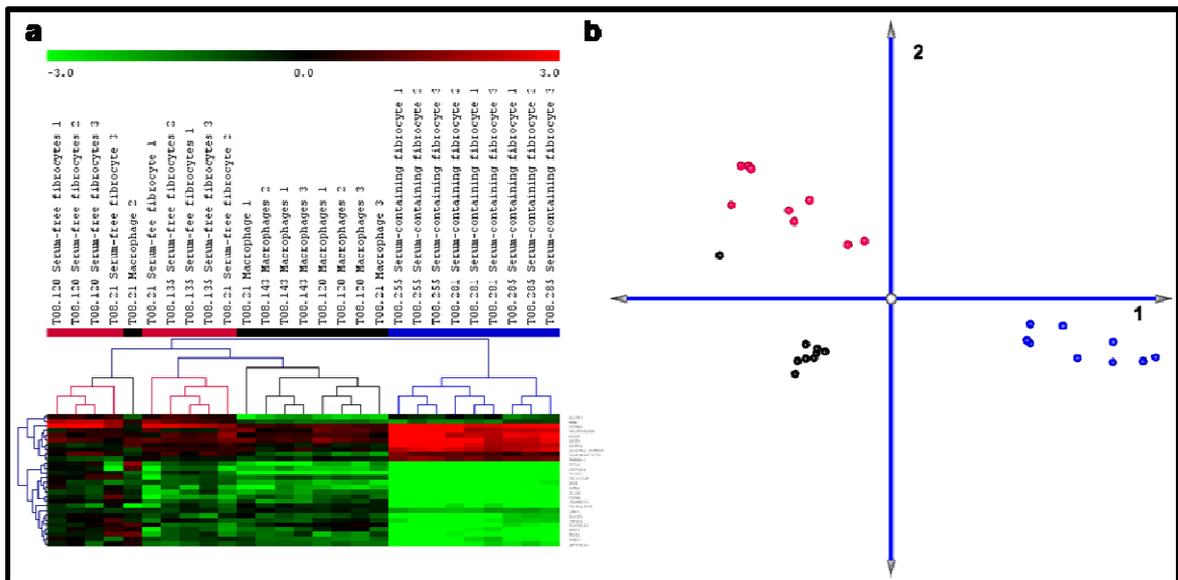
Gene	Full name
ALPK3	Alpha kinase 3
ALOX5	Arachidonate 5-lipoxygenase
BRE	Transcription Factor II B Recognition Element
C19orf59	Chromosome 19 open reading frame 59
CCL2	Chemokine (C-C motif) ligand 2
CCNB1	Cyclin B1
CD300LF	CD300 molecule-like family member f
CYBB	Cytochrome b-245, beta polypeptide
CYP1B1	Member of cytochrome p450 family
DLK1	Delta-like 1 homolog (Drosophila),
GJB2	Gap junction protein, beta 2
GPC4	Glypican 4
HIST1H2AH	Histone cluster 1, H2ah
hsa-mir-27b	Homo sapiens miR-27b stem-loop
IL1B	Interleukin 1, beta
JAKMIP2	Janus kinase and microtubule interacting protein 2
KRT8	Keratin 8
KRT81	Keratin 81
LMO4	LIM domain only 4
MMD	Monocyte to macrophage differentiation-associated
PSD3	Pleckstrin and Sec7 domain containing 3
PTGS1	Prostaglandin-endoperoxide synthase 1
Q6ZUB2_HUMAN	Unknown
SLC6A12	Solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12
TIE1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TMEM54	Transmembrane protein 54
VSIG4	V-set and immunoglobulin domain containing 4
ZFYVE16	Zinc finger, FYVE domain containing 16,

**Table 6.3 List of genes used eight times and above in the ten iterations of PAM to classify the 28 samples.** Ten different iterations of PAM analysis were performed on the serum-free, serum-containing fibrocytes and macrophages. Using a predictive set of samples in a PAM analysis a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in the ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were listed above. The shortened and full gene name are shown.



**Figure 6.14 Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the 27 samples.** Ten different iterations of PAM analysis were performed on the serum-free, serum-containing fibrocytes and macrophages. Using a predictive set of samples in a PAM analysis a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in the ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen. Log(2) ratio data for each cell population for each gene is shown. Each sample is shown as a dot, with the median depicted by a line, serum-free fibrocytes are colour coded red, serum-containing fibrocytes blue and macrophages black. The population data for each gene was statistically tested using Friedman non-parametric statistical test, all are significantly different with at least  $P < 0.05$ . NB This is ratio data of reference divided by sample therefore the more negative a reading the higher the expression level of that gene.

Using just the 28 genes identified by the PAM analysis hierarchical cluster and Principal component analyses were performed (Figure 6.15, log(2) ratio data in appendix 5). As with figure 6.11 all three cell populations clustered separately, other than one macrophage sample (T08.21 Macrophage 3), however in tighter clusters than was seen in figure 6.11. These analyses indicate that the genes selected for their predictive nature are able to cluster the three different cell populations. They also show that although all three populations cluster separately the macrophages and serum-free fibrocytes appear to be more closely related to each other than either is to the serum-containing fibrocytes.



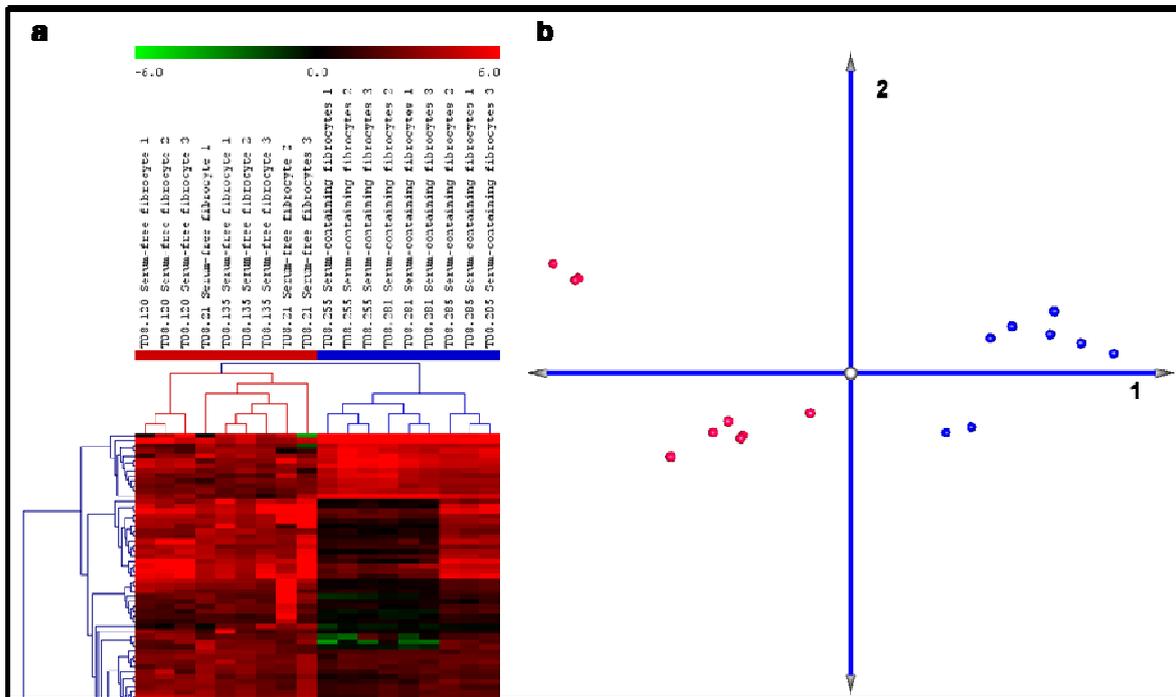
**Figure 6.15 Cluster analysis of serum-free fibrocyte, serum-containing fibrocyte and macrophage samples based on 28 predictive genes from PAM analysis.** Samples are colour coded, serum-free fibrocytes in red, serum-containing fibrocytes in blue and macrophages in black. **a.** Hierarchical cluster analysis was performed on the 28 predictive genes. The hierarchical cluster analysis tree and the log(2) ratio information on all of the genes is shown. Samples are clustered based on their similarities, with the length of the branches between them being important, rather than their proximity of the samples to each other. **b.** Principal component analysis was performed on the same data set.

## **6.9 Analysis of serum-free fibrocytes versus serum-containing fibrocytes**

Although the gene list provided by comparing all three *in-vitro* derived cells is informative, supporting the hypothesis that serum-free and serum-containing fibrocytes are different it does not provide any genes that are significantly different between just these two populations, therefore analyses of just those was performed.

### **6.9.1 SAM analysis of serum-free fibrocytes versus serum-containing fibrocytes**

An unpaired SAM analysis of the two fibrocyte populations at 0.1% FDR provided 6614 significantly different genes (appendix 6). Using these genes hierarchical cluster and Principal component analyses were performed, figure 6.16, and in both cases a clear separation can be seen between the two populations. However the samples T08.120 serum-free fibrocytes are still on a separate branch from the rest of the serum-free fibrocytes.



**Figure 6.16 Cluster analysis of serum-free fibrocyte and serum-containing fibrocyte samples based on 6614 significantly different genes from SAM analysis.** Samples are colour coded, serum-free fibrocytes in red and serum-containing fibrocytes in blue. **a.** Hierarchical cluster analysis was performed on the 6614 significantly different genes found between the two cell populations. The hierarchical cluster analysis tree and information on 50 genes are shown, although all 6614 genes were used to determine the tree. Samples are clustered based on their similarities, with the length of the branches between them being important, rather than their proximity of the samples to each other. **b.** Principal component analysis was performed on the same data set.

### 6.9.2 Functional annotation of genes identified by SAM analysis of serum-free versus serum-containing fibrocytes

The database for annotation, visualization and integrated discovery, or DAVID, is a web-based tool that can be used to put biological meaning behind lists of genes determined by microarray analysis<sup>186</sup>. It maps the genes contained in the list to an associated biological annotation, then statistically highlights the most over-expressed or enriched biological annotations<sup>187</sup> to perhaps

give some idea of the potential role of these genes in biological systems. The ideal gene number to analyse with DAVID is between 200-2000 genes<sup>187</sup>, the SAM of serum-free versus serum-containing fibrocytes provided 6614 significantly different genes, which is too high. To reduce this a three-fold difference cut off was introduced, only those genes whose ratio to reference was three fold different or more between the two cell populations were used (appendix 7). This provided 808 genes, which were split into two gene lists, using the mean  $\log_2$  ratio reading across the samples for each population. The two lists contained those higher in serum-free fibrocytes (appendix 8) and those higher in serum-containing fibrocytes (appendix 9) and were inputted separately into DAVID. DAVID highlighted many biological processes however a cut off of an enrichment score of greater than 2 and a FDR of less than 5 were used. Table 6.4 shows the six processes that were highlighted by the gene list of just those genes higher in serum-free fibrocytes, compared to serum-containing. The gene count indicates the number of genes out of the inputted list involved in each process; and the percentage indicates those genes as a percentage of the number of genes put in. Table 6.5 shows the thirty seven processes enriched by the genes higher in serum-containing fibrocytes compared to serum-free. These gene ontology terms can act as a guide to which processes may be important when determining the roles of a cell population in the body.

Gene Ontology term	Gene Count	Percentage (%)	FDR
Chromatin assembly or disassembly	10	0.9	0
Chromatin assembly	8	0.7	0.1
Nucleosome assembly	7	0.6	0.3
Protein-DNA complex assembly	8	0.7	0.9
Establishment and/or maintenance of chromatin architecture	10	0.9	3.9
DNA Packaging	10	0.9	4.4

**Table 6.4 Processes enriched in DAVID when looking at those genes that were higher in serum-free fibrocytes than serum-containing fibrocytes.** The gene list provided by a 2 way SAM analysis of serum-free versus serum-containing fibrocytes was cut down to 808 genes by a three-fold difference threshold. Of those genes just those with more expressed in serum-free fibrocytes than serum-containing fibrocytes were inputted into DAVID (appendix 7). The processes classified as being enriched with this gene list, based on an enrichment score of 2, with an FDR of less than 5, are listed above with the number of genes out of the imputed list involved in the process, what percentage of imputed genes this is and the FDR.

Gene Ontology term	Gene Count	Percentage (%)	FDR
Localisation	96	26.1	0
Establishment of localisation	78	21.2	3.8
Cell proliferation	33	9	0.3
Regulation of cell proliferation	23	6.2	1
Defence response	29	7.9	0
Inflammatory response	16	4.3	2.4
Response to wounding	20	5.4	2.4
Chemical homeostasis	18	4.9	0.1
Cellular cation homeostasis	14	3.8	0.2
Cation homeostasis	14	3.8	0.2
Cellular chemical homeostasis	15	4.1	0.2
Cellular ion homeostasis	15	4.1	0.2
Ion homeostasis	15	4.1	0.6
Cellular homeostasis	17	4.1	1.3
Homeostatic process	20	5.4	1.7
Cellular di-,tri-,valent inorganic cation homeostasis	11	3	3
di-,tri-,valent inorganic cation homeostasis	11	3.0	3.1
Carboxylic acid metabolic process	29	7.9	0
Organic acid metabolic process	29	7.9	0
Icosanoid metabolic process	8	2.2	0
Icosanoid biosynthetic process	7	1.9	0
Cellular lipid metabolic process	30	8.2	0.1
Monocarboxylic acid metabolic process	17	4.6	0.1
Organic acid biosynthetic process	8	2.2	1.4
Carboxylic acid biosynthetic process	8	2.2	1.4
Alkene metabolic process	5	1.4	2.4
Leukotriene metabolic process	5	1.4	2.4
Fatty acid biosynthetic process	7	1.9	3.5
Signal Transduction	100	27.2	3.4
Localisation of cell	19	5.2	4.5
Cell motility	19	5.2	4.5
Tricarboxylic acid cycle intermediate metabolic process	5	1.4	4.3
Hydrogen Transport	10	2.7	0.2
Proton Transport	9	2.4	0.8
Coenzyme metabolic process	12	3.3	4.3
Vitamin Metabolic process	9	2.4	0.2
Water soluble vitamin metabolic process	6	1.6	4.7

**Table 6.5 Processes enriched in DAVID when looking at those genes that were higher in serum-containing fibrocytes than serum-free fibrocytes.** The gene list provided by a 2 way SAM analysis of serum-free versus serum-containing fibrocytes was cut down to 808 genes by a three-fold difference threshold. Of those genes just those with more expressed in serum-containing fibrocytes than serum-free fibrocytes were inputted into DAVID (appendix 8). The processes classified as being enriched with this gene list, based on an enrichment score of 2, with an FDR of less than 5, are listed above with the number of genes out of the imputed list involved in the process, what percentage of imputed genes this is and the FDR.

