IMMOBILISED GROWTH FACTORS FOR SCALABLE CELL THERAPY MANUFACTURING PLATFORMS

Matthew James Worrallo

A thesis submitted to Loughborough University for the degree of

Doctor of Philosophy.

March 2017.

Centre for Biological Engineering

Healthcare Engineering

Wolfson School for Mechanical and Manufacturing Engineering

Loughborough University

Abstract

Regenerative medicine has the potential to establish or restore normal function in defective tissues and organs. The realisation of such therapies is restricted due to costs, lack of scalability and inefficient manufacturing process controls. A major contributor to cost is the use of expensive growth factors supplemented into media at high concentrations.

In vivo, growth factors exist in soluble, immobilised and transmembrane forms, expressed in a spatiotemporal fashion within the stem cell niche. In comparison to soluble equivalents, immobilised growth factors exhibit increased potency, distinct functional activities, improved cell phenotypic control and act in synergy with other soluble and immobilised ligands. To date, most research into immobilised growth factors has been restricted to planar cell culture surfaces such as tissue culture plastics which have limited scalability.

To address the scalability limitations, a novel growth factor immobilisation technology was developed using magnetic microparticles which can be scaled with respect to surface area to volume ratio in standard stirred tank bioreactors. Three clinically relevant growth factors, SCF, TPO and GM-CSF were immobilised and were shown to remain functionally active where surface concentration could be manipulated in a number of ways. Through a series of experiments, it was demonstrated that immobilised growth factors exhibited ~10-fold increase in potency compared with soluble equivalents and remain stable for up to 192 hours following recycling during multiple media passages. Immobilised growth factors were able to expand more cells over a longer period of time after transient exposure and finally, the immobilisation technique was successfully applied to the expansion of umbilical cord derived haematopoietic stem cells using immobilised SCF.

The immobilisation method described here has the potential to significantly reduce media costs in large scale cell manufacturing processes.

Publications and Patents Arising from PhD

Publications

Worrallo, M. J., Moore, R. L.L., Glen, K. E. and Thomas, R. J. (2017), **Immobilized hematopoietic growth factors onto magnetic particles offer a scalable strategy for cell therapy manufacturing in suspension cultures.** Biotechnol. J., 12: n/a, 1600493. doi:10.1002/biot.201600493.

Moore, R. L.L., Worrallo, M.J., Mitchell, P.D., Harriman, J.; Glen, K.E., Thomas, R.J. (2017). **Immobilisation of Delta-Like 1 ligand for the scalable and controlled manufacture of hematopoietic progenitor cells in a stirred bioreactor**. BMC Biotechnology. Submitted 21/03/2017.

Patents

Improved cell culture using beads. Filed May 2016. GB1608847.8

Acknowledgments

First and foremost I would like to acknowledge my supervisors, Rob and Katie, for their support, encouragement and patience throughout the undertaking of this PhD.

I would like to thank Katie for her help and guidance particularly in the lab when I first joined, she taught me the "ways" of a PhD and was there to crack the whip in times when I had lost motivation. Rebecca's input during the final stages of the PhD was instrumental in producing some very interesting data!

I have made many great memories at Loughborough and have met some lifelong friends along the way. Preeti, Maz and Emma have been instrumental in keeping me sane, a laugh-a-day was almost certainly guaranteed with you guys and I wonder how many cups of tea we made together (or I have made for you)?

Undoubtedly, I could not have completed this PhD without the love, encouragement and support from my family over the last 4 years. Hopefully I can now live up to the nickname you gave me when I first told you I had secured a place at Loughborough (Dr Nobby).

Last but not least, thank you to Loughborough Graduate School for the financial support over the course of the four years; I most certainly wouldn't have been able to pursue this PhD without such incentive.

Contents

Abstract I

Publications and Patents Arising from PhD II

Acknowledgments III

Contents IV

List of Figures IX

List of Tables XV

List of Abbreviations XVI

1 Introduction 1

1.1 Cell and Gene Therapies 1

1.1.1 Cell Therapies 1

1.1.2 Gene Therapies 2

1.1.3 The Cell and Gene Therapy Landscape 3

1.1.4 Clinically Relevant Cells 4

1.2 Haematopoietic Stem Cells 7

1.2.1 Characterisation 8

1.2.2 The Stem Cell Niche(s) 11

1.2.3 Sources of Haematopoietic Stem Cells 12

1.3 Considerations and Challenges with Cell Expansion 13

1.4 Hurdles and Solutions to the Commercialisation of Cell and Gene Therapy 15

1.4.1 Automation 16
1.4.2 Quality by Design 17
1.4.3 Safety and Efficacy 18
1.5 Growth Factors 20
1.5.1 Immobilised Growth Factors 21
1.6 Applications of Magnetic Microparticles in Cell and Gene Therapy 22
1.7 Thesis Objectives 25
2 Materials and Methods 26
Methods 27
2.1 Cell Culture 28
2.1.1 TF-1 Human Erythroleukemia Cell Line 28
2.1.2 M-07e Cell Line 30
2.1.3 Umbilical Cord Blood Derived CD34+ Cells 31
2.2 Cell Counting 34
2.2.1 ViCell (Trypan Blue Exclusion) 34
2.2.2 NC-3000 34
2.2.3 Flow cytometry 35
2.3 Agitated Cell Culture 35
2.4 Functional Assays 37
2.4.1 TF-1 Functional Assay 37

2.4.2 M-07e Functional Assay 37

2.4.3 CD34+ Functional Assay 37

2.4.4 Dose-response Assay (AlamarBlue®) 38

2.5 Growth Factor Immobilisation 39

2.5.1 Streptavidin Method 39

2.5.2 Carboxyl Method 41

2.5.3 Fibronectin Method 42

2.5.4 Solulink Method 42

2.6 Nutrient and Metabolite Analysis 43

2.7 Protein Quantification 43

2.7.1 Immobilised Growth Factor Concentration 43

2.7.2 Soluble Growth Factor Concentration 44

2.8 Immunophenotyping 44

2.9 Magnetic removal 45

2.9.1 Magnetic pipette tips 45

2.9.2 Magnetic rack 45

2.9.3 Magnetic plate 46

2.10 Equations 46

2.11 Statistical Analysis 46

Materials 48

3 The Development of a Growth Factor Immobilisation Technology for use in Cell Culture Systems 52

3.1 Introduction 53

3.1.1 Protein Immobilisation Methods 54

3.1.2 Cell Counting Methods 61

3.1.3 Protein Quantification Methods 61

3.2 Results 64

3.2.1 Development of a Functional Assay to Assess Biological Activity of Immobilised Growth Factors 64

3.2.2 Selection of a Growth Factor Immobilisation Method 79

3.2.3 Development of a Quantification Assay 84

3.3 Discussion 93

4 Functional Properties of Immobilised Growth Factors 95

4.1 Introduction 96

4.2 Results 97

4.2.1 Scalability of Immobilisation method - Proof of Concept 97

4.2.2 Effects of immobilised growth factor surface concentration 99

4.2.3 Functional Properties of immobilised Growth Factors 108

4.2.4 Recycling immobilised GM-CSF 110

4.2.5 Transient exposure of iGM-CSF 113

4.3 Discussion 124

5 Application of Immobilisation Technology in a Primary Cell Culture System 128

5.1 Introduction.....129

5.2 Results 135

5.2.1 Functional Response of Immobilised and Soluble Growth Factors in M-07e Cell Line 135

5.2.2 Immobilisation Preferences of Growth Factors.....139

5.2.3 Expansion of CD34+ Cells using Immobilised SCF.....141

5.2.4 Particle Internalisation.....149

5.3 Discussion.....152

6 Final Discussion and Future Work 156

7 References 163

8 Appendix A – Optimisation of AlamarBlue Assay.....179

8.1 Introduction.....180

8.2 Results and Conclusions.....180

List of Figures

Figure 1. The differences between CAR and TCR therapies. TCR cells recognise antigens presented by MHCI molecules and are therefore HLA specific cells. CAR cells recognise cell surface antigens and generally cannot recognise intracellular tumour antigens. 6

Figure 2. A schematic of haematopoiesis showing the lineages produced with different cytokine combinations. Image taken from [21].....8

Figure 3. Characterisation of haematopoietic lineages by cluster of differentiation, genetic and biochemical markers[28], [32]–[36]. 10

Figure 4. A model of the stem cell niche, highlighting the complexity of signalling pathways involved in HSC maintenance. Diagram taken from [41] 11

Figure 5. The fully automated cell therapy processing platform Prodigy by Miltenyi Biotec..
.....17

Figure 6. An overview of Quality by Design process to aid cell therapy manufacturing..18

Figure 7. Magnetic particles are widely used in for biomedical applications due to their biocompatibility and low immunogenicity. 24

Figure 8. Growth factor immobilisation reaction using the 'Streptavidin Method'.....40

Figure 9. Custom made magnetic pipette tip..45

Figure 10. A magnetic stand to accommodate multiple 1.5 mL eppendorf tubes.....45

Figure 11. A) A magnetic plate for 96 well plates B) capture of magnetic particles during a media change in a AMBR vessel. 46

Figure 12. Carboxyl immobilisation technique chemical reaction. 57

Figure 13. Streptavidin immobilisation technique chemical reaction.....58

Figure 14. Solulink immobilisation technique chemical reaction.....59

Figure 15. Fibronectin immobilisation technique chemical reaction. 60

Figure 16. Dose-response profile of soluble GM-CSF in a TF-1 cell line. 66

Figure 17. Flow cytometry event count correlates with membrane integral TF-1 cell count. 68

Figure 18. The AMBR 15 with 24 micro-bioreactors. 69

Figure 19. Effect of TF-1 cell seeding density on cell growth until media exhaustion in a stirred tank bioreactor.. 73

Figure 20. Effect of TF-1 cell seeding density and GM-CSF replenishment on cell growth until media exhaustion in a stirred tank bioreactor.. 74

Figure 21. Overlay of figure 14 and figure 15 showing that GM-CSF extends cell growth. GM-CSF utilisation is predominantly cell density dependent. 75

Figure 22. Effect of phenol red free media and dO2 on TF-1 cell growth until media exhaustion in a stirred tank bioreactor. 76

Figure 23. Overlay of figure 15 and figure 17 showing improved cell growth with the withdrawal of phenol red from cell culture media. 77

Figure 24. Effect of glutamine source on TF-1 cell growth until media exhaustion in a stirred tank bioreactor. 78

Figure 25. Screen of immobilized GM-CSF prepared using four different conjugation methods and three different GM-CSF-biotin concentrations. 83

Figure 26. Functional screen of immobilized GM-CSF prepared with two different conjugation methods and three final bead concentrations. 84

Figure 27. Exemplar work flow for the quantification of immobilised proteins using flow cytometry. 86

Figure 28. Quantification of immobilised GMSCF using flow cytometry A) Fluorescence histograms of igg control and positively stained samples.....87

Figure 29. Flowchart showing the growth factor immobilisation and quantification process highlighting areas of risk (blue circles) which could impact the efficacy of the immobilisation process or functionality. 90

Figure 30. Fishbone diagram of immobilisation and quantification method showing the variables that impact reproducibility and accurate quantification of the immobilisation method. Numbers in green circles are references to figures in this thesis where the variables were tested. 91

Figure 31. Optimisation of quantification assay parameters A) Optimisation of antibody concentration B) Effect of particle concentration at a fixed antibody concentration. Data points show mean immobilised GM-CSF per particle ± S.D (n=2). 92

Figure 32. Functional activity of immobilised GM-CSF in a stirred tank culture system....98

Figure 33. The effect of surface concentration and surface area of immobilised GM-CSF on TF-1 growth in a stirred tank bioreactor. 104

Figure 34. The surface concentrations of three growth factors were controlled by regulating the NHS-(PEG)n-Biotin (Biotin) molar excess during step 1 of the immobilization reaction. Maximum surface concentrations were achieved with a 1:1 Biotin to Lysine molar ratio for each of the growth factors investigated (n=3 mean ± S.D). 105

Figure 35. The effect of soluble GF-biotin concentration at optimal GF:biotin molar ratios were investigated to regulate the surface concentration of immobilized growth factors ...106

Figure 36. Reaction duration for SCF Biotinylation. 107

Figure 37. (A) The response of TF-1 cells to a range of immobilized GM-CSF surface concentrations and particle concentrations were investigated. 109

Figure 38. The effects of a single dose of immobilized GM-CSF (0.3 ng/mL) retained throughout the culture period was investigated in comparison to a soluble control (soluble GM-CSF was replaced every 48 hours at media change – vertical dashed lines) 111

Figure 39. Degradation of soluble GM-CSF is cell density dependant 112

Figure 40. Effects of GM-CSF exposure period on functional response in stirred culture. Growth curve of TF-1 cells in response to short exposure periods of immobilised GM-CSF in a stirred tank bioreactor. 117

Figure 41. Particle counts as determined by flow cytometry for immobilised GM-CSF conditions shows sufficient particle removal after 0.5 hours and maintenance of particles in the continuous condition (n=2). 118

Figure 42. 30 minutes transient exposure of soluble GM-CSF elicits no functional response in TF-1 cells (n=3 ±S.D). 119

Figure 43. Effects of growth factor exposure period and recycling of immobilised GM-CSF in stirred culture 120

Figure 44. Analysis of media samples shows no leaching of immobilised GM-CSF across the entire culture period. 121

Figure 45. Functionality, stability, and manipulation of immobilized GM-CSF in stirred tank bioreactors and effects of transient exposure to growth factor. 122

Figure 46. Representative flow cytometry plots indicating efficient particle removal and sustained cell growth in transient iGM-CSF conditions was not due to residual particles. ..123

Figure 47. The Dynamag™ CTS magnet for removal of T-cell activation beads 127

Figure 48. Soluble dose-response assays of SCF, GM-CSF and TPO in a cell proliferation assay using a factor dependent cell line, M-07e 137

Figure 49. Functionality and dose response of immobilized GM-CSF, SCF and TPO.....138

Figure 50. The immobilization preferences for a mixture of two or more growth factors at equal molar concentrations were investigated 140

Figure 51. Expansion and phenotype of cryopreserved CD34+ cells with low concentrations of iSCF. 144

Figure 52. Exemplar flow cytometry plots and gating strategy for CD34+ cells incubated with soluble SCF at 100 ng/mL concentration. 145

Figure 53. Exemplar flow cytometry plots and gating strategy for CD34+ cells incubated with immobilised SCF at $5x10^5$ particles per mL 146

Figure 54. Example of a non-stained CD34 sample used to gate negative and positive samples 147

Figure 55. Representative photomicrographs of CD34+ cells cultured with various concentrations of soluble or immobilised SCF (40x magnification) 148

Figure 56. Growth curve of non-cryopreserved CD34+ cells and phenotype (at 96 hours) with high concentrations of iSCF 149

Figure 57. Flow cytometry dot plots showing unique populations of CD34+ cells incubated with iSCF for 30 seconds or 72 hours 151

Figure 58. A schematic of the Streptavidin immobilisation method using the standard 'forward' method and proposed 'reverse' method which may increase NHS susceptibility to hydrolysis. 155

Figure 59. The effect of PEG length on spatial presentation and surface concentration of immobilised growth factors 161

Figure 60. AlamarBlue assay read-out using Fluorescence 181

Figure 61. AlamarBlue assay read-out using Absorbance 182

Figure 62. A GM-CSF dose-response assay in a GM-CSF dependent cell line, TF-1, using an AlamarBlue assay. 183

List of Tables

Table 1. A summary of benefits and disadvantages of Autologous and Allogeneic stem cell treatments. 2

Table 2. List of reagents 48

Table 3. List of consumables 50

Table 4. List of instruments and equipment 50

Table 5. A comparison of commercially available and customised protein immobilisation methods. 55

Table 6. A comparison of widely used cell viability assays. The table shows that widely used assays are not high-throughput and therefore can not generate the high-resolution cell growth curves required to investigate the functional effects of immobilised growth factors. 63

Table 7. CD34+ Expansion Technologies 130

Table 8. Haematopoietic growth factors, their effects on cell lineages and applied concentrations. 132

Table 9. Comparison of growth factor costs in an example stirred tank manufacturing process as described by Collins et. Al, 1998 and potential costs savings using immobilised growth factors, assuming 1.5% of the total soluble growth factor is required [171]. 157

List of Abbreviations

Abbreviation	Description
4FB	4-Formylbenzamide
BBC	Biotin Binding Capacity
BCA	Bicinchoninic Acid
bFGF	Basic Fibroblast Growth Factor
BM	Bone Marrow
CAR	Chimeric Antigen Receptor
CD	Cluster of Differentiation
cGMP	Current Good Manufacturing Practise
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
CPP	Critical Process Parameters
CQA	Critical Quality Attributes
CV	Coefficient of Covariance
DMSO	Dimethyl Sulfoxide
dO_2	Dissolved Oxygen
DOE	Design of Experiments
DOE	Design of Experiments
ECM	Extracellular Matrix
EDC	1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride
EMA	European Medicines Agency
FACS	Fluorescent Activated Cell Sorting
FBS	Foetal Bovine Serum
FDA	Food and Drug Association
Flt-3L	fms related tyrosine kinase 3 ligand
FSC	Forward Scatter
GMax	Glutamax™
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GMP	Granulocyte-Monocyte Progenitor
GvHD	Graft vs Host Disease
HC(s)	Haematopoietic Cell(s)
HLA	Human Leukocyte Antigen
HSC(s)	Haematopoietic Stem Cell(s)
iGF	Immobilised Growth Factor
IMS	Industrial Methylated Spirits
iPSC	Induced pluripotent stem cell
mAb	Monoclonal Antibody
MACS	Magnetic Activated Cell Sorting
MEP	Megakaryocyte-Erythroid Progenitor
MESF	Molecules of Equivalent Soluble Factor
MNCs	Mononuclear Cells
MPB	Mobilised Peripheral Blood
MSC	Mesenchymal Stem Cell

NHS	N-Hydroxysuccinimide
PBS	Phosphate Buffered Saline
PD(s)	Population Doubling(s)
PDT	Population Doubling Time
PE	R-Phycoerythrin
PEG	Poly Ethylene Glycol
PFA	Paraformaldehyde
QTPP	Quality Target Product Profile
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute 1640
SCF	Stem Cell Factor
SD	Standard Deviation
sGF	Soluble Growth Factor
S-HyNic	Succinimidyl-6-hydrazinonicotinamide
SME	Small-Medium Enterprise
SPADE	Spanning tree Progression of Density normalised Events
SSC	Side Scatter
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride
TCR	T Cell Receptor
TNC	Total Nucleated Cells
TPO	Thrombopoeitin
UCB	Umbilical Cord Blood

1 Introduction

Regenerative medicine is an umbrella term for therapies which may involve molecular therapy, gene therapy, stem cell transplantation, tissue engineering and reprogramming of cell and tissue types or a combination thereof[1]. Due to the broad spectrum of therapies involved in regenerative medicine, attempts at defining regenerative medicine have led to lengthy and ambiguous definitions however, in 2008 a brief definition was published; "Regenerative medicine replaces or regenerates human cells, tissues or organs, to restore or establish normal function"[2]. Regenerative medicine is often considered the pinnacle of 21st century medicine due to its potential to restore or establish function in disorders where traditional pharmaceuticals have failed to deliver.

1.1 Cell and Gene Therapies

Cell therapies consist of the transplantation of cells with therapeutic potential. Often these cells are stem cells which can reconstitute a defective organ or tissue in order to restore or establish normal function, but they can also involve more specialised cells such as T-cells. In addition the transplanted cells can be genetically modified to correct for inherited genetic disorders such as Sickle Cell Anaemia.

1.1.1 Cell Therapies

A stem cell can be defined as a cell that has the capacity to self-renew and has the potential to produce differentiated progeny with more specialised function or a more mature phenotype[3]. Patients can be treated with their own stem cells (autologous) or by donor

stem cells (allogeneic treatment). Both types of treatments have benefits and risks as summarised in Table 1.

Table 1. A summary of benefits and disadvantages of Autologous and Allogeneic stem cell treatments.

Benefits		**Risks**	
Autologous	**Allogeneic**	**Autologous**	**Allogeneic**
No GvHD	Off-the-shelf product.	Higher manufacturing costs [4]	Potential for GvHD and or pathogen transfer.
	Scalable.	Delayed treatment time	
	Quicker release due to pre-approved QC	Variability between patients due to stem cell quality	

Despite the benefits associated with allogeneic therapies, the risk of Graft versus Host Disease due to HLA mismatches is a major challenge for the adoption in clinical settings.

1.1.2 Gene Therapies

The origins of gene therapy can be traced back to 1928 where Frederick Griffith's demonstrated the principle of genetic transformation of pneumonia from a non-virulent form to a virulent form. It wasn't until 1962 that the first documented gene transfer in mammalian cell lines was reported and in 1989 the first gene transfer into humans took place [5].

Gene transfer can be achieved using viral and non-viral vectors, the most common vectors being adenoviruses, retroviruses and naked plasmids (non-viral) which are reviewed elsewhere [6].

Gene therapies can be combined with cell therapies, for example where the host cell for the gene construct is transfected to become stem cells which have the ability to reconstitute entire tissues with cells expressing normal function (i.e. Strimvelis™, GSK).

1.1.3 The Cell and Gene Therapy Landscape

Some recent successes for the industry include a gene therapy for ADA-SCID patients (Strimvelis™, GSK), which has been granted market approval by the European Medicines Agency (EMA) and will cost around 594,000 euros per patient. Cell and gene therapy for immuno-oncology targets (particularly for CD19 malignancies) have many promising products in late stage clinical trials (e.g. Juno Therapeutics, Bluebird Bio, Kite Pharma, Celgene).

Cell and gene therapy start-ups and small-medium sized enterprises (SMEs) have achieved some of the biggest IPOs in recent biotech history (BlueBird Bio $101 Million, Kite Therapeutics $127 Million, NantKwest $207 Million). The Alliance for Regenerative Medicine estimates public and private companies have raised $10 Billion in 2015 with over 70 treatments in late stage clinical trials. Significant investments and collaboration between industry and academia are key to moving the field forward.

The safety of cell and gene therapies particularly with T-cell engineered therapies is a real concern for the industry. In March 2017, it was reported that there were multiple deaths

associated with the infusion of T-cell engineered constructs in a pivotal trial from Juno Therapeutics (JCAR-015).

1.1.4 Clinically Relevant Cells

Multiple cell types are being investigated in the clinic for cell and gene therapies, with the majority being Haematopoietic Stem Cells (HSCs), Mesenchymal Stem Cells (MSCs), T-cells (CAR T-cells and or TCR T-cells) and induced pluripotent stem cells (iPSCs). Cells can be classified by their differentiation potential:

- Totipotent - A cell that has the capacity to develop into any type of human cell. An example is a Zygote.
- Pluripotent – A cell that can differentiate into all three germ layers. Examples are embryonic stem cells or iPSCs.
- Multipotent – A cell that can develop into more than one type of cell but does not have the potential to differentiate into all types of cells. Examples are HSCs and MSCs.
- Unipotent – has the capacity to develop into one cell type. Examples are Hepatoblasts.

A brief overview of common cell types for cell and gene therapies are given below however, the focus of this thesis will involve HSCs since they are the most widely characterised and utilised cells in transplant settings.

1.1.4.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are adherent cells capable of tri-lineage differentiation into bone, cartilage and adipose tissues derived from the perivascular niche. Therefore, MSCs

suited towards musculoskeletal indications. Recently MSCs have been shown to exhibit immuno-modulatory properties (by the secretion of anti-inflammatory paracrine factors) making them suitable for immune disorders such as Crohn's disease and other inflammatory bowel syndromes [7]. MSCs have also demonstrated repair of cardiac function following myocardial infarction [8].

1.1.4.2 Induced Pluripotent Stem Cells

The pioneering work conducted by Shinya Yamanaka's lab in 2006 showed that adult stem cells could be reprogrammed into pluripotent stem cells by genetically engineering the cells to express four transcription factors; oct4, sox2, cymyc and klf4[9]. The work has the potential to revolutionise allogeneic therapies where the donors own stem cells are not of adequate quality for cell therapies. However, safety concerns have arisen with the formation of tumours in multiple independent studies [10]. A recent pivotal clinical trial for wet age-related macular generation (AMD) has been put on hold because mutations were observed in transplanted cells (Rikagaku Kenkyūsho Institute, Japan).

1.1.4.3 T-Cell Therapies

T-cell therapies may comprise genetically engineered and expanded T-cell populations with modified T-cell receptors (TCR) or chimeric antigen receptors (CAR) and are mainly utilised to target clinical indications involving liquid or solid tumours. Naive or central memory T-cells are thought to correlate with long-term activity and increased anti-tumour activity in pre-clinical models[11], [12].

Both CAR and TCR T-cells have shown clinical efficacy since the first proof of concept studies in 2005 [13]. The main differences between CAR and TCR cells are summarised below in Figure 1.

Despite many clinical trials taking place globally with TCR and CAR therapies, off-target toxic effects continue to restrict the progression of the therapies into the clinic. Recently, a leading candidate was withdrawn from Juno Therapeutics' pipeline due to several patient deaths in their Rocket clinical trials (JCAR015).

Advances are being made to improve safety of t-cell therapies with next generation therapies having suicide switches' so that implanted cells can be killed in a short period of time should any untoward toxic side-effects occur[14]. Such advances will inevitably accelerate therapy development.

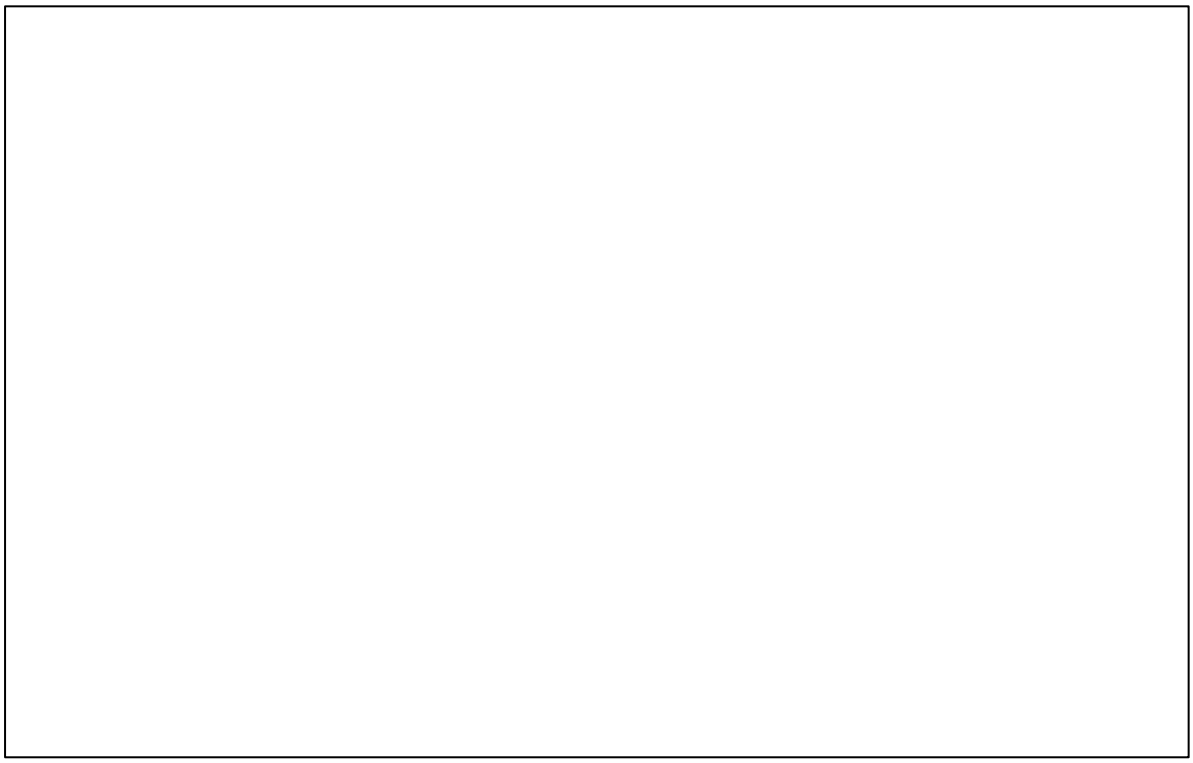


Figure 1. The differences between CAR and TCR therapies. TCR cells recognise antigens presented by MHCI molecules and are therefore HLA specific cells. CAR cells recognise cell surface antigens and generally cannot recognise intracellular tumour antigens. Image modified from [15].

1.2 Haematopoietic Stem Cells

HSCs (or CD34+ cells) were first shown to have colony-forming potential in *in vitro* cultures and were later shown to have the capacity to repopulate immunocompromised mice [16], [17]. Today, over 42,000 HSC transplants take place every year, with the majority being performed to treat haematological malignancies [18]. More recently, HSCs have also been used as a 'host cell' for genetic engineering to successfully cure genetic disease such as ADA-SCID and sickle cell anaemia [19], [20].

Haematopoietic stem cells (HSCs) have the capacity to differentiate into all mature blood cell types, through a process known as haematopoiesis (Figure 2). Haematopoiesis occurs throughout the entire human life-span, where cell lineages are directed by a complex interaction of physicochemical and biological cues.

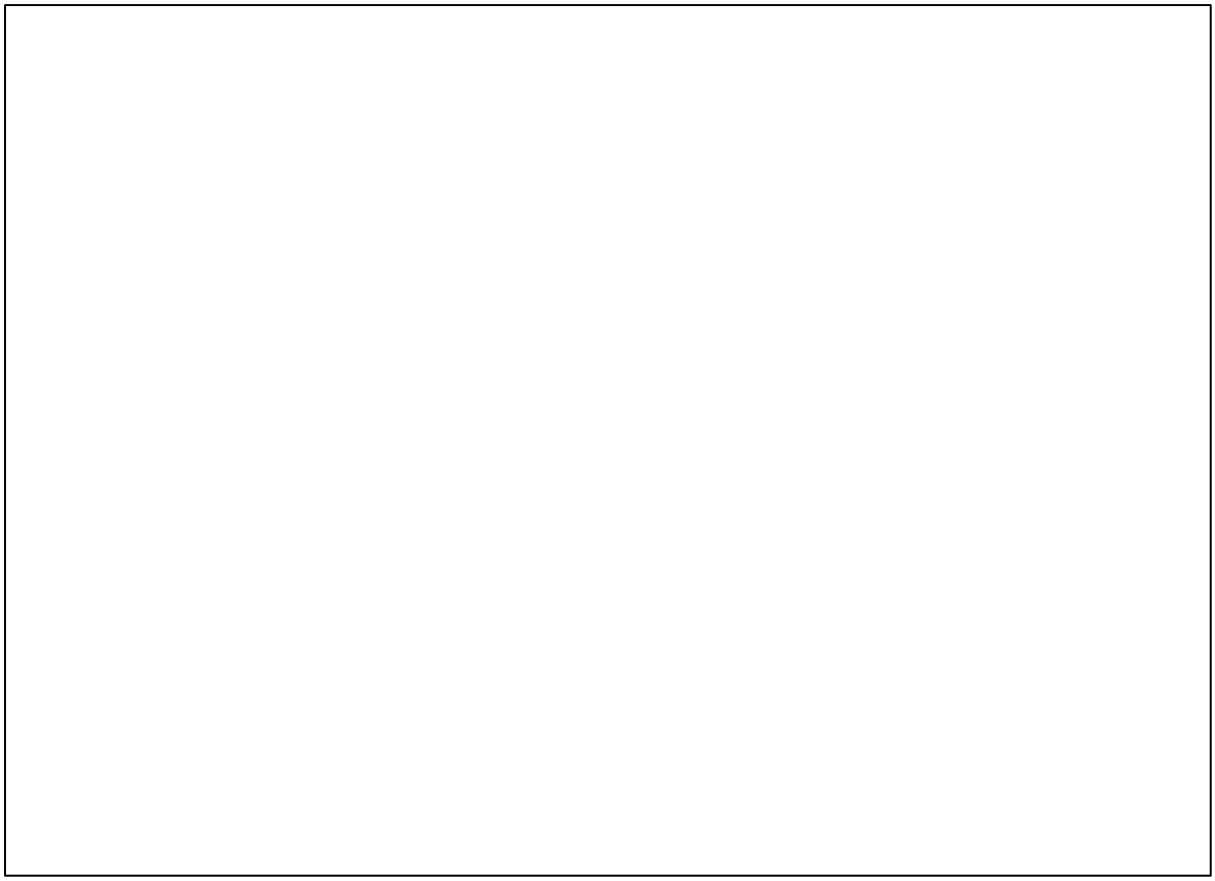


Figure 2. A schematic of haematopoiesis showing the lineages produced with different cytokine combinations. Image taken from [21]

1.2.1 Characterisation

Characterisation of HSCs is difficult due to their complexity, plasticity and lack of sensitive characterisation assays. Immunophenotyping using flow cytometry is the most widely used method for characterisation, but is usually limited to 7-8 cluster of differentiation (CD) markers with standard equipment due to optic limitations (i.e. spill-over of fluorescence into other channels). Emerging equipment such as Mass Cytometry can increase those numbers of markers to 50+ with the use of radioactive CD tags. With increasing marker numbers; tools such as Spanning tree Progression of Density normalised Events (SPADE) become important in visualising high-dimensional data and automatically organising cells into

hierarchies of related phenotypes. SPADE is increasing our understanding of haematopoiesis and cancer biology[22].

CD markers remain the preferred method for phenotypic identification of cells using flow cytometry. Over 30 years ago, cells with 'repopulating activity' were identified by expression of the CD34+ marker, which remains the most common method for identifying HSCs today[23]. Since the discovery of CD34+, many more markers have been identified based on their short term or long term repopulating activity[24]. A sub-population of CD34+ cells expressing little or no CD38 were identified with extended self-renewal activity [25]–[27]. Whereas, some of the most primitive (and rarest) HSCs have been found to express CD133 [24].

'Definitive' HSCs can be identified by expression of a large combination of CD markers, for example CD34+/CD38-/CD90+/CD45RA-/CD49f+/Rho123^{low}/Lin- [28]. Markers such as CD49f may be more representative of those cells with good engraftment potential due to its involvement in niche homing. Large marker combinations become limited by the optic configuration of most modern day flow cytometers (due to fluorescence spill-over into detector channels). Simplified marker panels have been proposed such as SLAM markers, CD150, CD244, and CD48 and may be more appropriate for phenotypic analysis[29].

Despite the complexities involved in HSC identification, it is widely accepted that CD34+, CD34+/CD38- or CD133+ cells incorporate the stem cell/progenitor HSC populations.

Genetic profiling amongst various stem cells has highlighted genes upregulated in stem cell/progenitor populations including STAT and c-myc genes. Furthermore biochemical analysis (for example ALDH) shows increased expression in stem cells, and may be a marker

for stem cell quality and engraftment success [30], [31]. A summary of marker expression throughout haematopoiesis is given in Figure 3.