Of the 808 genes to be imputed into DAVID 151 had no name attached to the gene ID, and so could not be included. Of the remaining genes, 225 (28%) were higher in serum-free than serum-containing fibrocytes and 432 (53%) were higher in serum-containing than serum-free fibrocytes.

### **6.9.3 PAM analysis of serum-free fibrocytes versus serum-containing fibrocytes**

Predictive analysis of microarrays was performed on the two fibrocyte populations, serum-free and serum-containing. As with the three way analysis performed earlier six samples from each population were classified into the predictive set and three as the test set. Ten different iterations of PAM were performed with the samples in the predictive and test sets changing. A summary of all the test probability results of the test sets for all 10 iterations of PAM is shown in table 6.6. In all ten PAM analyses the gene list that had been determined classified all the samples correctly.

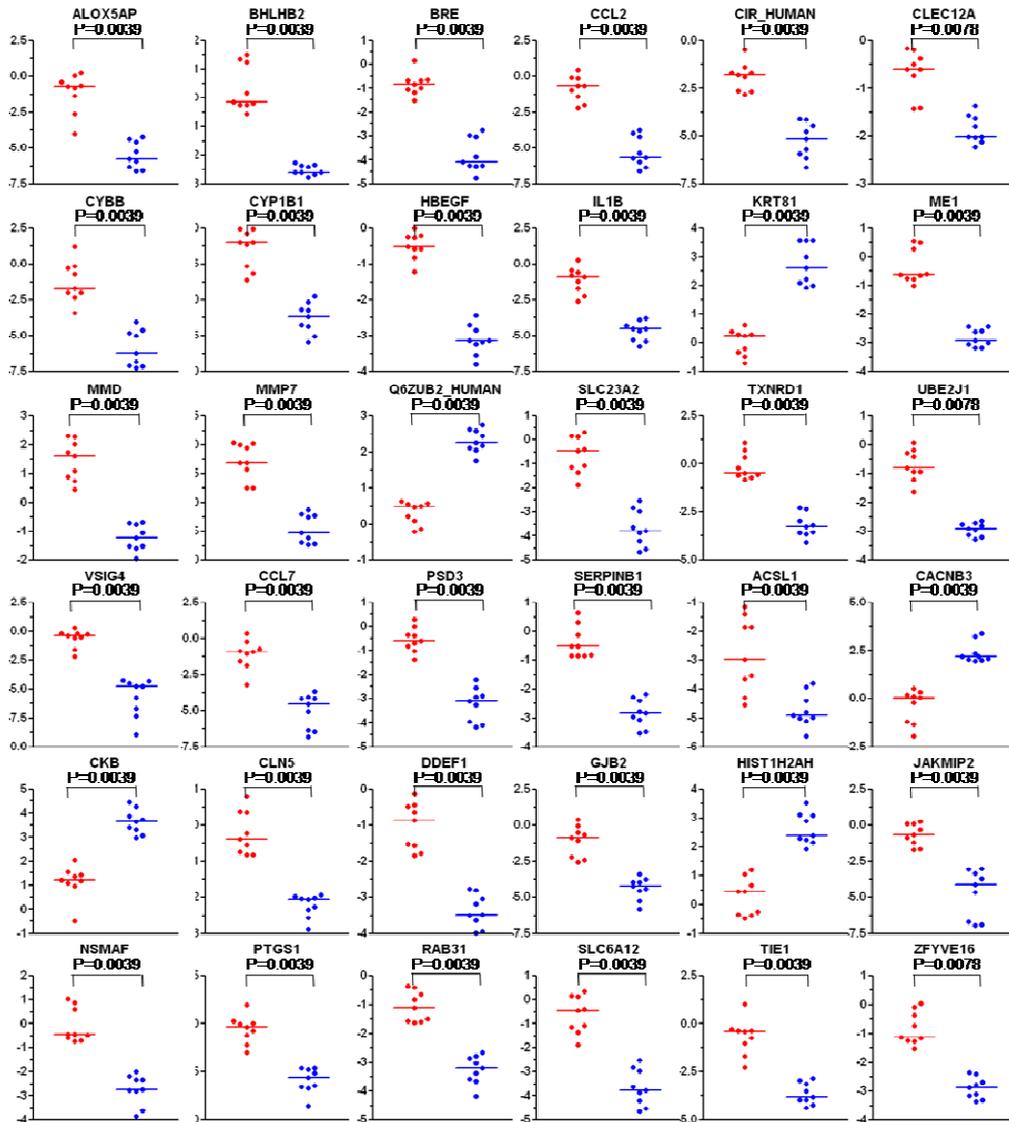
		Predicted Population	
		Serum-free fibrocyte	Serum-containing fibrocyte
True Population	Serum-free fibrocyte	30/30	
	Serum-containing fibrocyte		30/30

**Table 6.6 Summary of test probability results for PAM analysis of serum-free and serum-containing fibrocytes.** Using the predictive set of samples in a PAM analysis a set of genes was determined that can be used to predict the population type of samples. To test the ability of this predictive set a set of samples (test set) not involved in determining the predictive set of genes, were used. This was performed on ten different iterations of the PAM analysis and the results tabulated above. The population predicted by the gene lists is shown across the top and the true population down the side.

The genes within the gene lists used in these ten different analyses were ranked by the number of times they appeared and those genes which had been used 8 times and above selected. There were 36 genes selected, listed in table 6.7 along with their full name. Figure 6.17 shows the  $\log_2$  ratio data for each gene and the results of a Wilcoxon signed rank non-parametric statistical test, all are significantly different between the two cell populations, with at least  $P < 0.05$ . These genes were also identified as being present in the 6614 genes listed as being significantly different by SAM analysis at 0.1% FDR. To note is that this is again ratio data therefore the more negative a reading the higher the expression level of that gene in those samples is, therefore the cell type with the lowest ratio reading has the highest expression.

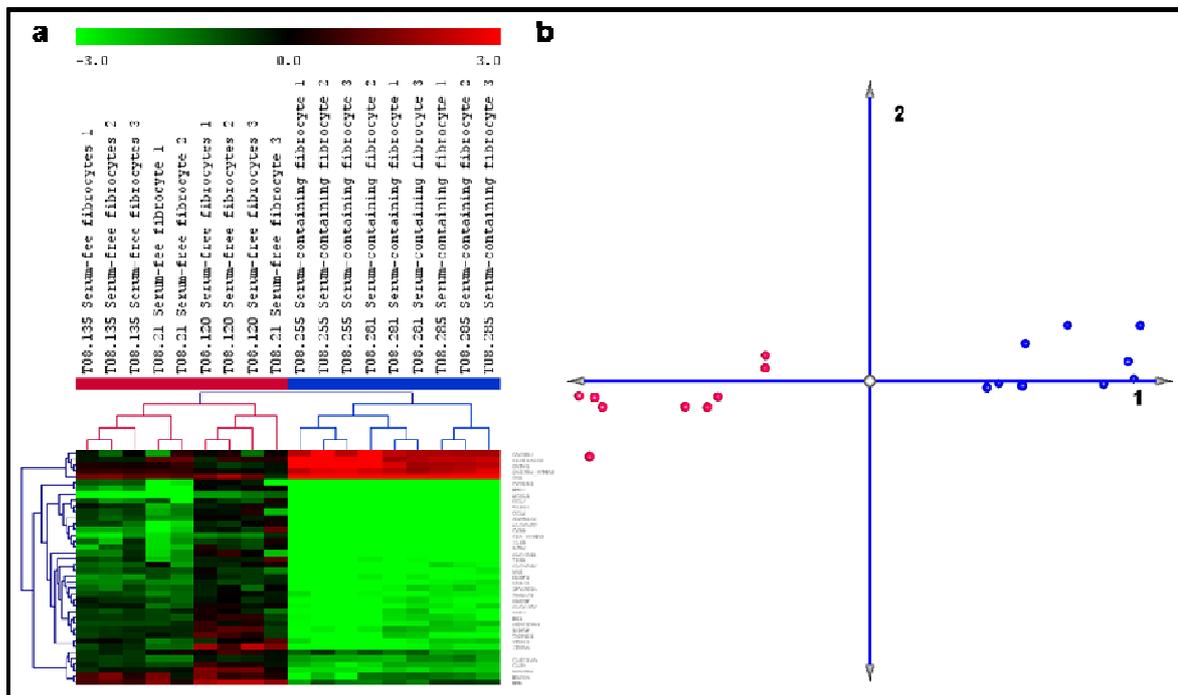
Gene	Full Name
ACSL1	Fatty acid coenzyme a ligase, long chain 1
ALOX5AP	Arachidonate 5-lipoxygenase-activating protein
BHLHB2	Basic helix-loop helix domain containing, class b, 2
BRE	Brain and reproductive organ-expressed
CACNB3	Calcium channel, voltage dependent, beta 3 subunit
CCL2	Chemokine (C-C motif) ligand 2
CCL7	Chemokine (C-C motif) ligand 7
CIR_HUMAN	CBF1-interacting corepressor
CKB	Cretine kinase, brain
CLEC12A	c-type lectin domain family 12, member a
CLN5	Ceroid-lipofuscinosis, neuronal 5
CYBB	Cytochrome b-245, beta polypeptide
CYP1B1	Member of cytochrome p450 family
DDEF1	Development and differentiation enhancing factor 1
GJB2	Gap junction protein, beta 2
HBEGF	Proheparin-binding EGF-like growth factor
HIST1H2AH	Histone cluster 1, H2ah
IL1B	Interleukin 1, beta
JAKMIP2	Janus kinase and microtubule interacting protein 2
KRT81	Keratin 81,
ME1	Malic enzyme 1
MMD	Monocyte to macrophage differentiation-associated
MMP7	Matrix metalloproteinase 7
NSMAF	Neutral sphingomyelinase (n-smase) activation associated factor
PSD3	Pleckstrin and Sec7 domain containing 3
PTGS1	Prostaglandin-endoperoxide synthase 1
Q6ZUB2_HUMAN	Unknown
RAB31	rab31, member ras oncogene family
SERPINB1	Serpinpeptidase inhibitor, clade b (ovalbumin)
SLC23A2	Solute carrier family 23 (nucleobase transporters), member 2
SLC6A12	Solute carrier family 6 (neurotransmitter transporter, betaine/GABA), member 12
TIE1	Tyrosine kinase with immunoglobulin-like and EGF-like domains 1
TXNRD1	Thioredoxin reductase 1
UBE2J1	Ubiquitin-conjugating enzyme e2,i1
VSIG4	V-set and immunoglobulin domain containing 4
ZFYVE16	Zinc finger, FYVE domain containing 16,

**Table 6.7 List of genes used eight times and above in the ten iterations of PAM to classify the serum-free and serum-containing fibrocyte samples.** Ten different iterations of PAM analysis were performed on the serum-free and serum-containing fibrocytes. Using a predictive set of samples in a PAM analysis a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in the ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen and listed above. The shortened and full gene names are shown.



**Figure 6.17** Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-free and serum-containing fibrocyte samples. Ten different iterations of PAM analysis were performed on the serum-free and serum-containing fibrocytes. Using a predictive set of samples in PAM a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in these ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen. Log(2) ratio data for each cell population for each gene is shown. Each sample is shown as a dot, with the median depicted by a line, serum-free fibrocytes are colour coded red and serum-containing fibrocytes blue. The population data for each gene was statistically tested using Wilcoxon signed rank non-parametric statistical test, all are significantly different with at least  $P < 0.05$ . NB This is ratio data of reference divided by sample therefore the more negative a reading the higher the expression level of that gene.

The  $\log_2$  ratio data of these 36 genes was used in hierarchical and Principal component analyses, (Figure 6.18). Even with these few genes, a clear separation of the two populations can be seen ( $\log_2$  ratio data in appendix 10). This provides further evidence that these are two different cell types, not the same cell type differentiated under different conditions.



**Figure 6.18 Cluster analysis of serum-free and serum-containing fibrocytes samples based on 36 predictive genes from PAM analysis.** Samples are colour coded, serum-free fibrocytes in red and serum-containing fibrocytes in blue. **a.** Hierarchical cluster analysis was performed on the 36 predictive genes. The hierarchical cluster analysis tree and the  $\log_2$  ratio information on all of the genes is shown. Samples are clustered based on their similarities, with the length of the branches between them being important, rather than their proximity of the samples to each other. **b.** Principal component analysis was performed on the same data set.