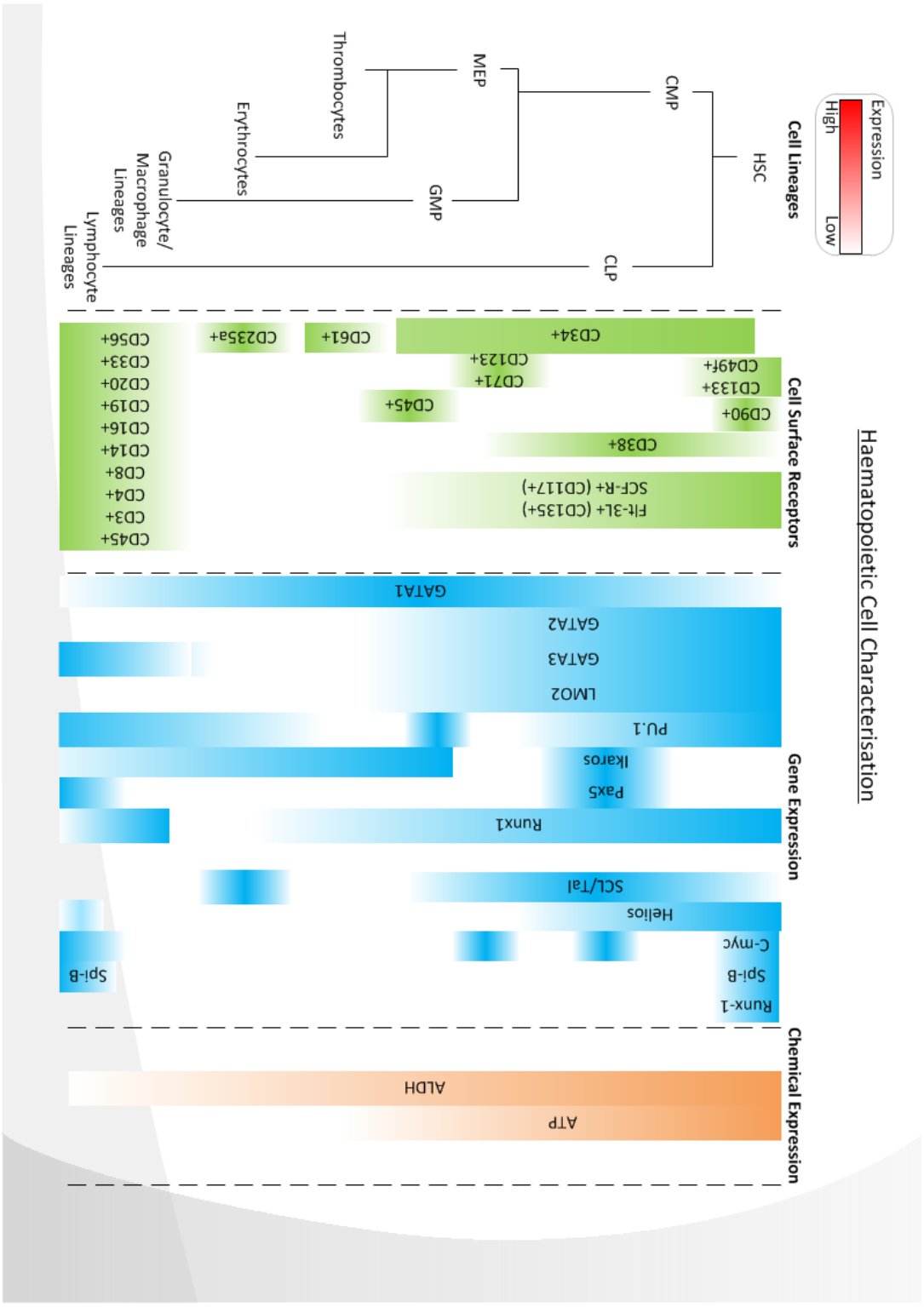


Figure 3. Characterisation of haematopoietic lineages by cluster of differentiation, genetic and biochemical markers[28], [32]–[36].

1.2.2 The Stem Cell Niche(s)

Localised microenvironments that maintain and regulate stem or progenitor cells are referred to as a 'Stem cell niche'[37]. The stem cell niche varies throughout human development, for example during embryogenesis; niches reside in the yolk and placenta where postnatally HSC niches reside within the bone marrow[38].

The bone marrow niche is a highly complex microenvironment, where the Extracellular Matrix (ECM), growth factors, hormones, adhesion molecules, integrins, cells (i.e. stromal cells) and physicochemical cues work in concert to maintain stem cell phenotype as shown in the model illustrated in Figure 4 [39]–[41]. Within the bone marrow, HSCs reside near to sinusoids where SCF and other paracrine factors (secreted by MSCs and endothelial cells) promote their maintenance [42].

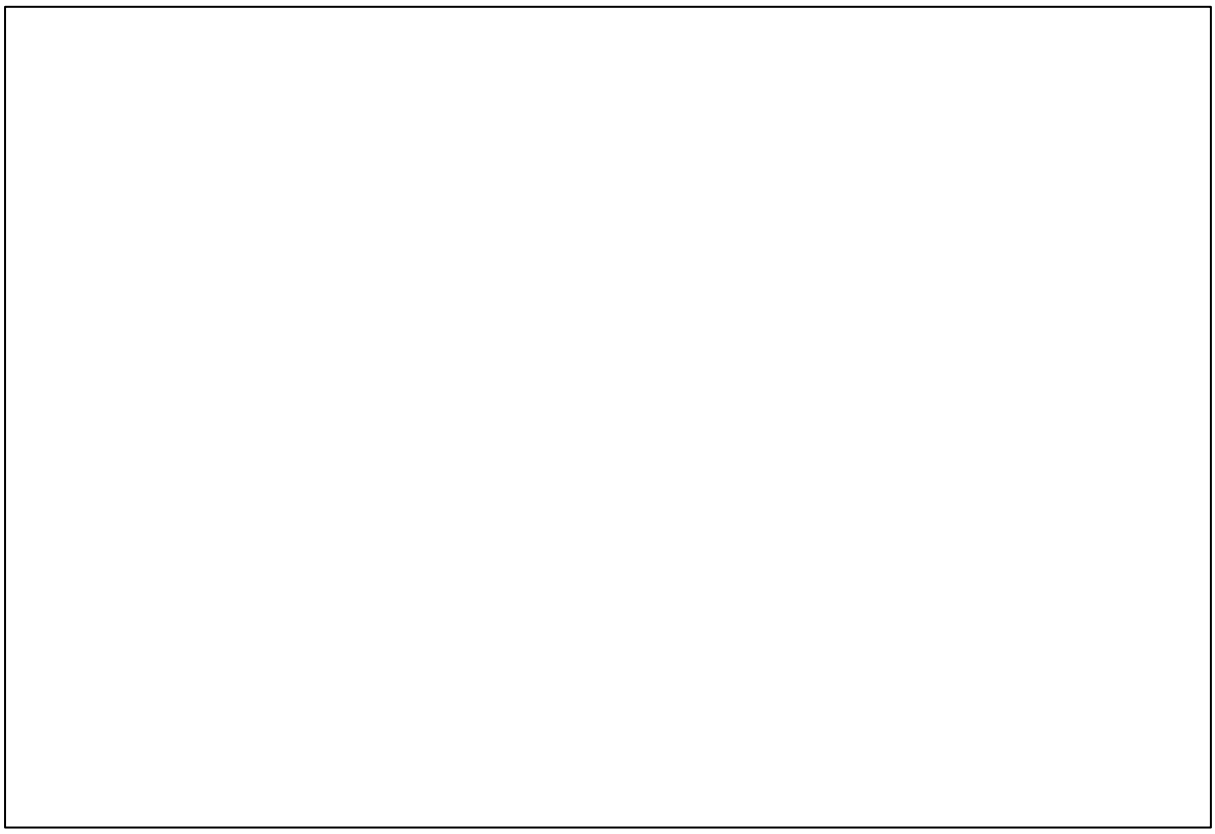


Figure 4. A model of the stem cell niche, highlighting the complexity of signalling pathways involved in HSC maintenance. Diagram taken from [41]

1.2.3 Sources of Haematopoietic Stem Cells

HSCs can be sourced directly from the bone marrow or can be temporarily mobilised into the blood stream. Alternative non-invasive sources include umbilical cord blood and placenta. Here bone marrow, peripheral blood and umbilical cord blood are reviewed since these are the most widely used sources in the clinic.

1.2.3.1 Bone Marrow

The bone marrow (BM) is the primary HSC niche in the adult human body where approximately 1% of total Mononuclear Cells (MNCs) are CD34+. BM transplants have been performed since the 1950's and consist of painful operations requiring general anaesthesia and long hospital stays for both patient and donor. Donor and patient have to be HLA matched to reduce the risk of GvHD, meaning those patients with no siblings are at great risk of not finding a suitable donor and will have to undergo a non-related BM transplant.

1.2.3.2 Mobilised Peripheral Blood

Stem cells can be temporarily mobilised from the bone marrow into the peripheral blood with G-CSF injections (sometimes in combination with Plerixafor) [43]. Mobilisation is now the preferred choice of stem cell isolation because the process is less painful for the donor and does not require administration of general anaesthesia or an operating theatre.

During the mobilisation process (up to 4 days) stem cells are mobilised into the peripheral blood stream and collected using equipment which separates and collects white blood cells from the bulk of the blood, whilst filtering out red blood cells and returning them to the donor. Higher concentrations of HSCs can be collected from mobilised peripheral blood

(MPB) compared with BM - over 1000 CD34+ cells/µL of blood can be achieved with G-CSF mobilisation alone [44].

1.2.3.3 Umbilical Cord Blood

Umbilical cord blood (UCB) is one of the preferred non-invasive sources of HSCs available. Historically the umbilical cord was disposed of after child birth, however the blood retained within the umbilical cord contains HSCs. Often low volumes of UCB mean relatively low numbers of HSCs are recovered from the cord which are insufficient to treat the average adult [45]. Increased levels of HLA disparity can be tolerated with UCB in comparison to other sources of HSCs meaning the availability of HSCs is increased to a wider population pool, which is of particular importance to ethnic minorities [46], [47].

1.3 Considerations and Challenges with Cell Expansion

HSCs derived from umbilical cord blood offer biological, clinical and ethical advantages over other sources. High total nucleated cell count (TNC) is correlated with engraftment success and good clinical outcome; with a TNC count of $2x10^9$ cells or $1.2x10^6$ CD34+ cells now widely accepted as a minimum threshold for a successful single cord blood transplant [48].

TNC counts are highly variable between cord blood units due to a number of factors (i.e. weight of foetus, size of placenta, processing method used) and low TNC counts are the primary reason why the utilisation of banked cord blood units are now estimated at 1-3% globally and utilisation rates are declining [49].

For cord blood banks to be economically sustainable there is now a focus to process only those cord blood units meeting the TNC threshold. Although efforts have been made to

improve processing methods or moving towards automated processing to minimise operator variability (such as the Sepax, GE Healthcare).

Double cord blood transplants have been explored where one unit does not meet the minimum TNC threshold, however finding two suitable matches at the same time is logistically challenging and economically impractical [50].

In addition to improving the processing and banking of cord blood units, many research groups are exploring *ex vivo* expansion of HSCs are in order to increase utilisation and patient access. Maintaining stem cell or progenitor phenotypes during CD34+ expansion *ex vivo* is challenging, namely due to rapid differentiation and slow engraftment times once transplanted.

Engraftment time can be problematic for HSC transplants particularly where HSCs are derived from UCB where CD34+ populations are naive in nature. Slow engraftment leaves patients at risk of infection due to the lack of an immune system and infection is one of the leading causes for mortality in UCB HSC transplants. Slow engraftment can also be caused by stem cell exhaustion following *ex vivo* expansion as described later. To overcome the issues involved in HSC transplants, some studies have looked at administering the patient with two fractions of cells; the expanded CD34+ fraction and a proportion of the original cord blood fraction which contains higher numbers of mature blood cells which were stored before *ex vivo* manipulation [51].

Stem cell exhaustion can occur during rapid *ex vivo* expansion where increases in reactive oxygen species (ROS) may lead to poor engraftment when transplanted [52], [53]. Increases in ROS arise due to increased metabolic activity upon *ex vivo* expansion and due to un-optimised dissolved oxygen in cell culture conditions.

A major challenge for cell and gene therapies (particularly T-cell therapies) is the sheer number of cells required in the drug product. Currently the consensus in the field is “more is better” however; manufacturing large numbers of cells becomes expensive due to the volume of media required and scalability of the manufacturing platform.

Whilst studies have looked at moving towards high density cell cultures and perfusion systems to reduce quantities of media required, it leaves the question of whether the quality or potency of the cells being administered are suboptimal. Furthermore, defining the rarer sub-populations that correlate better with good therapeutic effect is likely to reduce the number of cells required for such therapies.

1.4 Hurdles and Solutions to the Commercialisation of Cell and Gene Therapy

The first gene therapy receiving market authorisation by the EMA was Strimvelis™ (GSK) in 2016, the gene therapy for ADA-SCID which is priced at ~600K euros per patient. The cost has received much criticism from the healthcare industry, however in relation to the life-long costs of treating an ADA-SCID patient in isolation; it is relatively low in comparison. This one dose treatment for a rare disease is unlikely to be a blockbuster medicine due to its high costs and low patient numbers.

If we take this cost for Strimvelis and extrapolate it to clinical indications with much higher patient numbers for example in the immuno-oncology field, a price this high is unlikely to be reimbursed by public health services or health insurance companies. ChondroCelect™ has recently been withdrawn from the market by its manufacturer, TiGenix as of November

2016 due to reimbursement issues. Treatment costs can be reduced through the use of automation, reducing raw material costs, scaling-up and using Quality by Design (QbD) principles.

1.4.1 Automation

Automation of cell processing reduces both costs (of raw materials and human resources) and reduces product variability by removing operator dependent variation. Automated (fully or semi-automated) systems exist for all sections of cell therapy processing, from cell washing to fill/finish. Cell washing equipment such as the COBE 2991 (Terumo BCT) have been around for many years and widely used in blood banks due to its ease of use and robustness. The equipment also uses disposable single-use kits which reduces the risk of patient-to-patient carry over and contamination. More sophisticated equipment (CompacT selecT, Sartorius and Sepax, GE Healthcare) is capable of handling and culturing of standard T-flasks with tractability using barcode readers, with comparability to manual processing [54].

Fully automated systems such as the Prodigy™ (Miltenyi Biotech) is changing the face of cell manufacturing processes – the closed-system allows manufacturing outside of cGMP suites; greatly reducing costs and processes are available for CAR/TCR manufacturing as well as CD34+ manufacturing (see Figure 5) [55]–[57]. The Prodigy is ultimate evolution of manufacturing equipment for autologous therapies due to its scale-out potential. However, autologous therapies are likely to manufactured using standard stirred tank bioreactors due to their scalability. Furthermore, systems such as the Prodigy are not a one size-fits-all solution; it requires significant process development for individual cell therapies and cannot yet formulate the drug product with standard formulations involving DMSO.

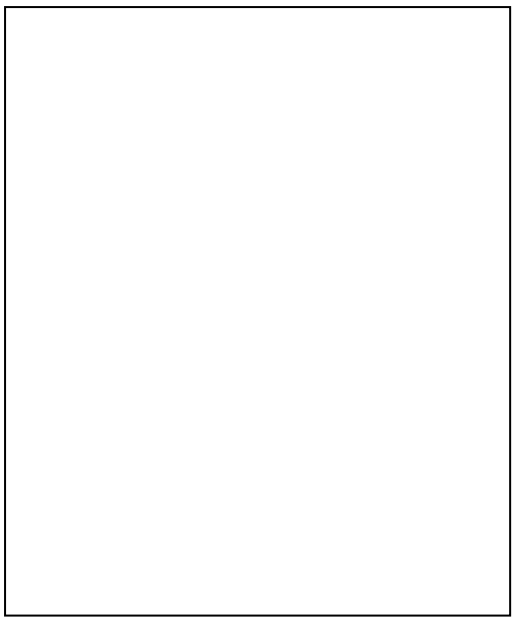


Figure 5. The fully automated cell therapy processing platform Prodigy by Miltenyi Biotec. Such automated systems are key to the realisation and commercialisation of cell and gene therapies.

1.4.2 Quality by Design

The Quality by Design (QbD) principle was introduced by the FDA in 2006 to address the variation in manufacturing processes of pharmaceuticals and to incorporate the International Conference on Harmonization guidelines. QbD integrates scientific knowledge and risk analysis into manufacturing process development to address drug manufacturing failures caused by manufacturing inefficiencies [58]. The process has been summarised in Figure 6.

Tools such as Design of Experiments (DoE) can help to elucidate complex interactions between manufacturing variables in cell and gene therapies and therefore help to define optimal conditions for both target cell numbers and desired cell quality. Scaled down micro-bioreactor platforms such as the ambr™ (Sartorius) will allow such DoE experiments to be performed.

The use of QbD principles for the manufacture of cell and gene therapies are likely to accelerate development, decrease process variability, reduce cost of goods and assist with regulatory compliance [58].

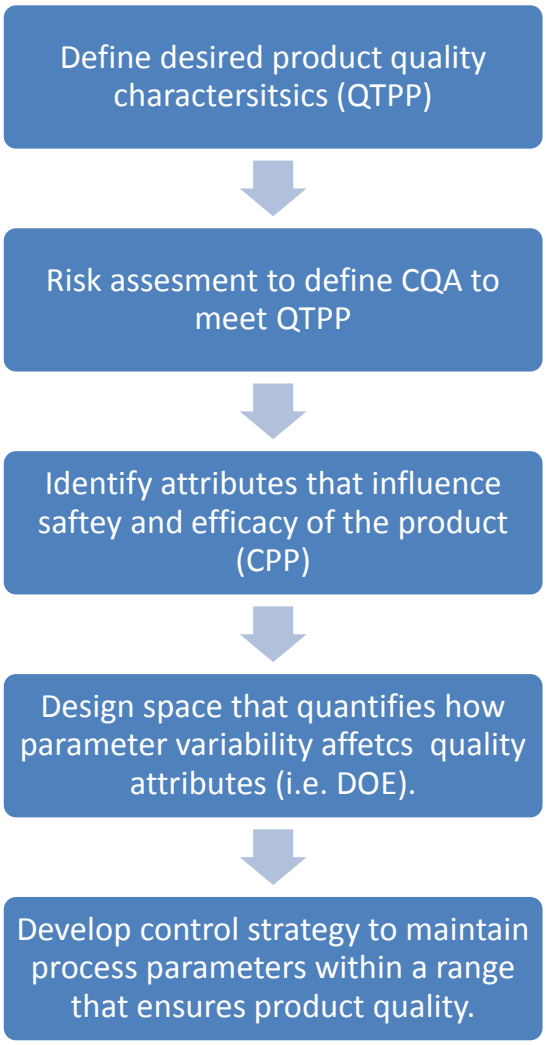


Figure 6. An overview of Quality by Design process to aid cell therapy manufacturing. Adopting QbD principles in cell therapy manufacturing processes will accelerate development.

1.4.3 Safety and Efficacy

Despite the potential of cell and gene therapies, the inherent complexity of such treatments gives rise to greater safety concerns when we compare them to conventional pharmaceuticals and biopharmaceuticals.

Purity of the cell therapy drug product is an important consideration. Contamination of non-target cell types poses safety and potency risks of the final drug product. Contaminating cell types, for example in HSC and T-cell therapies, can affect the potency of the therapy by competitively repopulating the blood system [59]. Furthermore, cell impurities may suggest inefficient manufacturing processes which may lead to variable clinical outcomes. Other impurities can stem from microorganisms that contaminate the culture. Microorganism contaminants can arise from the donor, during *ex vivo* manipulation and from raw material sources such as serum and animal derived supplements such as growth factors. Due diligence can be taken by moving towards serum free manufacturing process and or recombinant proteins produced in non-mammalian cell types – such steps also reduce batch-to-batch manufacturing variability. Release testing should extensively screen for microorganism contamination which adds to the cost of cell therapies and increases the time to administration.

Graft versus host disease for allogeneic treatments remains a significant risk for patients where suitable matches are not available. This is particularly an issue for patients from ethnic minorities where rare HLA types mean donors are not readily available.

Tumorigenicity of cell and gene therapy is a major concern for the industry and the long term effects of therapies with market approval are still widely unknown. Tumourgeneticity can arise from the method of genetic modification of cells, where insertion of genes via viral vectors can cause mutations causing uncontrolled growth of progeny cells. As little as 0.025% of undifferentiated pluripotent cells can lead to teratoma formation [60]. Studies have also shown that injection of autologous stem cells into kidneys have developed into tumours;

highlighting the need for the field to better understand the effects of administration sites on tumour formation [61].

Due to the risks associated with cell and gene therapies and unknown long-term effects, regulatory authorities require extensive pre-clinical and clinical safety data and analytical testing methods for IND applications. Such extensive data not only increases development time lines, but also adds to development costs. Albeit, the FDA is committed to less rigorous safety testing by working with developers throughout the life-cycle of a product. The FDA have introduced programmes such as 'breakthrough therapy designation' and 'accelerated approval' expedite therapy approvals[62].

1.5 Growth Factors

Growth factors were first discovered during the 1950-1960s by Cohen and Levi-Montalcini during their research into the growth of nerves. Their research led to the discovery of Nerve Growth Factor (NGF) and Epidermal Growth Factor (EGF) for which they won a Nobel Prize in 1986[63]–[65]. Growth factors are defined as proteins that are secreted by cells that regulate a wide range of biological effects including cell proliferation, migration, differentiation, morphogenesis and angiogenesis. The terms cytokine and growth factor are often interchanged however, cytokines are proteins which are secreted by immune cells that induce inflammatory responses. The first cytokine, interferon, was discovered by Isaacs and Lindenmann in 1957[66].

Defined growth factor 'cocktails' are supplemented to cell culture media in order to support the growth and differentiation of cells *in vitro.* The first successful attempts of cell culture was achieved by Harrison in 1907 who realised the nutritional and growth support that

clotted lymph provided to explanted tissues[67], [68]. Building on from this work, it was quickly realised that plasma or serum was essential for the growth and nutritional support of cells[69]–[71]. However, it wasn't until 1980 that growth factors were found to be the component in plasma or serum which provided growth and support of cells; furthermore, the requirements of growth factors were found to be different for every cell type[72].

1.5.1 Immobilised Growth Factors

In vivo, growth factors can exist in both soluble and matrix bound forms. Matrix bound growth factors associate with glycosaminoglycan's (heparin or heparan sulfate) or proteins of the extracellular matrix (ECM) including fibronectin and collagen[73], [74], some growth factors such as SCF and Flt-3L have distinctly separate soluble and membrane bound forms *in vivo*[75], [76].

The ECM provides a storage mechanism for growth factors as they are protected from degradation by proteolytic enzymes, receptor-mediated endocytosis and other physicochemical cues for which soluble growth factors are susceptible to degradation. Soluble growth factors consequently have very short half-lives with bFGF having a half-life of just three minutes *in vivo*[74], [77]. Rapid release of matrix associated growth factors can be achieved by proteolytic activation (a growth factor which is in its latent state when immobilised but becomes biologically active when released in a soluble form), leading to a localised concentrated growth factor microenvironment in response to tissue injury for example. Without proteolytic activation, a response to tissue injury would be a timely process as growth factors would need to be produced through gene expression and transcription.

Matrix bound proteins often retain their biological potency however some exist in a latent state (e.g. TGF-β, HGF and CSF-1)[78]–[80]. The literature also suggests that some matrix bound growth factors (i.e. VEGF) have demonstrated an increased biological potency as compared to soluble counterparts[81]; additionally a prolonged response can also be achieved in comparison to their soluble counterparts[82]–[84]. Increased potency can be achieved by ligand multivalency and receptor clustering which has been shown to produce distinct signalling pathways from their soluble counterparts[85].

Numerous recent studies have shown the importance of growth factors (SCF) and notch-ligands for the expansion of UCB derived HSCs [86], [87].

1.6 Applications of Magnetic Microparticles in Cell and Gene Therapy

Micro-Particles are an ideal surface for the immobilised growth factors since they offer a large surface area to volume ratio, meaning high localised concentrations can be achieved, but also so they can be used in scalable manufacturing systems such as stirred tank bioreactors. The magnetic element of the particles will also allow manipulation of the growth factors independently of the media bulk.

Magnetic particles in biomedical research are becoming increasingly popular since they are versatile and biocompatible materials which have many potential applications.

Magnetic microparticles typically range from 0.1-100µm in diameter which consist of a polymer core (usually polystyrene) and coated with a magnetic material such as magnetite or maghemite (both are iron oxides which have ferrimagnetic properties). Iron coatings

result in the particles having low levels of agglomeration and are highly biocompatible because Fe^{2+} ions are endogenous inside of the human body where metabolic pathways exist for the adsorption, distribution, metabolism and excretion of the metal[88].

Magnetic microparticles have paramagnetic properties meaning that electrons within the iron atoms are able to align with the magnetic field - they hold no residual magnetism but when placed in a magnetic field they exhibit magnetic properties.

Magnetic particles have received regulatory approval for some biomedical applications such as, MRI contrast reagents (i.e. Ferucarbotran and Ferumoxtran-10) and cell isolation (CliniMACS™, Miltenyi Biotec). More applications of magnetic particles can be seen in Figure 7.

Activation of T-cells is induced by the formation of T-cell receptor complexes[89]. *In* vivo, the complexes often involve the presentation of anti-CD3 and anti-CD28 by dendritic cells leading to a cascade of events leading to T-cell activation and proliferation. Dynabeads™ (Life Technologies) are the leading technology for *ex vivo* T-cell expansion, where CD3 and CD28 antibodies are conjugated onto Protein A functionalised magnetic particles – recapitulating the presentation by dendritic cells *in vivo*. Dynabeads™ have been widely used over many years for the activation of T-cells in an ever increasing number of oncology clinical trials[90]. Similar technologies include the capture of proteins in a ‘quickgel’ on the surface of magnetic microparticles (MagCloudz™, Quad Technologies).

The safety, biocompatibility and current usage of magnetic particles in numerous biomedical fields means magnetic microparticles are an attractive substrate for growth factor immobilisation.

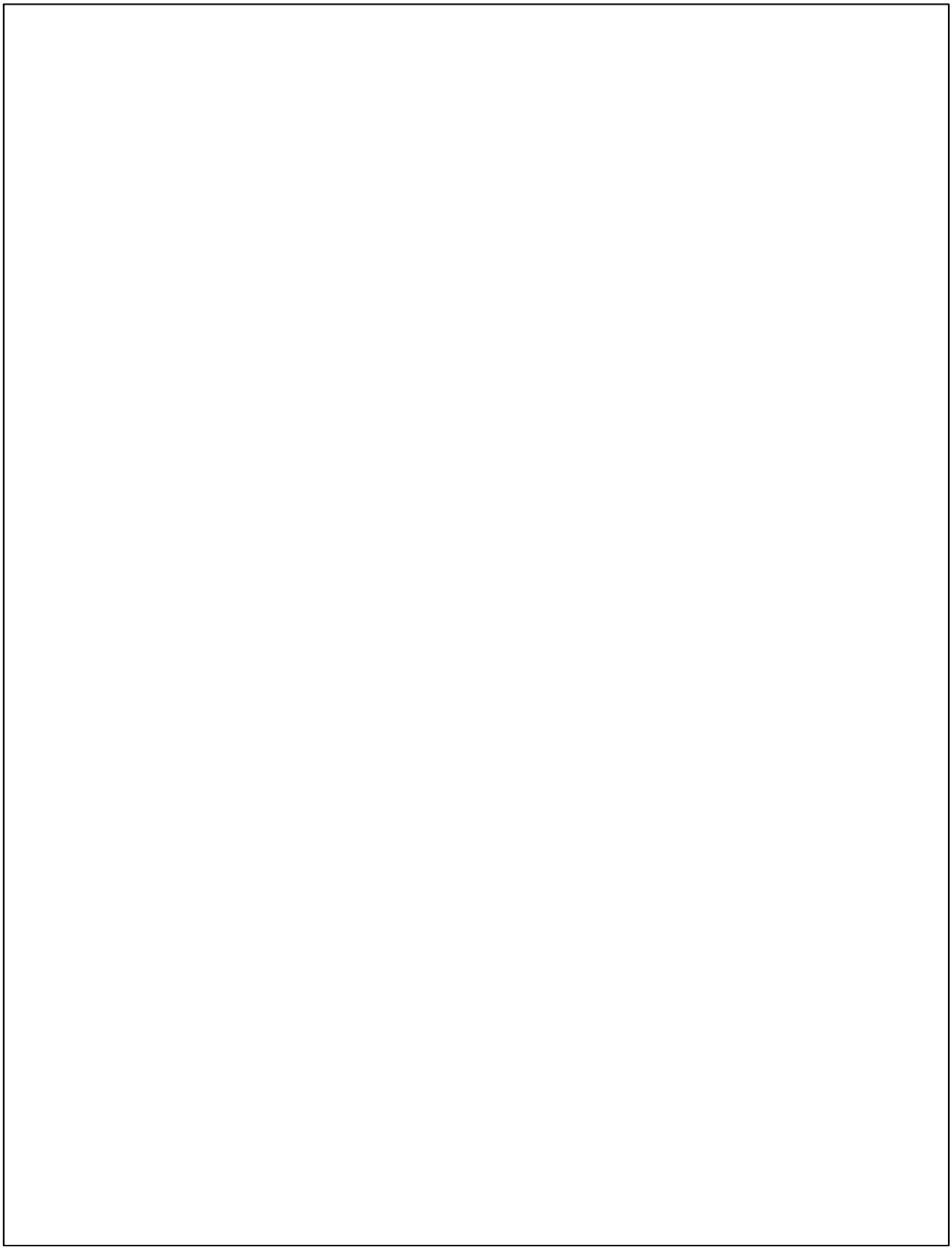


Figure 7. Magnetic particles are widely used in for biomedical applications due to their biocompatibility and low immunogenicity.

1.7 Thesis Objectives

The objective of this project was to develop a scalable growth factor immobilisation technique in order to address some of the raw material cost issues surrounding the manufacturing of cell and gene therapies and to demonstrate some of the functional and process benefits of using immobilised growth factors as opposed to standard soluble growth factors. This thesis aims to:

- Develop a novel growth factor immobilisation technology using magnetic microparticles to allow scalability in standard stirred tank bioreactor platforms.
- Develop functional assays to assess and optimise the activity of immobilised growth factors in comparison to soluble equivalents.
- Demonstrate the unique functional activities of immobilised growth factors in comparison to soluble equivalents.
- Demonstrate the scalability and process benefits of immobilisation using magnetic particles.
- Finally, demonstrate the immobilisation technology in a model (clinically relevant) system for the expansion of umbilical cord blood derived haematopoietic stem cells.

2 Materials and Methods

Methods

2.1 Cell Culture

The following protocols were used to culture cell lines and primary cells unless stated otherwise.

2.1.1 TF-1 Human Erythroleukemia Cell Line

TF-1 cells are dependent on GM-CSF and therefore chosen to investigate and compare the activity of soluble and immobilised GM-CSF. TF-1 cells were obtained frozen from ATTC (ATTC, UK) and cultured according to manufacturer's recommendations. A description of media formulations are presented in Table 2.

TF-1 cells were maintained in culture at a density of 2×10^5 to 8×10^5 cells/mL with complete media in a humidified incubator (5% CO_2, 37^{o}C). Complete media was replenished every two to three days.

A description of cryopreservation and thawing of TF-1 cells are described in section 2.1.1.1.

Table 2. TF-1 Media formulations

Media Type	**Description**
Complete Media	RPMI 1640 (Phenol Red Free, Glutamine Free; ThermoFisher Scientific) 10% v/v Heat Inactivated Foetal Bovine Serum (Life Technologies) 5mM (final concentration) Glutamax (ThermoFisher Scientific) 5 ng/mL carrier free GM-CSF (R&D Systems)
Assay Media	As Complete Media without GM-CSF
Freezing Media	As Complete Media with 10% DMSO

2.1.1.1 TF-1 Cryopreservation and Thawing

Cryopreservation

Cells were concentrated to $1x10^6$ cells/mL in cold freeze media and 0.5 mL was transferred to a 1.5 mL cryo-vial. The cryo-vials were placed in a Mr Frosty (controlled rate freezer) at -80^oC for 24 hours before transferring to the vapour phase of liquid nitrogen for long term storage.

Thawing

A vial of cells were removed from storage in the vapour phase of liquid nitrogen and transferred to a water bath at 37ºC. The vial was removed from the water bath when a miniscule ice crystal was all that remained in the vial and was sprayed with 70% IMS before placing into a Biological Safety Cabinet (BSC). The cells were removed from the vial using a sterile Pasteur pipette and transferred into 5mL of pre-warmed complete medium, washing the inside of the vial to remove all cells. Cells were centrifuged (300g, 5 minutes) and the supernatant was aspirated to remove DMSO. Cells were then re-suspended in a volume of complete medium to achieve $2x10^5$ cells/mL and transferred to a T25 Culture Flask (Fisher Scientific) and placed in a humidified incubator (5% CO_2, 37^oC).

2.1.2 M-07e Cell Line

M-07e cells are dependent on GM-CSF, SCF and TPO, therefore chosen to investigate and compare the activity of multiple soluble and immobilised growth factors. Frozen M-07e cells were obtained from Creative Bioarray and cultured according to manufacturer's recommendations. A description of media formulations are presented in Table 3.

Table 3. M-07e media formulations.

Media Type	**Description**
Complete Media	RPMI 1640 (Phenol Red Free, Glutamine Free; ThermoFisher Scientific) 10% v/v Heat Inactivated Foetal Bovine Serum (Life Technologies) 5mM (final concentration) Glutamax (ThermoFisher Scientific) 5 ng/mL carrier free GM-CSF (R&D Systems) 10 ng/mL carrier free SCF (R&D Systems)
Assay Media	As Complete Media without GM-CSF and SCF
Freezing Media	As Complete Media with 10% DMSO

M-07e cells (Creative Biosystems, USA) were maintained in culture at a density of 4×10^5 to 1×10^6 cells/mL with complete media in a humidified incubator (5% CO_2, 37^{o}C). Media was replenished every two to three days.

A description of cryopreservation and thawing of TF-1 cells are described in section 2.1.1.1.

2.1.3 Umbilical Cord Blood Derived CD34+ Cells

Umbilical cord blood not suitable for clinical transplant was received from Anthony Nolan (Nottingham, UK). Blood was processed fresh, or incubated on a tube rotator overnight at 3^{o}C before processing.

Mononuclear cells (MNCs) were isolated by density gradient separation with Ficoll Paque. CD34+ cells were isolated from the MNCs fraction by magnetic separation using a CD34 microbead kit, LS columns and quadroMACS magnet (Miltenyi Biotec) as per the manufacturers protocol.

Cells were cultured in complete media. Cells were seeded at 5x10^{5}/mL and were reset back to initial seeding density every 3 days through centrifugation and re-suspension of cells in a 1:1 replacement of spent medium:fresh medium, cytokines were added at double concentration in fresh medium to replace degraded cytokines in the spent fraction.

A description of media formulations are listed in Table 4.

Table 4. CD34+ Media Formulations.

Media Type	**Description**
Complete Media	Iscove's Modified Dulbecco's Medium (IMDM with Glutamax, Invitrogen) 20% v/v BIT 9500 Serum Substitute (Stem Cell Technologies) 100 ng/mL SCF, IL-3 and Flt-3L (R&D Systems) 50 ng/mL TPO (R&D Systems)
Assay Media	As Complete Media without growth factors
Freezing Media	80 % IMDM (with Glutamax, Invitrogen) 10 % Heat Inactivated Foetal Bovine Serum (Life Technologies) 10% DMSO

2.1.3.1 CD34+ Cryopreservation and Thawing

Cryopreservation

Cells were concentrated to $1x10^6$ cells/mL in chilled freeze media and 0.5 mL was transferred to a 1.5 mL cryo-vial. The cryo-vials were placed in a Mr Frosty (controlled rate freezer) at -80ºC for 24 hours before transferring to the vapour phase of liquid nitrogen for long term storage.

Thawing

A vial of CD34+ cells were removed from storage in the vapour phase of liquid nitrogen and transferred to a water bath at 37ºC. The vial was removed from the water bath when a miniscule ice crystal remained in the vial and was sprayed with 70% IMS before placing into

a Biological Safety Cabinet (BSC). The cells were removed from the vial using a sterile Pasteur pipette and transferred into 5mL of pre-warmed complete medium, washing the inside of the vial to remove all cells. Cells were centrifuged (300g, 5 minutes) and the supernatant was aspirated to remove DMSO. Cells were then re-suspended in a volume of complete medium to achieve $5x10^5$ cells/mL and transferred to a T25 Culture Flask (Fisher Scientific) and placed in a humidified incubator (5% CO_2, 37^oC).

2.2 Cell Counting

The ViCell counting method was used for ambr™ experiments, whereas the NC-3000 was used for static culture experiments unless otherwise stated.

2.2.1 ViCell (Trypan Blue Exclusion)

For AMBR experiments, 500µl of cell suspension (concentrated or diluted with medium) was taken for analysis, and placed into the sampling cup of the automated cell counter. Counts were an average of 50 images per sample. Counting settings were:

- Minimum Diameter (µm): 6
- Maximum Diameter (µm): 20
- Number of images: 50
- Trypan blue mixing cycles: 3
- Cell brightness (%): 85
- Cell Sharpness: 100
- Viable cell spot brightness (%): 65
- Viable cell spot area (%): 10
- Minimum circularity: 0.8
- Decluster degree: None

2.2.2 NC-3000

A 100 µL cell suspension sample was taken and dispensed into a 1.5 mL eppendorf tube. Approximately 60 µl of cell suspension was aspirated using a Via-1 cassette. The cassette was immediately placed into the NC-3000 and the automated mammalian cell count and viability protocol was selected and run. Acridine Orange and DAPI dyes are used to determine cell viability.

2.2.3 Flow cytometry

Typically 50 µL of cell suspension were sampled by the AMBR and dispensed into a 96 well plate containing 50 µL of 4% paraformaldehyde (PFA). Spare wells and in between wells were filled with sterile water and the plate was lidded to prevent evaporation of samples. A plate was often left for 10 hours at room temperature depending on the frequency of sampling. Plates were analysed by mixing wells with a multichannel pipette prior to analysis on a flow cytometer. A fixed volume of 50 µL was analysed with a fixed medium flow rate for all wells. To calculate an event count, cell populations were gated to remove debris in flow cytometry analysis software (FlowJo).

2.3 Agitated Cell Culture

A micro-scale stirred tank bioreactor platform (ambr®; Sartorius Stedim, UK.) was used to determine the scalability of the immobilization method. Unless otherwise stated, physicochemical parameters were set to: 100% DO_2 (oxygen delivery), pH 7.4, temperature 37°C, stir speed 350 rpm. Sparged vessels were used with 1% antifoam additions (Antifoam C, Sigma Aldrich, UK) performed every 24 h beginning at 0 h. The ambr® was programed to sample from vessels as required, dispensing into a microtiter plate containing 4% paraformaldehyde (Sigma Aldrich, UK) which was then analysed on a flow cytometer to determine cell and particle number. A complete media change was performed every 48–72 h and cells were reset to a density of 2×10^5 cells/mL. For iGF conditions, particles were retained within the vessel by holding a magnet against the vessel, whilst performing media changes.

For particle removal; The AMBR 1mL pipette tips were modified to accommodate magnets (first 4 magnets; neodymium 0.58kg pull 4mmx4mm) and autoclaved. AMBR vessel caps were removed and a sterile magnetic pipette tip was manually inserted into vessels for 5 minutes with continuous stirring.

2.4 Functional Assays

Growth factor dependent cell lines were used to assess the functional activity of immobilised and soluble growth factors.

2.4.1 TF-1 Functional Assay

TF-1 cells were used to assess the function of GM-CSF. Unless otherwise stated, TF-1 cells were washed three times in GM-CSF free media and starved of GM-CSF for 24 hours. Either soluble GM-CSF (sGM-CSF) or immobilized GM-CSF (iGM-CSF) was supplemented to starved TF-1 cells and a growth rate was determined thereafter using flow cytometry.

2.4.2 M-07e Functional Assay

M-07e cells were used to assess the function of GM-CSF, SCF, Flt-3L and TPO. Unless otherwise stated, M-07e cells were washed three times in GF free media and starved of GF for 24 h. Either soluble GF (sGF) or immobilized GF (iGF) was supplemented to starved M-07e cells and a growth rate was determined thereafter using flow cytometry.

2.4.3 CD34+ Functional Assay

CD34+ cells were used to assess the function of SCF. Unless otherwise stated, CD34+ cells were washed three times in GF free media and starved of GF for 24 h. Either soluble GF (sGF) or immobilized GF (iGF) was supplemented to starved CD34+ cells and a growth rate was determined thereafter using flow cytometry.

2.4.4 Dose-response Assay (AlamarBlue®)

The AlamarBlue® assay was optimised as per Appendix A – Optimisation of AlamarBlue Assay. In brief, cells were cultured for 48 hours in a 96 well plate, where 10% (v/v) AlamarBlue was dispensed into each well and incubated for 3.5 hours in a humidified incubator (37^{o}C, 5% CO_2). Afterwards, the plate was removed from the incubator and allowed to cool to room temperature (protecting from direct light) for 0.5 hours before measuring the fluorescence on a plate reader.

The effective concentration 50 (EC50) was calculated in GraphPad Prism software using a four-parameter variable slope curve.

2.5 Growth Factor Immobilisation

The following protocols were used for four different protein immobilisation methods unless otherwise stated.

2.5.1 Streptavidin Method

The immobilization method involves a two-step reaction (summarised in Figure 8):

Step 1 – Growth factor biotinylation

1) Lyophilized carrier free GFs (R&D Systems, UK) were re-suspended in PBS at a concentration of 50 µg/mL.
2) NHS-PEG2000-biotin (Nanocs Inc., USA) was suspended in anhydrous DMSO at the required molar concentration for the individual experiment being carried out and used immediately.
3) Equal volumes of GF and NHS-PEG2000-biotin were mixed in LoBind Protein Eppendorf tubes and vortexed for 2 h at room temperature.

Step 2 - Immobilization of biotinylated growth factors

4) 2.8 µm streptavidin coated magnetic particles (Solulink, USA) were placed on a magnetic stand (MagnaRack™ Magnetic Separation Rack, ThermoFisher Scientific) and the supernatant removed.
5) Biotinylated GF solution from step 1.3 was added to the prepared particles and vortexed overnight at 4–8 ºC.
6) iGFs were washed three times in 500 µL of PBS using a magnetic stand and used immediately.

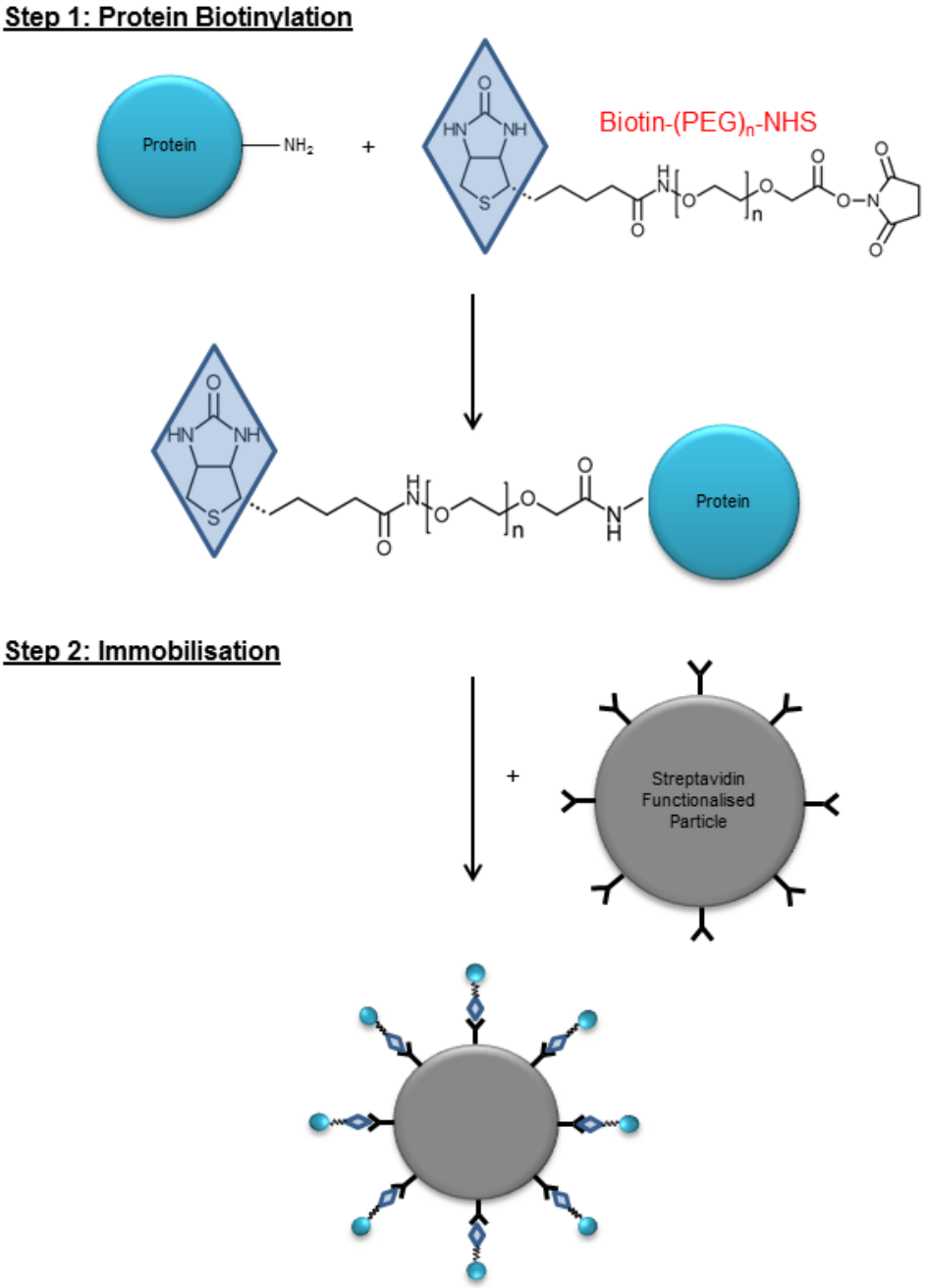


Figure 8. Growth factor immobilisation reaction using the 'Streptavidin Method'. The immobilisation method consists of a two-step reaction, where first the GF is biotinylated and secondly immobilised onto streptavidin functionalised magnetic microparticles.

2.5.2 Carboxyl Method

Primary amines of GM-CSF were modified to thiol functional groups using TCEP reagent as per the manufacturer's instructions. The modified GM-CSF (R&D Systems) was then pegylated using a heterobifunctional PEG molecule, NH2-PEG2000-Maleimide (Nanocs Inc., USA). The PEG molecule was re-suspended at 10 mg/mL. Three 50 µL aliquots of the PEG were mixed with three separate 50µL aliquots of GM-CSF of different concentrations (10 µg/mL, 2.5 µg/mL and 0.3 µg/mL) at room temperature for two hours using a tube rotator.

The pegylated GM-CSF suspension was supplemented to dry pre-functionalised 2.0-2.9um carboxyl magnetic particles (Spherotech Inc., USA); $1x10^6$ particles per 100µl of PEGylated GM-CSF. EDC reagent was then supplemented at a final concentration of 1mM. The suspension was mixed overnight at 3-4ºC using a tube rotator.

The particles were washed three times using nuclease free water, centrifuging briefly at 10,000rpm and placing on a magnetic stand whilst removing the supernatant. The final immobilised GM-CSF particles were re-suspended in assay media.

2.5.3 Fibronectin Method

Lyophilised human fibronectin (R&D Systems) was suspended in PBS at a final concentration of 10 mg/mL. 50µL of fibronectin was supplemented to dry plain (non-functionalised) magnetic particles (Spherotech Inc., USA) and mixed at room temperature on a tube rotator for 2 hours. Fibronectin coated particles were washed three times with nuclease free water, centrifuging briefly at 10,000rpm and placing on a magnetic stand whilst removing the supernatant.

$1x10^6$ particles per 100µl of PEGylated GM-CSF Dry fibronectin coated particles were re-suspended in three 50µL aliquots of GM-CSF of different concentrations (10 µg/mL, 2.5 µg/mL and 0.3 µg/mL) at 3-8ºC overnight using a tube rotator. The particles were then washed three times using nuclease free water, centrifuging briefly at 10,000rpm and placing on a magnetic stand whilst removing the supernatant. The final immobilised GM-CSF particles were re-suspended in assay media.

2.5.4 Solulink Method

Immobilisation of GM-CSF was achieved using the MagnaLink BeadLink Kit (Solulink Inc, USA). The manufacturer's immobilisation protocol was followed except for the following:

All of the reagents were split into thirds and the yellow and red spin columns were re-used three times for each of the GM-CSF concentrations (10 µg, 2.5 µg, and 0.63 µg/mL). The spin columns were washed once with nuclease free water before re-use.

The final immobilised GM-CSF particles were re-suspended in nuclease free water at 3-8ºC until use.

2.6 Nutrient and Metabolite Analysis

Media supernatants (1mL) were sampled and stored in 1.5 mL eppendorf tubes at -80^{o}C until time of analysis. Samples were thawed at room temperature and vortexed briefly before analysis on the BioProfile Flex (Nova Biomedical). QC tests and calibrations were performed as per the manufacturer's recommendations.

2.7 Protein Quantification

Flow cytometry based methods were used to quantify immobilised growth factors and ELISA based methods were used to quantify soluble growth factors.

2.7.1 Immobilised Growth Factor Concentration

Surface Concentration

2×10^5 iGF particles were incubated in 50 µL of flow cytometry stain buffer containing the required concentration of conjugated monoclonal antibodies (R&D Systems, UK) for 1 h at room temperature. Where conjugated antibodies were unavailable, antibodies were custom conjugated using an APC-Antibody conjugation kit (Solulink, USA). In parallel, iGF particles were also stained with an antibody isotype control. iGF particles were then washed once with PBS and re-suspended in 100–500 µL of PBS. Fluorescence intensity was measured using FACS Canto II flow cytometer (BD Biosciences, UK) by taking a minimum of 10,000 events at medium flow rate. Flow cytometry data was analysed using FlowJo software, where a singlet population was discriminated. Fluorescence reference standards (Quantum

MESF; Bangs Labs Inc., USA) were run on the same day to determine the mean mass of iGF per particle using the net geometric of the singlet population.

Total Concentration

Final particle concentration was determined using flow cytometry where a fixed volume of sample was analysed using medium flow rate. Flow cytometry data was analysed using FlowJo software (Treestar, USA) and cells/mL was generated from the particle population gate. The particle count per mL was then multiplied by the mean surface concentration of iGF.

2.7.2 Soluble Growth Factor Concentration

Soluble GF concentrations were determined with a Bio-Plex Magpix Multiplex Reader (Bio-Rad Laboratories, UK) using pre-mixed Human Magnetic Luminex assay kits with SCF and GM-CSF analytes (R&D Systems, UK) as per the manufacturer's instructions. Samples were prepared by sampling cell suspensions and removing cells (by centrifugation), and storing the supernatant in 1.5 mL protein LoBind eppendorf tubes at -20°C until use.

2.8 Immunophenotyping

Approximately $1x10^5$ cells were stained with the appropriate CD antibody in a total volume of 100µL per well at room temperature for 30 minutes. Cells were pelleted by centrifugation and re-suspended in 100µL of PBS to remove non-bound antibody. IgG isotype controls were used in parallel to assist in gating of the positive cell population. Stained cells were analysed on the FACS Canto II HT. Antibodies were titrated before analysis.

2.9 Magnetic removal

A variety of methods were used to remove and retain magnetic particles in culture. The methods used are listed below.

2.9.1 Magnetic pipette tips

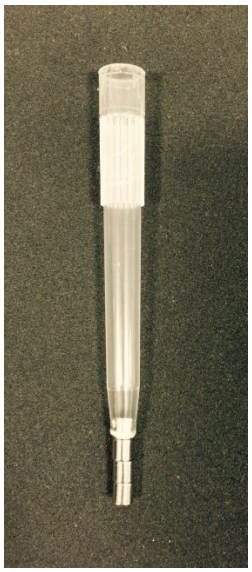


Figure 9. Custom made magnetic pipette tip. A 1 mL pipette tip was trimmed and four small magnets were placed inside of the tip and autoclaved to ensure sterility.

2.9.2 Magnetic rack

Figure 10. A magnetic stand to accommodate multiple 1.5 mL eppendorf tubes.

2.9.3 Magnetic plate

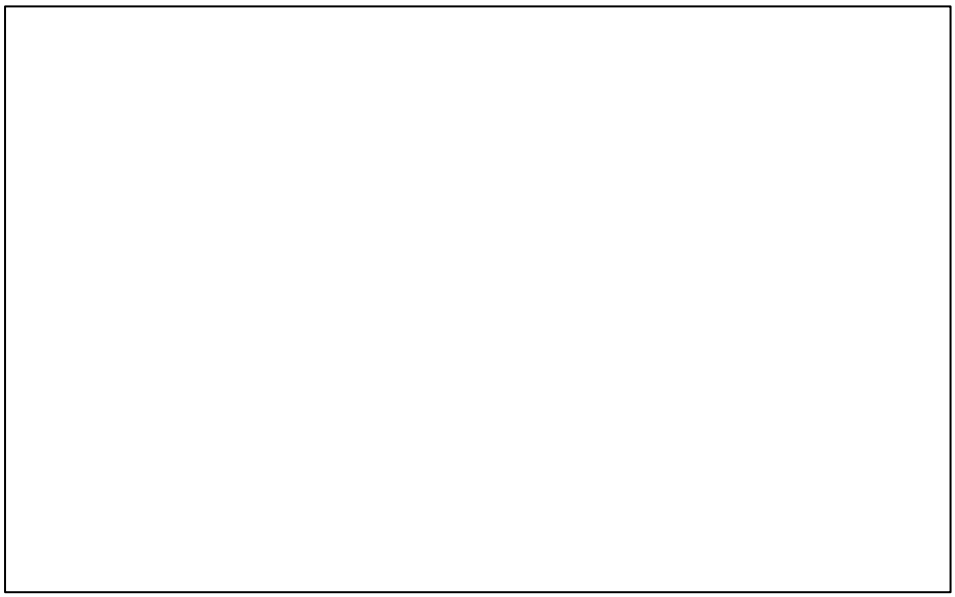


Figure 11. A) A magnetic plate for 96 well plates B) capture of magnetic particles during a media change in an AMBR vessel.

2.10 Equations

Equation 1. Cell Population Doublings (PD).

$$PD = \frac{\log 10\left(\frac{FN}{SD}\right)}{\log 2}$$

Where PD = population doublings; FN= final cell concentration (cells/mL); SD = starting cell concentration (cells/mL).

2.11 Statistical Analysis

To determine the statistical significance of one variable between two groups, a student's two-tailed T-Test was performed. To determine the statistical significance of one variable

between three or more groups a one-way ANOVA was performed followed by Dunnett's test for multiple comparisons. To determine the statistical significance of two variables between three or more groups a two-way ANOVA was performed followed by Sidak's test for multiple comparisons.

For all tests a $P \leq 0.05$ was deemed significant. All statistical tests were performed using GraphPad Prism 6 (GraphPad Software Inc., USA).

Materials

Table 5. List of reagents

Description	Supplier	Cat/Model Number
Anti-Human CD133-APC	R&D Systems	FAB11331A-025
Anti-Human CD34-FITC	BD Biosciences	555821
Antifoam A	Sigma-Aldrich	A6582-100G
Carboxylated Functionalised Particles	Spherotech	CM-20-10
CD34 Microbead Kit, Human	Miltenyi Biotec	130-046-702
Distilled Water	Life Technologies	10977-035
DMSO	Sigma Aldrich	471267-1L
EDC	Thermo Scientific	22980
Ficoll-Paque	GE Healthcare	17-1440-02
FLT-3L	R&D Systems	308-FK-025/CF
Glutamax	Life Technologies	35050-038
Heat Inactivated FBS (US Origin)	Life Technologies	10082-147
Human recombinant EPO	R&D Systems	307-ER-050/CF
Human recombinant GM-CSF	R&D Systems	215-GM-050/CF
Human recombinant SCF	R&D Systems	255-SC-200/CF
Human recombinant TPO	R&D Systems	288-TP-200/CF
IMDM	BioChrom	F 0465
L-glutamine	Fisher Scientific	11500626
LS separation columns	Miltenyi Biotec	130-042-401

MagnaLink BeadLink Kit	Solulink	A-9404-002
MagnaLinkBeadLink Kit	Solulink	A-9404-002
MagnaRack™ Magnetic Separation Rack	ThermoFisher	CS15000
Mal-PEG-NHS	Nanocs Inc	PG2-MLNS-2k
NC-3000 Via-1 Cassettes	Sartorius	16704-000070
NH2-PEG(2000)-Mal	Nanocs Inc.	PG2-AMML-2k
NHS-PEG(10000)-Biotin	Nanocs Inc.	PG2-BNNS-10k
NHS-PEG(2000)-Biotin	Nanocs Inc.	PG2-BNNS-2k
NHS-PEG-Biotin	Nanocs Inc	PG2-BNNS-2k
PE Rat Anti-Human GM-CSF	BD Biosciences	554507
PE Rat IgG2a K Isotope Control	BD Biosciences	554689
PFA	Sigma Aldrich	F8775-25ML
Phosphate Buffered Saline	SLS	BE17-516F
Plain Magnetic microparticles	Spherotech	PM-20-10
Quantum MESF R-PE	Bangs Laboratories	899
RPMI 1640	Life Technologies	32404-014
Serum Substitute (BIT 9500)	Stem Cell Technologies	09500
Streptavidin Functionalised Particles	Solulink	M-1003-020
TCEP	Thermo Scientific	20490
TF-1 Human Erythroleukaemia	Public Health England	93022307
TRIS Buffer	Life Technologies	AM9855G

Table 6. List of consumables

Description	Supplier	Cat/Model Number
24 Deep Well Plate	Tap Biosystems	A-0038
Costar 48 Well Plate	SLS	3548
96 well black walled plate	BD Falcon	353219
96 Well Plate	SLS	3799
AMBR 15 Vessels (Sparged)	TAP Biosystems	001-0B80
Protein LoBind Eppendorfs (1.5mL)	Fisher Scientific	10708704
T25 Culture Flask	Fisher Scientific	TKT-130-170F
T75 Culture Flask	Fisher Scientific	TKT-130-210T

Table 7. List of instruments and equipment

Description	Supplier	Cat/Model Number
AMBR 15	TAP Biosystems (Sartorius)	N/A
Biological Safety Cabinet	Herasafe KS Class II	N/A
Bioprofile FLEX	Nova Biomedical	N/A
Centrifuge	Sigma	3-16 pk
Countess Automated Cell Counter	Life Technologies	C10227
FACS Canto II HT Flow Cytometer	BD Biosciences	338962
FLUOstar Omega Plate Reader	BMG Labtech	N/A
Guava Easycyte 8HT Flow Cytometer	Merck Millipore	0500-4005
Incubator	Thermo Scientific	Heraeus HERA Cell 150
Magnetic Rack	Life Technologies	CS15000

Magnets for pipette tip modification (neodymium 0.58kg pull 4mmx4mm)	First4magnets	F340SH-25
Micro-centrifuge	Sigma	1-14
Microscope	Nikon	N/A
NucleoCounter NC-3000	Chemometec	16704-000060
QuadroMACS magnetic separation column	Miltenyi Biotec	130-090-976
ViCell Automated Cell Counter	Beckman Coulter	Vi-Cell XR
Water Bath	Grant	Sub Aqua 26 Plus

3 The Development of a Growth Factor Immobilisation Technology for use in Cell Culture Systems

3.1 Introduction

The costs of cell therapies have been identified as a major hurdle commercialisation and patient access[91]. Costs can be attributed largely to the quantities of cell culture media and supplements such as growth factors which are required in high concentrations (e.g. SCF is usually supplemented at concentrations ≥100 ng/mL).

Steps have been taken to move towards perfusion systems which maintain levels of nutrients and supplements whilst minimising metabolic by-products and inhibitory paracrine signals [92], [93]. However, perfusion systems are limited by a lack of on-line feedback sensors and mechanisms, meaning there is often a delay between manual measurement, analysis of results and adjusting feeding regimes to compensate for cell number and or metabolic activity [45], [94]. Furthermore, cell expansion protocols for HSCs and T-cells can typically be 7-14 days meaning media requirements can remain a significant cost even in perfusion systems [45], [94]–[98]. Consequently, reducing total quantities or improving the performance of media or supplements (primarily expensive growth factors) would represent a significant advancement for the reduction in cost of goods for cell and gene therapies.

The extracellular matrix plays an important role is presenting a variety of factors in an immobilised form, including adhesion molecules, integrins and growth factors. Maintenance of stem cell phenotype or differentiation into more specialised cell types is regulated by a number of physicochemical factors in the stem cell niche working in concert in a spatiotemporal fashion[99]. The niche comprises of short-term cyclical and injury mediated regenerative responses and is far from a static environment [100].

The following chapter discusses the development of a novel growth factor immobilisation method using magnetic microparticles as an immobilisation substrate. Immobilisation methods were designed by evaluating and building upon current available methods.

A selection of methods was assessed using a functional assay utilising a growth factor dependent cell line. The quantity of immobilised growth factor was determined using a custom developed flow cytometry assay that allowed a direct comparison between functional effects and immobilised concentrations.

3.1.1 Protein Immobilisation Methods

Immobilisation of growth factors for the purpose of cell culture can be achieved via a wide range of methods comprehensively described elsewhere [101]. Methods were shortlisted and screened on the basis of meeting the following criteria:

- A widely used and accepted technique (or derivative thereof),
- Clinically compatible reagents and materials,
- Mild chemical reactions to prevent protein degradation and 'click' chemistry (no extensive modification of functional groups),
- Inexpensive and rapid process with a low number of process steps,
- And (or) a commercially available protein immobilisation kit.

Table 5 summarises a selection of immobilisation techniques along with their advantages and limitations.

Table 8. A comparison of commercially available and customised protein immobilisation methods.

Technique	Description	Advantages	Limitations
Commercially available immobilisation techniques			
Bead Link Kit (Solulink)	Conjugation occurs by a bis-arylhydrazone bond (covalent bond).	• Quantitative bond. • Purification of modified proteins. • High immobilisation yields.	• Expensive. • No spatial presentation modifications.
InnovaCoat (Innova Biosciences)	Covalent binding to Gold nanoparticles via primary amines of proteins.	• Rapid conjugation in 15 minutes. • 100% conjugation efficiency.	• Nanometre diameter is susceptible to cellular endocytosis. • No spatial presentation modifications.
BioMag Plus (Bangs Labs)	Amine or Carboxyl functionalised, coupling via primary amines.	• Technically simple.	• Expensive • No spatial presentation modifications. • Irregular particle morphology
Customised immobilisation techniques			
Passive Absorption	Electrostatic interactions between the surface of the particle and the growth factor.	• Technically simple. • Recapitulates growth factor presentation in vivo.	• Hydrophobic surfaces can denature proteins. • Leaching of protein common due to weak interactions. • Non-specific protein immobilisation.
Streptavidin Particles	Biotinylated proteins bind to streptavidin groups on the particles.	• Protein specific conjugation chemistry. • Spatial presentation of proteins can be tuned with spacer molecules • Strongest non-covalent bond.	• N/A
Carboxylated Particles	NHS esters are formed by EDC activation of Carboxylic acid groups. The NHS esters undergo nucleophilic attack from the nitrogen within primary amine groups forming an amide bond.	• Strong amide conjugation bond • Most commonly used crosslinking method.	• Blocking required (amine containing buffers such as TRIS). • Hydrolysis of the NHS esters is a competitive reaction and water can inactivate EDC. • Hydrophobic surface can denature proteins. • Non-specific protein conjugation.
Amine Particles	Carboxylic acids are first activated with EDC before introducing the amine functionalised particles to form an amide bond.	One/two step conjugation reactions.	• Hydrolysis of the NHS esters is a competitive reaction and water can inactivate EDC. • Non-specific protein immobilisation. • Protein polymerisation is possible.
Hydroxyl Particles	Formation of a reactive carbonyl group which react with primary amines of proteins.	• Low non-specific protein binding	• Susceptible to hydrolysis. • Use of toxic conjugation reagents • Long immobilisation process.

3.1.1.1 Shortlisted immobilisation methods for initial screening experiment

In an initial screening experiment a selection of immobilisation techniques were investigated to compare their functional performance in a growth factor dependent cell line. The following four conjugation methods were shortlisted by meeting the critea listed in section 3.1.1.

3.1.1.1.1 Carboxyl

One of the most common ways of immobilising proteins is to use a reducing agent (TCEP) to modify disulphide bonds to thiol groups which spontaneously react with maleimide groups. Incorporating a PEG linker molecule between the protein and surface allows the steric presentation of proteins to be controlled by altering the PEG linker length. A forward and reverse version of the reaction can be performed by PEGylating either the particle or protein first as shown in Figure 12.

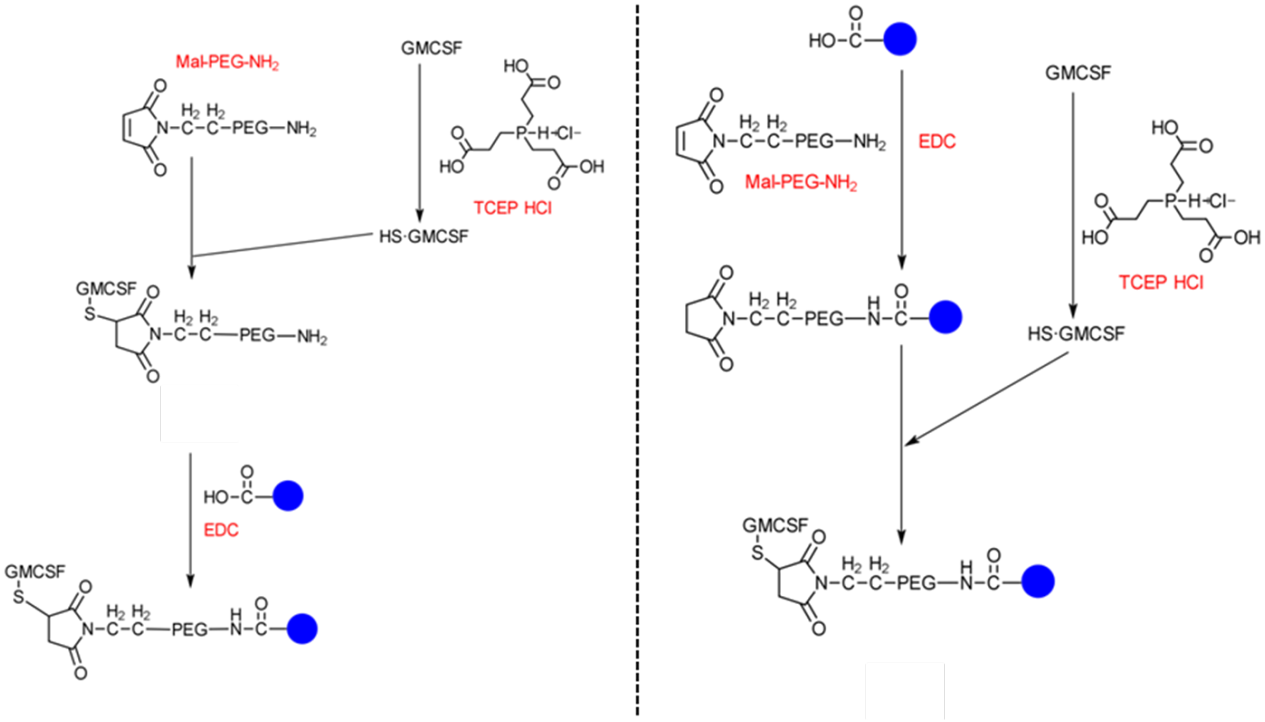


Figure 12. Carboxyl immobilisation technique chemical reaction. (Left) *Forward Reaction* - Disulphide bonds within the GM-CSF molecule are transformed to thiol functional groups that spontaneously react with the Maleimide functional group of the heterobifunctional PEG compound, resulting in the loss of hydroxide ions. Secondly, the pre-functionalised carboxylated magnetic particles are activated with a carbodiimide reagent (EDC) resulting in a dehydration reaction (loss of water). The activated carboxylic acid groups undergo nucleophilic attack from the primary amine group of the PEG compound where the growth factor is immobilised and water is released as a by-product. (Right) *Reverse Reaction* – The reverse reaction involves similar chemical reactions to the forward reaction however, the carboxyl particle is first conjugated to the PEG linker molecule and finally the PEGylated particle is conjugated to the GM-CSF molecule.

3.1.1.1.2 Streptavidin

The streptavidin-biotin method is one of the most widely used conjugation methods in biotechnology due to its bond strength (strongest non-covalent bond), simplicity of the reaction and specificity. Incorporation of a PEG molecule allows the steric presentation of the growth factor to be tuned by altering the PEG molecular weight as illustrated in Figure 13.