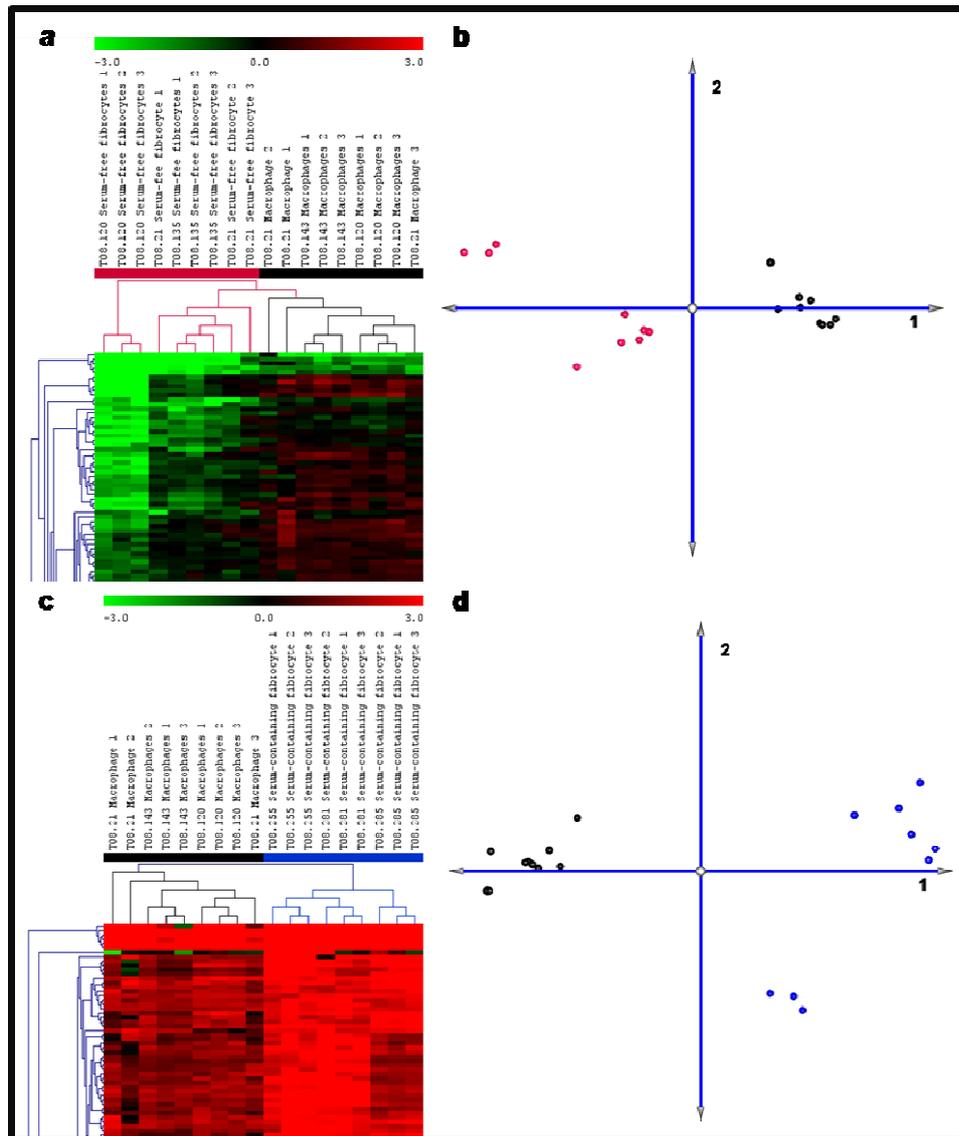
## 6.10 Analysis of fibrocytes versus macrophages

Although in some cases the literature discusses that fibrocytes are a type of macrophage<sup>85;86</sup> the majority of literature and data in this thesis does suggest that they are separate cell types as they have different characteristic morphologically, phenotypically and mechanistically<sup>84;87;88;145</sup>.

To determine that the differences noted between serum-free and serum-containing fibrocytes by this microarray analysis are enough to be able to ascertain that these are different cell types a comparison between both serum-free and serum-containing fibrocytes to macrophages was also performed. If the level of separation seen between the fibrocyte populations and macrophages is clear then there can be confidence in the differences seen between the two types of fibrocytes.

### 6.10.1 SAM analysis of fibrocytes versus macrophages

Unpaired SAM analysis of serum-free or serum-containing fibrocytes and macrophages, at 0.1% FDR provided 2419 or 5565 significantly different genes respectively (appendices 11 and 12). Using these genes hierarchical cluster and Principal component analyses were performed, figure 6.19, and in both cases a clear separation can be seen between both types of fibrocytes and macrophages. These results indicate that although related both types of fibrocytes are different cell populations to macrophages.



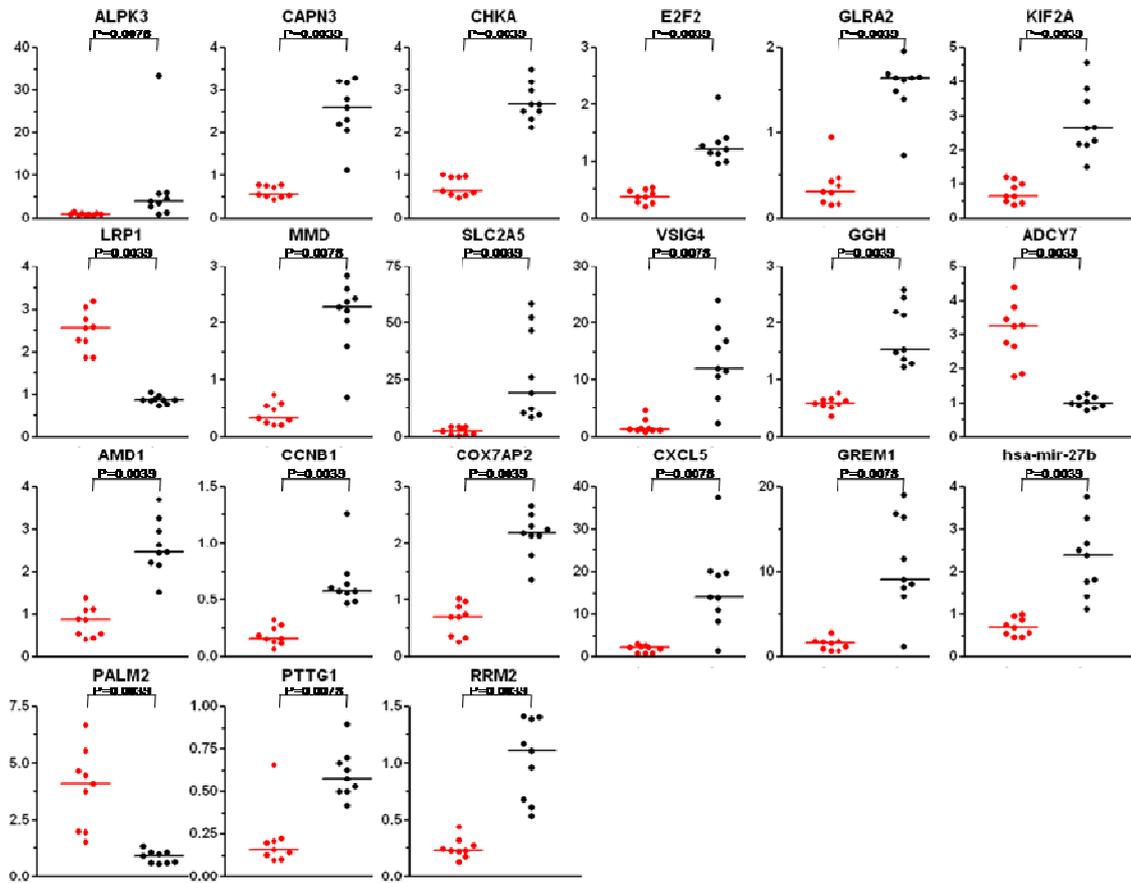
**Figure 6.19 Cluster analysis of fibrocytes and macrophage samples.** Samples are colour coded, serum-free fibrocytes in red, serum-containing fibrocytes in blue and macrophages in black. **a.** Hierarchical cluster analysis was performed on the 2419 significantly different genes found between serum-free fibrocytes and macrophages. The hierarchical cluster analysis tree and information on 50 genes are shown, although all 2419 genes were used to determine the tree. **b.** Principal component analysis was performed on the same data set. **c.** Hierarchical cluster analysis was performed on the 5565 significantly different genes found between serum-containing fibrocytes and macrophages. Again the hierarchical cluster analysis tree and information on 50 genes are shown, although all 5565 genes were used to determine the tree. **d.** Principal component analysis was performed on the same data set. Samples are clustered based on their similarities, with the length of the branches between them being important, rather than their proximity of the samples to each other.

### 6.10.2 PAM analysis of fibrocytes versus macrophages

Predictive analysis of microarrays was performed on both serum-free and serum-containing fibrocytes with macrophages. As previously six samples from each population were classified into the predictive set and three as the test set. Ten different iterations of PAM were performed with the samples in the predictive and test sets changing. In all ten PAM analyses the gene list that had been determined classified all the samples correctly in both comparisons. The genes within the gene lists used in these ten different analyses were ranked by the number of times they appeared and those genes which had been used 8 times and above selected. For serum-free fibrocytes versus macrophages 21 genes were selected, listed in table 6.8 along with their full name. Figure 6.20 shows the  $\log_2$  ratio data for each gene and the results of a Wilcoxon signed rank non-parametric statistical test, all are significantly different between the two cell populations, with at least  $P < 0.05$ . To note is that this is ratio data of reference divided by sample, therefore the more negative a reading the higher the expression level of that gene in those samples is, therefore the cell type with the lowest ratio reading has the highest expression. These genes were also identified as being present in the 2419 genes listed as being significantly different by SAM analysis at 0.1% FDR.

Gene	Full name
ADCY7	Adenylate cyclase 7
ALPK3	Alpha kinase 3
AMD1	Adenosylmethionine decarboxylase 1
CAPN3	Calpain 3
CCNB1	Cyclin B1
CHKA	Choline kinase alpha
COX7AP2	Cytochrome c oxidase subunit via pseudogene 2
CXCL5	Chemokine (c-x-x motif) ligand 5
E2F2	e2f transcription factor 2
GGH	Gamma-glutamyl hydrolase
GLRA2	Glycine receptor, alpha 2
GREM1	Gremlin 1, cysteine knot superfamily
hsa-mir-27b	Homo sapiens miR-27b stem-loop
KIF2A	Kinesin heavy chain member 2
LRP1	Low density lipoprotein-related protein 1
MMD	Monocyte to macrophage differentiation-associated
PALM2	Paralemmin 2
PTTG1	Pituitary tumour transforming 1
RRM2	Ribonucleotide reductase m2 polypeptide
SLC2A5	Solute carrier family 2 (facilitated glucose/fructose transporter), member 5
VSIG4	V-set and immunoglobulin domain containing 4

**Table 6.8 List of genes used eight times and above in the ten iterations of PAM to classify the serum-free fibrocyte and macrophage samples.** Ten different iterations of PAM analysis were performed on the serum-free fibrocytes and macrophages. Using a predictive set of samples in a PAM analysis a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in the ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen and listed above. The shortened and full gene names are shown.

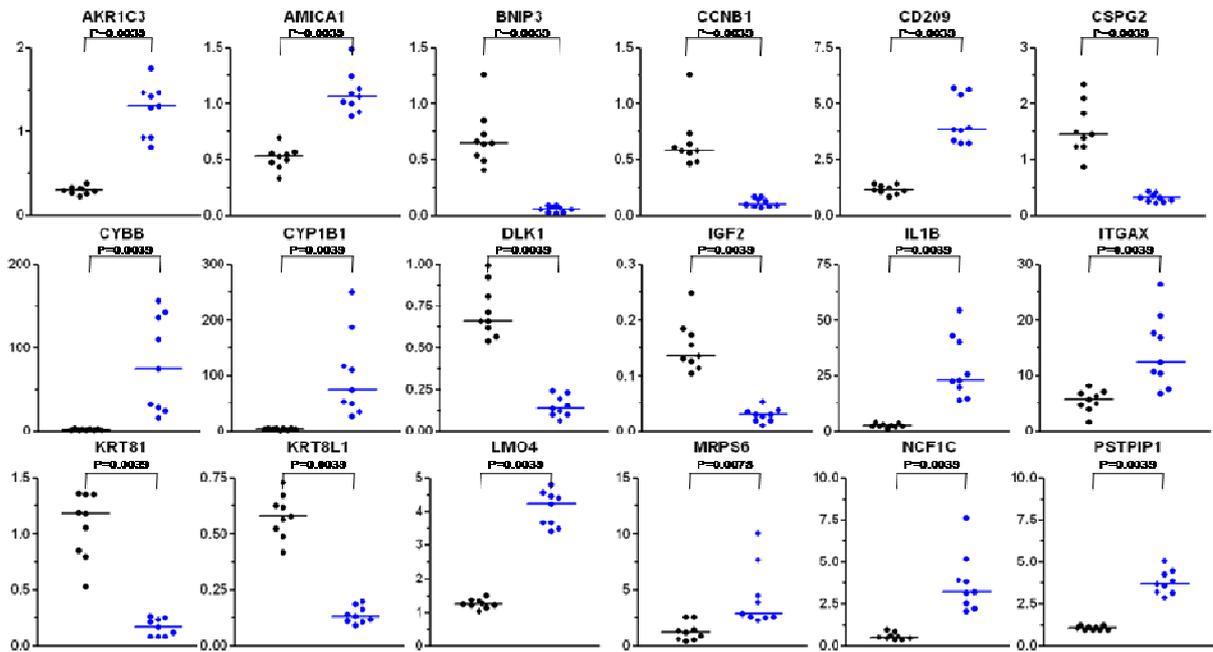


**Figure 6.20** Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-free fibrocytes and macrophage samples. Ten different iterations of PAM analysis were performed on the serum-free fibrocytes and macrophages. Using a predictive set of samples in PAM a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in these ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen. Log(2) ratio data for each cell population for each gene is shown. Each sample is shown as a dot, with the median depicted by a line, serum-free fibrocytes are colour coded red and macrophages black. The population data for each gene was statistically tested using Wilcoxon signed rank non-parametric statistical test, all are significantly different with at least  $P < 0.05$ . NB This is ratio data of reference divided by sample therefore the more negative a reading the higher the expression level of that gene.