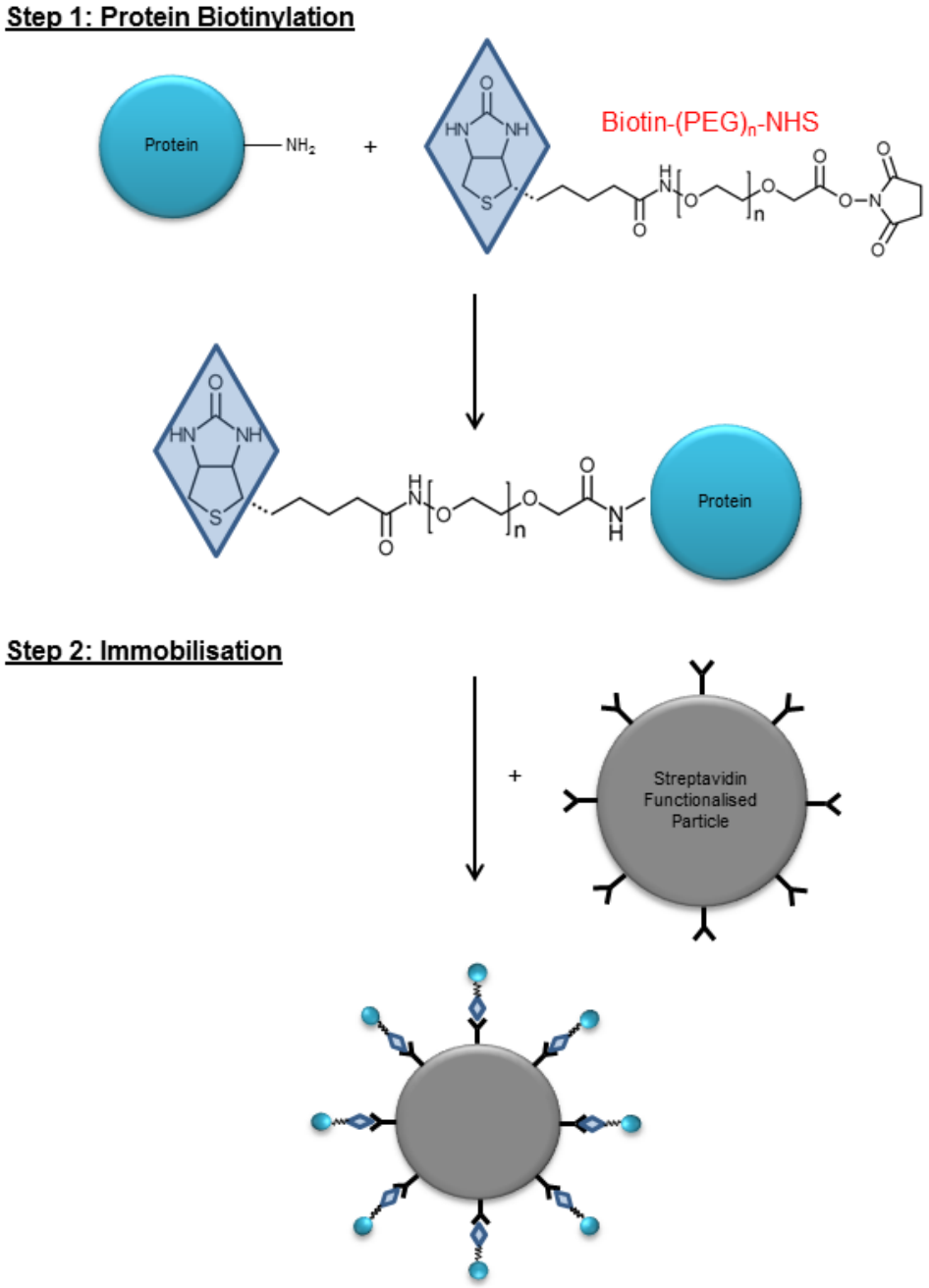


Figure 13. Streptavidin immobilisation technique chemical reaction. (Left) Reverse Reaction - N-hydroxysuccinimide (NHS) within the heterobifunctional PEG compound reacts with the primary amines of the GM-CSF molecule forming a stable amide bond and releasing NHS. The GM-CSF-PEG-Biotin conjugate is then introduced to a streptavidin pre-functionalised magnetic particle where a stable (spontaneous) bond is formed between the streptavidin and biotin groups. The red circle represents a magnetic particle functioned with streptavidin groups and having a mean diameter of 2.8µm. (Right) Forward Reaction – The forward reaction involves a similar chemical process to the reverse reaction however, the GM-CSF molecule is first PEGylated before being conjugated to the streptavidin functionalised particle.

3.1.1.1.3 Solulink

Solulink is a commercially available magnetic microparticle protein conjugation kit. Proteins are immobilised by a quantifiable bond through modification of the protein using a proprietary chemical process illustrated in Figure 14.

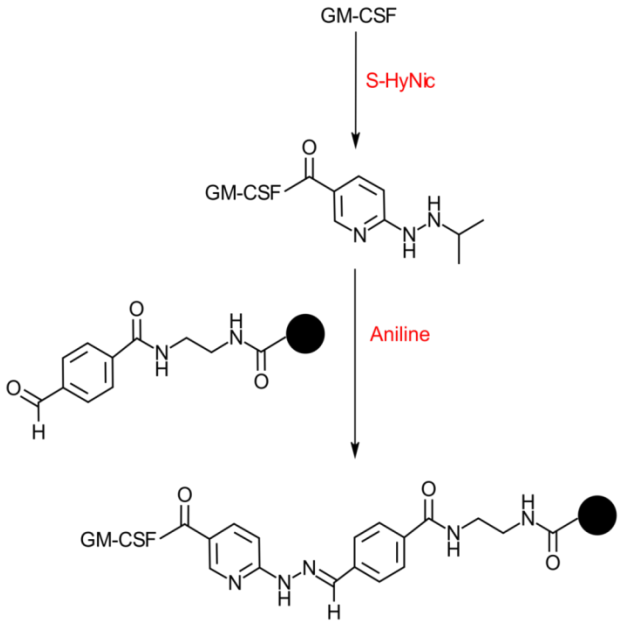


Figure 14. Solulink immobilisation technique chemical reaction. GM-CSF is modified to incorporate S-HyNic by targeting the primary amines of the protein. The modified GM-CSF molecule is incubated with 4FB pre-functionalised magnetic particles in the presence of a catalyst (aniline). GM-CSF is specifically immobilised by the formation of a stable covalent bis-arylhydrazone bond. The black circle represents a magnetic particle functioned with 4FB groups and having a mean diameter of 2.8µm.

3.1.1.1.4 Fibronectin

The fibronectin immobilisation technique is a passive adsorption technique where proteins are passively adsorbed onto the fibronectin molecules through electrostatic interactions. This method is most similar to the natural immobilisation of growth factors and proteins *in vivo* by the extracellular matrix.

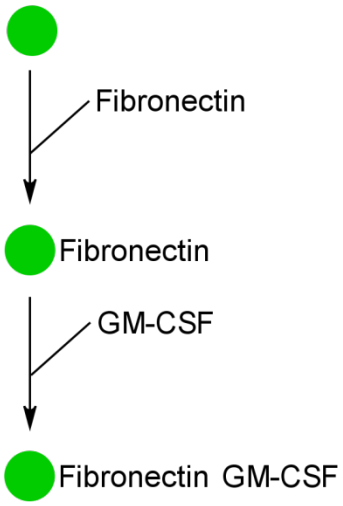


Figure 15. Fibronectin immobilisation technique chemical reaction. Fibronectin is incubated with plain magnetic particles (non-functionalised) to enable passive adsorption. GM-CSF is then immobilised by incubation with the fibronectin coated particles through passive adsorption. The blue circle represents a plain magnetic particle having a diameter of 2.0-2.9µm.

3.1.2 Cell Counting Methods

To investigate the functional effects of immobilised growth factors (specifically the effects on cell growth rates in comparison to soluble growth factors), high resolution growth curves were required using a high-throughput cell counting assay. A number of cell viability assays are available, however as highlighted in Table 6, the majority of current cell counting assays do not offer the high-throughput element required to construct high resolution cell growth curves.

After evaluating the available cell counting methods, flow cytometry (i.e. the BD FACS Canto II) was selected as a high-throughput cell counting method that could achieve cell growth curves with a high degree of time resolution.

3.1.3 Protein Quantification Methods

3.1.3.1 Immobilised Proteins

A custom quantification method was developed using flow cytometry and is described in full in 3.2.3. In brief, immobilised growth factors were incubated with fluorescently labelled antibodies and quantified from a standard curve using fluorochrome matched fluorescent standards. Molecules of equivalent soluble factor (MESF) values could be determined from fluorescence intensity.

3.1.3.2 Soluble Proteins

Concentrations of soluble growth factors were determined using the Luminex MAGPIX™ multiplex system as per the manufacturer's protocol. Multiplex or singleplex kits were

purchased from R&D Systems. Media samples were stored at -80^{o}C, thawed at room temperature and vortexed directly prior to analysis.

Table 9. A comparison of widely used cell viability assays. The table shows that widely used assays are not high-throughput and therefore can not generate the high-resolution cell growth curves required to investigate the functional effects of immobilised growth factors.

Counting Method	Type	Supplier	Accurate Cell Concentration (/mL)	Sample Volume (uL)	Analysis Time (Seconds)	CV %	Viability ?	High-Throughput?
Trypan Blue	Haemocytometer	Various	N/A	>10	210	41.8	Yes	No
	ViCell	Beckman Coulter	5E4-1E7	500	75	6	Yes	No
	Countess	Invitrogen	5E4-5E6	10	30-300	3.5-10	Yes	No
DNA Stain	ViaCount	Merck Millipore	1E3-1E4 (/Test)	20	N/A	3-7	Yes	Semi
	NC-3000	Chemometec	5E4-5E6	10-60	35-180	3.5-10	Yes	Semi
Electrical Impedance	Scepter 2.0	Merck Millipore	1E4-5E5	100	14-25	9.1	No	Semi
Other	FACSCanto	Beckman Coulter	1E4-5E6	>72	Variable	N/A	Yes	Yes

3.2 Results

The following results section evaluates the short-listed (refer to section3.1.1.1) growth factor immobilisation techniques using a functional assay and aims to develop a quantification assay so that the potency of growth factors can be determined and the immobilisation method can be optimised.

3.2.1 Development of a Functional Assay to Assess Biological Activity of Immobilised Growth Factors

To investigate the biological activity of immobilised growth factors, a functional assay was developed consisting of a model (growth factor dependent) haematopoietic cell line to reduce the complexities involved in primary cell systems (i.e. donor variability and cell differentiation). The cell line is a Human erythroblast derived from leukaemia – TF-1 Erythroleukemia.

3.2.1.1 Activity of Soluble GM-CSF in a GM-CSF Dependent Cell Line.

Since TF-1 cells proliferate dependently on Granulocyte Macrophage Stimulating Colony Stimulating Factor (GM-CSF) a dose-response assay of GM-CSF was performed in order to determine the dose required for the maximal biological response using an AlamarBlue® assay.

Cell seeding density, growth factor incubation period, AlamarBlue incubation period and phenol red interference were predetermined in assay optimisation studies (results of the optimisation studies can be found in Appendix A).

Figure 16 shows the dose-response profile of soluble GM-CSF in the TF-1 cell line. The maximal biological response was achieved with 2.3 ng/mL of soluble GM-CSF where the EC_{50} was calculated at 0.25 ng/mL.

The results from the dose-response assay are in agreement with the supplier QA results for the TF-1 cell line, where a recommended concentration of GM-CSF is 2-5ng/mL. To ensure that the maximum biological response was achieved across experiments, 5 ng/mL of soluble GMSCF was used in all future experiments as a positive control.

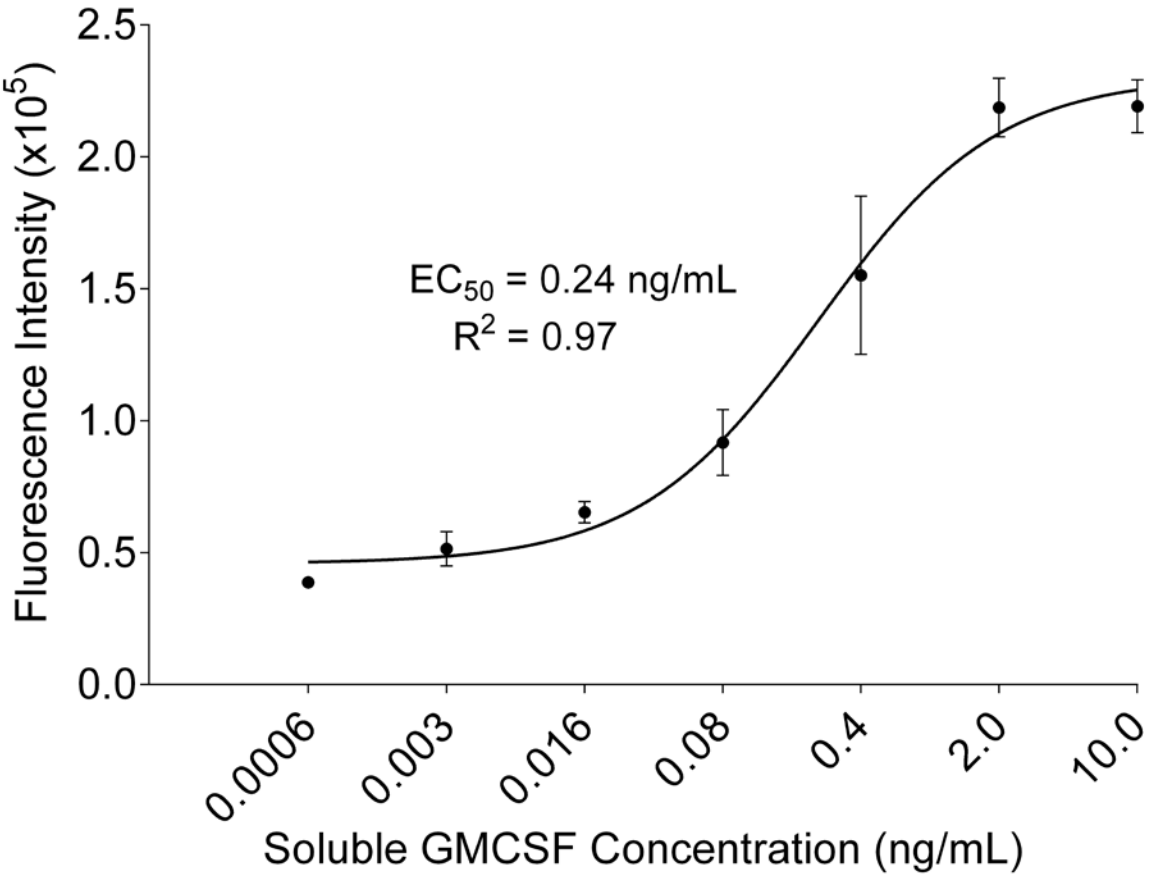


Figure 16. Dose-response profile of soluble GM-CSF in a TF-1 cell line. The response profile shows that concentrations of 2 ng/mL and above provide the maximal TF-1 cell number after 48 hours of culture. The EC50 was calculated at 0.24 ng/mL. Data points represent the mean fluorescence intensity of a cell viability assay reagent - AlamarBlue (n=5±S.D).

3.2.1.2 Flow Cytometry for High Resolution Cell Growth Curves

It was hypothesised that cell suspensions could be sampled and dispensed into a 96 well plate containing a cell fixative solution (2% final concentration paraformaldehyde), where the plate could later be analysed using high-throughput flow cytometry. Automated sampling and high-throughput analysis allows continuous sampling over a 24 hour period enabling high resolution growth curves to be constructed.

Analysing a fixed volume of the wells at a fixed flow rate using flow cytometry would allow relative cell growth curves to be established between conditions by gating around viable cells on a standard forward v side scatter dot plot and obtaining an event count for that viable cell gate.

To validate the gating strategy on flow cytometry, serial dilutions of viable TF-1 cells were performed and calculated viable cell counts were compared between flow cytometry and a standard automated membrane exclusion assay (acridine orange and DAPI; NC-3000, Chemometec).

Viable cell counts displayed high correlation (R^2=0.99) between those calculated using the custom flow cytometry assay and the automated cell membrane exclusion assay, across a wide range of cell densities; $1.5x10^3$ cells/mL – $3.1x10^6$ cells/mL (see Figure 17).

The custom flow cytometry cell counting assay was carried forward in cell proliferation based assays to investigate the functional differences between soluble and immobilised growth factors.

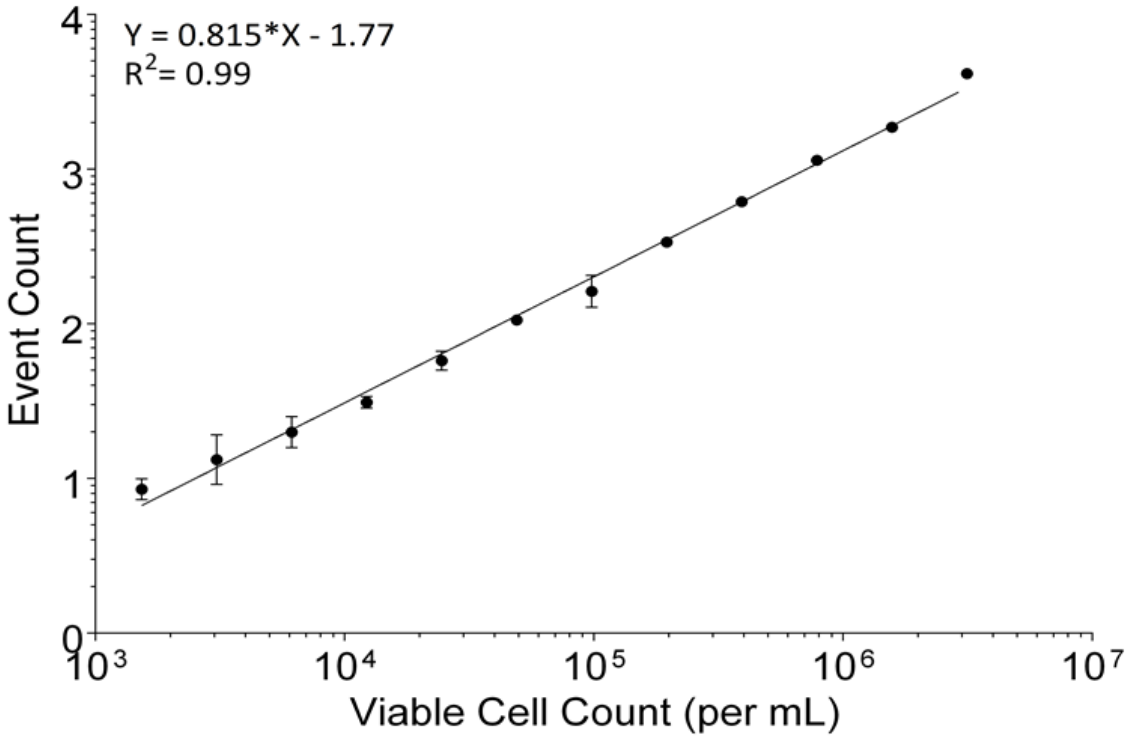


Figure 17. Flow cytometry event count correlates with membrane integral TF-1 cell count. A high correlation between viable cell count per mL (membrane exclusion method) and event count (flow cytometry) was observed (R-Sq = 0.99). Flow cytometry can be used as a high resolution cell counting method. Data points are representative of mean viable cells per mL and event count (n=5±S.D)

3.2.1.3 Optimisation of TF-1 Culture Conditions.

The following experiments were performed to optimise the culture conditions of TF-1 cells in an automated stirred tank micro-bioreactor ambr™ 15 (Figure 18). The ambr was chosen as a small scale model of a standard stirred tank bioreactor since it has been shown to scale well up to 200 Litres with CHO cell culture[102]. The aims of the optimisation studies were to prolong cell growth by reducing limiting factors of cell growth i.e. cell density, metabolic waste products, maintenance of pH, nutrient and growth factor supplementation. Optimisation of the culture conditions will enable good experimental control when the functional assay is used to evaluate the activity of immobilised growth factors.

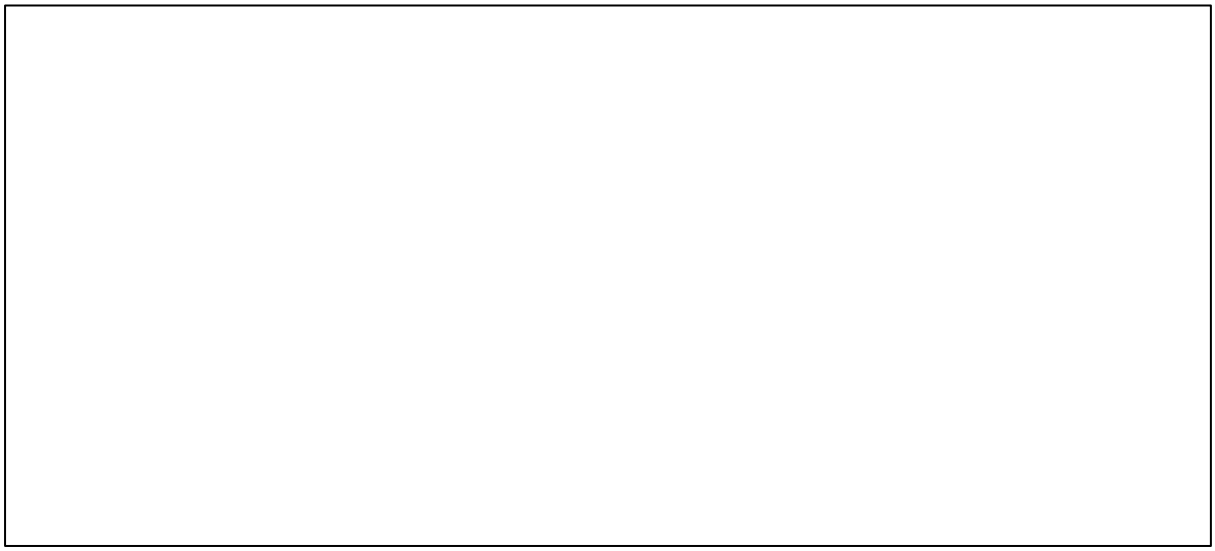


Figure 18. The AMBR 15 with 24 micro-bioreactors.

It was hypothesised that a bell shaped curve relationship would exist between cell seeding density and final density over a given duration; where low seeding densities would limit cell growth due to a lack of secreted paracrine factors and cell-to-cell contact and high cell seeding densities would limit final cell density due to medium exhaustion and induced cell death due to excessive contact inhibition [103].

The optimal cell seeding density was investigated to determine the density for prolonged cell growth until media exhaustion. TF-1 cells were starved of GM-CSF for 24 hours prior to the experiment to prevent historic exposure of cytokines impacting the results of the assay, so that it is clear that the functional response is from the exposure of cytokines presented at t=0. Figure 19 shows the effect of cell seeding density on cell growth over 96 hours until media exhaustion. A lag in cell growth was observed for the initial 22 hours of culture, this was due to the GM-CSF starvation for 24 hours before the beginning of the experiment (t=0). Cells entered log phases of growth following 22 hours, where each condition reached a different peak cell density before cell numbers decreased thereafter.

Since all conditions reached different peak cell densities it was hypothesised that a factor other than final cell density was becoming inhibitory for cell growth (i.e. GM-CSF degradation, inhibitory paracrine signals or a build-up of toxic metabolic by-products).

The effects of re-supplementing GM-CSF on TF-1 cell growth were investigated since it is recommended that GM-CSF be supplemented every 2-3 days. Figure 20 shows cell growth was sustained for longer durations with GM-CSF supplementation where peak final cell densities were achieved at similar time-points for most conditions (approximately 100 hours) with the exception of the highest cell seeding density condition which peaked around 48 hours (Figure 21).

Nutrient and metabolite analysis of spent media at 168 hours shows glucose concentrations decreased by 7% and 62% in 1.90×10^6 and 6.08×10^6 cells/mL cell seeding densities respectively. Glucose was found to be depleted in the four lowest seeding densities and >1.5 g/L of glucose remained in the highest cell seeding density condition. In addition, the final

concentrations of glutamate and ammonium decreased linearly with an increase in seeding density.

The nutrient and metabolite results show intermediate cell densities are dividing at a higher rate and therefore their metabolic demands are increased – the utilisation of more glucose coincides with increased production of lactate (via anaerobic respiration).

It has been well documented that the hypoxic nature of the HSC niche is critical for cell proliferation and differentiation, therefore the effect of hypoxic conditions on TF-1 proliferation was investigated[104]. Furthermore, phenol red can affect the regulation of pH in the AMBR bioreactor, thus phenol red free medium was used in this experiment[105].

Figure 22 and Figure 23 show a general increase in cell growth with the absence of phenol red. Increased growth rates could be due to an inhibitory effect of phenol red on TF-1 cells or that the pH of the media was better controlled by the AMBR bioreactor (Phenol red interferes with the optical sensors that analyse media pH in the AMBR) . Additionally, the glutamine source was changed from L-glutamine to a more stable form (Glutamax) to prevent degradation into ammonia which could also be a cause of increased growth rates.

In contrast to the literature, no differences in cell growth profiles were observed between 21% or 5% dO_2 for similar cell seeding densities[95].

Similar growth rates were observed for the various seeding densities until approximately 72 hours, with the exception of the highest seeding density ($1x10^6$ cells/mL). A decline in cell growth is observed earlier in this experiment compared with the previous experiment, most likely due to faster cell proliferation rates and therefore media exhaustion.

L-glutamine rapidly degrades into ammonia and pyrrolidone carboxylic acid in cell culture media [106], [107]. A dipeptide form of glutamine (L-alinine-L-glutamine, commercially

known as GLUTAMAX) is often used as a direct substitute to L-glutamine in cell culture since it is less prone to degradation. It was hypothesised that substituting L-glutamine with GLUTAMAX would increase cell growth by maintaining a stable concentration and reducing ammonia production.

Figure 24 shows that glutamax improves growth rates in the $2x10^4$ cells/mL seeding densities in comparison to L-glutamine, particularly after 48 hours of culture but no significant differences were observed for higher seeding densities.

Glucose depeletion and ammonium concentrations were shown to be limiting factors at the end of the culture period for conditions with GM-CSF supplementation. Conditions free of GM-CSF demonstrated no cell growth and therefore glucose was not metabolised.

The series of experiemnts in this section have defined a well charactersied cell line culture system which will assist in evaluating the functional effects of soluble versus immobilised GM-CSF. Furthermore, experiments can be designed and conclusions drawn with the variables that affect growth limitations in mind at different cell densities.

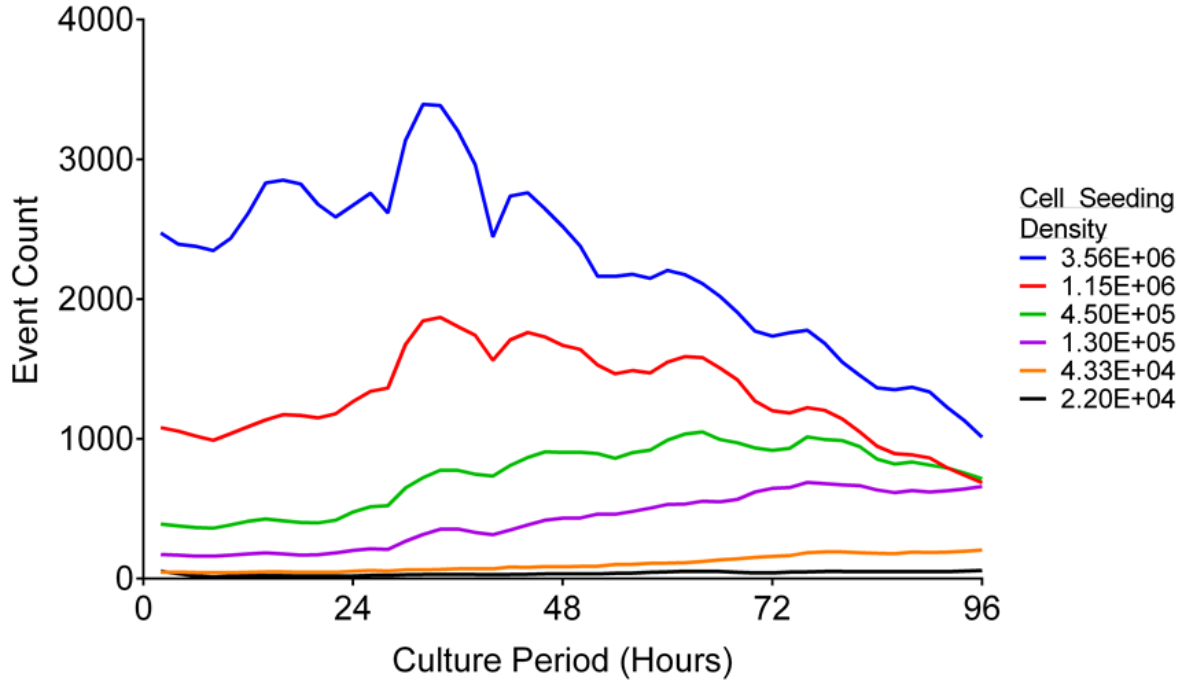


Figure 19. Effect of TF-1 cell seeding density on cell growth until media exhaustion in a stirred tank bioreactor. Conditions show an initial lag period of 22 hours where cells enter a log period of growth thereafter (with the exception of the highest cell seeding density). Cell growth diminishes at various time points depending on initial seeding density with highest densities diminishing soonest. Optimal seeding densities for prolonged growth phases are between $4x10^4$ – $5x10^5$ cells/mL. Data lines are representative of mean smoothed data where an average of 3 time points were taken (n=2). Sampling occurred every 2 hours.

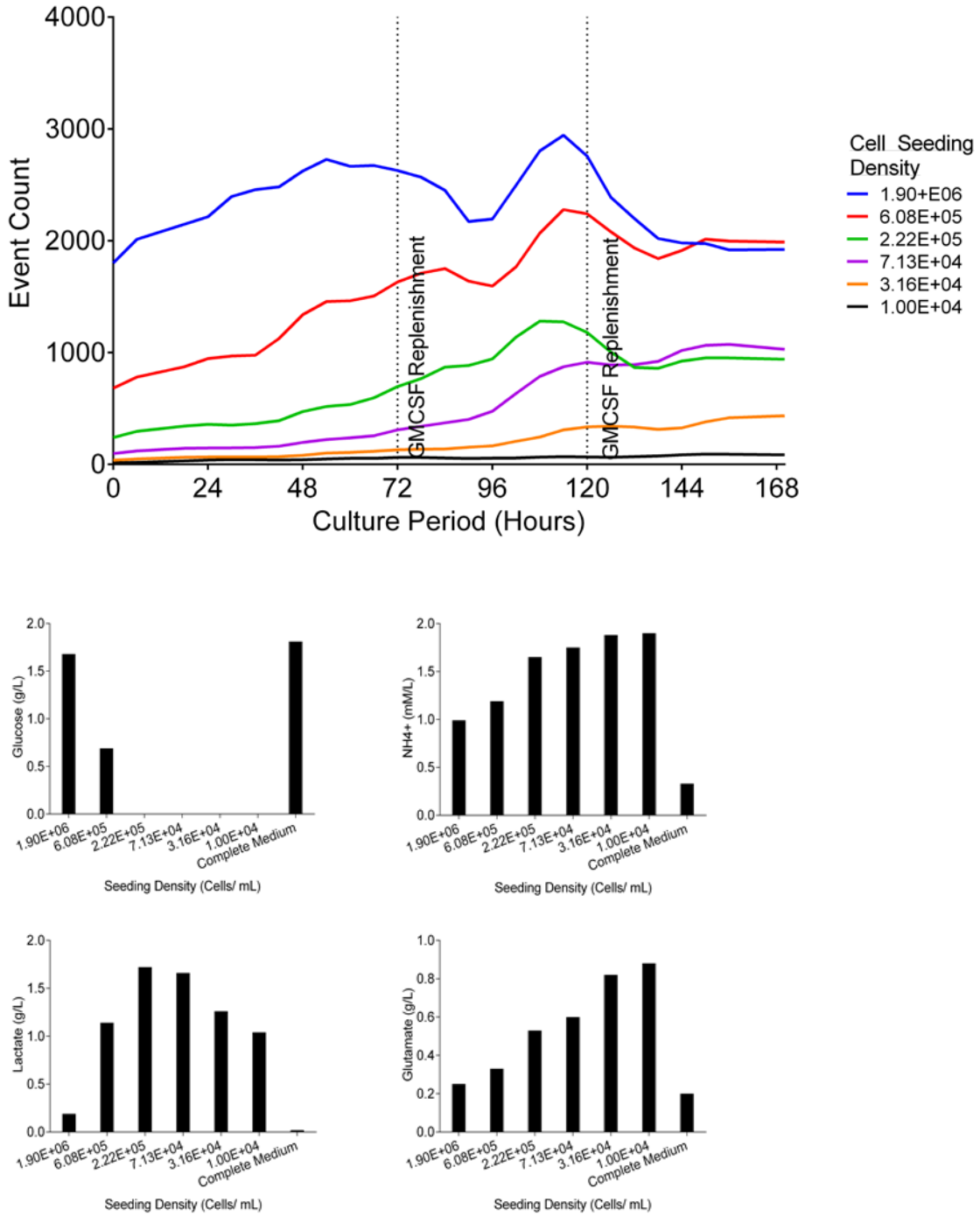


Figure 20. Effect of TF-1 cell seeding density and GM-CSF replenishment on cell growth until media exhaustion in a stirred tank bioreactor. Top Graph - Exponential cell growth phase is significantly extended with GM-CSF replenishment; cells continue to grow after 150 hours without a medium exchange ($3.16x10^4$ cells/mL). Data lines are representative of mean smoothed data where an average of 3 time points were taken (n=2). Sampling occurred every 6 hours. Vertical dotted lines indicate time points of soluble GM-CSF replenishment. Bottom Graph - End-point nutrient and metabolite analysis of spent media identifies glucose depletion and metabolic waste products as limiting factors for cell growth at later stages of the culture period.

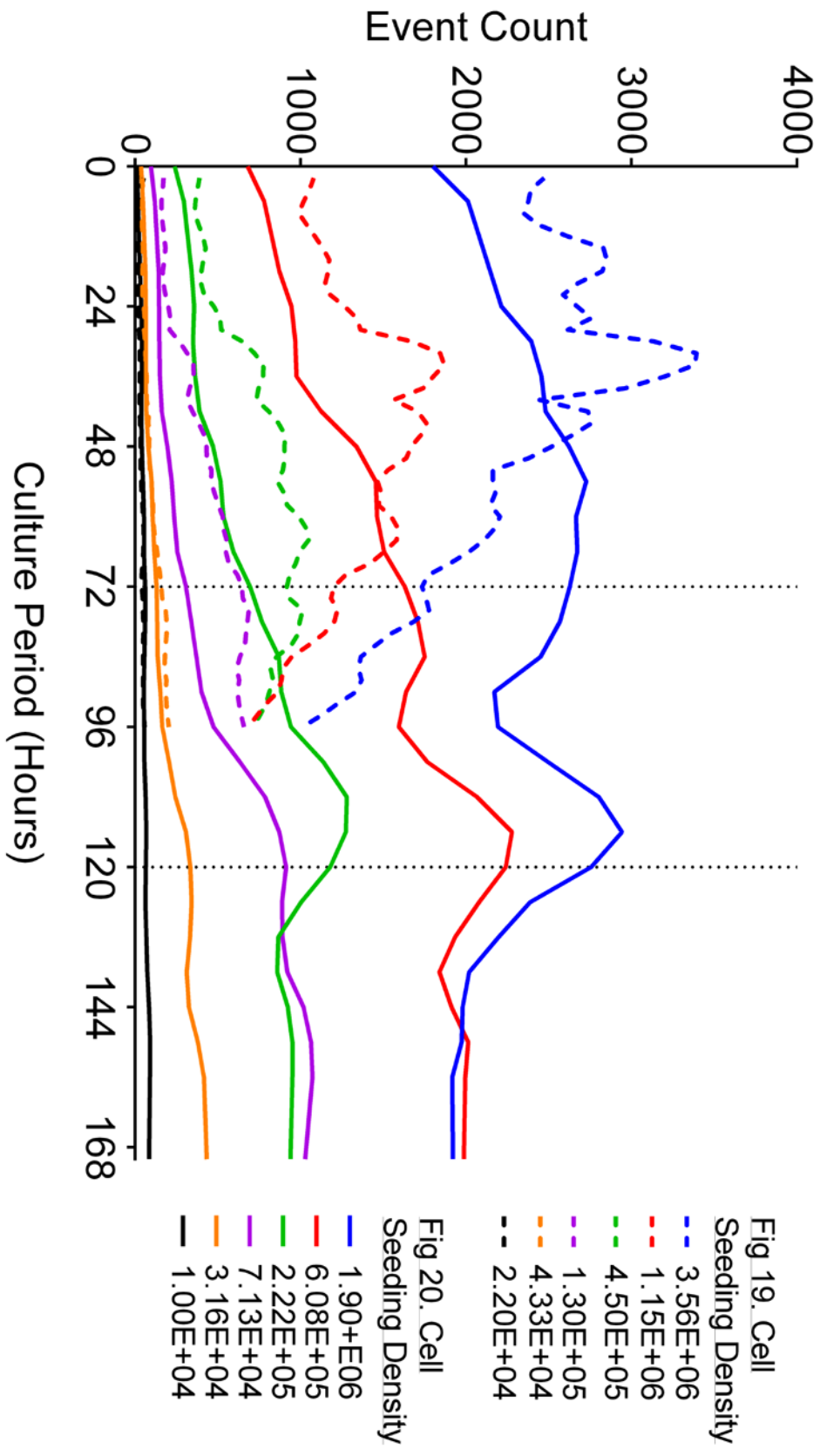
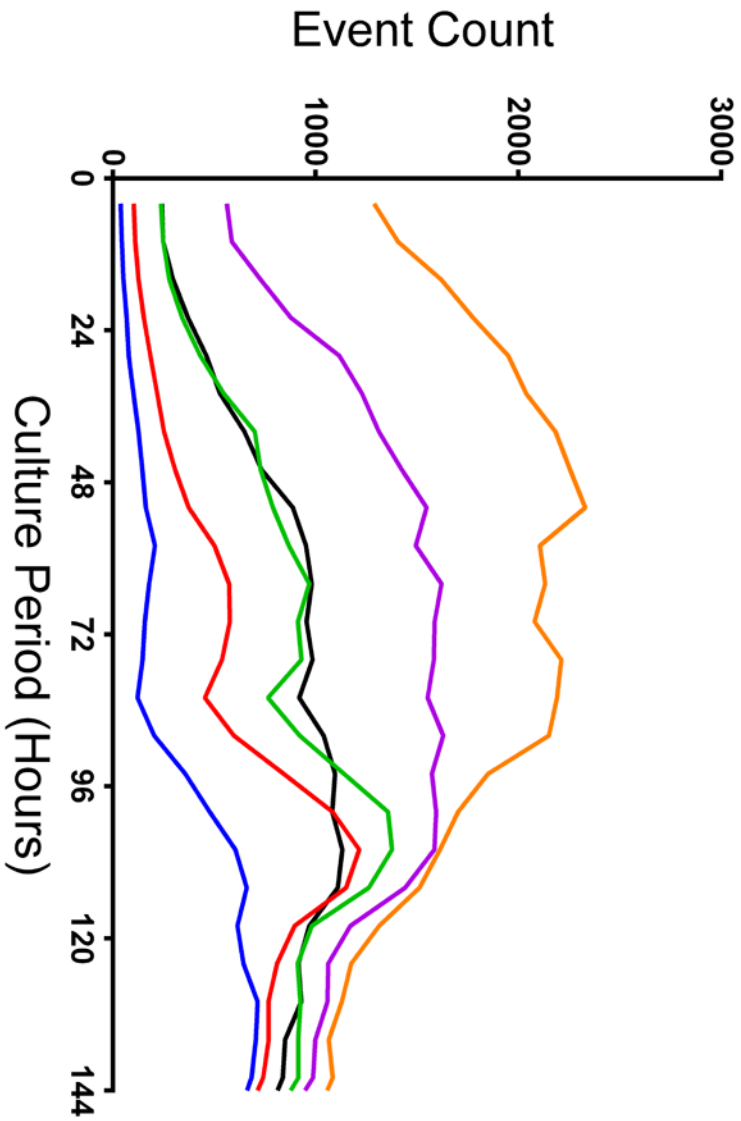


Figure 21. Overlay of figure 19 and figure 20 showing that GM-CSF extends cell growth. GM-CSF utilisation is predominantly cell density dependent.



	Seeding Density	dO2
1	3.00E+04	21
2	7.50E+04	21
3	1.88E+05	21
4	4.69E+05	21
5	1.17E+06	21
6	1.18E+05	5

Figure 22. Effect of phenol red free media and dO2 on TF-1 cell growth until media exhaustion in a stirred tank bioreactor. Phenol red free media improves cell growth particularly in the mid stages of culture. Low concentrations of dissolved oxygen display no benefit for cell growth. Data lines are representative of mean smoothed data where an average of 3 time points were taken (n=2).

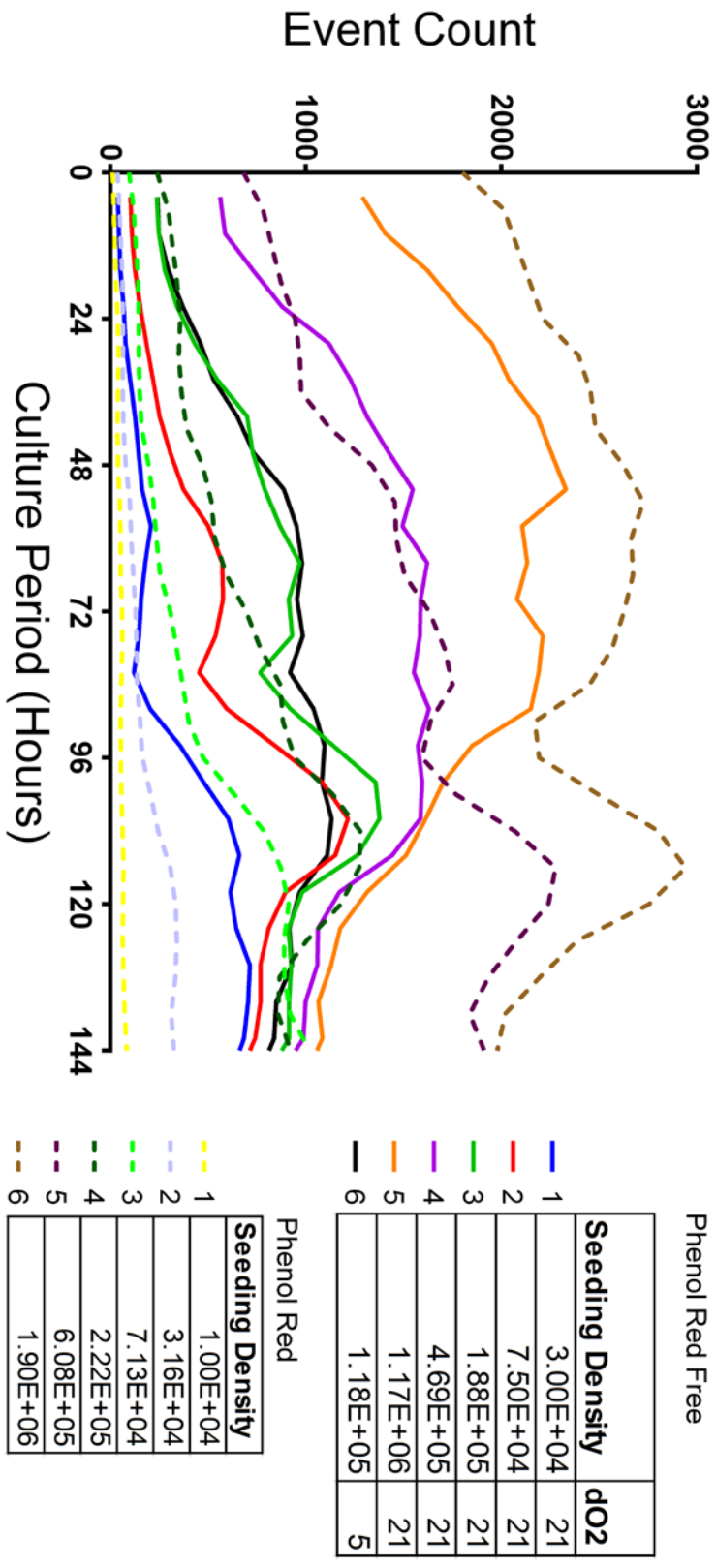
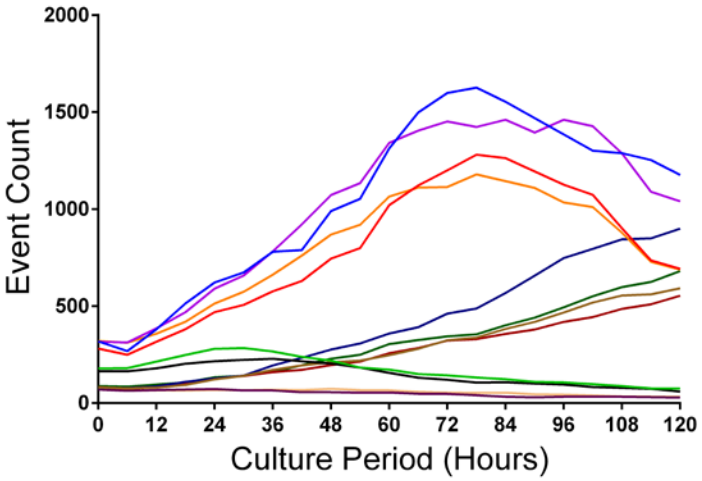


Figure 23. Overlay of figure 20 and figure 22 showing improved cell growth with the withdrawal of phenol red from cell culture media.



	SD	Gmax/Lglu	GMCSF Start	GMCSF Supplementation
V1	2.00E+05	GMAX	Y	Y
V2	2.00E+05	GMAX	Y	N
V3	2.00E+05	GMAX	N	N
V4	2.00E+05	LGLU	Y	Y
V5	2.00E+05	LGLU	Y	N
V6	2.00E+05	LGLU	N	N
V7	2.00E+04	GMAX	Y	Y
V8	2.00E+04	GMAX	Y	N
V9	2.00E+04	GMAX	N	N
V10	2.00E+04	LGLU	Y	Y
V11	2.00E+04	LGLU	Y	N
V12	2.00E+04	LGLU	N	N

Figure 24. Effect of glutamine source on TF-1 cell growth until media exhaustion in a stirred tank bioreactor. A) Cell growth is improved with glutamine supplemented as a stable dipeptide - Glutamax. Data lines are representative of smoothed data where an average of 3 time points were taken (n=2). B) Glucose was completely depleted except for conditions which showed no cell growth. Ammonia did not significantly differ between Glutamax and L-glutamine conditions. Columns show nutrient and metabolite concentration of spent medium at hour 120.

3.2.2 Selection of a Growth Factor Immobilisation Method

The shortlisted immobilisation techniques were screened to assess their performance on key experimental outcomes which informed the selection of a single immobilisation technique. The selection criteria were:

- Technical ease,
- Cost,
- Biological potency,
- Reproducibility,
- Clinical compliant (i.e. potential GMP grade reagents).

The following series of experiments were performed in order to demonstrate the feasibility of immobilising growth factors in order to induce a functional response (in this case - cell proliferation) and to select the most suitable technique to carry forward into further experiments.

3.2.2.1 Growth factor concentration

The functional effects of four different protein immobilisation methods were investigated using the TF-1 functional assay developed previously (see section 3.2). Due to a problem with the flow cytometer, cell counts were determined using the NC-3000 method. In addition to the immobilisation method - GM-CSF concentration was also investigated for each of the methods to determine if surface concentration could be manipulated and if surface concentration had an effect on cell growth.

Figure 25 that shows a biological response was observed in two out of the four immobilisation techniques – Streptavidin and Fibronectin. The Streptavidin and Fibronectin

immobilisation techniques significantly (P=≤0.0001) induced cell proliferation in comparison to the negative control (GM-CSF free). The population doublings per day for the Streptavidin and Fibronectin conditions did not significantly deviate from the positive control (5ng/ml soluble GM-CSF) which has previously been shown to achieve the maximum biological response (Figure 16). The stimulation of cell proliferation in the Streptavidin and Fibronectin immobilisation techniques provides strong evidence for the successful immobilisation of GM-CSF, since this cell line proliferates dependently on GM-CSF.

No increases in population doublings were achieved using the Carboxylated and Solulink immobilisation techniques, where the mean population doublings demonstrated no deviations from the negative control condition (GM-CSF free). The failure of the Solulink and Carboxylated immobilisation techniques were likely methodology related as explained below:

- *Failure of Carboxyl method:* The disulphide bonds of the GM-CSF molecule were targeted with TCEP to transform the bond into a thiol group, which would later spontaneously react with the maleimide group of the PEG linker in the carboxylated immobilisation technique (Figure 12). Disulphide bonds are critical for maintenance of the tertiary structure of proteins, thus disrupting disulphide bonds could have denatured the protein causing it to lose its function and hence cell proliferation was not induced[108].
- *Failure of Solulink method:* Solulinks' immobilisation technique is considered a single use kit. In this experiment the conjugation reagents were split into three equal parts to allow the conjugation of three different concentrations of GM-CSF where the de-salting spin columns were re-used. It is likely that splitting those conjugation

reagents had altered the specific molarities (or volume) required for the conjugation reaction to take place and therefore the splitting of reagents will be avoided in future experiments.

- In addition, the streptavidin and fibronectin immobilisation techniques both included large protein molecules (Streptavidin = 52.8kDa; Fibronectin = 440kDa) within the GM-CSF conjugated bond whereas, the Carboxylic and Solulink immobilisation techniques contained short covalent bonds. Since the two methods that demonstrated functional activity, in culture, involved large protein linkers this supports the hypothesis that the steric presentation of GM-CSF has an important role to play in functional activity. Large protein linkers disperse the GM-CSF more than smaller chemical bonds on the particle surface and they introduce flexible bonds which may help in receptor accessibility and multiple growth factor-receptor complexes in turn having improved synergistic signalling effects.

In general no significant differences in population doublings were observed by altering the concentration of soluble GM-CSF. This result could be due to two reasons. 1) The surface concentration is saturated at the lowest level of initial GM-CSF concentrations and further increases in GM-CSF concentration do not increase surface concentration or 2) The functional response is saturated at the lowest GM-CSF concentration. However there was a tendency for the population doublings to increase with increasing initial GM-CSF concentrations for the Streptavidin immobilisation technique.

Quantifying the surface concentration would allow us to determine if the surface was saturated at lower GM-CSF concentrations. In addition, a series of particle concentrations at

fixed GM-CSF concentrations would assist in determining if the functional response was saturated.

This experiment has demonstrated that a growth factor can be immobilised onto magnetic particle and retains its biological activity to stimulate a biological response (in this instance, cell proliferation).

A following experiment was conducted to investigate the effect of particle concentration on cell proliferation as opposed to the previous GM-CSF concentration variable and to correct any methodological errors previously encountered.

Figure 26 illustrates that the Solulink and streptavidin conditions produced a statistically significant increase ($P=\leq 0.0001$; 4E5 particles/mL) in population doublings compared with the negative control (GM-CSF free). Albeit lower than the positive control.

The streptavidin immobilisation technique performed less well in comparison to the previous experiment, with a 1.75 fold reduction in mean population doublings between experiments. This observed difference in response is likely due to different culture conditions and batch variation of the Streptavidin particles and (or) control over final particle concentration. The culture plate conditions differed from the first experiment by the use of a deep well plate (1mL well volume) as opposed to a standard 96well plate (max. well volume 300 µL).

Larger differences in functional response were observed by altering particle number as opposed to GM-CSF concentration (in the previous experiment). This suggests that functional response is saturated at the surface concentrations previously used.

The functional response to the fibronectin particles was unable to be investigated in this experiment due to particle aggregation following incubation of the plain particles with

fibronectin. Also, the carboxylated immobilisation technique was not investigated due to loss of particles during washing stages.

To further investigate the properties of immobilised growth factors using magnetic particles, the Streptavidin immobilisation method was selected to carry forward because it met the criteria listed in section 3.2.2.

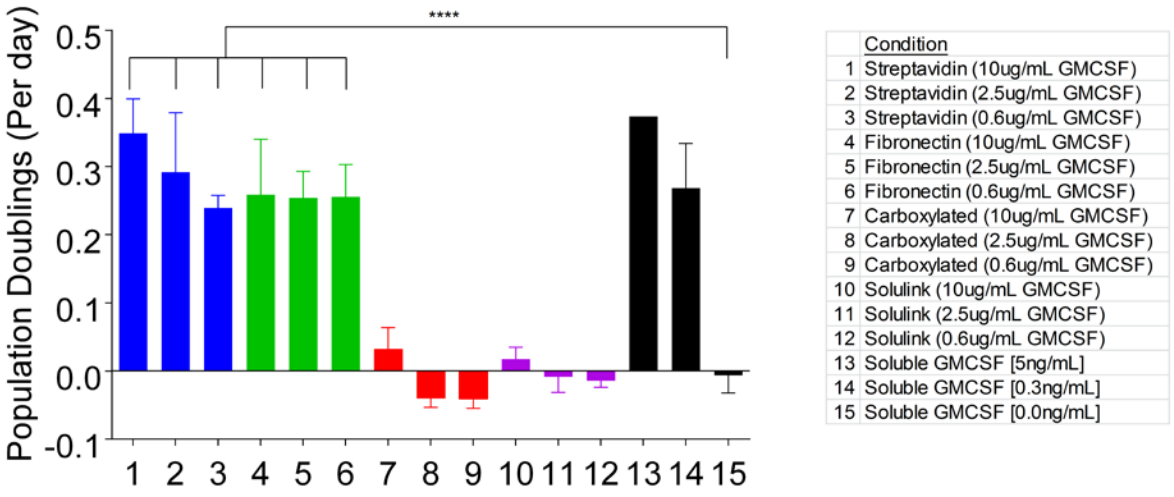


Figure 25. Screen of immobilized GM-CSF prepared using four different conjugation methods and three different GM-CSF-biotin concentrations. Significant TF-1 cell growth was achieved in streptavidin and fibronectin preparation methods. Carboxylated and solulink conditions were not statistically different from the negative control (GM-CSF free). Higher GM-CSF-Biotin concentrations induced the highest population doublings per day in the streptavidin and fibronectin conditions. Columns represent mean population doublings per day (n=3±S.D). **** = $P \geq 0.001$

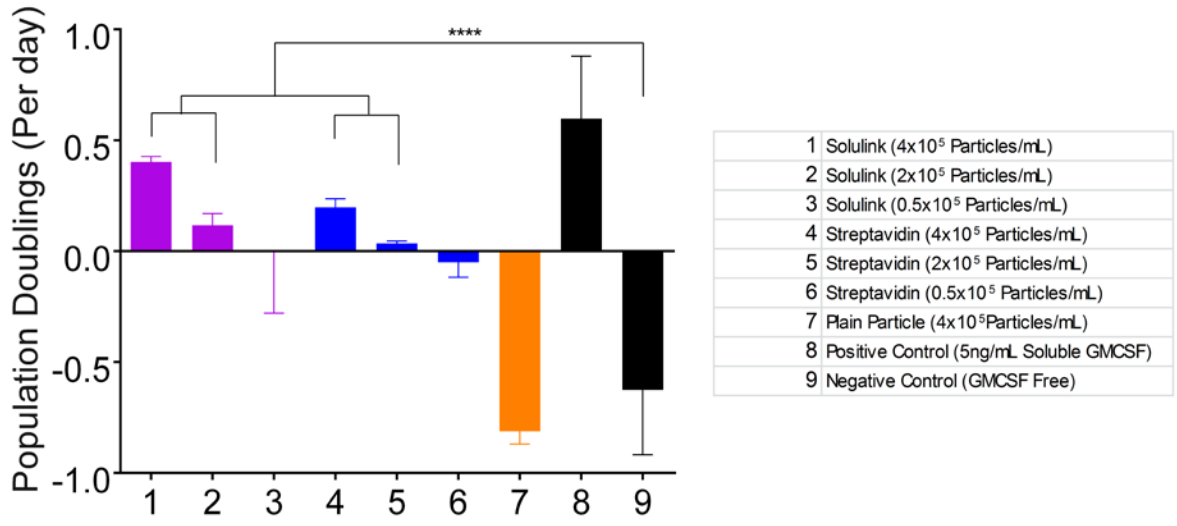


Figure 26. Functional screen of immobilized GM-CSF prepared with two different conjugation methods and three final bead concentrations. Statistically significant increases in population doublings were seen for the two highest bead concentrations in the solulink and streptavidin conditions. The lowest bead concentration conditions did not induce cell proliferation and the plain particle condition exhibited similar response to the GM-CSF free condition demonstrating no non-specific binding of GM-CSF. Columns represent mean population doublings per day (n=3±S.D). **** = $P \geq 0.001$

3.2.3 Development of a Quantification Assay

As discussed in the introduction, many quantification methods are not applicable to immobilised growth factors. Consequently, this section explores the use of flow cytometry and fluorescent reference standards as a specific method for quantification of immobilised growth factors. A quantification method will allow a direct comparison to the relative potencies of growth factors in immobilised or soluble formats and it will also allow the effects of changing variables within the immobilisation reaction to be investigated in order to optimise surface concentration.

It was hypothesised that immobilised growth factors could be quantified with fluorescently labelled antibodies and fluorescent reference standards using flow cytometry. An overview of the flow cytometry quantification assay is illustrated in Figure 27.

The following experiment was performed in order to determine if the sensitivity of flow cytometry was sufficient to quantify immobilised GM-CSF. Figure 28 shows small increases in fluorescence intensity between the positive stained sample and IgG control. A net increase in the geometric mean of fluorescence was 17 and 15 for technical replicate 1 and replicate 2 respectively. Net fluorescence was converted to 246 and 217 Molecules of Equivalent Soluble Factor (MESF).

A Students t-test was conducted in order to determine if the small differences in fluoresce between the IgG controls and positively stained sample were within the noise of the measurement system or whether this was representative to immobilised GM-CSF. Statistical analysis of the MESF values were shown to be significantly different from the IgG controls ($P<0.05$).

The reported biotin binding capacity of the particles were $2x10^{-4}$ grams per particle. However, the calculated mass of immobilised GM-CSF was an average of 0.005 femtograms per particle. However the maximal functional response was achieved with 0.005 fg/particle at $2x10^{5}$ particles/mL (or a total of 1.08±0.10pg/mL immobilised GM-CSF).

A reduced concentration of GM-CSF could be due to multiple biotins binding per GM-CSF molecule (maximum of 6 biotins per GM-CSF), potentially reducing the maximum binding capacity 6 fold ($3.3x10^{-5}$ grams per particle) and this could be reduced further by steric hinderance of large GM-CSF-PEG conjugates.

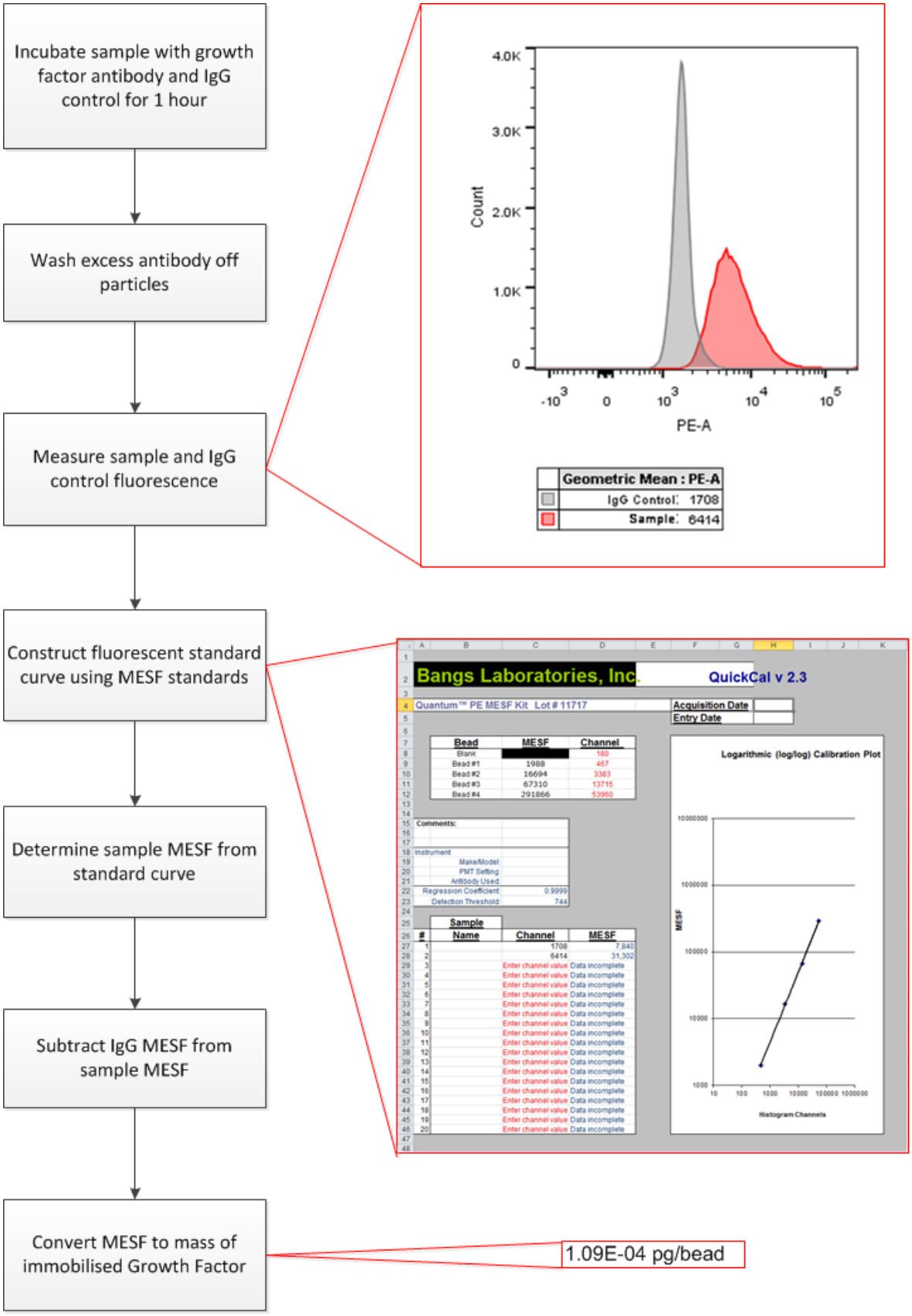


Figure 27. Exemplar work flow for the quantification of immobilised proteins using flow cytometry. Molecules of equivalent soluble factor (MESF) are calculated from fluorescence intensity and used to calculate a mass of growth factor per particle.

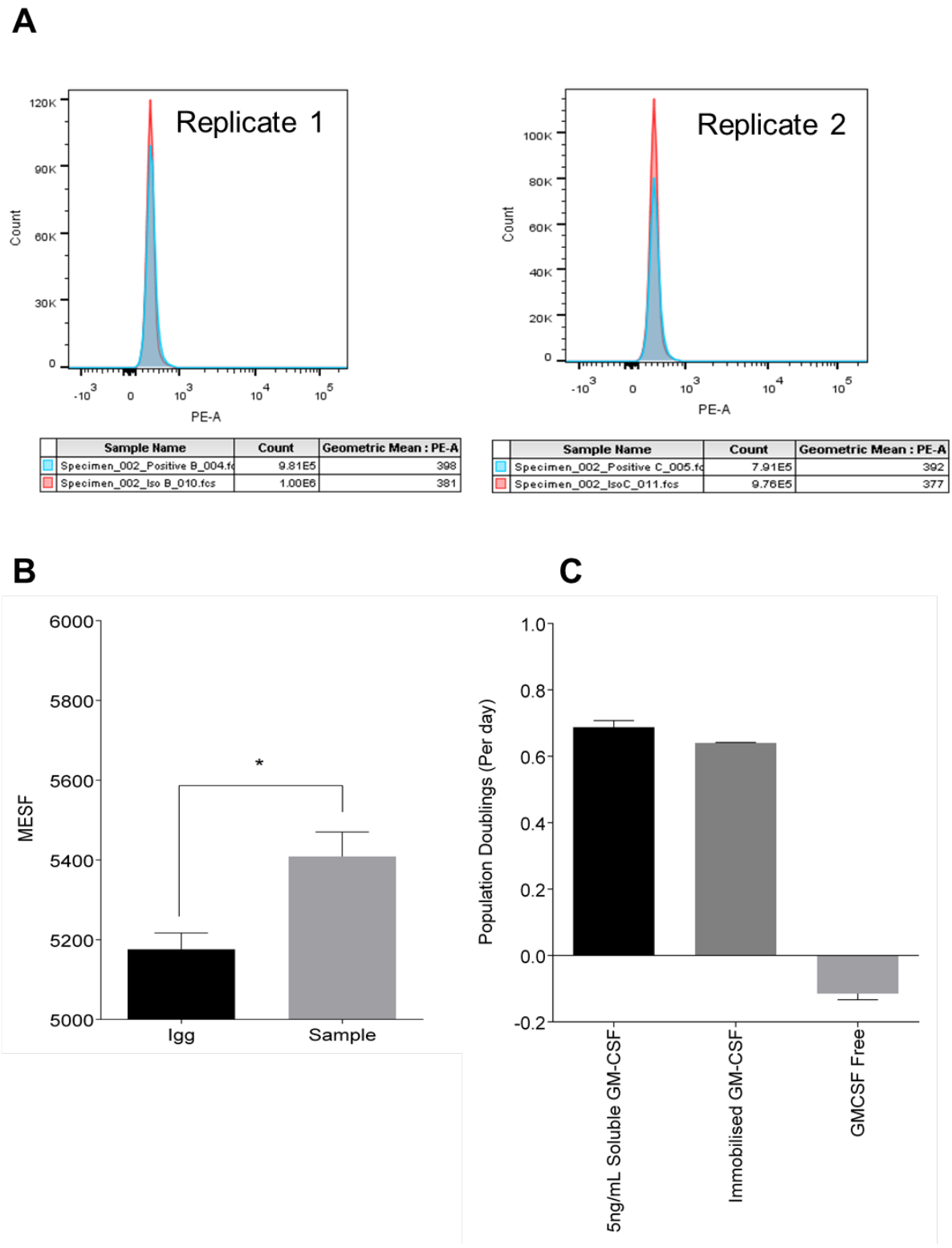


Figure 28. Quantification of immobilised GMSCF using flow cytometry A) Fluorescence histograms of igg control and positively stained samples. B) Statistical significance of calculated MESF values between IgG controls and positively stained samples. C) Functional response as determined by the NC-3000 cell counting method. *= $P<0.05$ (n=2±S.D).

3.2.3.1 Optimising the flow cytometry quantification assay

The entire immobilisation and quantification process was mapped out using a flow diagram and fishbone diagram (Figure 29 and Figure 30) in order to highlight variables which are most likely to impact on the final surface concentration and quantifiation accuracy of immobilised GM-CSF. Highlighting those variable that have an impact on suface concentration or accuarcy of the quantification method could then be controlled or optimised in future experiments.

Antibody (Ab) concentration, particle concentration and surface concentration of iGFs will have an impact on the calculated iGF concentration. The initial quantification assay was performed using an unknown amount of particles and a standard 1:10 ab dilution was used as a final concentration during incubation (with no prior ab titration). Since the iGF concentration in the initial quantification assay was lower than expected – it suggests that optimising or controlling all three factors could improve the assay accuracy. Therefore a series of particle concentrations and ab concentrations were analysed.

Figure 31 shows antibody saturation at >50 µL of antibody per mL of stain buffer. Previously only 10 µL of antibody was used which gave move than a 6 fold difference in the calculated immobilised GM-CSF per particle.

The streptavidin particles have a certificate of analysis (CofA) which reports a biotin binding capacity i.e. the mean quantity of biotin the particles can bind as determined by the manufacturers assay. However, even with Ab titration this is still significantly less than the reported biotin binding capacity and may suggest that there is further optimisation required from the immobilisation method itself. In addition, the quantification assay was shown to be

sensitive to particle number. A linear increase in surface concentration was observed with decreasing particle numbers.

There is a requirement for high ab concentrations to ensure the ab is in excess of iGF for accurate quantification of iGFs. Using high concentrations of ab would become a very expensive assay, particularly if multiple iGFs were quantified simultaneously. There are further means in which the antibody concentration could be reduced for example; by lowering the staining volume, increasing incubation times and ensuring that there is sufficient mixing during ab incubation. However, these steps would introduce longer process times, more process steps, and increased assay variation/error – all undesirable traits for a manufacturing process. Therefore, it was decided to allow an event count that would be considered sufficient for flow cytometry analysis and to reduce the quantity of antibody required that the number of particles for the assay would be fixed at $2x10^5$ particles in a staining volume of 100µL.

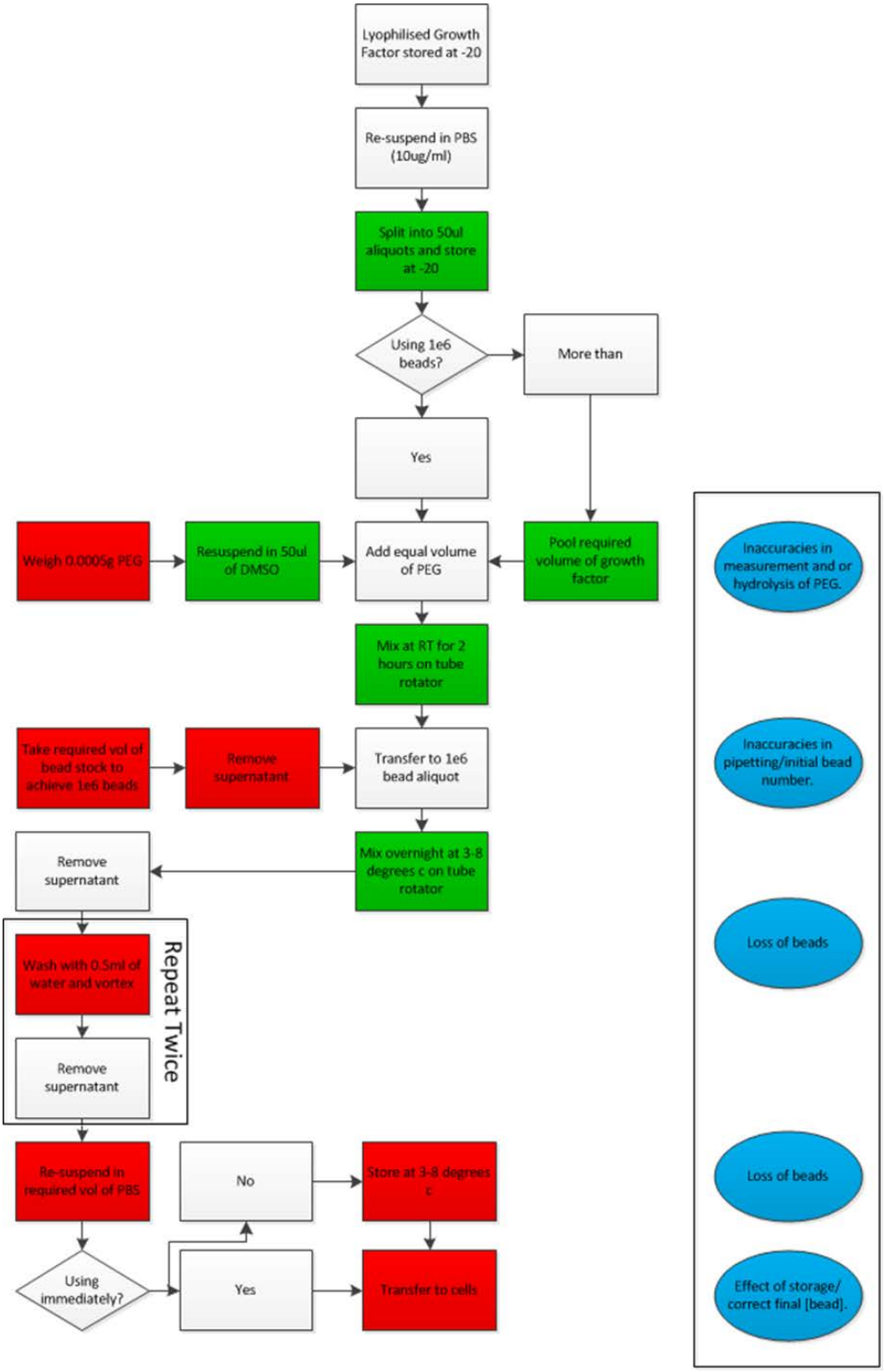


Figure 29. Flowchart showing the growth factor immobilisation and quantification process highlighting areas of risk (blue circles) which could impact the efficacy of the immobilisation process or functionality.