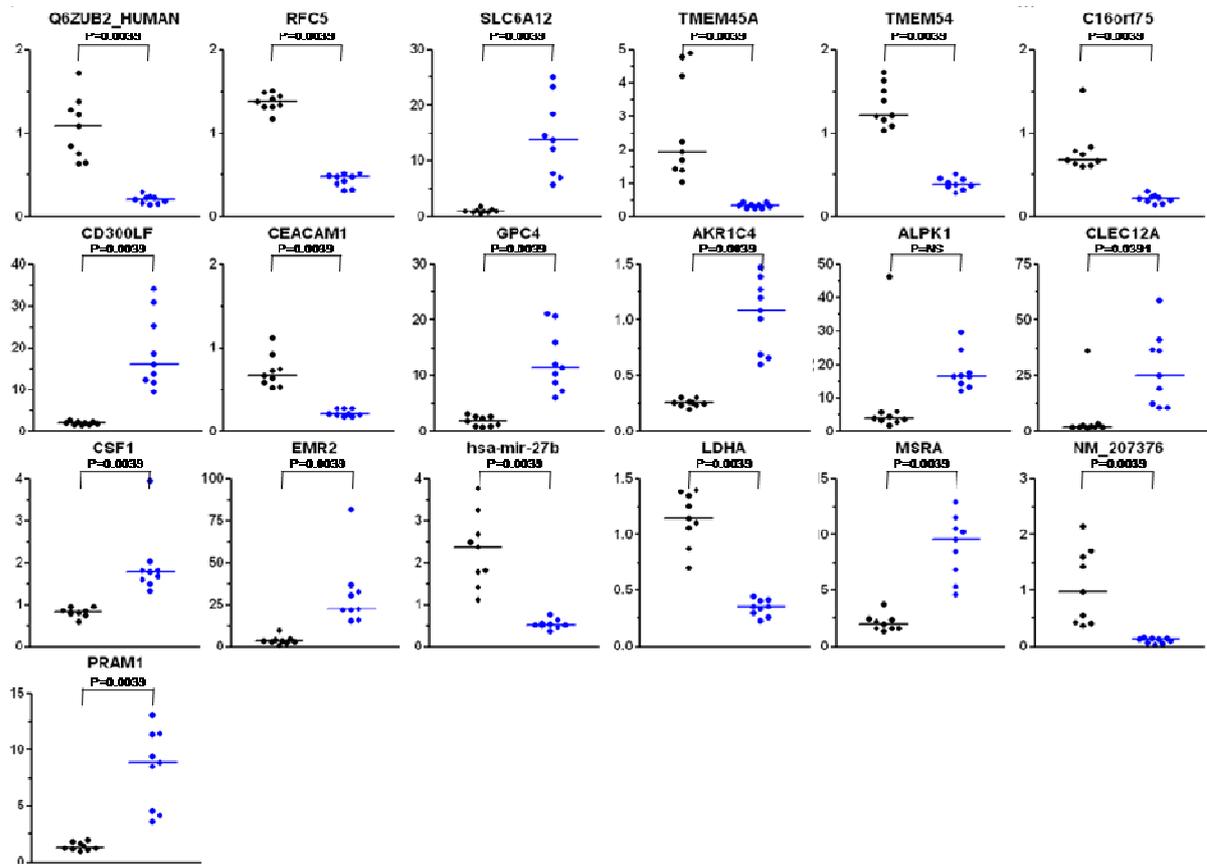
For serum-containing fibrocytes versus macrophages 37 genes were selected, listed in table 6.9 along with their full name. Figures 6.21a and 6.21b show the  $\log(2)$  ratio data for each gene and the results of a Wilcoxon signed rank non-parametric statistical test, all are significantly different between the two cell populations, with at least  $P < 0.05$ . To note is that this is ratio data of reference divided by sample, therefore the more negative a reading the higher the expression level of that gene in those samples is. These genes were also identified as being present in the 5565 genes listed as being significantly different by SAM analysis at 0.1% FDR.

Gene	Full name
AKR1C3	Aldo-keto reductase family 1, member c3
AMICA1	Adhesion molecule, interacts with cxadr antigen 1
BNIP3	bcl2/adenovirus elb 19kda interacting protein 3
CCNB1	Cyclin b1
CD209	CD209
CSPG2	Chondroitin sulfate proteoglycan core protein 2
CYBB	Cytochrome b-245, beta polypeptide
CYP1B1	Cytochrome P450, family 1, subfamily b, polypeptide
DLK1	Delta-like 1 homolog
IGF2	Insulin-like growth factor 2
IL1B	Interleukin 1, beta
ITGAX	Integrin, alpha x
KRT81	Keratin 81
KRT8L1	Keratin 8 pseudogene 11
LMO4	Lim domain only 4
MRPS6	Mitochondrial ribosomal protein s6
NCF1C	Neutrophil cytosolic factor 1c
PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1
Q6ZUB2_HUMAN	Unknown gene
RFC5	Replication factor c5
SLC6A12	Solute carrier family 6 member 12
TMEM45A	Transmembrane protein 45a
TMEM54	Transmembrane protein 54
C16orf75	Hypothetical protein
CD300LF	CD300 antigen like family member f
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
GPC4	Glypican-4
AKR1C4	Aldo-keto reductase family 1, member c4
ALPK1	Alpha protein kinase 1
CLEC12A	c-type lectin domain 12, member A
CSF1	Colony stimulating factor 1 (macrophage)
EMR2	egf-like module containing, mucin-like hormone receptor-like 2
hsa-mir-27b	Homo sapiens miR-27b stem-loop
LDHA	Lactate dehydrogenase a
MSRA	Methionine sulfoxide reductase a
NM_207376	Hypothetical gene
PRAM1	pm1-rara regulated adaptor molecule 1

**Table 6.9 List of genes used eight times and above in the ten iterations of PAM to classify the serum-containing fibrocyte and macrophage samples.** Ten different iterations of PAM analysis were performed on the serum-containing fibrocytes and macrophages. Using a predictive set of samples in a PAM analysis a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in the ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen and listed above. The shortened and full gene names are shown.



**Figure 6.21a** Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-containing fibrocytes and macrophage samples. Ten different iterations of PAM analysis were performed on the serum-containing fibrocytes and macrophages. Using a predictive set of samples in PAM a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in these ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen. Log(2) ratio data for each cell population for each gene is shown. Each sample is shown as a dot, with the median depicted by a line, serum-containing fibrocytes are colour coded blue and macrophages black. The population data for each gene was statistically tested using Wilcoxon signed rank non-parametric statistical test, all are significantly different with at least  $P < 0.05$ . NB This is ratio data of reference divided by sample therefore the more negative a reading the higher the expression level of that gene.



**Figure 6.21b** Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-containing fibrocytes and macrophage samples. Ten different iterations of PAM analysis were performed on the serum-containing fibrocytes and macrophages. Using a predictive set of samples in PAM a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in these ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen. Log(2) ratio data for each cell population for each gene is shown. Each sample is shown as a dot, with the median depicted by a line, serum-containing fibrocytes are colour coded blue and macrophages black. The population data for each gene was statistically tested using Wilcoxon signed rank non-parametric statistical test, all are significantly different with at least  $P < 0.05$ . NB This is ratio data of reference divided by sample therefore the more negative a reading the higher the expression level of that gene.

The log(2) ratio data of these genes were used in hierarchical and Principal component analyses, figure 6.22, in which, even with these few genes, a clear separation of the two populations can be seen with macrophages (log(2) ratio data for serum-free and serum-containing fibrocytes in

appendices 13 and 14 respectively). Although the macrophage sample which was not clustering with the other macrophages previously is again distant from the macrophage cluster.



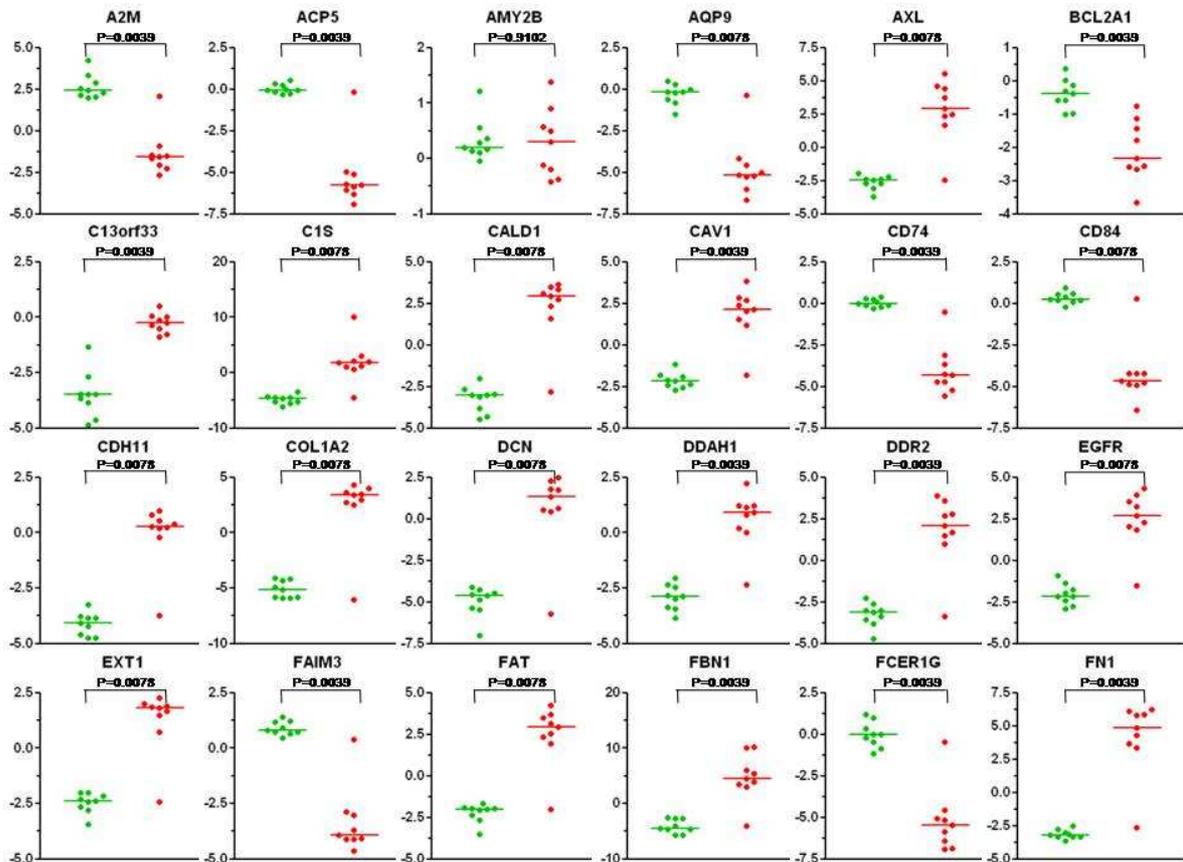
## **6.11 Predictive analysis of fibroblasts versus fibrocytes and macrophages.**

Using the normalised data of all four cell populations predictive analysis of microarrays was performed between fibroblasts and each of the in vitro derived cell types, serum-free, serum-containing fibrocytes and macrophages. For each PAM analysis PAM six samples of each cell type were classified into the predictive set and three as the test set. Ten different iterations of PAM were performed with the samples in the predictive and test sets changing. In all ten PAM analyses the gene list that had been determined classified all the samples correctly in both comparisons. The genes within the gene lists used in these ten different analyses were ranked by the number of times they appeared and those genes which had been used 8 times and above selected.

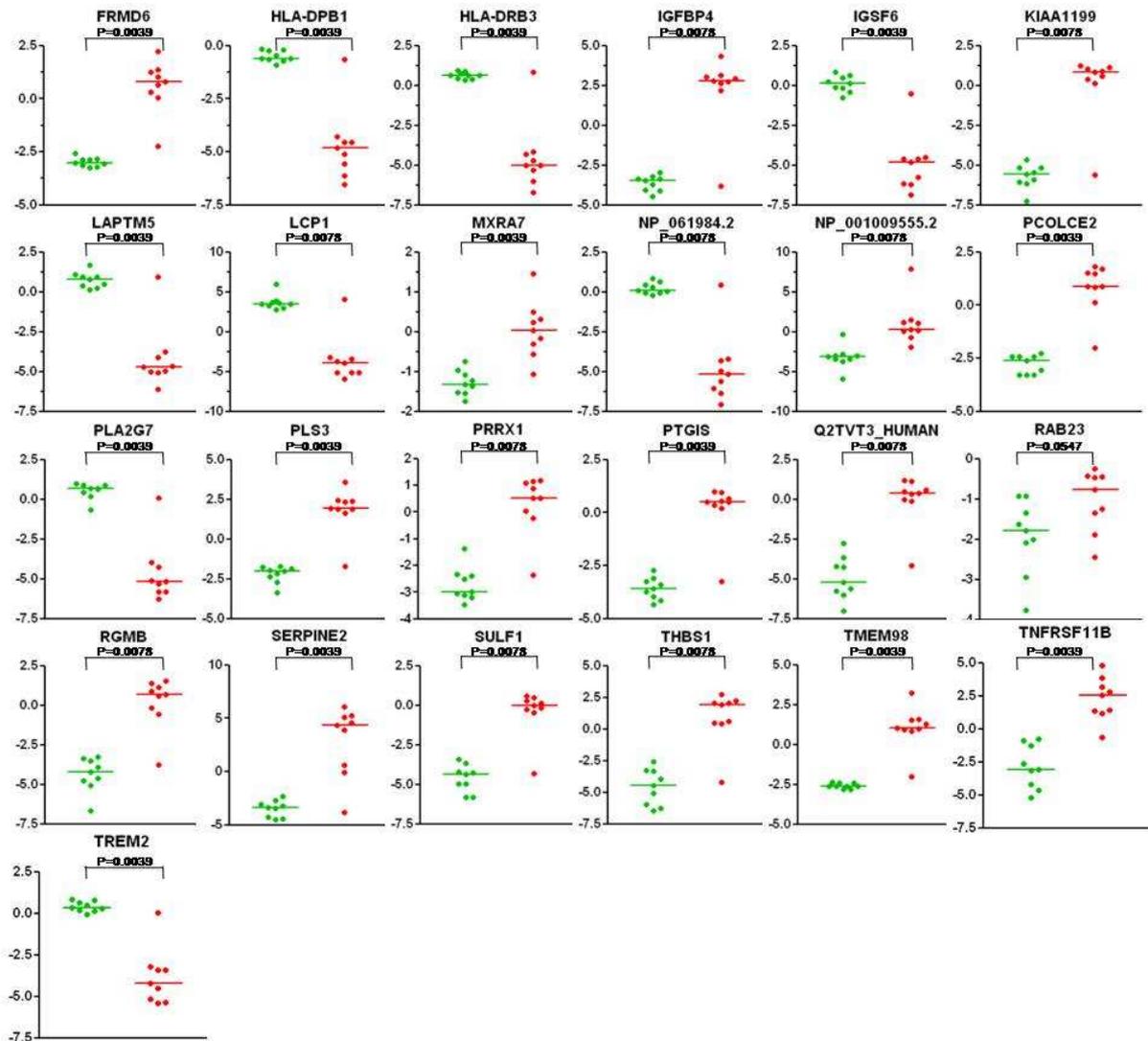
For serum-free fibrocytes versus fibroblasts 49 genes were selected, listed in table 6.10 along with their full name. Figures 6.23a and 6.23b shows the  $\log(2)$  ratio data for each gene and the results of a Wilcoxon signed rank non-parametric statistical test, all are significantly different between the two cell populations, with at least  $P < 0.05$ . To note is that this is ratio data of reference divided by sample, therefore the more negative a reading the higher the expression level of that gene in those samples is.