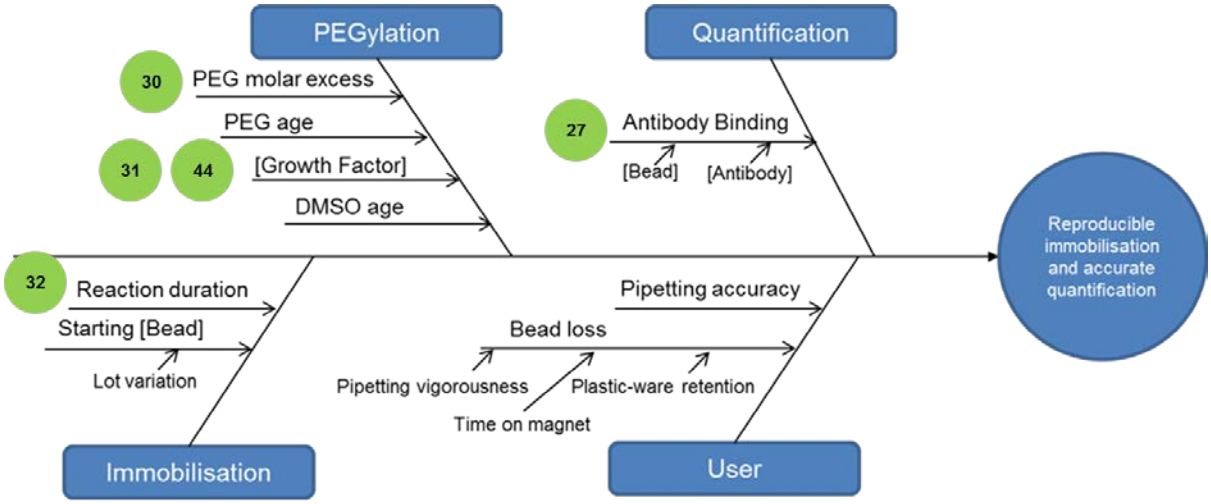


Figure 30. Fishbone diagram of immobilisation and quantification method showing the variables that impact reproducibility and accurate quantification of the immobilisation method. Numbers in green circles are references to figures in this thesis where the variables were tested.

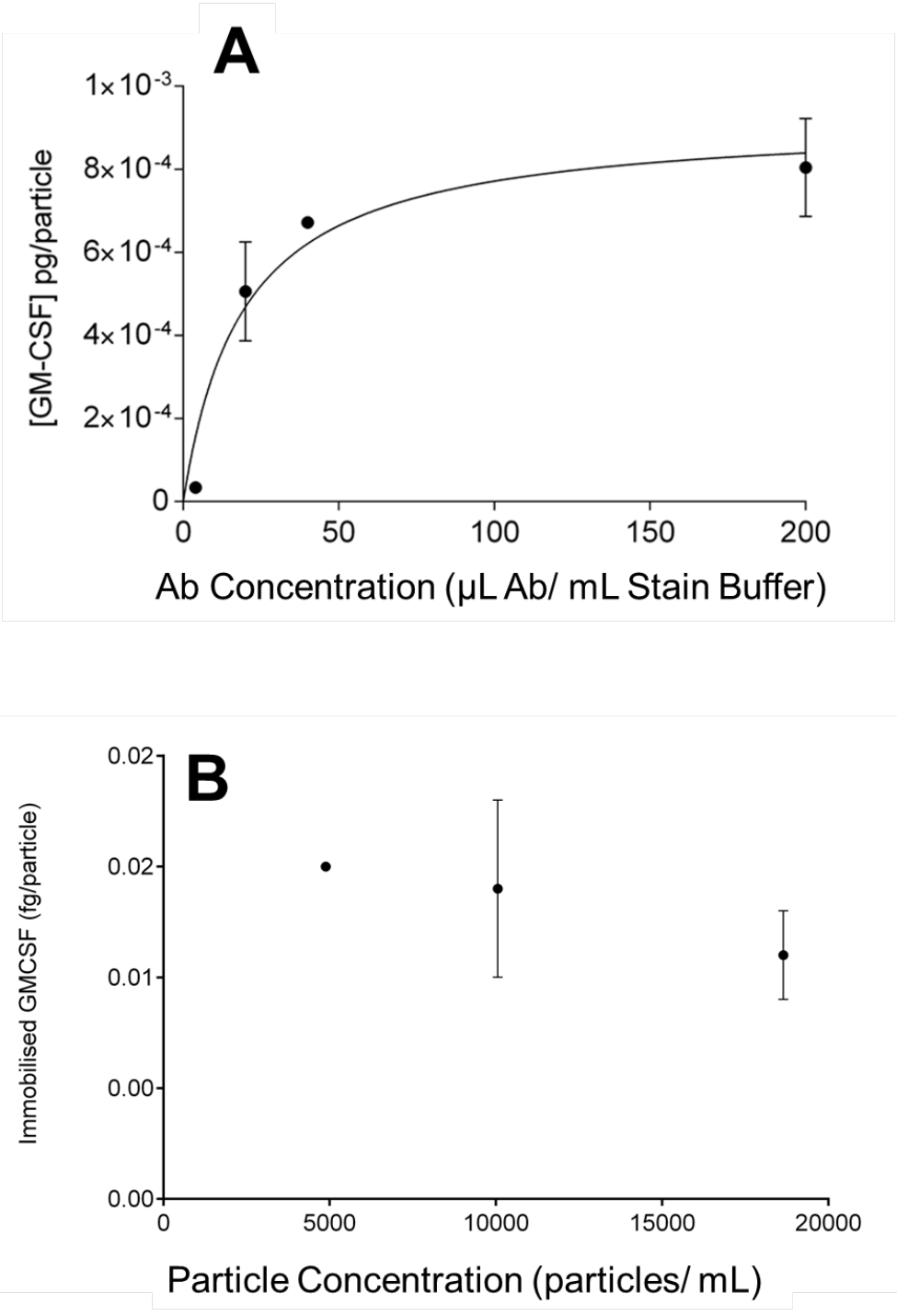


Figure 31. Optimisation of quantification assay parameters A) Optimisation of antibody concentration B) Effect of particle concentration at a fixed antibody concentration. Data points show mean immobilised GM-CSF per particle ± S.D (n=2).

3.3 Discussion

This chapter aimed to develop a novel and scalable protein immobilisation method using magnetic microparticles.

In order to screen and evaluate immobilisation techniques, a functional assay was developed using a growth factor dependent cell line. The culture conditions of the cell line were optimised before screening the immobilisation methods to ensure that maximum sensitivity could be achieved in the assay.

Four short-listed immobilisation methods were screened by assessing their biological activity in the functional assay. The streptavidin immobilisation technique was carried forward because it consistently showed good functionality and met the desirable criteria such as: technical ease, low cost, reproducibility and being clinical compatible. Efforts to correct the failure of other immobilisation techniques could have been carried out, however in the interests of pursuing the experiments to look at functional effects (and because the results of the streptavidin method were promising) a decision was made to take forward the streptavidin method so that the functional effects and process benefits could be investigated.

To determine the potency of immobilised growth factors in relation to their soluble equivalents and in order to optimise the surface concentration of immobilised growth factors, a quantification assay was developed using flow cytometry since current protein quantification methods were not suitable.

Process mapping and tools such as fish-bone diagrams have enabled the identification of variables in the immobilisation process which may result in irreproducibility and inaccurate

quantification of immobilised growth factors. Reducing the variation in the growth factor immobilisation method will enable consistent cell growth between experiments.

The methods developed in this chapter enabled the properties of the immobilisation to be investigated further and also allows the method to be optimised further before applying to primary cell culture systems.

4 Functional Properties of Immobilised Growth Factors

4.1 Introduction

In the previous chapter it was demonstrated that a single growth factor (GM-CSF) retained its biological activity once immobilised onto magnetic microparticles as demonstrated in a static functional assay.

Soluble growth factors (sGF) will diffuse down a concentration gradient in static culture systems; whereas iGFs have a fixed locus (iGFs will settle) leading to potentially heterogeneous exposure of individual cells.

A major advantage of the immobilisation method means the particles have the potential to be used in agitated (scalable stirred tank) cell culture platforms. Achieving controlled cell fate outcomes from immobilised growth factors (iGFs) is dependent on a suitably well mixed system and that the cell to iGF interaction scales predictably with scale of the manufacturing platform.

Stirred tank bioreactors are well characterised and bode well for the manufacture of haematopoietic cell therapies due to the non-adherent nature of the cells and such culture formats have extensive literature supporting control of culture conditions and scalability [96], [98].

This chapter aims to explore the scalability of the GF immobilisation method developed in the previous chapter, demonstrate the process benefits the method may entail (e.g. the ability to re-use growth factors) and to demonstrate additional functional benefits.

4.2 Results

The following results section will look at the scalability of the immobilisation technology, look at optimising the surface concentration of multiple clinically relevant GFs, and demonstrate the functional and process benefits of iGFs.

4.2.1 Scalability of Immobilisation method - Proof of Concept

The aim of this experiment was two-fold; 1) to demonstrate that the immobilisation technology is scalable in stirred tank bioreactors and 2) that the particles (and therefore growth factors) could be retained and recycled during a media passage and continue to elicit a biological response thereafter.

Figure 32 shows the growth curves of TF-1 cells over the course of 6 days with a media passage performed at day 3. Growth curves were determined using the Vicell cell counting method and magnetic particles were retained using method 2.9.2. The growth curves for the positive control and immobilised GM-CSF condition were comparable for the first three days of culture with population doublings of 2.7 and 2.5 respectively over the three days. Following a media exchange at day 3, cell growth diminished for the immobilised GM-CSF condition whereas the positive control continued to grow in log phase.

Diminished cell growth could be due to a decline in immobilised GM-CSF activity or that the retention of particles was not efficient during the media exchange process; the sensitivity of cell growth to particle concentration was demonstrated in the previous chapter. To ensure particle retention is efficient, in future experiments a magnet will be placed onto the side of vessels whilst slowly aspirating cell suspensions to minimise particle loss.

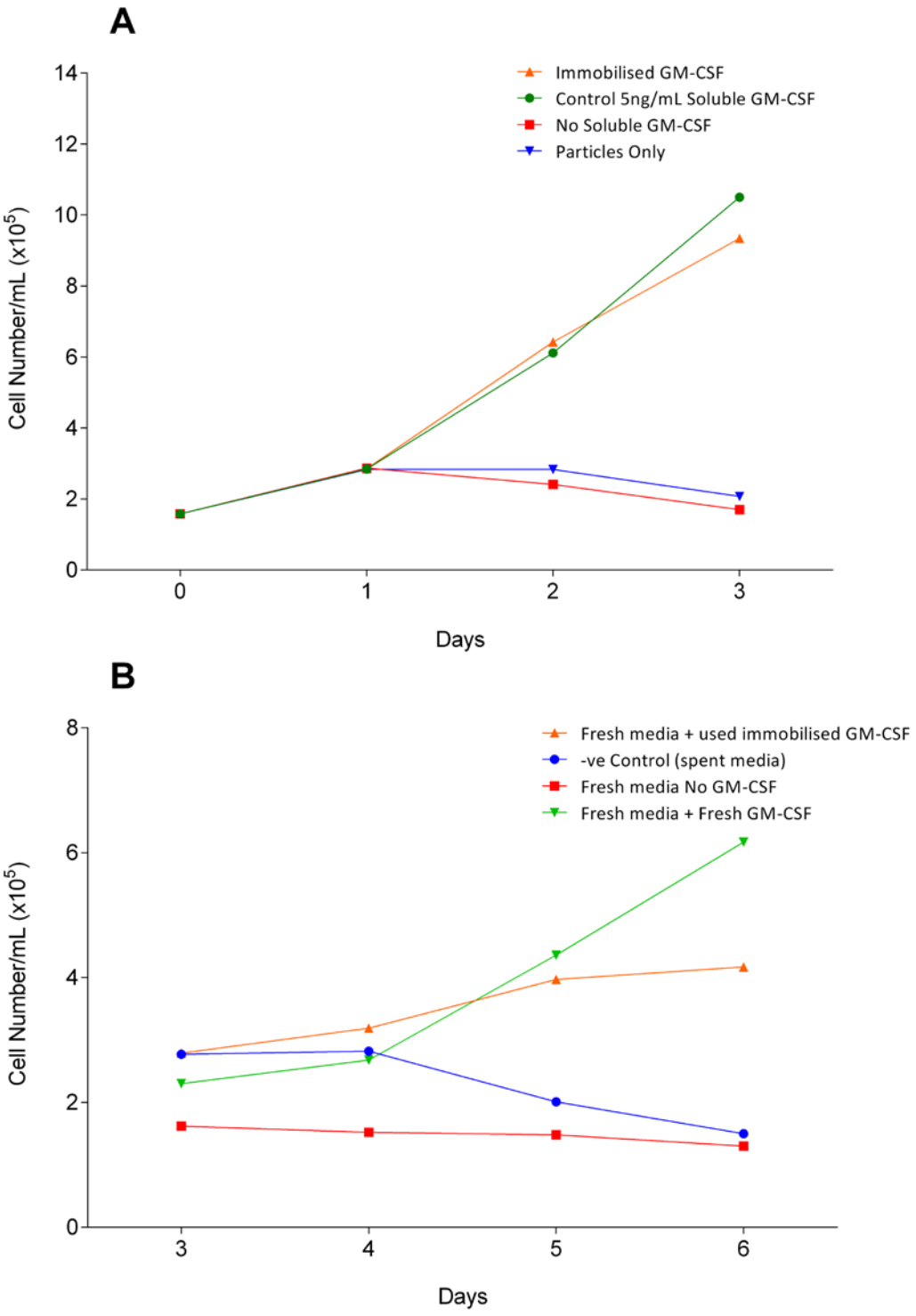


Figure 32. Functional activity of immobilised GM-CSF in a stirred tank culture system. A) Immobilised GM-CSF induced TF-1 cell growth at equal levels to the positive soluble GM-CSF control conditions for up to 3 days in culture. B) Following a media passage at day 3 and a cell reset to a seeding density of $2x10^5$ cells/mL the functional response of immobilised GM-CSF was reduced where cell growth plateaued at day 5. Data points represent viable cells per mL (n=1).

4.2.2 Effects of immobilised growth factor surface concentration

It was hypothesised that different surface concentrations and total surface area of immobilised growth factors would have distinct functional effects. Immobilised concentration was varied in two ways; 1) by changing the surface concentration by using an unconjugated PEG molecule (PEG 10,000) during the preparation of the growth factors for competitive binding and 2) by varying the concentration of particles at a fixed surface concentration. The rationale for changing surface concentration was due to the reported functional differences between ligand spacing as reported in the literature [109].

Figure 33 shows cells did not grow for the initial ~20 hours of culture for all conditions, due to the starvation of GM-CSF for 24 hours before t=0. Following ~20 hours of culture, cells began to proliferate exponentially for the positive soluble control (5 ng/mL of soluble GM-CSF) and the two highest immobilised GM-CSF concentrations (total concentration, blue and purple conditions) where the positive soluble control and 0.092 fg/particle ($2x10^5$ particles/mL) concentrations exhibited similar growth curves up to 50 hours of culture. Intermediate cell growth was induced with the 0.092 fg/particle ($2x10^4$ particles/mL) and no cell growth was observed for the lowest two immobilised GM-CSF concentrations.

Notably, conditions with similar total concentrations of immobilised GM-CSF but different surface concentrations (conditions 3 and 5) exhibited opposing functional effects, where lower surface concentrations failed to induce cell growth. This is in line with the literature in that that there may be optimal ligand spacing for the desired functional response[109].

In this experiment surface concentration of immobilised GM-CSF was controlled by the use of an unconjugated PEG molecule (PEG 10,000) which was hypothesised to competitively

bind with biotinylated GM-CSF onto the streptavidin particles. Surface concentration could be varied with the use of an unconjugated PEG molecule; however the surface concentration was unpredictable in this experiment. Conditions 4 and 5 were predicted to be 10% concentration of conditions 2 and 3, however were closer to 1% of the concentration. It is possible that the PEG 10,000 sterically blocked the binding of biotinylated GM-CSF to give lower vales than predicted as illustrated in Figure 33B.

It was then hypothesised that surface concentration could be controlled by varying the molar concentration of PEG during the conjugation stage. Until now, it was calculated that PEG was used at an 8000 fold excess to GM-CSF during conjugation. 8000 fold excess was used because this was the re-constitution concentration recommended by the manufacturer of the PEG and an equal PEG:GF volume was kept the same to ensure sufficient mixing volumes. The high PEG excess could be decreasing the reaction efficiency by a number of ways:

1. Free biotin will competitively bind with biotinylated growth factor for streptavidin ligands on the particles. The reaction will favour immobilisation of free biotin over PEGylated growth factor.

2. An excess of free biotin will also increase the number of PEG molecules per growth factor reducing its effectiveness in two ways:

- More PEG molecules per growth factor will decrease its biological potency by coating and disrupting its ability to bind to the growth factor receptor on the cell surface.
- One growth factor with multiple PEG molecules has the potential to “blanket” coat multiple streptavidin ligands, therefore reducing the quantity of immobilised PEGylated growth factor molecules.

It was anticipated that biotin–PEG_{2000}NHS in excess of a 1:1 ratio with target lysine would result in free-biotin being carried forward to competitively blank streptavidin on the particles and therefore reduce GF-biotin bound to the particles.

A Biotin-PEG2000-NHS below a 1:1 ratio would result in the number of biotinylated GFs available during the particle binding step would become a limiting factor. It was therefore hypothesised that a 1:1 molar ratio of PEG:GF would result in the highest surface concentration of immobilised growth factor.

In the next chapter the growth factor immobilisation technology will be used for proof of concept in a primary cell model culture system – expansion of umbilical cord derived HSCs. With this is mind, three growth factors were chosen for their use in the maintenance of HSC and progenitor populations.

Figure 34 shows that the surface concentration of three different growth factors could be controlled by altering the PEG:lysine ratio. As hypothesised, for each of the three growth factors the optimal PEG:lysine ratio was 1:1.

Maximum mean surface concentrations were 2.5, 1.1 and 0.7 fg/particle for GM-CSF, SCF and TPO respectively. The maximum surface concentration was not correlated to the growth factor's molecular weight (molecular weight for GM-CSF, SCF and TPO are 14, 18.6 and 18.6 kDa respectively) or lysine number (Lysine number for GM-CSF, SCF and TPO are 7, 14 and 5 respectively). It is likely that the 'availability' of lysine residues has an impact on the maximum surface concentration.

It was hypothesised that the surface concentration of immobilised GF could be manipulated by varying the absolute concentration of biotin–PEG2000-NHS and GF during GF biotinylation, at a fixed molar ratio of 1:1 biotin-PEG2000-NHS to lysine residues. In previous

experiments the GF concentration during the Biotinylation stage has been 10 µg/mL (10 µg/mL is the recommended concentration for reconstitution of lyophilised GFs as per manufacturer guidelines).

Figure 35 shows a sigmoidal relationship between soluble GF-biotin concentration and immobilised GF concentration with an R^2 of 0.98 for both GM-CSF and SCF. The mechanism underpinning the sigmoidal relationship is likely to be NHS degradation (via hydrolysis) at a constant rate and a rate of NHS binding with GF which is proportional to reagent concentration.

In contrast with the previous experiment – the highest concentration of immobilised SCF was approximately 3 times higher than GM-CSF at 200 µg/mL of soluble GF-Biotin concentrations..

50 µg/mL of soluble GF-biotin was taken forward into future experiments primarily for cost-effectiveness and because functional responses are likely to be saturated above this taking into account the results from previous experiments.

A standard reaction time of 2 hours has been implemented for the Biotinylation reaction (see previous chapter) in line with the literature, however it was hypothesised that immobilised GF concentrations could be increased by increasing the duration of the Biotinylation reaction [101].

Figure 36 shows the results of two reaction durations for SCF Biotinylation at two SCF-biotin concentrations. As expected, a 2 fold increase in SCF-biotin concentration increased the subsequent immobilised surface concentration 2 fold, from ~3 fg/bead to ~6 fg/bead.

Surprisingly, increasing the reaction duration from 2 to 4 hours decreased the immobilised surface concentration slightly for both SCF-biotin concentrations. This is because the biotin-

PEG molecule is dissolved in DMSO to prevent hydrolysis of the NHS terminal group. When the biotin solution is mixed with the GF at room temperature for Biotinylation – the DMSO denatures the α-helix of proteins at high concentrations and therefore it is likely that the immobilised GF is denatured to such an extent that the antibody cannot recognise the GF epitope [110], [111].

Consequently, a 2 hour reaction period was implemented for future experiments to limit denaturation of immobilised GFs.

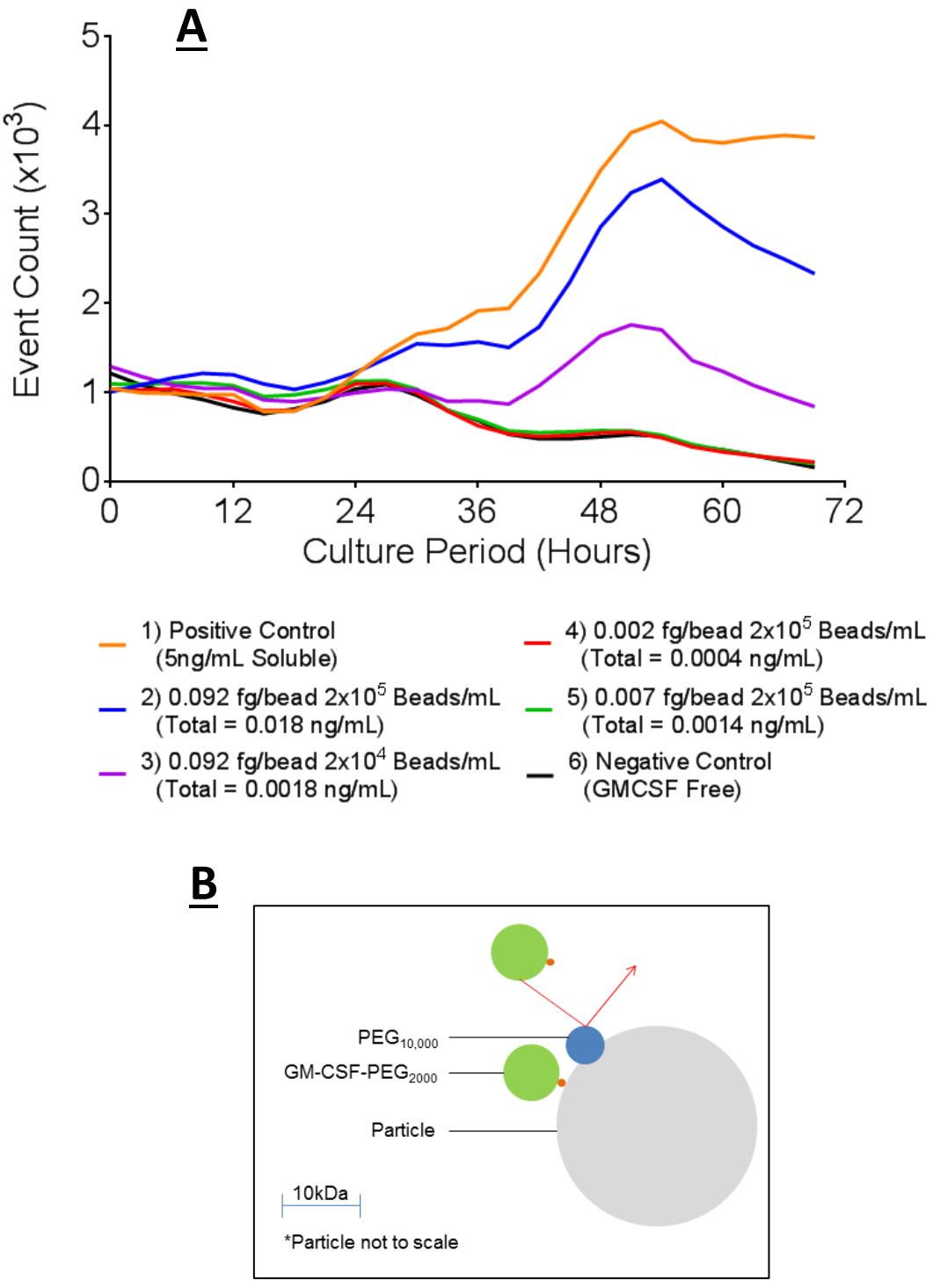


Figure 33. The effect of surface concentration and surface area of immobilised GM-CSF on TF-1 growth in a stirred tank bioreactor. A) Growth curve of TF-1 cells in a stirred tank bioreactor in response to different surface concentrations and different bead concentrations of immobilized GM-CSF. The graph illustrates that functional response is reduced by one quarter with a log decrease in bead concentration (conditions 2 & 3). In addition, functional response may cease to exist when total immobilized GM-CSF is equal but surface concentrations are different (conditions 3 & 5). Data lines are representative of mean smoothed data where an average of 3 time points were taken (n=2). B) Schematic of steric hindrance using PEG10K to modulate surface concentration of immobilised GM-CSF

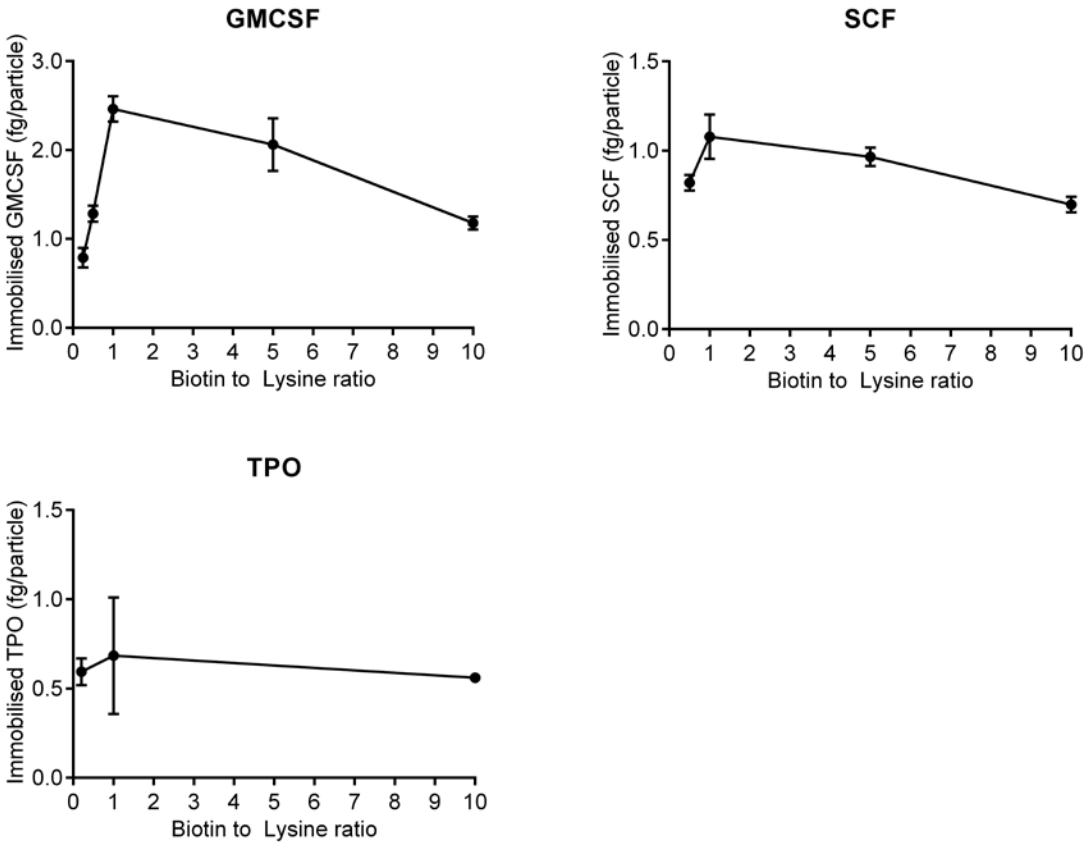


Figure 34. The surface concentrations of three growth factors were controlled by regulating the NHS-(PEG)n-Biotin (Biotin) molar excess during step 1 of the immobilization reaction. Maximum surface concentrations were achieved with a 1:1 Biotin to Lysine molar ratio for each of the growth factors investigated (n=3 mean ± S.D).

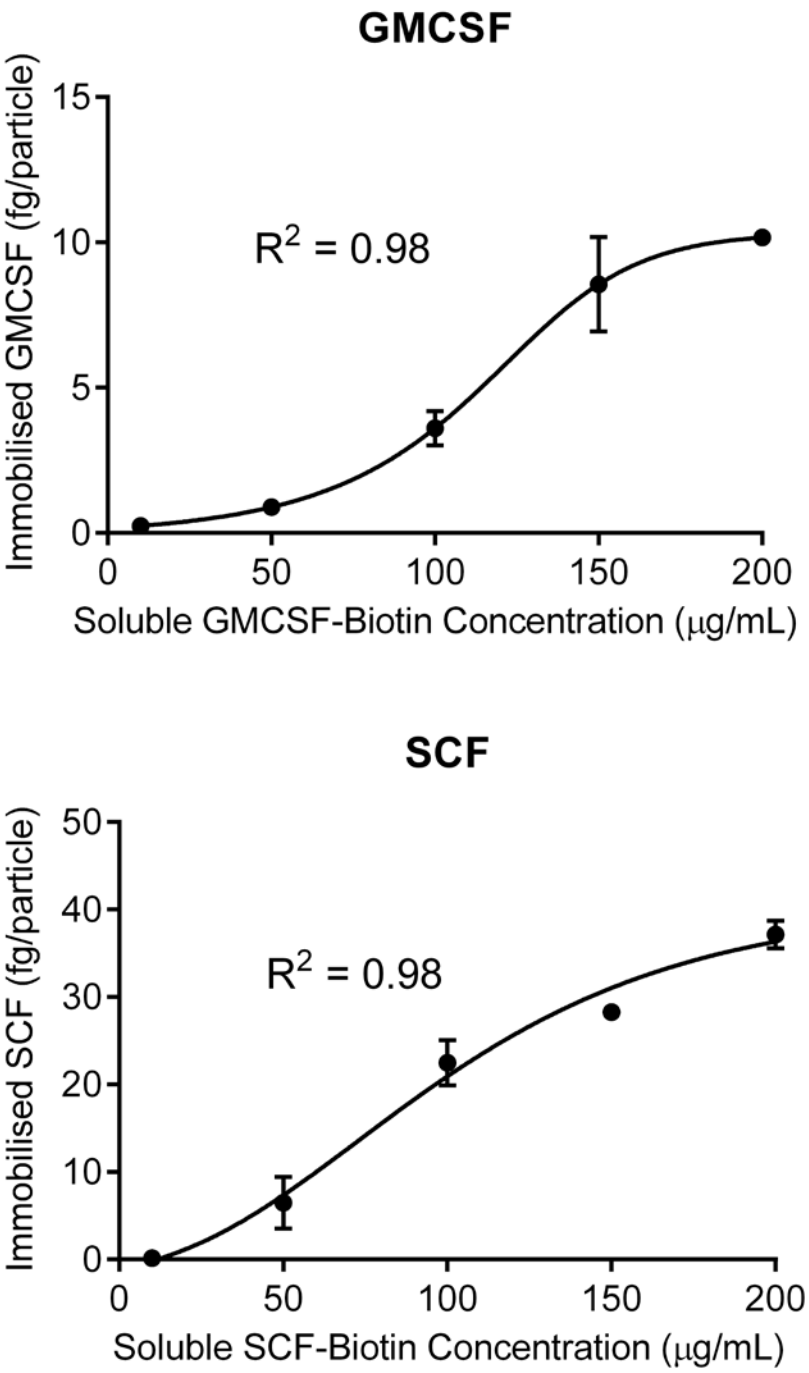


Figure 35. The effect of soluble GF-biotin concentration at optimal GF:biotin molar ratios were investigated to regulate the surface concentration of immobilized growth factors. A sigmoidal relationship between soluble GF-biotin and iGF concentration for (i) GM-CSF and (ii) SCF (n = 3 mean ± SD).

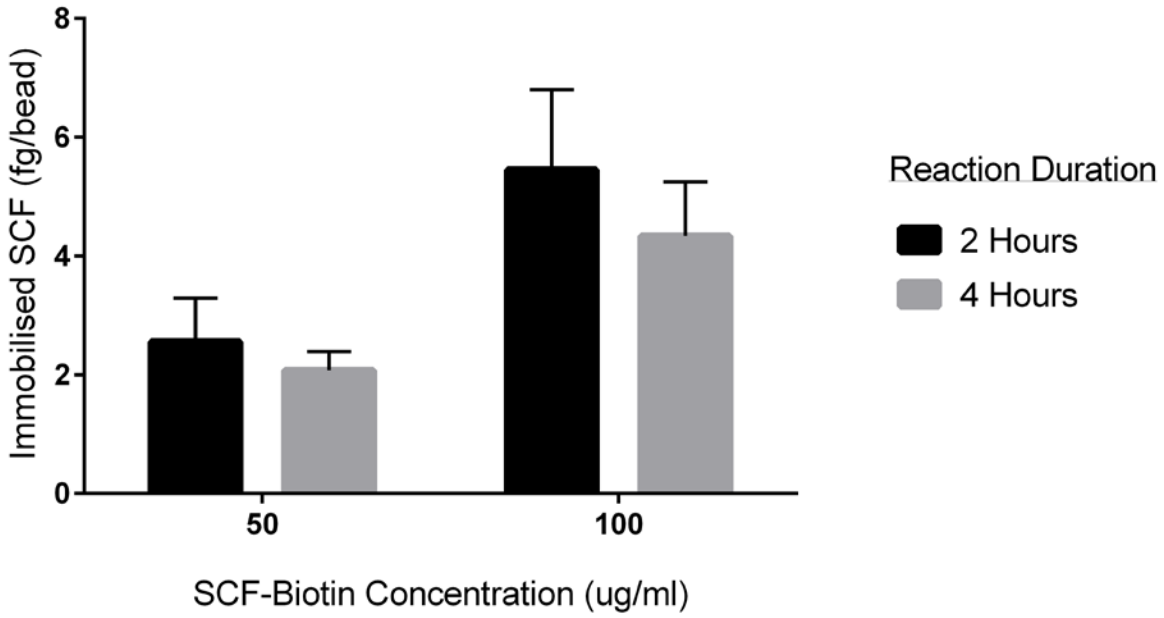


Figure 36. Reaction duration for SCF Biotinylation. A 2 hour reaction period provides maximum immobilised SCF concentrations following subsequent immobilisation. Data points represent mean femtograms of immobilised SCF per bead (n=3±S.D).

4.2.3 Functional Properties of immobilised Growth Factors

Now the immobilisation method has been established and various methods for controlling surface concentrations of immobilised GFs has been demonstrated, the following series of experiments aimed to investigate the functional differences and processes benefits of GF immobilisation.

A method for maximising and manipulating surface concentrations of growth factors was demonstrated in the previous section by altering the PEG molar concentration relative the lysine content of the growth factor. The functional activity of a range of iGM-CSF concentrations could therefore be investigated *in vitro*.