Gene	Full name	Gene	Full name
A2M	Alpha-2-microglobulin	HLA-DPB1	Major histocompatibility complex, class II, DR beta 1
ACP5	Acid Phosphatase 5	HLA-DRB3	Major histocompatibility complex, class II, DR beta 3
AMY2B	Amylase, alpha 2	IGFBP4	Insulin-like growth factor binding protein 4
AQP9	Aquaporin 9	IGSF6	Immunoglobulin superfamily member 6
AXL	AXL receptor tyrosine kinase	KIAA1199	Transmembrane protein 2-Like
BCL2A1	BCL2-related protein A1	LAPTM5	Lysosomal protein transmembrane 5
C13orf33	Chromosome 13 open reading frame 33	LCP1	Lymphocyte cytosolic protein 1
C1S	Complement component 1	MXRA7	Matrix-remodelling associated 7
CALD1	Caldesmon 1	NP_001009555.2	Unknown
CAV1	Caveolin 1	NP_061984.2	Major histocompatibility complex, class II, DR alpha. (HLA-DRA)
CD74	CD74 molecule	PCOLCE2	Procollagen C-endopeptidase enhancer 2
CD84	CD84 molecule (member of CD2 subgroup of Ig superfamily)	PLA2G7	Phospholipase A2
CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	PLS3	Plastin 3
COL1A2	Collagen type I, alpha 2	PRRX1	Paired related homeobox 1
DCN	Decorin	PTGIS	Prostaglandin 12 synthase
DDAH1	Dimethylarginine dimethylaminohydrolase 1	Q2TVT3_HUMAN	Unknown
DDR2	Discoidin domain receptor tyrosine kinase 2	RAB23	RAB23, member RAS oncogene family
EGFR	Epidermal growth factor receptor	RGMB	RGM domain family, member B
EXT1	Exostosin 1	SERPINE2	Serpin peptidase inhibitor, clade E, member 2
FAIM3	Fas apoptotic inhibitory molecule 3	SULF1	Sulfatase 1
FAT	FAT tumour suppressor homolog	THBS1	Thrombospondin 1
FBN1	Fibrillin 1	TMEM98	Transmembrane protein 98
FCER1G	Fc fragment of IgE. High affinity receptor 1. receptor for; gamma polypeptide	TNFRSF11B	Tumour necrosis factor receptor superfamily, member 11b
FN1	Fibronectin 1	TREM2	Triggering receptor expressed on myeloid cells 2
FRMD6	FERM domain containing 6		

**Table 6.10 List of genes used eight times and above in the ten iterations of PAM to classify the serum-free fibrocyte and fibroblast samples.** Ten different iterations of PAM analysis were performed on the serum-free fibrocytes and fibroblasts. Using a predictive set of samples in a PAM analysis a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in the ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen and listed above. The shortened and full gene names are shown.



**Figure 6.23a** Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-free fibrocytes and fibroblast samples (Part one). Ten different iterations of PAM analysis were performed on the serum-free fibrocytes and fibroblasts. Using a predictive set of samples in PAM a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in these ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen. Log(2) ratio data for each cell population for each gene is shown. Each sample is shown as a dot, with the median depicted by a line, serum-free fibrocytes are colour coded red and fibroblasts green. The population data for each gene was statistically tested using Wilcoxon signed rank non-parametric statistical test, all are significantly different with at least  $P < 0.05$ . NB This is ratio data of reference divided by sample therefore the more negative a reading the higher the expression level of that gene.

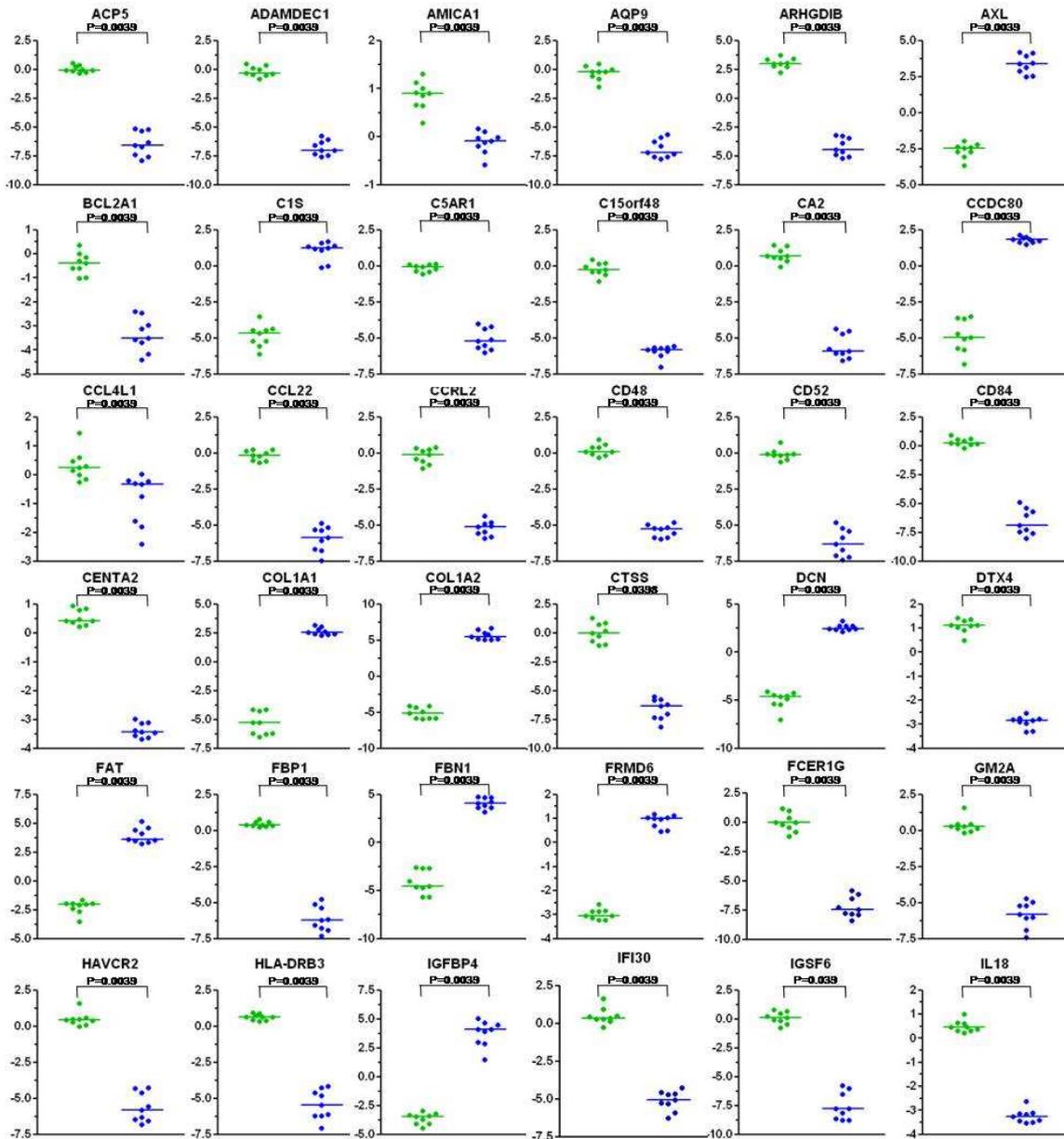


**Figure 6.23b** Log<sub>2</sub> ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-free fibrocytes and fibroblast samples (Part two). Ten different iterations of PAM analysis were performed on the serum-free fibrocytes and fibroblasts. Using a predictive set of samples in PAM a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in these ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen. Log<sub>2</sub> ratio data for each cell population for each gene is shown. Each sample is shown as a dot, with the median depicted by a line, serum-free fibrocytes are colour coded red and fibroblasts green. The population data for each gene was statistically tested using Wilcoxon signed rank non-parametric statistical test, all are significantly different with at least  $P < 0.05$ . NB This is ratio data of reference divided by sample therefore the more negative a reading the higher the expression level of that gene.

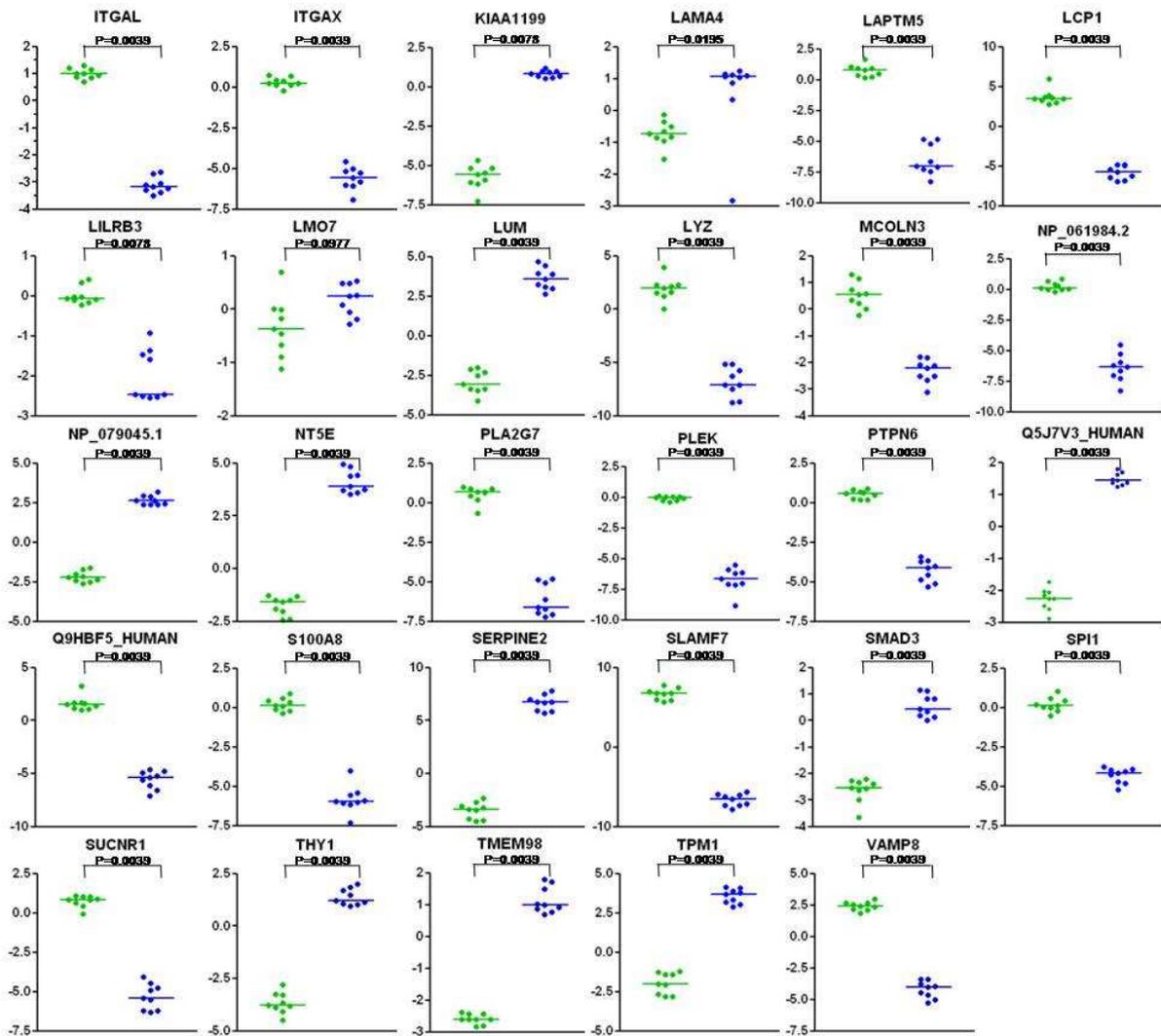
For serum containing fibrocytes versus fibroblasts 65 genes were selected, listed in table 6.11 along with their full name. Figures 6.24a and 6.24b shows the  $\log_2$  ratio data for each gene and the results of a Wilcoxon signed rank non-parametric statistical test, all are significantly different between the two cell populations, with at least  $P < 0.05$ . To note is that this is ratio data of reference divided by sample, therefore the more negative a reading the higher the expression level of that gene in those samples is.

Gene	Full name	Gene	Full name
ACP5	Acid Phosphatase 5	IGFBP4	Insulin-like growth factor binding protein 4
ADAMDEC1	ADAM-like, decysin 1	IGSF6	Immunoglobulin superfamily member 6
AMICA1	Adhesion molecule	IL18	Interleukin 18
AQP9	Aquaporin 9	ITGAL	Integrin, alpha L
ARHGDI3	Rho GDP dissociation inhibitor (GDI) beta	ITGAX	Integrin, alpha x
AXL	AXL receptor tyrosine kinase	KIAA1199	Transmembrane protein 2-Like
BCL2A1	BCL2-related protein A1	LAMA4	Laminin, alpha 4
C15orf48	Chromosome 15 open reading frame 48	LAPTM5	Lysosomal protein transmembrane 5
C1S	Complement component 1	LCP1	Lymphocyte cytosolic protein 1
C5AR1	Complement component 5a receptor 1	LILRB3	Leukocyte immunoglobulin-like receptor, subfamily B
CA2	Carbonic anhydrase II	LMO7	LIM domain 7
CCDC80	Coiled-coil domain containing 80	LUM	Lumican
CCL22	Chemokine ligand 22	LYZ	Lysozyme
CCL4L1	Chemokine ligand 4-like 1	MCOLN3	Mucolipin 3
CCRL2	Chemokine receptor-like 2	NP_061984.2	Major histocompatibility complex, class II, DR alpha. (HLA-DRA)
CD48	CD48 molecule	NP_079045.1	Adipocyte-specific adhesion molecule
CD52	CD52 molecule	NT5E	5'nucleotidase, ecto (CD73)
CD84	CD84 molecule (member of CD2 subgroup of Ig superfamily)	PLA2G7	Phospholipase A2
CENTA2	ArfGAP with dual PH domains 2	PLEK	Pleckstrin
COL1A1	Collagen type 1 alpha 1	PTPN6	Protein phosphatase, non-receptor type 6
COL1A2	Collagen type I, alpha 2	Q5J7V3_HUMAN	Unknown
CTSS	Cathepsin S	Q9HBF5_HUMAN	Unknown
DCN	Decorin	S100A8	S100 calcium binding protein A8
DTX4	Deltex homolog 4	SERPINE2	Serpin peptidase inhibitor, clade E, member 2
FAT	FAT tumour suppressor homolog	SLAMF7	Slam family member 7
FBN1	Fibrillin 1	SMAD3	SMAD family member 3
FBP1	Fructose-1, 6-biphosphatase	SPI1	Spleen focus forming virus (SFFV) proviral integration oncogene spi1
FCER1G	Fc fragment of IgE. High affinity receptor 1. receptor for; gamma polypeptide	SUCNR1	Succinate receptor 1
FRMD6	FERM domain containing 6	THY1	Thy-1 cell surface antigen
GM2A	GM2 ganglioside activator	TMEM98	Transmembrane protein 98
HAVCR2	Hepatitis A virus cellular receptor 2	TPM1	Tropomyosin 1
HLA-DRB3	Major histocompatibility complex, class II, DR beta 3	VAMP8	Vesicle-associated membrane protein 8
IFI30	Interferon, gamma inducible protein 30		

**Table 6.11 List of genes used eight times and above in the ten iterations of PAM to classify the serum-containing fibrocyte and fibroblast samples.** Ten different iterations of PAM analysis were performed on the serum-containing fibrocytes and fibroblasts. Using a predictive set of samples in a PAM analysis a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in the ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen and listed above. The shortened and full gene names are shown.



**Figure 6.24a** Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-containing fibrocytes and fibroblast samples. Ten different iterations of PAM analysis were performed on the serum-containing fibrocytes and fibroblasts. Using a predictive set of samples in PAM a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in these ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen. Log(2) ratio data for each cell population for each gene is shown. Each sample is shown as a dot, with the median depicted by a line, serum-containing fibrocytes are colour coded blue and fibroblasts green. The population data for each gene was statistically tested using Wilcoxon signed rank non-parametric statistical test, all are significantly different with at least  $P < 0.05$ . NB This is ratio data of reference divided by sample therefore the more negative a reading the higher the expression level of that gene.

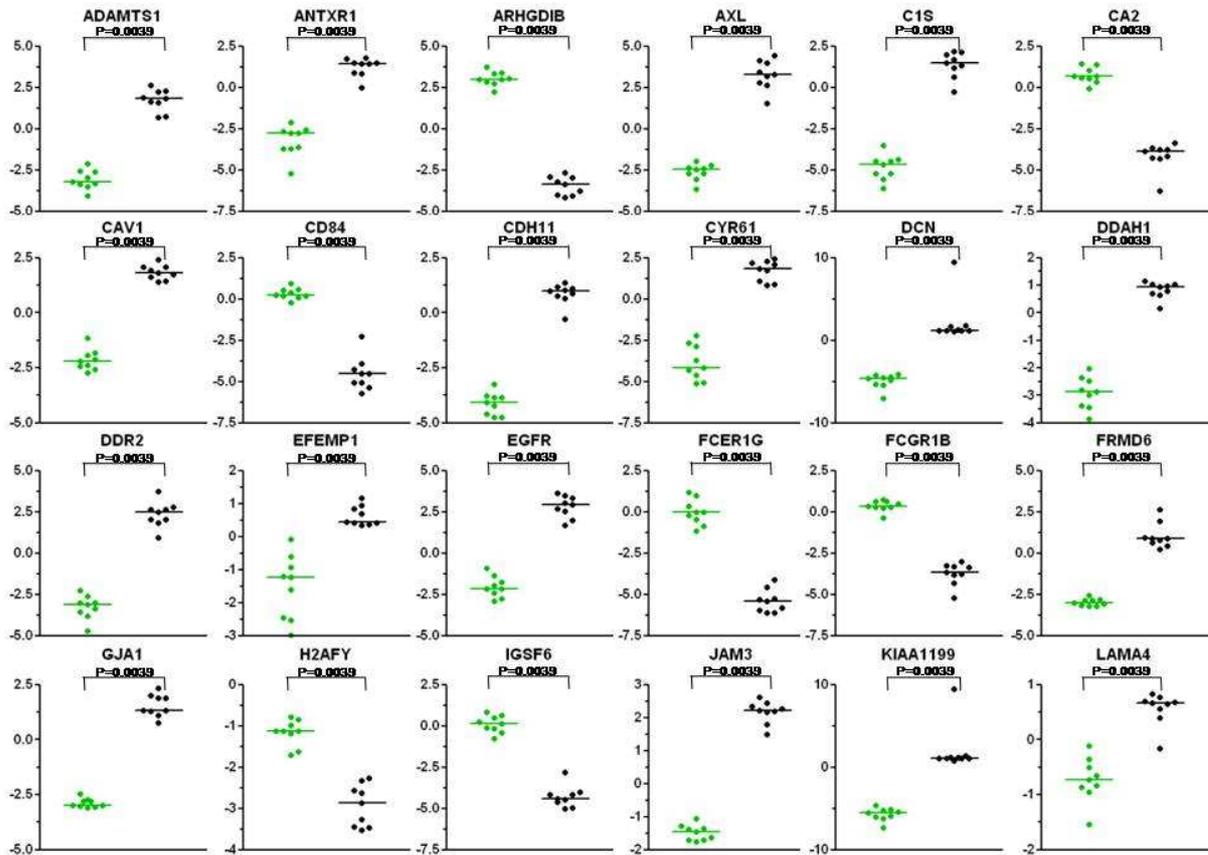


**Figure 6.24b** Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the serum-containing fibrocytes and fibroblast samples. Ten different iterations of PAM analysis were performed on the serum-containing fibrocytes and fibroblasts. Using a predictive set of samples in PAM a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in these ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen. Log(2) ratio data for each cell population for each gene is shown. Each sample is shown as a dot, with the median depicted by a line, serum-containing fibrocytes are colour coded blue and fibroblasts green. The population data for each gene was statistically tested using Wilcoxon signed rank non-parametric statistical test, all are significantly different with at least  $P < 0.05$ . NB This is ratio data of reference divided by sample therefore the more negative a reading the higher the expression level of that gene.

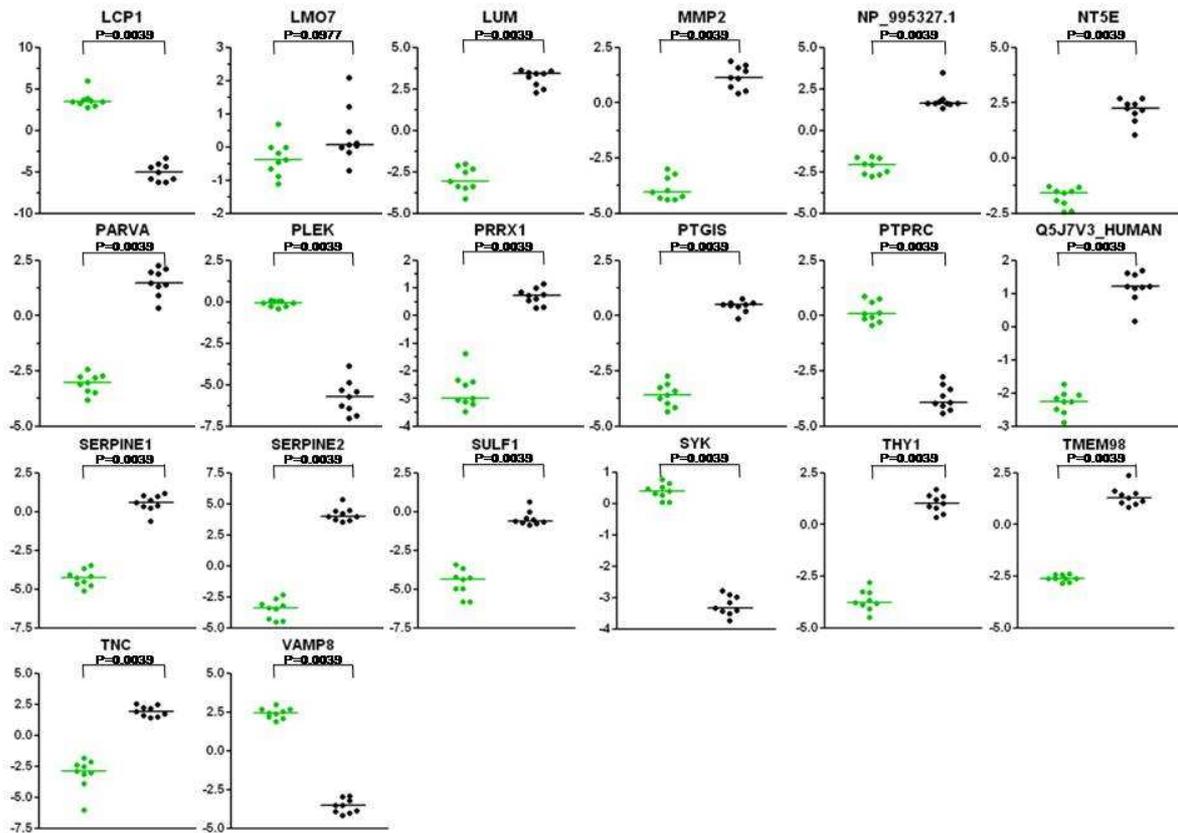
For macrophages versus fibroblasts 44 genes were selected, listed in table 6.12 along with their full name. Figures 6.25a and 6.25b shows the log(2) ratio data for each gene and the results of a Wilcoxon signed rank non-parametric statistical test, all are significantly different between the two cell populations, with at least  $P < 0.05$ . To note is that this is ratio data of reference divided by sample, therefore the more negative a reading the higher the expression level of that gene in those samples is.

Gene	Full name	Gene	Full name
ACP5	Acid Phosphatase 5	KIAA1199	Transmembrane protein 2-Like
ANTXR1	Anthrax toxin receptor 1	LAMA4	Laminin, alpha 4
ARHGDI3	Rho GDP dissociation inhibitor (GDI) beta	LCP1	Lymphocyte cytosolic protein 1
AXL	AXL receptor tyrosine kinase	LMO7	LIM domain 7
C1S	Complement component 1	LUM	Lumican
CA2	Carbonic anhydrase II	MMP2	Matrix metalloproteinase 2
CAV1	Caveolin 1	NP_995327.1	FAM180A family with sequence similarity 180, member A
CD84	CD84 molecule (member of CD2 subgroup of Ig superfamily)	NT5E	5'nucleotidase, ecto (CD73)
CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	PARVA	Parvin, alpha
CYR61	Cysteine-rich, angiogenic inducer, 61	PLEK	Pleckstrin
DCN	Decorin	PRRX1	Paired related homeobox 1
DDAH1	Dimethylarginine dimethylaminohydrolase 1	PTGIS	Prostaglandin 12 synthase
DDR2	Discoidin domain receptor tyrosine kinase 2	PTPRC	Protein tyrosine phosphatase, receptor type C
EFEMP1	EGF-containing fibulin-like extracellular matrix	Q5J7V3_HUMAN	Unknown
EGFR	Epidermal growth factor receptor	SERPINE1	Serpin peptidase inhibitor, clade E, member 1
FCER1G	Fc fragment of IgE. High affinity receptor 1. receptor for; gamma polypeptide	SERPINE2	Serpin peptidase inhibitor, clade E, member 2
FCGR1B	Fc fragment of IgG, high affinity 1b, receptor (CD64)	SULF1	Sulfatase 1
FRMD6	FERM domain containing 6	SYK	Spleen tyrosine kinase
GJA1	Gap junction protein, alpha 1	THY1	Thy-1 cell surface antigen
H2AFY	H2A histone family	TMEM98	Transmembrane protein 98
IGSF6	Immunoglobulin superfamily member 6	TNC	Tenascin C
JAM3	Junctional adhesion molecule 3	VAMP8	Vesicle-associated membrane protein 8

**Table 6.12 List of genes used eight times and above in the ten iterations of PAM to classify the macrophage and fibroblast samples.** Ten different iterations of PAM analysis were performed on the macrophage and fibroblasts. Using a predictive set of samples in a PAM analysis a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in the ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen and listed above. The shortened and full gene names are shown.



**Figure 6.25a** Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the macrophages and fibroblast samples. Ten different iterations of PAM analysis were performed on the serum-free fibrocytes and macrophages. Using a predictive set of samples in PAM a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in these ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen. Log(2) ratio data for each cell population for each gene is shown. Each sample is shown as a dot, with the median depicted by a line, macrophages are colour coded black and fibroblasts green. The population data for each gene was statistically tested using Wilcoxon signed rank non-parametric statistical test, all are significantly different with at least  $P < 0.05$ . NB This is ratio data of reference divided by sample therefore the more negative a reading the higher the expression level of that gene.



**Figure 6.25b** Log(2) ratio data on genes used eight times and above in the ten iterations of PAM to classify the macrophages and fibroblast samples (Part two). Ten different iterations of PAM analysis were performed on the serum-free fibrocytes and macrophages. Using a predictive set of samples in PAM a set of genes was determined that could be used to predict the population type of samples. These were then tested on a test set of samples. The genes in the gene lists created in these ten iterations were ranked according to the number of times a gene was used and any genes used in eight lists or above were chosen. Log(2) ratio data for each cell population for each gene is shown. Each sample is shown as a dot, with the median depicted by a line, macrophages are colour coded black and fibroblasts green. The population data for each gene was statistically tested using Wilcoxon signed rank non-parametric statistical test, all are significantly different with at least  $P < 0.05$ . NB This is ratio data of reference divided by sample therefore the more negative a reading the higher the expression level of that gene.

## 6.12 Discussion of chapter

The aims of this transcriptome analysis were to try to determine the transcriptional relationship between *in vitro* derived, donor-matched serum-free and serum-containing fibrocytes to each other, and to donor matched macrophages and fibroblast cell lines together, or in various combinations. Ultimately this was to help determine whether serum-free and serum-containing fibrocytes are the same cell types, differentiated under different conditions, or two different cell types with similar phenotypic and morphologically characteristics. The first unsupervised cluster analyses (Figures 6.5 and 6.6) show a degree of separation between all four cell populations. This is a very important observation as it shows that despite 37632 genes being used to do the clustering there are enough differences to allow the division of samples into the different cell types. Especially when many of the 37632 genes are likely to be involved in defining a cell as just being a cell, and therefore whose expression is expected to be very similar among all cells. Not every sample clusters perfectly, however the general degree of clustering between samples of the same cell type is very promising. Once SAM had been performed and the genes significantly different between the populations (12602 genes) identified, the clustering was repeated and even tighter clustering was seen within the cell types (figures 6.8 and 6.9), indicating that all four cell types can be separated. It also needs to be noted that the purity of the T08.255 serum-containing fibrocytes, based on cell morphology in table 6.1 was very low, however was still used for consistency between the donors. Despite this all serum-containing fibrocytes samples cluster very closely together, indicating perhaps that those cells without a fibroblast like morphology are still a form of fibrocyte.