Figure 37A shows iGM-CSF created at a range of very low surface concentrations (0.008-0.09 fg/bead) stimulated a linear increase in growth rate with a logarithmic increase in particle concentration, matching the soluble response at approximately $1x10^5$ particles/mL at 0.09 fg/particle or 0.009 ng/mL.

Figure 37B shows the conversion of the different particle concentrations and surface concentrations into total iGF presented per mL, allowing the calculation of a single dose response. This indicated a maximal immobilized dose response of approximately 1-10 pg/mL, some 3 orders of magnitude more potent than the soluble equivalent. The formation of a relatively unified dose response curve from a range of different surface and particle concentrations suggest modulation of either surface concentration or particle number can be used to alter concentration of presented GF to a similar degree.

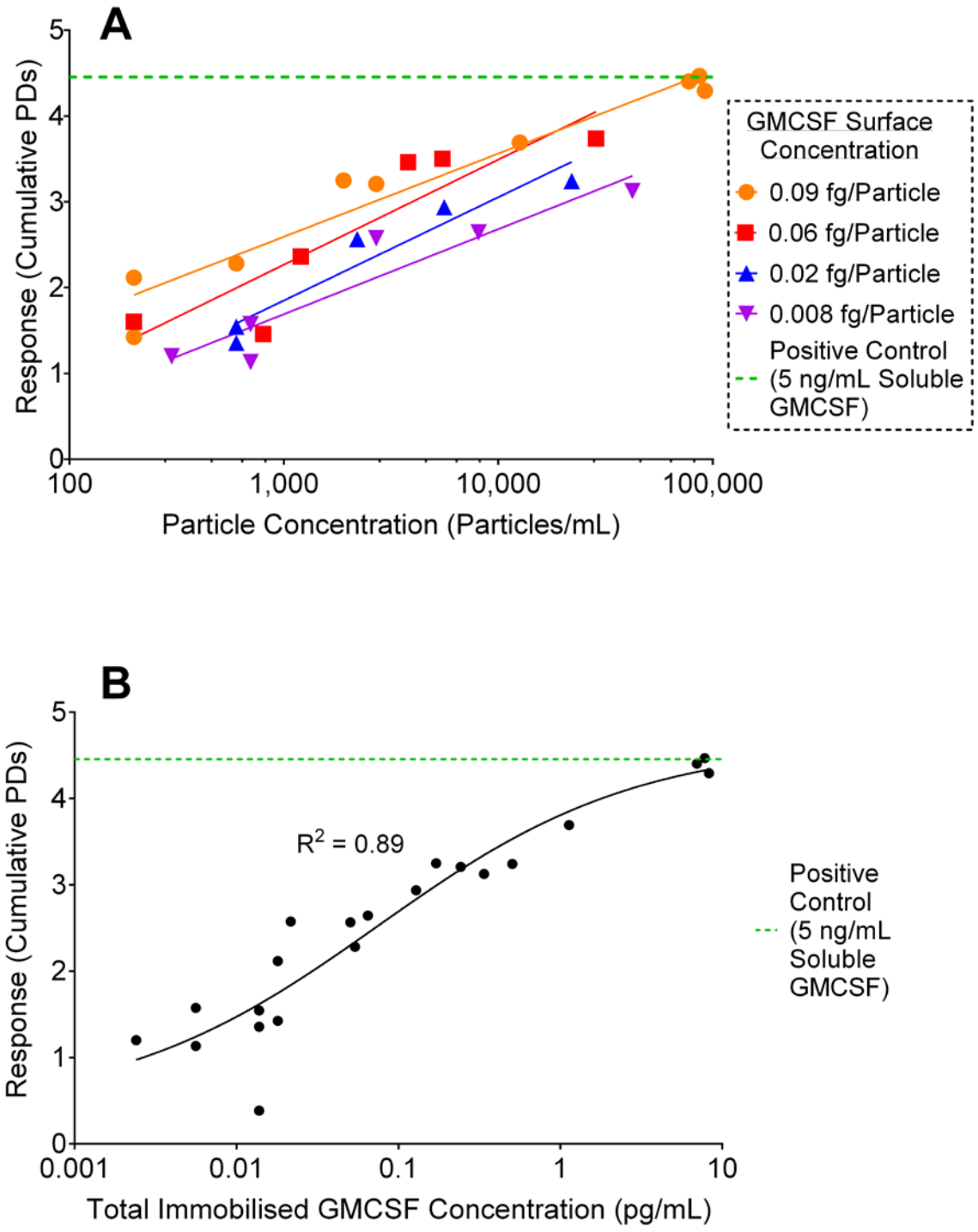


Figure 37. (A) The response of TF-1 cells to a range of immobilized GM-CSF surface concentrations and particle concentrations were investigated. The maximum soluble response was achieved with $1x10^5$ particles/mL at a surface concentration of 0.09 fg/particle (B) Particle concentration and surface concentration was converted to total immobilized GM-CSF/mL to construct a single dose-response curve. The maximum immobilized response is achieved with 10 pg/mL of immobilized GM-CSF (n=3).

4.2.4 Recycling immobilised GM-CSF

Since immobilised growth factors can be manipulated independently of the media bulk it was hypothesised that immobilised GM-CSF could be retained upon a media change. The functional stability of iGM-CSF was tested by culturing TF-1 cells with a single set of GM-CSF coated particles magnetically retained through serial bulk medium changes in a static well plate system.

Figure 38 shows the growth curve of TF-1 cells in static culture over 192 hours with a media passage every 48 hours. A positive control was treated with a media exchange containing sGM-CSF every 48 hours; a negative control had no GM-CSF. The graph shows the cell growth rate for the iGMSCF and sGM-CSF conditions were equivalent for the experimental duration of 192 hours with three media exchanges where approximately 2 population doublings occurred every 48 hours.

The surface concentration of iGM-CSF was quantified at 1.56 fg/particle or 0.3 ng/mL at $2x10^5$ particles/mL – an order of magnitude lower than required for the soluble equivalent. The quantity of iGM-CSF used in this experiment was equivalent to a 94% decrease in total GF required to stimulate the maximal biological response. Including the three media changes the immobilized culture used 1.5% of the GF over the 192 hours relative to the soluble control.

It was hypothesised that soluble GM-CSF degrades at a greater rate than iGM-CSF in line with literature[112], [113]. The concentrations of soluble GM-CSF were investigated over 96 hours during static TF-1 culture in two different cell seeding densities ($2x10^4$ and $2x10^5$ cells/mL). Figure 39 shows that GM-CSF concentrations remained stable at cell seeding

densities of $2x10^4$ cells/mL, whereas concentrations of GM-CSF decreased by 33% at cell seeding densities of $2x10^5$ cells/mL.

The results suggest that degradation is likely catalysed by paracrine factors (e.g. proteases) and highlights the need for re-supplementation of soluble growth factors to maintain constant concentrations for optimal cell growth.

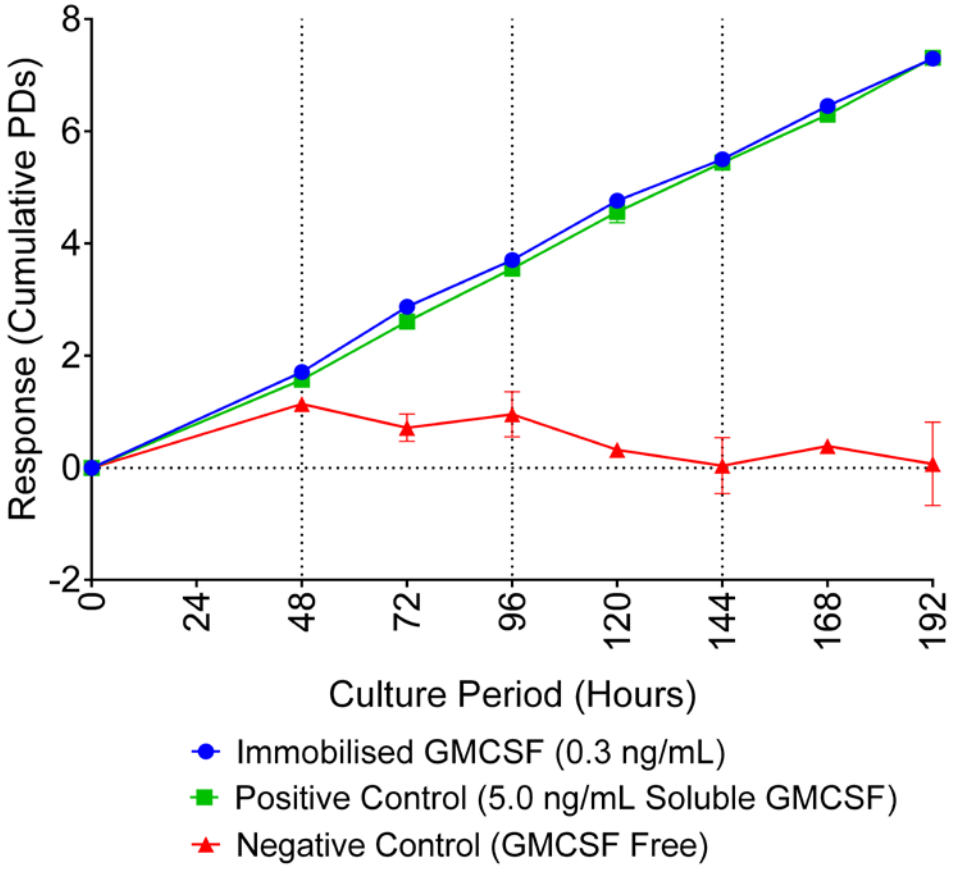


Figure 38. The effects of a single dose of immobilized GM-CSF (0.3 ng/mL) retained throughout the culture period was investigated in comparison to a soluble control (soluble GM-CSF was replaced every 48 hours at media change – vertical dashed lines). The cell growth rate for the immobilized and soluble GM-CSF conditions were equivalent for the experimental duration of 192 hours over three media exchanges (n=2±S.D).

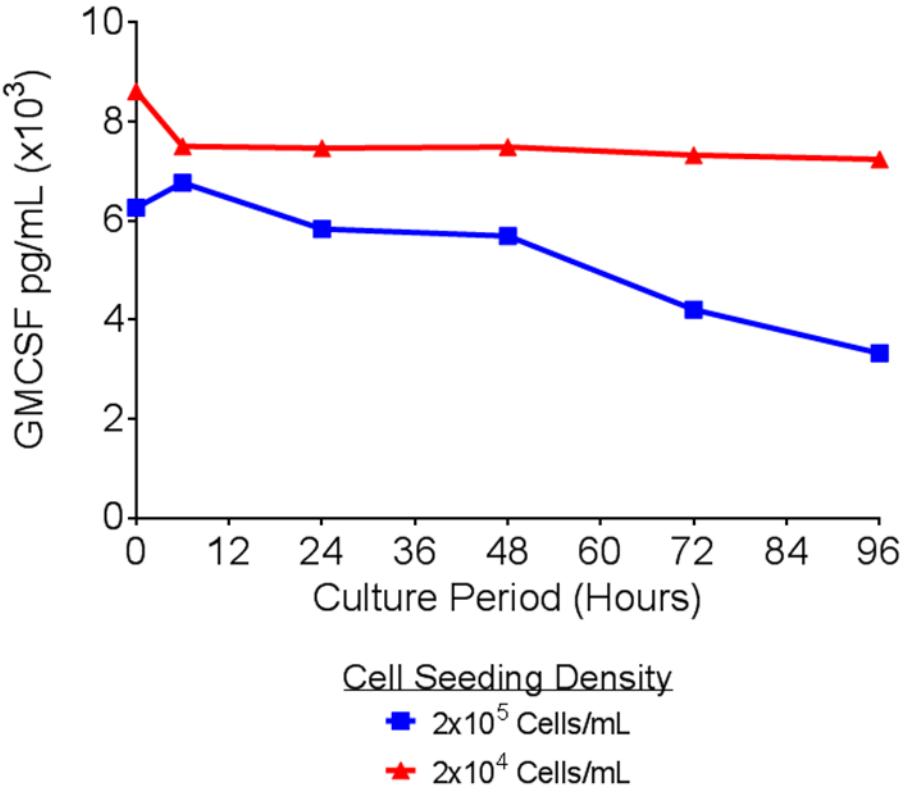


Figure 39. Degradation of soluble GM-CSF is cell density dependant. The graph illustrates stable GM-CSF concentrations at lower cell seeding densities over a 96 hour culture period. In contrast, GM-CSF concentrations decrease by ~33% over 96 hours with higher cell seeding densities. Data points represent mean concentration of GM-CSF in cell supernatant as determined by Magpix™ analysis (n=2).

4.2.5 Transient exposure of iGM-CSF

The immobilised growth factor technique was shown to elicit a full functional response in a growth factor dependent cell line in a stirred tank bioreactor. An impeller speed of 350 rpm in previous experiments shows that very short collision times are required between the receptor and growth factor in order to initiate the signalling cascade – enzyme-substrate interactions have been reported as short as 50 ms^{-1} [114]. Therefore it was hypothesised that a continuous exposure to growth factors is unnecessary, and could even be unfavourable for cell growth due to receptor desensitization[113–115].

Figure 40 shows the growth curve of TF-1 cells in response to different exposure durations to immobilised GM-CSF.

Cell growth was maintained for 36 hours in all conditions containing GM-CSF in both immobilised (0.5 hours and continuous exposure) and soluble form. Following 36 hours cell death occurred likely due to media exhaustion (i.e. glucose depletion as seen in the previous chapter) since a media change was not performed. The negative control displayed growth up to ~20 hours due to pre-historic culture with soluble GM-CSF.

Interestingly, the 0.5 hour exposure to iGM-CSF achieved approximately the same response (~1 population doubling at hour 36) as the continuous exposure to iGM-CSF and the positive control. This suggests that continuous exposure to iGM-CSF is unnecessary for a full functional response. Sufficient particle removal was shown by flow plots in Figure 41 and continued cell growth in the 0.5 hour condition was therefore not due to particle carry-over.

It was then hypothesised that soluble GM-CSF does not induce the same functional response due to a more dilute localised concentration.

Figure 42 shows the growth response of TF-1 cells after transient exposure to soluble GM-CSF in static culture. The graph shows that an equivalent response (~-0.7 PDs/day) for 0.5 hour exposure and the GM-CSF free condition, whereas continuous exposure resulted in ~1.2 PDs/day.

This experiment highlights the distinct functional responses immobilised and soluble growth factors have; this is likely due to the differences in localised surface concentrations – where immobilised growth factors can initiate receptor clustering for magnified stimulation of cell signalling[85], [117].

In final proof of concept experiments, the process benefits (ability to recycle iGFs during a media exchange and benefits of transient exposure) of the immobilisation method were demonstrated.

Figure 43A shows the growth curve of TF-1 cells in response to iGM-CSF and sGM-CSF with either transient exposure (6 hours) or continuous exposure with two media changes performed at 48 hours and 96 hours where particles were recycled. sGM-CSF was replenished at 5 ng/mL after a media change for the soluble positive control and a negative control was GM-CSF free. iGM-CSF was quantified at 1.06 fg/particle (0.2 ng/mL).

Equivalent growth rates were observed for the positive control and iGM-CSF continuous exposure condition for the initial 24 hours of culture. Following a media passage at 48 hours the positive control continued to grow exponentially reaching a cumulative population doubling of ~5.8 at 120 hours, whereas the iGM-CSF continuous exposure condition unexpectedly peaked at 3 cumulative population doublings at 48 hours and levelled-off for the remainder of the culture period.

Diminished performance in the iGM-CSF continuous exposure condition was thought to be caused by particle loss during the initial media change at 48 hours. Figure 43C shows cell suspensions were sampled every ~3 hours and analysed using flow cytometry. Counting particles using flow cytometry showed a decrease in particle numbers over time, where half of the particles were removed before the first media passage and the majority of the remaining particles were lost after the 48 hour media change. Particle loss was attributed to static interaction with the pipette tips as shown in Figure 43B.

Exponential cell growth was observed for 6 hour exposure period for iGM-CSF and sGM-CSF for ~36 hours, where the iGM-CSF condition peaked at approximately 1 cumulative population doubling in contrast to the soluble 6 hour exposure condition which peaked at approximately 0.5 cumulative population doublings. The results suggest an amplified response is achieved with iGM-CSF with a short exposure period in comparison to its soluble counterpart.

In order to demonstrate that the immobilisation technique is not a slow-release mechanism for iGFs, media supernatant was sampled and analysed over the duration of the culture period using ELISA. Figure 44 shows that no soluble GM-CSF was present in the iGM-CSF conditions over the culture period, providing assurance that the functional response is due to the immobilised GM-CSF solely. Effective removal of sGM-CSF was achieved in the 6 hour soluble GM-CSF condition and the positive soluble control showed a 50% decrease in concentration from 48 hours to 120 hours.

In order to minimise particle loss the previous transient exposure experiment was repeated but with less frequent sampling points. Figure 45A shows TF-1 cells responded equivalently to continual exposure of both sGM-CSF and iGM-CSF over the 120 hour culture period under

stirred conditions. A total of ~4 population doublings was achieved over 120 hours with the continuous exposure conditions, with an initial lag time of 24 hours.

At sequential time intervals (2,4,8 and 20 hours) after initiation of culture, GF was withdrawn to determine if stimulation and decay of cell growth was equivalent for GM-CSF in both soluble and immobilized forms. Continued cell growth was observed for a significant time period after removal of sGM-CSF or iGM-CSF, however the duration and magnitude of growth was dependent on time of exposure and form of presentation (Figure 45B). Cell growth measured at 50 hours showed a significantly (P = 0.01–0.05) higher response had been achieved with short exposure (2 and 4 hours) to iGM-CSF compared to equivalent short exposure to sGF, showing a heightened response to immobilised GM-CSF.

Similarly, cell numbers continued to significantly increase ($P \leqslant 0.05$) for a longer period after a short exposure (2 and 4 h) to iGM-CSF compared to the equivalent exposure to sGM-CSF (Figure 45C), showing immobilised GM-CSF initiates a longer lasting signalling potential.

Flow cytometry analysis of cell suspensions over the duration of culture show efficient particle removal, and therefore sustained growth in transient iGM-CSF conditions was not attributed to residual particles (Figure 46).

iGM-CSF concentrations were quantified at 1.96 fg/particle or 0.4 ng/mL ($2x10^5$ particles/mL) which equates to a 96% reduction in GF requirements for the continuous culture conditions (2 x 5 ng/mL supplements for the continuous soluble control).

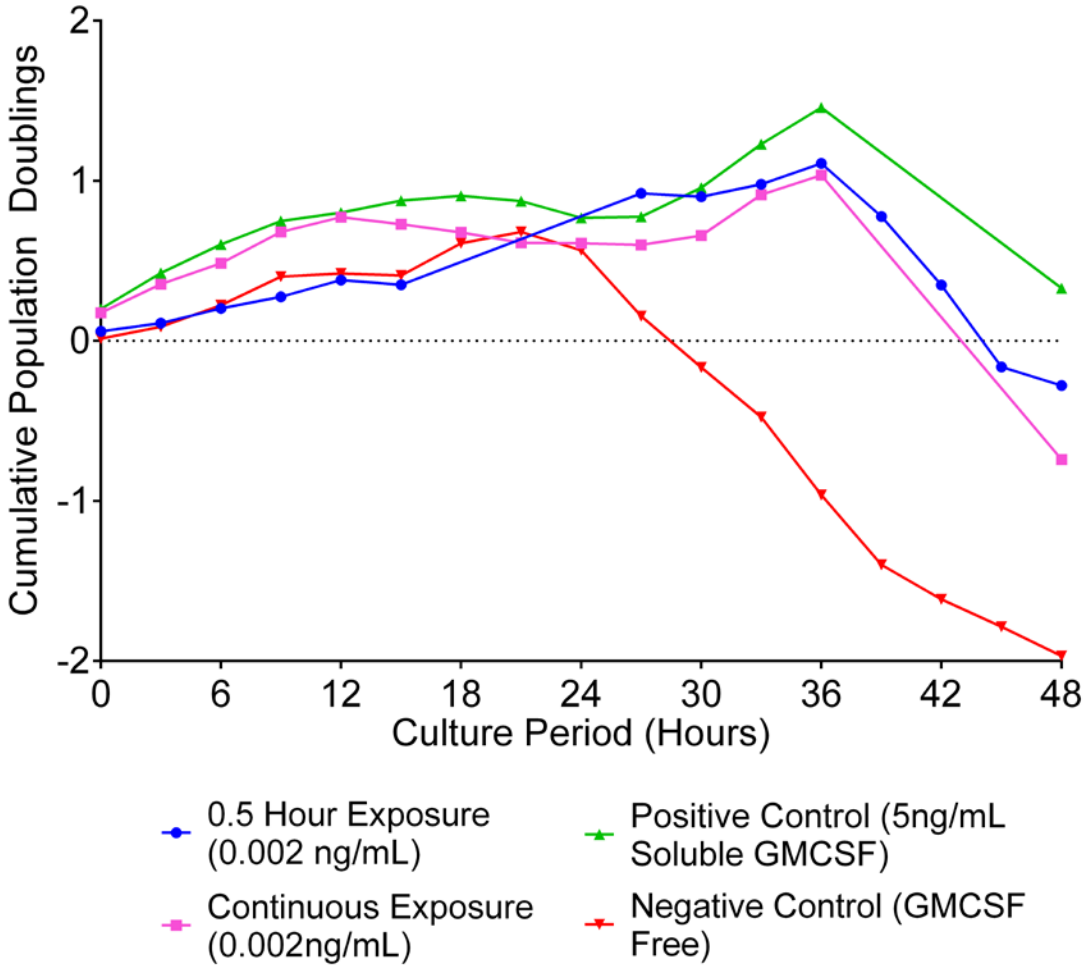


Figure 40. Effects of GM-CSF exposure period on functional response in stirred culture. Growth curve of TF-1 cells in response to short exposure periods of immobilised GM-CSF in a stirred tank bioreactor. A 0.5 exposure period exceeds the functional response of positive control where faster growth rates were also achieved. Continuous exposure provides no benefit and may have negative effects on cell growth (n=2).

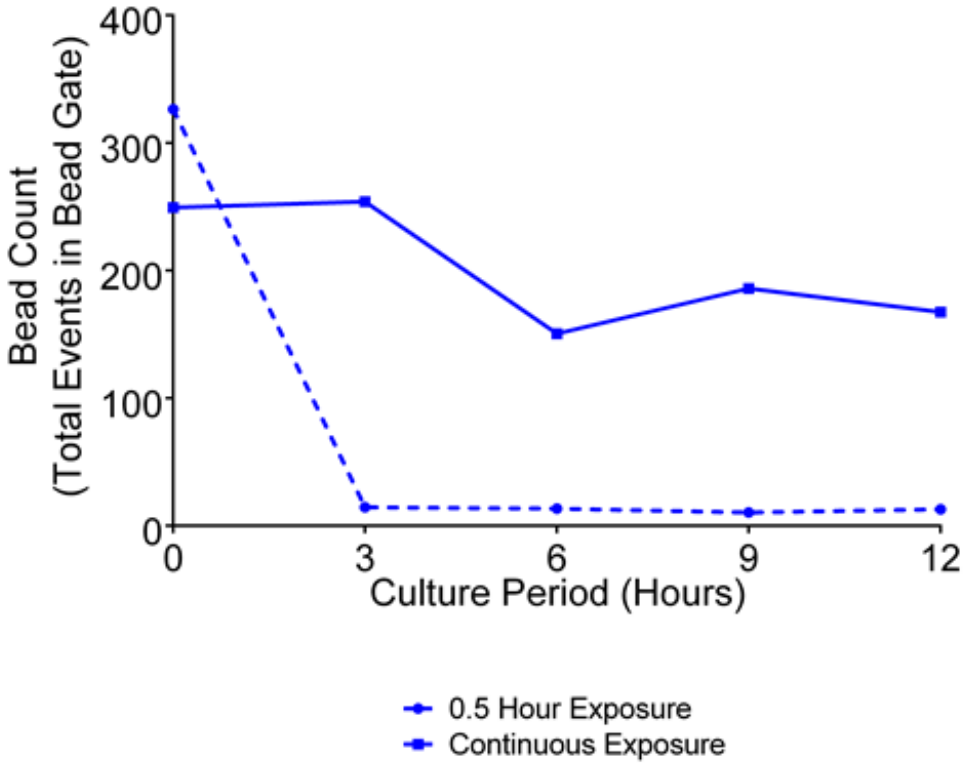


Figure 41. Particle counts as determined by flow cytometry for immobilised GM-CSF conditions shows sufficient particle removal after 0.5 hours and maintenance of particles in the continuous condition (n=2).

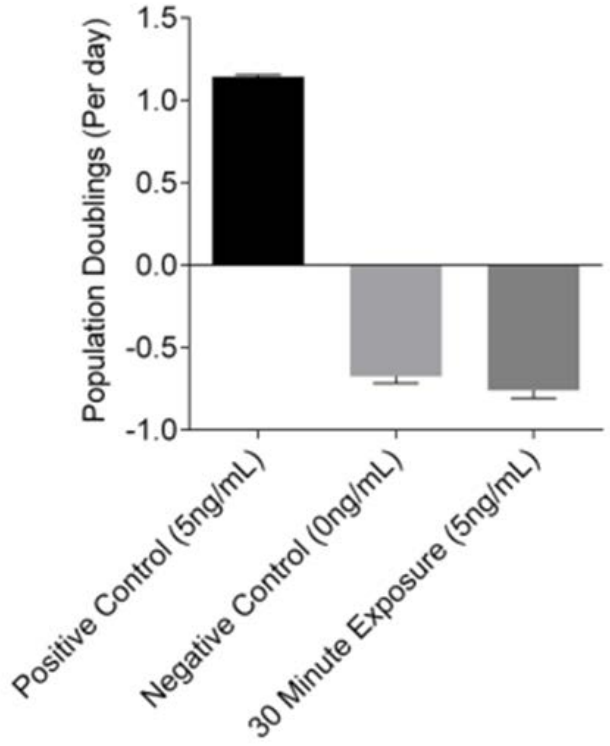


Figure 42. 30 minutes transient exposure of soluble GM-CSF elicits no functional response in TF-1 cells (n=3 ±S.D).

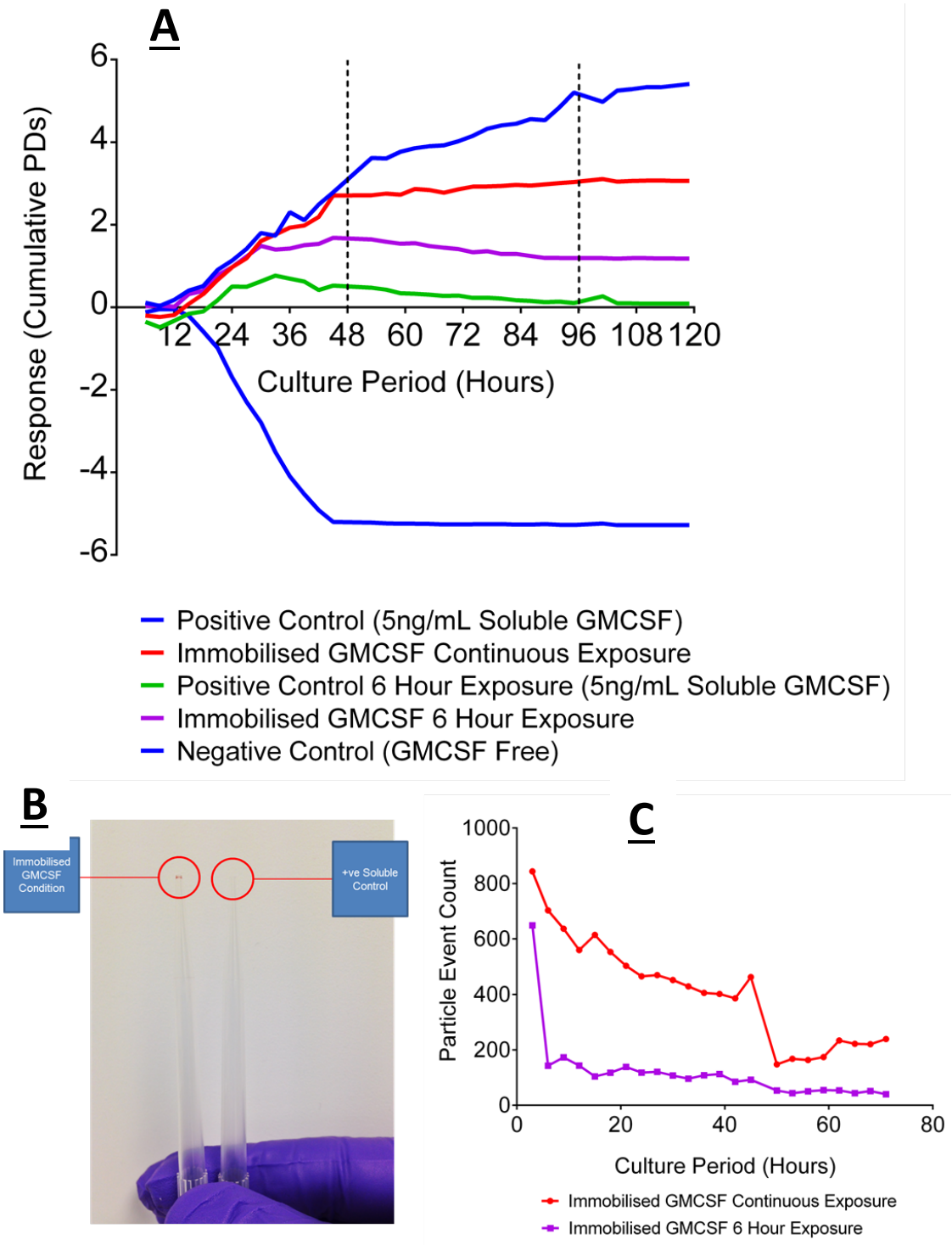


Figure 43. Effects of growth factor exposure period and recycling of immobilised GM-CSF in stirred culture. (A) Growth curve of TF-1 cells in response to continuous and 6 hour exposure periods of immobilised and soluble GM-CSF. The graph shows continuous exposure to immobilised and soluble GM-CSF produce identical functional responses. 6 hour exposure periods produce short periods of growth where the immobilised GM-CSF condition produced faster growth rates than the soluble counterpart. Diminished cell growth following 36 hours of culture was due to significant bead loss. (B) Beads were removed from culture by static interaction with pipette tips during sampling of the cell suspension (C) Analysis of media samples shows no leaching of immobilised GM-CSF across the entire culture period. Effective removal of soluble GM-CSF in the 6 hour exposure condition was demonstrated. In addition, soluble GM-CSF degraded by ~50% from hours 48-120 (n=2).

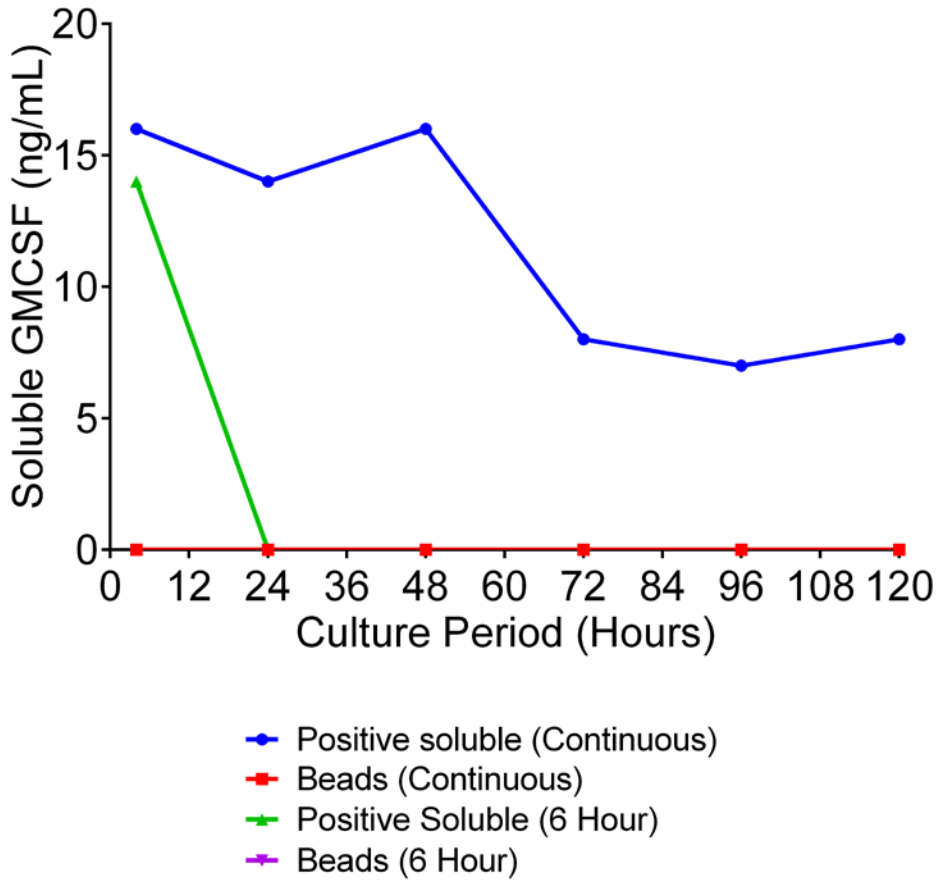


Figure 44. Analysis of media samples shows no leaching of immobilised GM-CSF across the entire culture period. Effective removal of soluble GM-CSF in the 6 hour exposure condition was demonstrated. In addition, soluble GM-CSF degraded by ~50% from hours 48-120 (n=2).