Although the 12602 significantly different genes identified by SAM could cluster the samples into four different populations the biggest difference between the samples appeared to be between the fibroblasts and everything else, with the exception being of one serum-free fibrocyte sample which clusters with the fibroblasts (Figures 6.8 and 6.9). Why this serum-free fibrocyte sample clustered with the fibroblasts is unknown as the replicates for that donor clustered with the other serum-free fibrocytes. Due to the major differences in the way the fibroblast and other cells are cultured we were concerned that this would be contributing largely to the effect seen between them. Fibrocytes and macrophages were differentiated from peripheral blood and cultured for a relatively short period of time *in vitro*. However the fibroblasts were proliferating cell lines, although only at passage four following isolation and culturing may have introduced a bias that was uncontrollable in this experimental set up. The presence of genes involved in proliferation in the fibroblasts could also mask more subtle differences seen between the other three cell types. Therefore the decision was made to exclude the fibroblasts from much of the further analysis to prevent any bias that they may introduce and to allow for a more refined analysis of the differences within the remaining three cell populations. Although it is important to compare the transcriptomes of fibrocytes and fibroblasts, due to the linkage between the two populations described in the literature<sup>124;145;183</sup>, comparing fibroblasts immediately after isolation, rather than as an established cell line may be more informative. This may also prevent the introduction of bias due to continuous cell culture as seen when using cell lines, however, until specific cell markers for both fibroblasts and

fibrocytes can be determined, so that populations of each one are not contaminated with the other, it will be not be possible to perform this investigation. However the data from the normalisation of all four populations was used to do predictive analysis of microarrays between fibroblasts and the other three cell populations. When doing this 49 genes were found to be sufficient to predict whether samples were serum-free fibrocytes or fibroblasts (table 6.10 figures 6.23a and 6.23b), 65 genes could predict whether samples were serum-containing fibrocytes or fibroblasts (table 6.11 and figures 6.24a and 6.24b) and 44 genes were able to predict macrophages and fibroblasts (table 6.12 and figures 6.25a and 6.25b).

Once the fibroblast samples had been removed unsupervised clustering of the three remaining cell types, serum-free, serum-containing fibrocytes and macrophages, was repeated. Again, despite being unsupervised a large amount of clustering between samples of the same cell type was seen (Figure 6.10). Once SAM analysis was performed and only those genes found to be significantly different between the three cell types used to perform cluster analysis (4563 genes), more stringent clustering was seen, in which there was only 1 macrophage sample which did not cluster as expected, it clustered with the serum-free fibrocytes in the hierarchical cluster analysis (Figure 6.11 a.), although in the Principal component plot all macrophage samples clustered closely together (Figure 6.11 b). Again it is unknown why this one macrophage sample did not cluster as expected, with its replicates, however as it clustered with the other macrophage samples in the PCA we were not concerned. Interesting to note is that the biggest difference observed (along principal component 1) is between serum-free fibrocytes

and serum-containing fibrocytes, giving more credence to the hypothesis that the two types of fibrocytes are not the same cell type. Also the macrophage samples seemed to cluster closer to the serum-free fibrocytes than to the serum-containing, perhaps suggesting the closer relationship between these two.

When performing PAM on these three populations just 28 genes were found to be sufficient to identify each cell population (Table 6.3). These 28 genes were also able to clearly separate the three populations by cluster analysis, HCL and PCA, other than the one macrophage sample which clustered with the serum-free fibrocytes (Figure 6.15). Of these 28 genes 24 showed the major difference in the expression level to be between the serum-containing fibrocytes and the other two populations (Figure 6.14), further suggesting that serum-free fibrocytes and macrophages are more closely related to each other than either are to the serum-containing fibrocytes. Also interesting to note is that of the remaining four genes three (ALPK3, CCNB1 and hsa-mir-27b) show macrophages being different from the two types of fibrocytes, and only one, MMD show serum-free fibrocytes being different from the other two cell populations, although this is because the level of expression of MMD in the serum-free fibrocytes is less than that in the other two populations, rather than a gene that is specific to this cell type. The role of MMD is not completely known, it was first described in 1995 as a macrophage maturation associated transcript, which, although having 7 transmembrane domains has little homology to G-protein receptors and has not been positively described as a receptor, however it has been suggested that it might be an ion channel protein<sup>188;189</sup>.

Although the three way analysis of the two types of fibrocytes and macrophages is informative, suggesting the presence of three different cell populations, a two way analysis of the fibrocytes would provide a more definitive answer and allow for specific transcriptome differences between them to be identified. A SAM analysis of serum-free and serum-containing fibrocytes at 0.1% FDR provided 6614 significantly different genes between them which, when used in cluster analysis, clearly separated out the two cell types (Figure 6.17). These results indicate that although morphologically and phenotypically very similar there is strong evidence to suggest that serum-free and serum-containing fibrocytes are not the same cell type.

The 6614 genes identified by SAM as being significantly different between serum-free and serum-containing were cut down to be entered into the web based computer programme DAVID. Of the 225 genes found to be higher in serum-free fibrocytes than serum-containing only 6 processes were found that these genes were enriched in (Table 6.4). Unfortunately all 6 appeared to be to do with chromatin and DNA mechanisms, giving no real insight into the potential roles of these cells in the body compared to serum-containing fibrocytes. However there were 432 genes higher in serum-containing fibrocytes compared to serum-free, providing 37 enriched processes (Table 6.5). These include processes involved in localisation, defence and inflammatory responses as well as a response to wounding. These results seemed biologically relevant as fibrocytes have been reported as being very important in wound healing<sup>45;87;90;142;170</sup>, as well as the immune system<sup>89;91</sup>, and may suggest the importance of serum-containing

fibrocytes, compared to serum-free, in localising to sites where they are needed and restoring homeostasis. It is important to remember that the biological processes provided by DAVID are based on previously published information on the roles of different genes in different processes, therefore just because the processes come up as being enhanced in these genes lists does not mean that these cell populations are definitely involved in these processes. It just means that these genes have been reported as being important in these processes at some point. Therefore processes provided by DAVID act only as a guide to the potential biological roles these cell populations may have within the body. There are quite a few processes enriched in the serum-containing fibrocyte genes list (Table 6.5) which are also involved in a form of homeostasis, as well as metabolic processes, which could suggest that as well as being involved in the defence system of the body and wound healing, these serum-containing fibrocytes may also be involved in returning the body to its normal homeostasis following an immune response.

PAM of serum-free fibrocytes versus serum-containing fibrocytes produced a list of 36 predictive genes (Table 6.7 and Figure 6.17), all of which were also seen within the 6614 genes found to be significantly different between the cell types by SAM. When used in cluster analysis these 36 genes were also able to clearly separate all 18 samples by HCL and PCA (Figure 6.18). Of these 36 genes both CCL2 and CCL7 were present and higher expression was observed in serum-containing fibrocytes compared to serum-free. CCL2 and CCL7 are chemokines that share the chemokine receptor CCR2, and the interactions of these chemokines and receptor have been reported as being important not only in wound healing and fibrosis, but also in the role of

fibrocytes in both of these processes as they are involved in their recruitment to sites of injury<sup>124;126;190;191</sup>. Also, although MMP7 and fibrocytes have not been associated within the literature, MMP7 has been reported as a signature protein found in peripheral blood, thought to indicate asymptomatic interstitial lung disease, or pulmonary fibrosis as well as disease progression<sup>192</sup>. As there is a reported role of fibrocytes in pulmonary fibrosis<sup>112;116;125;131;183</sup> the expression of MMP7 may also be related to fibrocytes.

Very few of the 36 genes highlighted by PAM between the two types of fibrocytes are reported in the literature as being related to fibrocytes, whether serum-free or serum-containing. However, of the genes highlighted some have been reported to be involved in inflammation. ALOX5AP is an activating protein which has a key role in the production of leukotrienes from leukocytes. Leukotrienes are fatty molecules involved in autocrine and paracrine signalling to regulate the immune response, often contributing to the inflammation in various inflammatory diseases including chronic obstructive pulmonary disease, asthma and rheumatoid arthritis<sup>193-195</sup>. Also, although only reported in endothelial cells to date, TIE1 has been reported as being important in inducing a pro-inflammatory response when expressed, as well as being up-regulated in inflammatory diseases such as atherosclerosis and rheumatoid arthritis<sup>196</sup>. IL1 $\beta$ , a member of the IL1 cytokine family, is known to be produced by activated macrophages and is an important mediator of the inflammatory response, as well as inducing the production of PTGS2, one of the two genes involved in the production of prostaglandins, however, in the case of this gene, only in times of inflammation.

As well as genes involved in inflammation genes involved in homeostasis were also highlighted in the PAM list. PTGS1 was listed and is the other gene involved in the production of prostaglandins, however rather than at times of inflammation as with PTGS2, it maintains baseline levels of prostaglandins for tissue homeostasis. The protein produced by the gene BRE, although initially thought to only be in the brain and reproductive systems, has now been reported as being expressed in many sites including the digestive system, respiratory organs as well as the lymphoid tissue and endocrine organs. As well as being reported as being involved in mediating anti-apoptosis<sup>197</sup>, it has also been suggested as possibly being a house-keeping gene for tissues constantly subjected to environmental hazards<sup>198</sup>. The gene CLEC12A produces a c-type Lectin also known as MICL known to be present on monocytes and macrophages<sup>199</sup> which has been suggested to play a role in homeostasis<sup>200;201</sup>. As has VSIG4, which is so far specific to macrophages and thought to be important in the maintenance of T cell unresponsiveness in healthy tissues<sup>202</sup>.

The protein produced by the gene HBEGF was first identified as a product secreted by macrophages like cells and has been implicated in angiogenesis as well as wound healing<sup>203;204</sup>, both processes of which fibrocytes are linked to<sup>45;87;90</sup>. Looking at the information on all of these gene we can see that the processes highlighted by the DAVID analysis as being different between the serum-free and serum-containing fibrocytes are backed up by the genes used by PAM to separate the two cell types.

The results of both DAVID and PAM suggest the importance of serum-containing fibrocytes in inflammation, tissue homeostasis and wound healing, but give very little insight into the role of serum-free fibrocytes in the body. Within the PAM gene list only five genes had a higher expression in serum-free fibrocytes compared to serum-containing fibrocytes, however very little is known about these five genes, other than CKB. The production of which is reported as being greatly increased in osteoclast formation<sup>205</sup> and its inhibition saw a suppression in bone-reabsorbing activity *in vitro* and *in vivo* in CKB knock-out mice<sup>205</sup>.

To investigate the relationship of both serum-free and serum-containing fibrocytes to macrophages two-way SAM and PAM were performed for both comparisons. In both cases each type of fibrocyte was found to be significantly different to macrophages, and clear separation was seen between the two cell populations (Figures 6.19).

When performing PAM 21 genes were found to successfully separate serum-free fibrocytes and macrophages (Table 6.8 and Figures 6.20 and 6.22). Of these 21 only 3 had a log(2) ratio higher in serum-free fibrocytes compared to the macrophages (figure 6.20). When looking at serum-containing versus serum-free fibrocytes only 5 of the 36 genes which could separate the two populations had a log(2) ratio value higher in serum-free fibrocytes. The fact that serum-free fibrocytes are mainly separated from other cell populations by the lack of genes rather than the expression of certain genes may suggest that they are potentially a type of default population

that monocytes differentiate into when there are no signals to differentiate into anything else, or to remain as a monocyte. As a default population they could retain some level of pluripotency, enabling them to continue to differentiate into other cell types if required, such as osteoclasts. As discussed in the introduction there are cells thought to be derived from CD14<sup>+</sup> peripheral blood cells that have the ability to differentiate into several mesenchymal type cell lineages<sup>147;148</sup>.

Further investigation of the gene lists provided by SAM and PAM is needed to elucidate potential candidate genes that could be used to both validate these results and to act as markers for separating out these populations in further investigations. Unfortunately the downside to using microarrays of the entire human genome are that very little is known about a large number of genes on the microarray, therefore when it comes to understanding the importance of these genes in context to the biological systems being looked at it can become very difficult, as in some cases very little may be published on them.

The results seen in this chapter indicate that serum-free and serum-containing fibrocytes are two different cell populations as 6614 genes were found to be significantly different between them by SAM and 36 genes found to be able to separate them by PAM.

## 7.0 FINAL DISCUSSION

Although fibroblasts-like cells that differentiate from a haematopoietic origin have been described for over a hundred years (reviewed in Postlewaite et al 2004<sup>84</sup>) it has only been in the last twenty years that people have really started investigating their roles in the human body and disease. In 1994 Bucala *et al* termed these cells fibrocytes and described them as being morphologically similar to fibroblasts, however expressing different surface markers, and most importantly being haematopoietic rather than mesenchymal<sup>87</sup> in origin. As well as their fibroblast-like morphology fibrocytes are identified by their expression of both stromal and haematopoietic makers, such as collagen, vimentin, CD45 and CD13, and are described as being important in both wound healing and fibrotic diseases<sup>47;87;113;134;142</sup>. The role of fibrocytes in both of these processes is not surprising as fibrotic diseases are thought to often be due in part to an excess of wound healing components<sup>106;108;206</sup>. In the majority of research into fibrotic diseases fibroblasts have been implicated as the critical cell of the disease<sup>104;106;108;122;206-208</sup>, however more recent studies have suggested that as well as the resident fibroblasts, fibrocytes have an important role in the disease process<sup>84;122;145;183</sup>. Mouse models of both pulmonary and hepatic fibrosis have implicated fibrocytes in the development of these fibrotic disorders<sup>111;116;134</sup> and fibrocytes have also been implicated in mouse models of wound healing<sup>87;111;142</sup>. However before the particulars of the role of fibrocytes in fibrosis and wound healing can be elucidated further investigation into the differentiation, development and recruitment of these cells is required to be able to understand their complex role in both wound

healing and fibrotic disorders, and so allow for therapeutic strategies of treatment to be determined.

In 2003 Pilling *et al* reported that fibrocyte differentiation is inhibited *in vitro* by the plasma protein SAP and its interaction with Fcγ receptors<sup>47;48</sup>, this was supported by a mouse model of fibrotic ischemia/reperfusion cardiomyopathy (I/RC) where the administration of SAP saw a reduction of cells with a fibrocyte-like morphology from the site of injury *in vivo* but did not suppress the development of inflammation or chemokine expression<sup>111;113</sup>. The inhibition of fibrocyte differentiation by SAP *in vitro* was determined by the study of fibrocytes in a serum-free environment<sup>47;48</sup> as these cells were seen to appear from PBMC after 3 days of cell culture. This is in contrast to many of the other reports on fibrocytes in which they are differentiated in a serum-containing environment, with differentiated cells appearing after 10-14 days<sup>45;87;89-91</sup>. Fibrocytes differentiated in both a serum-free and serum-containing environment morphologically look very similar and have the same expression profile, which has been shown in this thesis (chapter 3) as well as reported<sup>47;87</sup>; this has led to the belief that that they are the same cell differentiated through different methods. It has also been reported that serum-free derived fibrocytes express high levels of the enzyme required for collagen synthesis, prolyl 4-hydroxylase<sup>48</sup>, whereas in a separate report fibrocytes generated in a serum-containing environment have a low level of prolyl 4-hydroxylase<sup>150</sup>, although a direct comparison has not been made. This thesis addresses whether serum-free and serum-containing fibrocytes are the same cell differentiated under different methods or whether they are actually two different,

although related cell types; and this has been investigated by using both *in vitro* cell culture and molecular microarray profiling.

The optimum culture conditions for fibrocyte differentiation in both serum-free and serum-containing environment were initially addressed in this thesis due to discrepancies seen throughout the literature. Slightly different culture conditions have been used across the various reports; such as different starting concentrations of PBMC, whether or not the non-adherent cells were washed off, and at what time point<sup>45-48;87;89-91;103;116;146</sup>. In our hands the optimum culture conditions for fibrocyte differentiation were PBMC at  $1 \times 10^6$  cell/ml, washing off the non-adherent cells 96 hours later; and to plate out CD14<sup>+</sup> selected cells at  $2 \times 10^6$  cell/ml (Figures 3.7-3.9). Once the optimum culture conditions had been determined the expression profile of these cells were examined for a limited number of markers to ensure consistency between the cells used in this thesis and the reported expression profile of fibrocytes. The expression profile of the two types of fibrocytes were identical and concurred with reported results (Figures 3.15-3.17, Table 3.1).

Despite reports that fibrocytes can be detached by ice cold 0.05% EDTA/ PBS<sup>45;46;89;91;146</sup>, this was not seen in this thesis when tested on serum-free derived fibrocytes (Figures 3.10 and 3.12). As fibrocytes could not be detached a method of quantifying results *in situ* was required. Following testing, counting the number of fibrocytes and none fibrocytes then taking a mean reading from three fields of view from three wells from each condition was found to be a

suitable protocol (Figures 3.3-3.6). Therefore for each condition when looking at fibrocyte differentiation or the effects agents have on them a minimum of three wells was required.

If serum-free and serum-containing derived fibrocytes are the same cell type differentiated under different conditions it was speculated that once differentiated they could be switched into the opposite media and survive. This thesis shows that serum-containing fibrocytes, once differentiated, will survive happily in a serum-free environment (Figures 4.8 and 4.9), however serum-free derived fibrocytes roll up in the presence of serum, at whatever time point it is added and do not return to their original morphology for at least two weeks afterwards (Figures 4.8-4.10, 4.18). This effect was found to occur in the presence of both bovine and human serum, and the effects seen within four hours (Figures 4.11- 4.16). These rolled-up fibrocytes kept a fibrocyte expression profile though, continuing to express fibronectin when rolled-up (Figure 4.19), the expression of which is not seen in other monocyte-derived progeny, such as the macrophage (Figure 3.15). When trying to elucidate the cause of this effect of the serum preliminary experiments suggest it is a very heat stable, large protein or particle (Figures 4.20-4.21), which fits with the description of the plasma protein SAP. However commercial SAP, although found to prevent fibrocyte differentiation (Figure 4.23), did not cause fibrocytes to roll-up when administered once fibrocyte differentiation had taken place (Figure 4.22), this observation however requires further investigation as is only based on preliminary work.

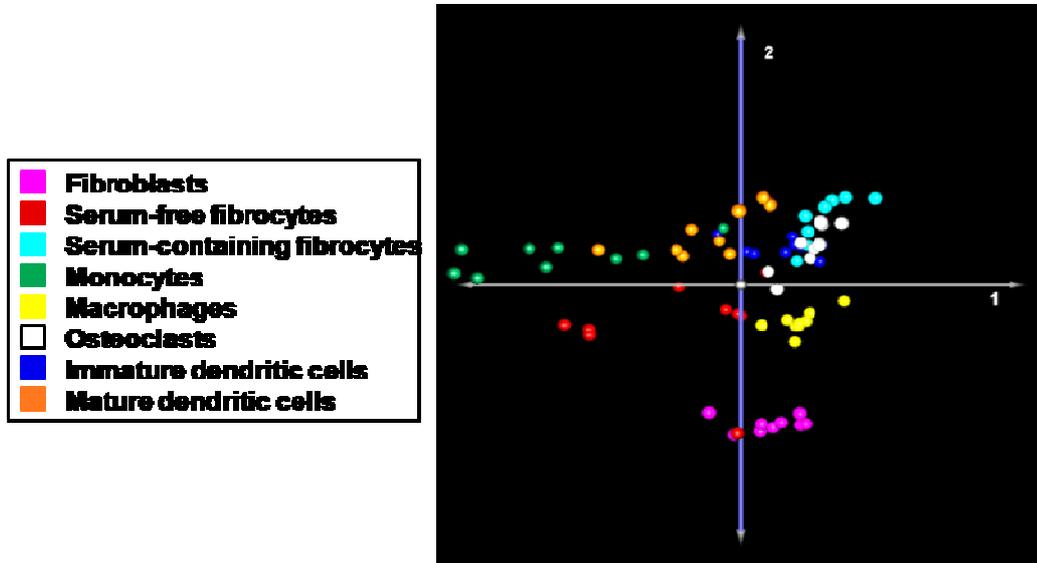
Fibrocyte differentiation is known to occur from CD14<sup>+</sup> cells of the peripheral blood or monocytes<sup>45;47-49;85;86</sup>. Despite reports that the presence of T cells, or at least T cell conditioned media are required for fibrocyte differentiation<sup>45;46</sup>, we found that both serum-free and serum-containing fibrocytes differentiated from PBMC and CD14 positively selected cells (although PBMC derived serum-containing fibrocyte numbers were very low) Interestingly however it was found that significantly more fibrocytes differentiated in a serum-free environment than a serum-containing environment from PBMC, and in contrast significantly more fibrocytes differentiated in a serum-containing environment than serum-free from CD14<sup>+</sup> selected cells (Figure 4.1). Further investigation into this is required to elucidate the causes of it whether it be due to factors present or the interaction of the CD14 molecule or both. However this observation is important as under the same culture conditions, from the same donors PBMC respond differently in the two culture environments. It is important to elucidate the cause of this phenomenon as if the hypothesis that these two related cells occur at different time points of a wound healing response is correct and subtly different signals are involved in their differentiation what these signals are would need to be known so that further work on these cells can be performed

Three populations of human monocytes have been described in the literature<sup>51;52</sup>, with the major population being CD14<sup>+</sup>CD16<sup>-</sup> and two other smaller populations identified as CD14<sup>+</sup>CD16<sup>+</sup> and CD14<sup>lo</sup>CD16<sup>+</sup>. As discussed in the introduction it has been suggested that these three different populations have different roles within the immune system<sup>40;53-55;61;179;180</sup>, and

due to the differences seen in this thesis between serum-free and serum-containing derived fibrocytes, whether the monocyte population they differentiated from was also different was investigated. Results showed that although macrophages will differentiate from all three monocyte populations both serum-free and serum-containing fibrocytes only differentiate from the CD14<sup>+</sup>CD16<sup>-</sup> and CD14<sup>+</sup>CD16<sup>+</sup>, and not CD14<sup>lo</sup>CD16<sup>+</sup> (Figures 5.3-5.4).

When examining these serum-free and serum-containing derived fibrocytes by molecular genes arrays, spotted microarrays of the complete human genome were used, containing approximately 37,000 genes. Ideally a comparison of the transcriptomes of the serum-free and serum-containing derived fibrocytes would be done on pure cultures of cells, however, as neither fibrocyte population differentiates in a homogeneous fashion *in vitro*, and a representative population of serum-free derived fibrocytes could not be detached, whole cultures had to be used to do the comparisons and were lysed *in situ*, despite the presence of non-morphological fibrocytes in the culture. However although under the criteria used in this thesis non-spindle shaped cells in a fibrocyte culture are not defined as fibrocytes 'rolled' up fibrocytes or round cells in figure 4.19 (serum-free fibrocytes which had been treated with serum) did still express fibrocyte markers. Also one of the serum-containing fibrocyte donors for the transcriptome analysis only produced cultures that were 8% pure based on morphology, however these went to on cluster well with the other serum-containing samples, therefore the lack of a spindle shaped morphology may not mean the absence of fibrocytes.

Following two way comparisons of the serum-free and serum-containing fibrocytes they were seen to cluster completely separately (Figures 6.17 and 6.19) and by statistical analysis 6614 genes were found to be different between them (Figure 6.17). Even when the clustering of the two populations was examined in the presence of other cell populations, namely macrophages and fibroblasts, in an unsupervised fashion, these two populations were still seen to separate (Figures 6.6, 6.7, 6.11, 6.12, 6.16). Further work within the department on the clustering of cell populations based on their transcriptome has included these samples plus other monocyte derived progeny, as well as monocytes themselves. They have found that even with other populations included (monocytes, osteoclasts, immature dendritic cells and mature dendritic cells) serum-free and serum-containing fibrocytes continue to cluster separately, supporting the hypothesis that these are two separate populations based on gene expression (Figure 7.1).



**Figure 7.1 PCA of Monocytes, monocyte derived progeny and fibroblasts.** Triplicate samples of donor matched monocytes and monocyte derived progeny, as well mismatched fibroblasts cell lines were analysed by transcriptome analysis and clustering based on all 37,000 genes, depicted by 3D Principal component analysis. Cell populations identified by the key, 3 separate donors/cell lines were used for each population.

The genes determined to be significantly different between serum-free and serum-containing fibrocytes by SAM analysis were used in the analysis programme DAVID to provide potential biological processes that these two cell populations are involved in (Tables 6.4-6.5). The list of genes found to have a higher expression in serum-containing fibrocytes compared to serum-free provided many more processes than the genes found to be more highly expressed in the serum-free fibrocytes. There were not only more processes but also processes that fibrocytes have already been reported to being involved in such as response to wounding and the inflammatory response (Table 6.5). As well as this PAM analysis provide a list of just 36 genes required to separate these two populations (Table 6.7), and of these genes the majority had a

higher expression value in serum-containing fibrocytes than serum-free (Figure 6.18). In fact rather than a set of genes or processes emerging as indicators of serum-free fibrocytes these cells actually appear to be identified by the lack of gene expression.

Although very little has been reported in the literature linking any of the genes highlighted by PAM to fibrocytes, by looking at what has been reported on these genes, the potential pathways determined from DAVID and the *in vitro* work in this thesis it could be suggested that although related serum-free and serum-containing derived fibrocytes are two different cell types that have very similar roles within the body. In the initial research by Bucala *et al* in a murine wound healing model a rapid influx of inflammatory cells prior to the emergence of fibrocytes was seen very soon after the initiation of the inflammation<sup>87</sup>. Further work on these cells *in vitro* went on to suggest their importance in not only angiogenesis but also in presenting antigen to T cells<sup>90</sup>; and all this work was done in a serum-containing environment.

One hypothesis that links all this data is that what are termed here as serum-containing fibrocytes appear very soon within sites of inflammation and wound healing to not only assist the macrophages and other immune cells in the removal of the cause of the inflammation, but also to begin the repair of the site with the production of extra cellular matrix components such as collagen and fibronectin. *In vitro* these serum-containing fibrocytes do take longer to appear than serum-free derived fibrocytes however there is currently no reported single signal that causes fibrocyte differentiation and it may be that the complex combination of signals a

monocyte receives on entering a site causes a more rapid differentiation of these cells than is seen *in vitro*. Plus it has also been suggested that monocytes are required to migrate through endothelial cells to be able to mature into fibrocytes rapidly<sup>111</sup>.

Once a site of inflammation or wound healing begins to recover and the environment changes, with the loss of serum factors 'serum-free derived' fibrocytes may enter the site and take over the role of repair, as they are more suited to that environment, in a less inflammatory environment they do not need the high expression of as many inflammatory mediating molecules, hence the lower expression of these factors in comparison to serum-containing fibrocytes. Although serum-containing derived fibrocytes can remain in the site it allows them to move on to other areas if required. It maybe the recruitment of these serum free derived fibrocytes that are involved in excessive fibrosis and so the development of fibrotic diseases, as SAP treatment in mouse models reduced the level of fibrosis seen<sup>112;113</sup>. This is all speculation and much more investigation would be required, however it would explain the need for two very similar cell types within the body and the sometimes conflicting reports in the literature seen.

A more precise transcriptome analysis between the two fibrocyte populations could be determined if a pure population of these two cell types could be obtained. Due to the difficulties in detaching these cells a protocol whereby individual cells could be laser microdissected and so a pure population obtained is being investigated. Using this technology a

sample of 20 or 30 cells can be collected for each population and RNA extracted. This RNA was found to be viable, as once reverse transcribed, product was seen following L27 gene PCR, however to be able to use this material on the human genome microarrays much more material would be required, and so cDNA amplification needed. So far testing of amplification protocols from this small amount of material have resulted in far too much non specific artefact being produced, making any microarray analysis of the material questionable. Therefore the current technology available does not allow for these more precise comparisons to be made at the moment.

However laser microdissected material could be used to validate the results of the transcriptome analysis performed so far, following up on the genes that have been found to be significantly different between the two types of fibrocytes. If a set of genes can be found to identify these two potential populations the role of serum-containing and serum-free fibrocytes in wound healing and disease could be further investigated. Cells of a 'fibroblast-like' morphology could be laser micro-dissected out of tissue samples of repairing or diseased tissue and the expression of these identifying genes looked at to see if a particular type of fibrocyte can be identified. Dependant on the tissue used a clearer idea of the role of these cells could be determined. As fibrocytes have been shown to be important factors involved in fibrosis and fibrotic diseases if there are two types the ability to determine them would be extremely important as it would lead to a clearer understanding of their role in the disease and consequently help in determine suitable treatments.

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