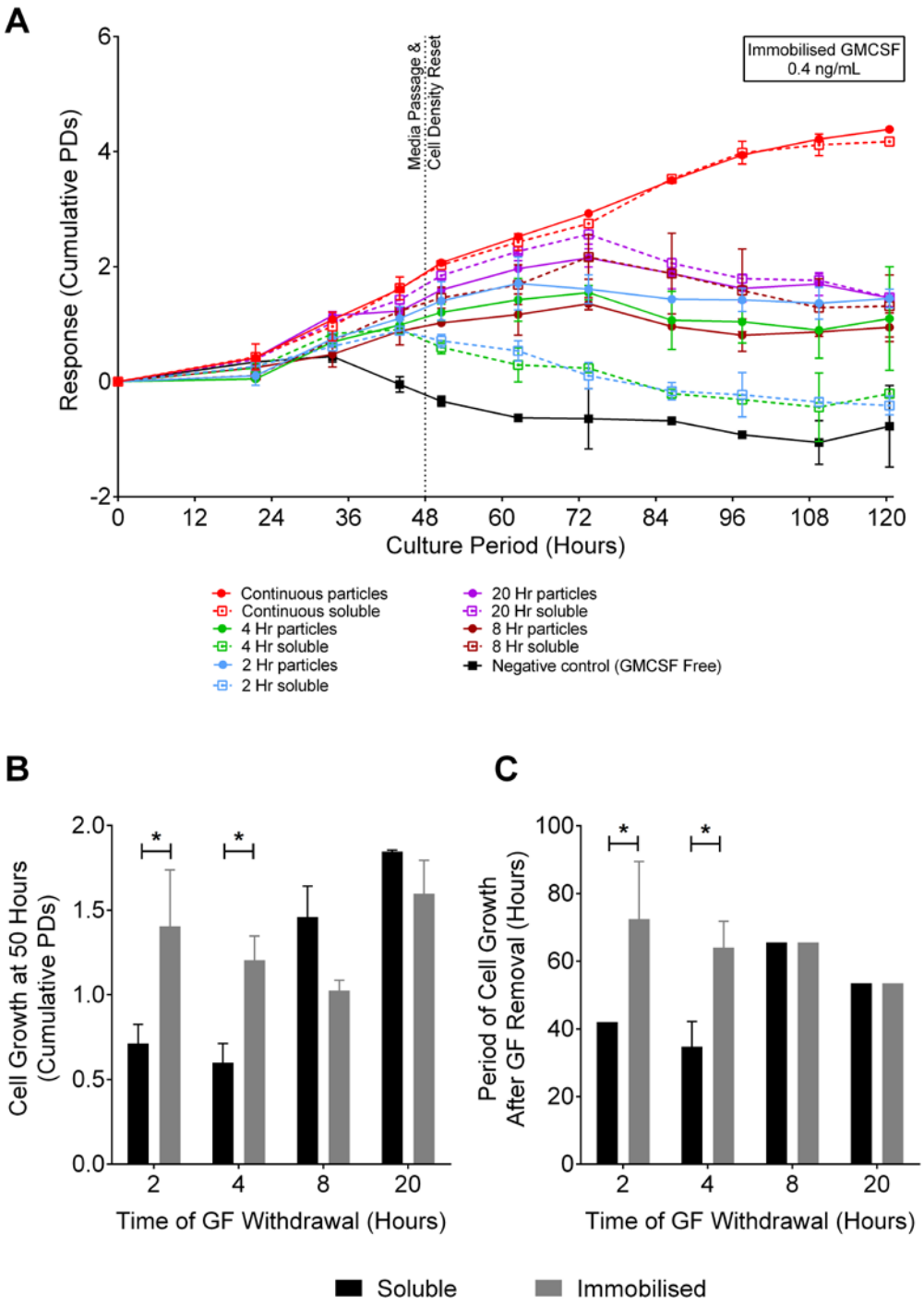


Figure 45. Functionality, stability, and manipulation of immobilized GM-CSF in stirred tank bioreactors and effects of transient exposure to growth factor. (A) The effects of immobilized and soluble GM-CSF exposure time on subsequent TF-1 cell growth rates were investigated in an agitated scalable cell culture bioreactor. Identical cell growth rates were achieved with continuous exposure to immobilized and soluble GM-CSF (n=2±SD). Vertical dashed line represents the time of a full media passage and cell reset to initial seeding density. (B) A comparison of cell growth rates at a single time-point from Figure 45A (50 hours) for immobilized and soluble GM-CSF were investigated. Cell growth was significantly increased at 2 and 4 hours of exposure to immobilized GM-CSF when compared to soluble GM-CSF (n=2±SD). Single asterisks represents a P value of 0.01-0.05. (C) The period of sustained cell growth following growth factor removal was calculated from Figure 45A. A significant increase in the sustained cell growth was achieved with 2 and 4 hours of exposure to immobilized GM-CSF (n=2±SD). Single asterisks represents a P value of 0.01-0.05.

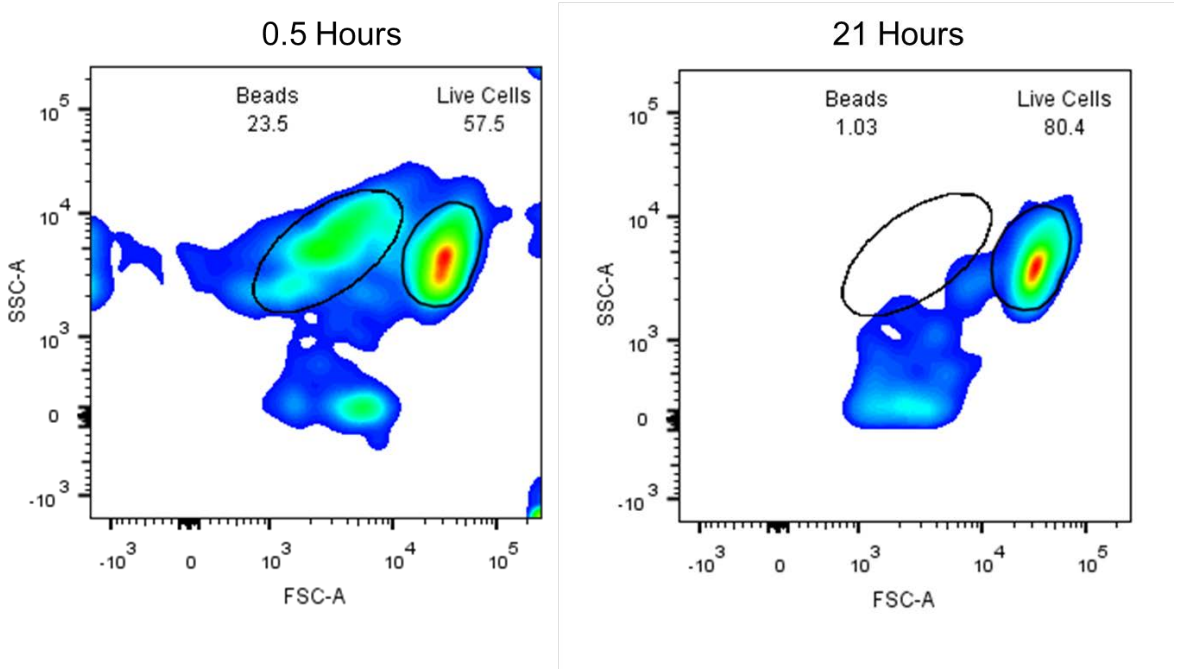


Figure 46. Representative flow cytometry plots indicating efficient particle removal and sustained cell growth in transient iGM-CSF conditions was not due to residual particles.

4.3 Discussion

Studies for numerous cell and gene therapies have shown that increasing cell numbers are correlated with good clinical outcomes [45]. In order to produce the required cell numbers, cell and gene manufacturing processes are likely to move towards scalable cell culture bioreactors such as stirred tank bioreactors. Therefore, the growth factor immobilisation technology developed in the previous chapter was developed with scalability built within the system. This chapter has demonstrated that the immobilisation technique was effective in a stirred tank micro-bioreactor platform and it exhibited a number of additional functional and process benefits.

Functionality of immobilised GM-CSF was retained in an agitated stirred tank culture system at relatively high stir speeds (350 rpm). Maximal functional responses were achieved in agitated culture systems at a stir speed of 350rpm (or ~70 cm/s). This suggests a very short interaction time between GF and receptor is required for a functional response. A response time of 50 ms is reported for enzyme-substrate interactions and blood flow in the human body can reach a velocity of 40 m/s within the aorta [118] [114]. Therefore, signalling cascades can be induced with brief interactions between ligands and receptors.

The ability for the GF immobilisation technique to retain maximal biological responses in stirred tanks bioreactors means the immobilisation method can be scaled with respect to surface area to volume ratio.

This chapter has highlighted the need to tailor PEG molar concentrations for individual growth factors in order to achieve maximum surface concentration. Three growth factors were immobilised, surface concentration optimised and shown to retain biological function. The ability to manipulate surface concentrations may be important for phenotype

maintenance of to induce cell differentiation by tightly controlling the ratio of growth factor combinations or total concentrations [119]. The activity of certain growth factors may be absent at low surface concentrations. A limitation of the immobilisation method is that the orientation of the GF cannot be controlled during immobilisation and it is expected that some of the GF will be inactive in its immobilised form, reducing the efficacy of the immobilisation method. Furthermore, the availability of lysine residues for Biotinylation of each individual GF should be given more consideration, in order to effectively titrate PEG molar ratios for optimal surface immobilisation.

Particle loss represents a major challenge for sustained and controlled functional responses particularly following media changes and frequent sampling – the sensitivity to particle concentration has been shown in a previous chapter. The use of strong magnets (see 2.9.3), slow removal of supernatant and reduced sampling frequencies however can overcome this issue and was shown to be effective for sustained cell growth.

Remarkably, iGM-CSF has been shown to retain consistent functionality following 192 hours of culture, whereas soluble GM-CSF can decrease as much as 50% over the course of 72 hours. Therefore the immobilisation method not only provides a method to recycle the growth factors, but the method also seemingly improves the stability of the growth factors which is in line with the reported literature[120], [121]. Increasing the stability of growth factors by immobilisation, together with the ability to recycle the growth factors following a media passage has the potential to greatly reduce the quantities required (and therefore costs) for cell and gene therapy manufacturing processes.

In this chapter a transient exposure to iGM-CSF, as little as 30 minutes, has been shown to provide a lasting functional response (over 20 hours). In addition, continuous exposure to

growth factors can be detrimental to cell signalling as it may lead to receptor down-regulation and desensitization.

A manufacturing system consisting of transient exposure would increase the number of manipulations in the manufacturing process. Various methods have been tested within this thesis to remove immobilised growth factors from culture and the methods are listed below with an explanation as to why they would or wouldn't be scalable in stirred tank bioreactors:

1. External magnet on the vessel wall
    - The immobilised growth factors would be drawn to the side of the vessel causing a 'mound' of particles. This would increase the shear stress that the cells were exposed to (particularly if the system remained agitated during particle removal). Furthermore, the risk of particle aggregation is increased, depending on the length of time the particles are held for.
2. A magnet in contact with the media supernatant
    - This process would entail the process being open, meaning it would have to take place in a Grade A clean room environment – increasing costs. Magnets would have to be sterilized prior to use if not disposable, the sterilization process would need to be validated and particles would not be reusable since they would be stuck to the magnet.

Clearly the two processes presented above would over complicate the process and the benefits of immobilised growth factors would be outweighed by the complexity.

Ideally the manufacturing system would consist of a disposable bioreactor and would be a 'closed' process. Therefore the Dynamag™ CTS magnet system could be applied to the

process where the product is drawn through tubing which is wrapped around a magnet, removing the particles and eventually the product is transferred into a secondary bag or vessel by gravity (Figure 47). The wrap-around magnet could be used in a scalable stirred tank bioreactor system which would be a simple, closed processing method to withdraw immobilised growth factors from the process.

Additional functional benefits have been demonstrated here, where iGFs have been shown to amplify functional responses and prolonged responses are achieved in comparison to soluble GFs. These functional benefits are likely achieved by assisting cytoskeletal changes via a physical anchor point and enhancing receptor clustering [85], [122], [123].

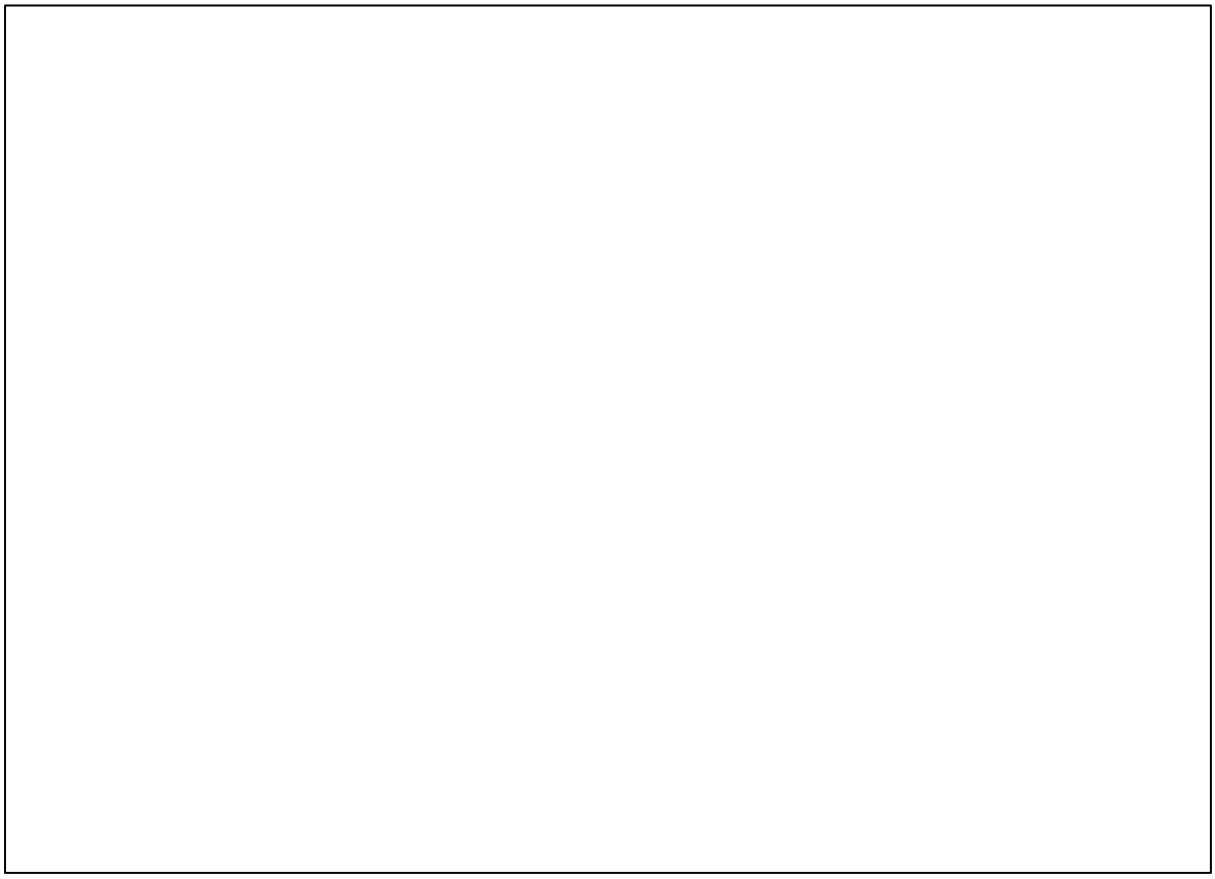


Figure 47. The Dynamag™ CTS magnet for removal of T-cell activation beads. A) The set up shows the product is transferred from its primary bag to a secondary bag via primary and secondary magnets in a closed system in order to remove particles from the cell suspension B) A close-up of the secondary magnet which the set-up could be transferred to a large scale bioreactor.

5 Application of Immobilisation Technology in a Primary Cell Culture System

5.1 Introduction

The previous results chapters have shown the successful development of a novel growth factor technology which was demonstrated using a model haematopoietic growth factor, GM-CSF and functionality was shown in a GM-CSF dependant cell line, TF-1.

The general introduction discussed the market for expansion of umbilical cord blood derived HSCs - since the majority of banked cord blood units do not contain enough cells to treat an adult patient. This section will evaluate the technologies reported for the expansion of UCB derived HSCs and growth factors which are fundamental for stem cell maintenance and expansion.

CD34+ cells derived from umbilical cord blood has added benefits over CD34+ cells derived from other sources such as bone marrow and peripheral blood. Some of the benefits are listed below:

- Lower incidence of GvHD – Higher HLA disparity results in lower incidence of GvHD in comparison with HSCs from other sources [124].
- Wider availability and readily available – umbilical cord blood is readily available, albeit specialist nurses are required to collect the material.
- Improved engraftment – increased long term repopulating stem cells (LT-HSC) [125].
- Non-invasive donation

Despite the many advantages of umbilical cord derived HSCs, low CD34+ percentages from the tissue means not enough cells are present for good clinical outcome for adult patients. $1x10^9$ total nucleated cells is often regarded as a minimum number of cells for transplant by the field[126]. This is the reason why current utilisation rates banked cord blood is only 1-3%

and declining. It is clear that there is a clinical requirement and commercial market for the expansion of CD34+ cells derived from umbilical cord blood.

Much research has been undertaken to increase the utilisation of cord blood for transplants. Research has involved the investigation of non-related cord blood transplants, double cord blood unit transplants and *ex vivo* expansion of cord blood units. Table 7 gives an overview of current *ex vivo* expansion technologies.

Table 10. CD34+ Expansion Technologies

Technology	**Description**
Copper Chelator [127]	Removal of intracellular copper which regulates HSC self-renewal and differentiation[128].
Notch [51], [86], [129]	Notch signalling has developmental roles and increases transcription of genes required for HSC expansion [130].
Perfusion [45], [93]	Dilution of inhibitory factors, consistent supplementation of growth factors and glucose.
Nicotinamide[131]	Inhibition of a SIRT1 deacetylase enhances proliferation potential of HPCs with decreased dependency on growth factors and increases telomerase activity via catalytic subunit of telomerase, hTERT [132].

Aryl Hydrocarbon receptor antagonist [133]	Inhibits receptor involved in regulating haematopoiesis[134].

Immobilised Notch with a defined soluble growth factor cocktail (SCF, TPO, Flt-3L) has shown some of the most promising results in the clinic and highlights the importance of immobilised ligands to recapitulate the stem cell niche and improve the expansion of HSCs *ex vivo*[86], [135].

Growth factors remain an essential supplement for the maintenance and expansion of CD34+ cells. An array of growth factors have been reported in the literature for haematopoietic cell culture summarised in Table 8. The table highlights no consensus on the growth factors and or concentrations of growth factors used for the culture of CD34+ cells, however it is clear that three or four growth factors (SCF, TPO, Flt-3L, Il-3) are commonly used in expansion protocols and are important for CD34 maintenance and expansion.

Highly cited CD34+ expansion journal publications routinely use SCF, TPO, Flt-3L and IL-3 [51], [129], [136], [137]. This chapter will focus on the immobilisation of three growth factors; SCF, TPO and Flt-3L for the expansion of UCB derived CD34+ cells.

Table 11. Haematopoietic growth factors, their effects on cell lineages and applied concentrations.

Cytokine	Effect on blood lineage	Applied Concentrations
SCF	Prevents HSC apoptosis[138]. HSC self-renewal[139].	10-100 ng/mL [140] [141] [142] [143] [144] [145] [146]
EPO	Directs erythrocyte lineage.	28 ng/mL[142]; 3-5 IU/mL[147] [144] [26
TPO	Regulates megakaryocyte and platelet development[148]. HSC Self-renewal[148][149]. HSC Expansion[148].	10-50 ng/mL [143][146][141]
IL-3	Regulates proliferation of HSCs[150]. Reduces *in vivo* reconstitution potential [151].	5-100 ng/mL [141][144][145] [147][142]
IL-6	Directs differentiation to mature lymphocytes. Increases proliferation of HSCs [152].	10-50 ng/mL[145][140][142]
IL-11	Regulates multi-lineage and committed progenitors[153]. Progresses cell cycle.	50 ng/mL [154]
Flt-3L	Regulates proliferation and differentiation of HSC progenitors [155][156].	10-50 ng/mL[144][145][141]

G-CSF	Proliferation and differentiation of Granulocytes. HSC mobilization.	100 ng/mL[142][145][144][157]
GM-CSF	Proliferation and growth of granulocytes and monocytes. Mobilisation of HSCs into the peripheral blood.	2-20 ng/mL[142][144][145]

The presentation mode of growth factors (soluble, membrane bound or immobilised) can have an impact on signalling fluxes and consequently impacts cell fate [120], [158], [159]. Fusion of unrelated growth factor molecules has shown the pleotropic and synergistic actions of growth factors in close proximity which influences downstream signalling and leading to increased mitogenic responses [160].

This chapter aims to investigate the immobilisation of multiple, clinically relevant growth factors, both individually and co-presented in a way that recapitulates the stem cell niche. In a proof of concept primary cell system, the growth factor immobilisation technology will be demonstrated in a clinically relevant system – the expansion of umbilical cord derived haematopoietic stem cells.

5.2 Results

Three growth factors (SCF, TPO, Flt-3L) were targeted for the expansion of UCB derived HSCs as discussed in the introduction to this chapter. This section follows the strategy developed in the previous two chapters, where the functionality of the immobilised growth factors was demonstrated in a growth factor dependent cell line and surface concentration maximised. A switch from the TF-1 cell line to an m-07e cell line was made, because the m-07e cell line is dependent on multiple growth factors that will be investigated in this chapter. Finally, the iGF method will be applied to a system for the expansion of UCB derived HSC.

5.2.1 Functional Response of Immobilised and Soluble Growth Factors in M-07e Cell Line

A dose-response profile of individual soluble growth factors (SCF, TPO, and GM-CSF) was generated to determine the working concentrations of soluble growth factors to compare with the functional response of immobilised growth factors. Figure 48 shows the dose-response profiles of soluble SCF, TPO and GM-CSF in the m-07e cell line. Maximum response was achieved with 100, 100 and 10 ng/mL of SCF, TPO and GM-CSF respectively. SCF induced cell proliferation most prominently out of the three growth factors investigated, however much higher concentrations of SCF (10 fold increase) were required for the maximal response in comparison to GM-CSF. As summarised in Table 8, the role of SCF is primarily for maintenance and proliferation of stem and progenitor cells, whereas GM-CSF and TPO have roles in directing cell differentiation and proliferation of stem cells to a lesser extent.

To determine the functional activity of additional clinically relevant growth factors, SCF, TPO and GM-CSF were immobilised and cultured in an M-07e functional assay. Figure 49 shows

that in addition to GM-CSF, SCF and TPO remain functionally active once immobilised using the streptavidin immobilisation technique. GM-CSF and TPO both significantly exceeded the maximum sGF response (P = <0.0001) whereas SCF did not achieve the soluble response despite reaching the top of a dose-response response curve. Maximum responses were achieved with $2.32x10^4$ particles/mL (1.4 fg/particle) for GM-CSF, $2.65x10^4$ particles/mL for TPO, and $8.42x10^6$ particles/mL (1.9 fg/particle) for SCF. Negative effects on cell viability were observed for highest concentrations of GM-CSF and TPO (>$5.35x10^4$ and >$8.82x10^4$ particles/mL respectively). This could be due to receptor down-regulation and or de-sensitization at high iGF concentrations as reported in the literature [115], [116]. In this cell line 0.003 ng/mL of iGM-CSF was required to accomplish the maximum soluble response achieved using 10 ng/mL of sGM-CSF, providing a similar orders of magnitude potency gain to that observed in the TF-1 line. Additionally, 16 ng/mL of iSCF accomplished 65% of the maximum response achieved with 200 ng/mL of sSCF. This is consistent with literature indicating distinct roles for soluble and bound SCF[161], [162].

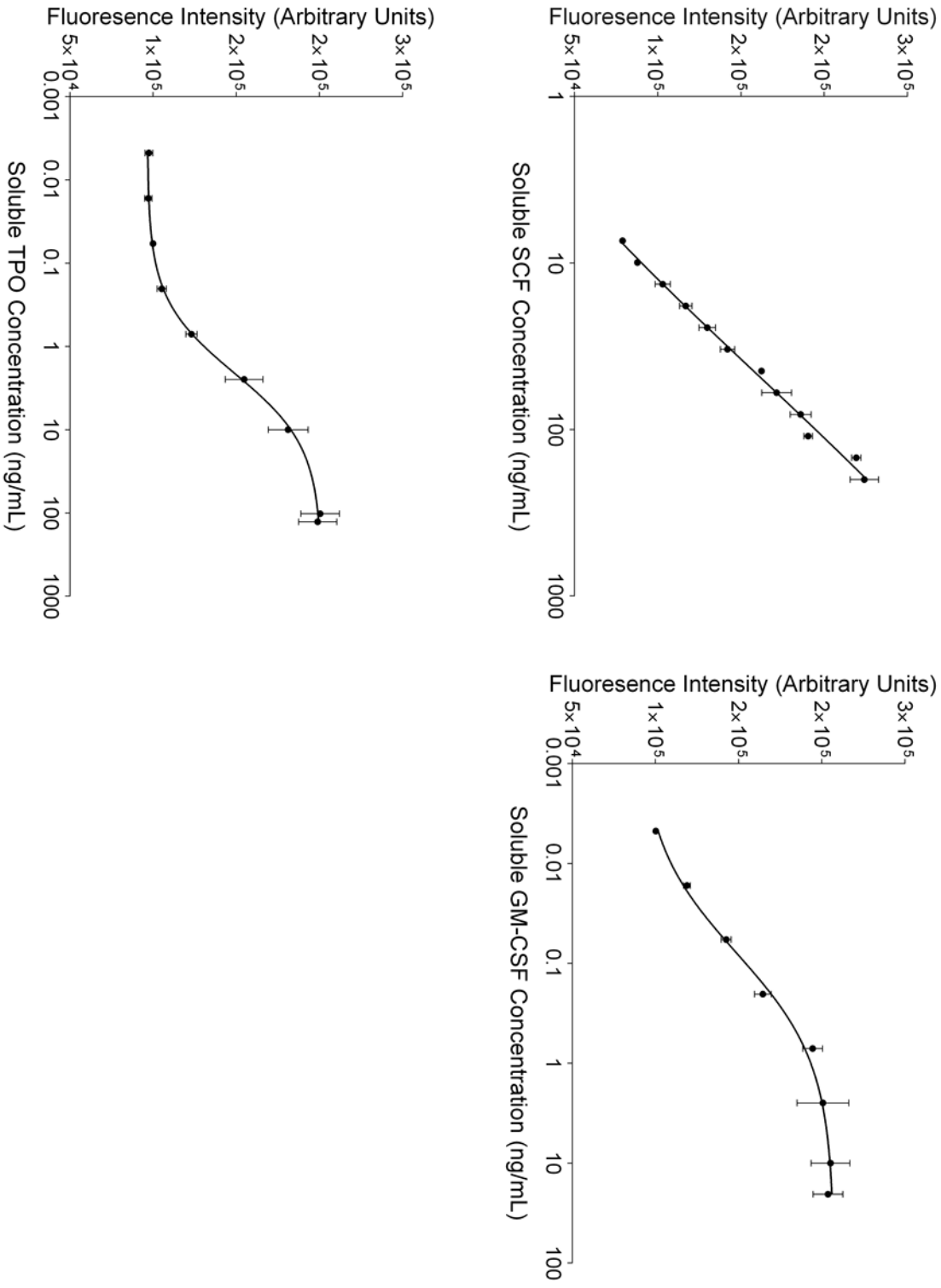


Figure 48. Soluble dose-response assays of SCF, GM-CSF and TPO in a cell proliferation assay using a factor dependent cell line, M-07e. Maximum response was achieved with 100, 10 and 100 ng/mL of SCF, GM-CSF and TPO respectively. Response was measured using an AlamarBlue assay. Data points represent mean fluorescent intensity (n=3±S.D).

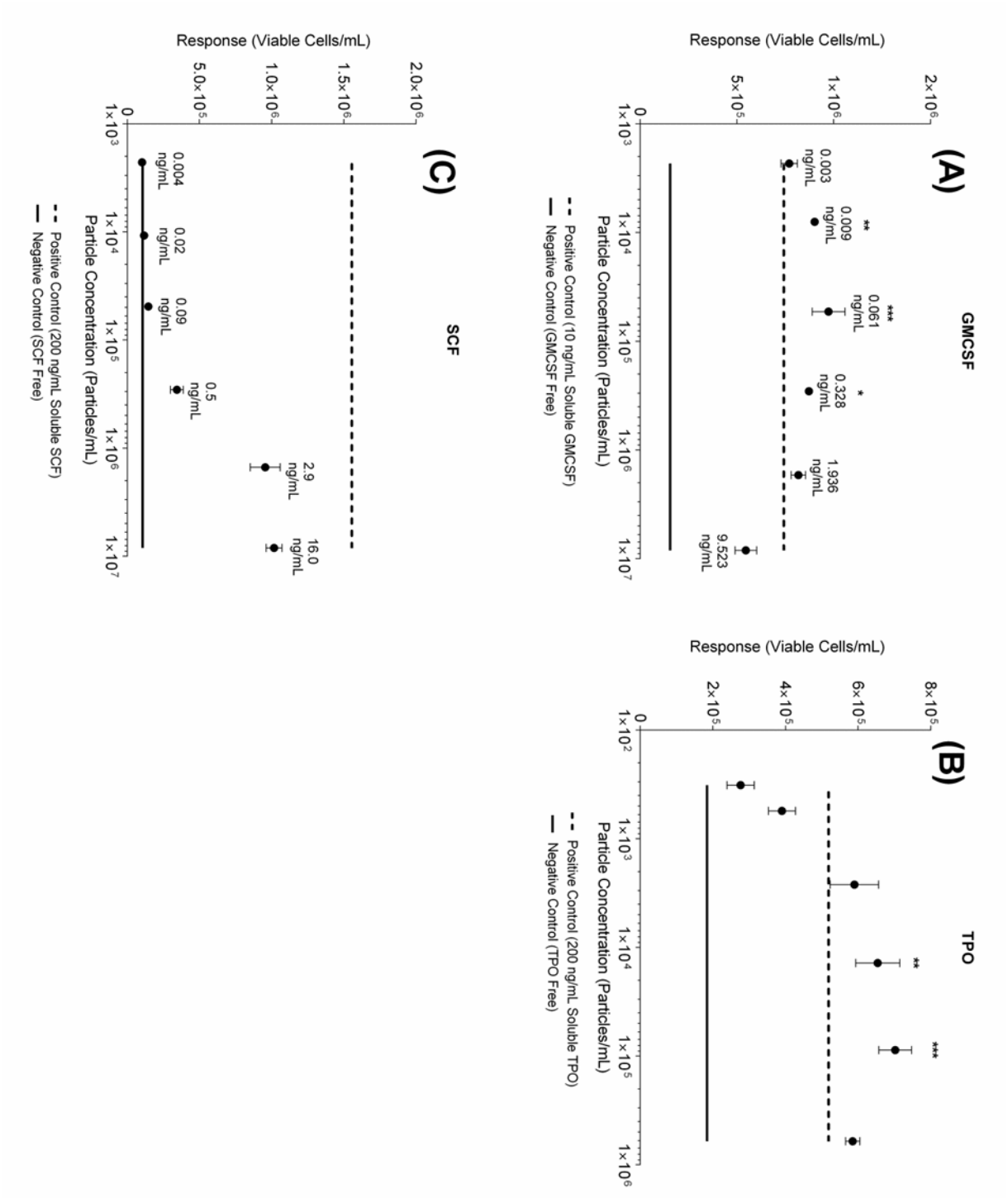


Figure 49. Functionality and dose response of immobilized GM-CSF, SCF and TPO. The function and dose-response of three immobilized growth factors were investigated in a multi-factor dependent cell line (M-07e). All three factors remain biologically active once immobilized. Immobilized GM-CSF and TPO both significantly exceed the maximal soluble response whereas SCF did not achieve the maximal soluble response. Where quantified, data points are labelled with total quantity of immobilized growth factor (n = 3 ± S.D.). * P = 0.01–0.05, **P = 0.001–0.01, ***P = 0.0001–0.001.

5.2.2 Immobilisation Preferences of Growth Factors

The complex nature of the HSC niche was described in the introduction. A combination of extracellular matrix, integrins and immobilised growth factors and other ligands make up the niche and are responsible for both phenotypic maintenance and homing of stem cells [39]–[41]. It was hypothesised that the immobilisation method developed here could be used to create microenvironments presenting multiple growth factors and therefore recapitulating the stem cell niche in a scalable culture system.

It was hypothesised that the binding preferences of multiple biotinylated GFs incubated with particles at equivalent concentrations would be correlated to lysine content of each growth factor. Figure 50A shows the binding preferences of GFs mixed at equal concentrations in pairs or in a combination of three growth factors. The growth factors were shown to preferentially immobilise in the following order of preference SCF>GM-CSF>TPO.

In concurrence with the hypothesis - GF binding preferences were correlated with GF lysine content (Figure 50B), where higher lysine number increases binding preference and total GF bound (Figure 50C). Co-presentation of multiple GFs could be controlled by compensating for lysine content, representing another method for controlling surface concentration of iGFs.

Notably, total bound GF was low in all cases in comparison to singly bound growth factors in previous experiments this is likely due to increased concentrations of non-reacted PEG which competitively binds for the biotinylated GFs. Purification of biotinylated growth factors following Biotinylation could overcome this issue, but adds further costs and processing steps to the immobilisation technique. However, synergistic effects of co-presentation of iGFs may compensate for lower surface concentrations.

Figure 50. The immobilization preferences for a mixture of two or more growth factors at equal molar concentrations were investigated. (A) The preference for immobilization was SCF>GM-CSF>TPO from high to low (B) Binding preference was linked to the residual lysine number of the growth factor (C) A linear relationship between lysine number and concentration of immobilized GF was shown (n = 3).

5.2.3 Expansion of CD34+ Cells using Immobilised SCF

SCF is a key growth factor for the expansion of CD34+ for its role in cell cycle progression [163]. In this final section, immobilised SCF was used to expand UCB derived HSCs as proof of concept in a primary cell culture system.

Expansion of Cryopreserved CD34+ UCB derived HSCs were incubated with iSCF to determine if CD34+ cells could be expanded in static culture following incubation for 72 hours. Figure 51 shows the response of CD34+ cells following 72 hours incubation with soluble or immobilised SCF. Approximately 0.7 population doublings per day were achieved with the highest concentration of soluble SCF (100 ng/mL) whereas no cell expansion was observed in all immobilised conditions.

Quantification of iSCF showed immobilisation of 0.72 femtograms per particle. Total concentrations of the immobilised SCF conditions were 108.7, 36.2, 3.6 picograms of SCF for $1.5x10^5$, $5.0x10^4$ and $5.0x10^3$ particles per mL respectively. Total concentrations are relatively low in comparison to soluble SCF and therefore higher concentrations may be required. Previously we have seen 10 fold less GM-CSF quantities used in comparison to SCF. Furthermore, CD34+ cells were cryopreserved prior to experiment meaning cell growth was sub-optimal. To reduce the complexities of cryopreservation the experiment should be conducted on fresh CD34+ cells.

Figure 55 shows representative pictomicrographs of cells incubated with soluble or immobilised SCF where cell morphology for iSCF conditions looked consistent with high concentrations of soluble SCF. In addition, phenotypic analysis (Figure 51) showed small quantities of iSCF can maintain stem cell phenotype comparable to high levels of sSCF. Example flow cytometry plots are shown in Figure 52 and Figure 53 for CD34 cells incubated

with soluble and immobilised SCF respectively. Positive and negative gates were determined using a non-stained CD34 sample (Figure 54).

Flow cytometry plots (Figure 53) show distinct population of cells when incubated with iSCF suggesting that there may be some degree of bead internalisation. Although, particles are widely regarded as being non-toxic and biocompatible it is important to understand the nature of internalisation, since this could pose a safety concern and affect regulatory approval [164]. It is also important to understand the effect of internalisation on the ability to recycle the particles during media passages.

The previous experiment was repeated using fresh, non-cryopreserved CD34+ cells and to incubate the cells with higher concentrations of iSCF since CD34+ cells require high concentrations (100 ng/mL soluble SCF) for maximal response.

Figure 56 shows the growth curve of CD34+ cells over a period of 96 hours when incubated with soluble or immobilised SCF. Cell growth improved from 0.6 population doublings in the previous experiment (with cryopreserved cells) to almost 4 population doublings at 96 hours with fresh non-cryopreserved CD34+ cells. This improved growth rate could be due to donor variability, but is likely due to un-optimised cryopreservation conditions. iSCF (140 and 70 ng/mL) saw the fastest cell growth at 24 hours in comparison to soluble SCF, where the growth rate decreased following 24 hours where all conditions began to merge. Decreased cell growth following 24 hours is likely due to a media exchange requirement (i.e. supplementation of glucose due to rapid cell expansion).

Quantification of iSCF particles showed 14.1 femtograms of immobilised SCF per particle. This surface concentration is one of the highest immobilisation surface concentrations

achieved with the immobilisation technology and iSCF was supplemented in excess, therefore the functional response could have been saturated at these levels.

Stem cell phenotype did not significantly change between SCF conditions – around 10% of total cells were CD34+ CD133+ for all conditions at 96 hours of culture.

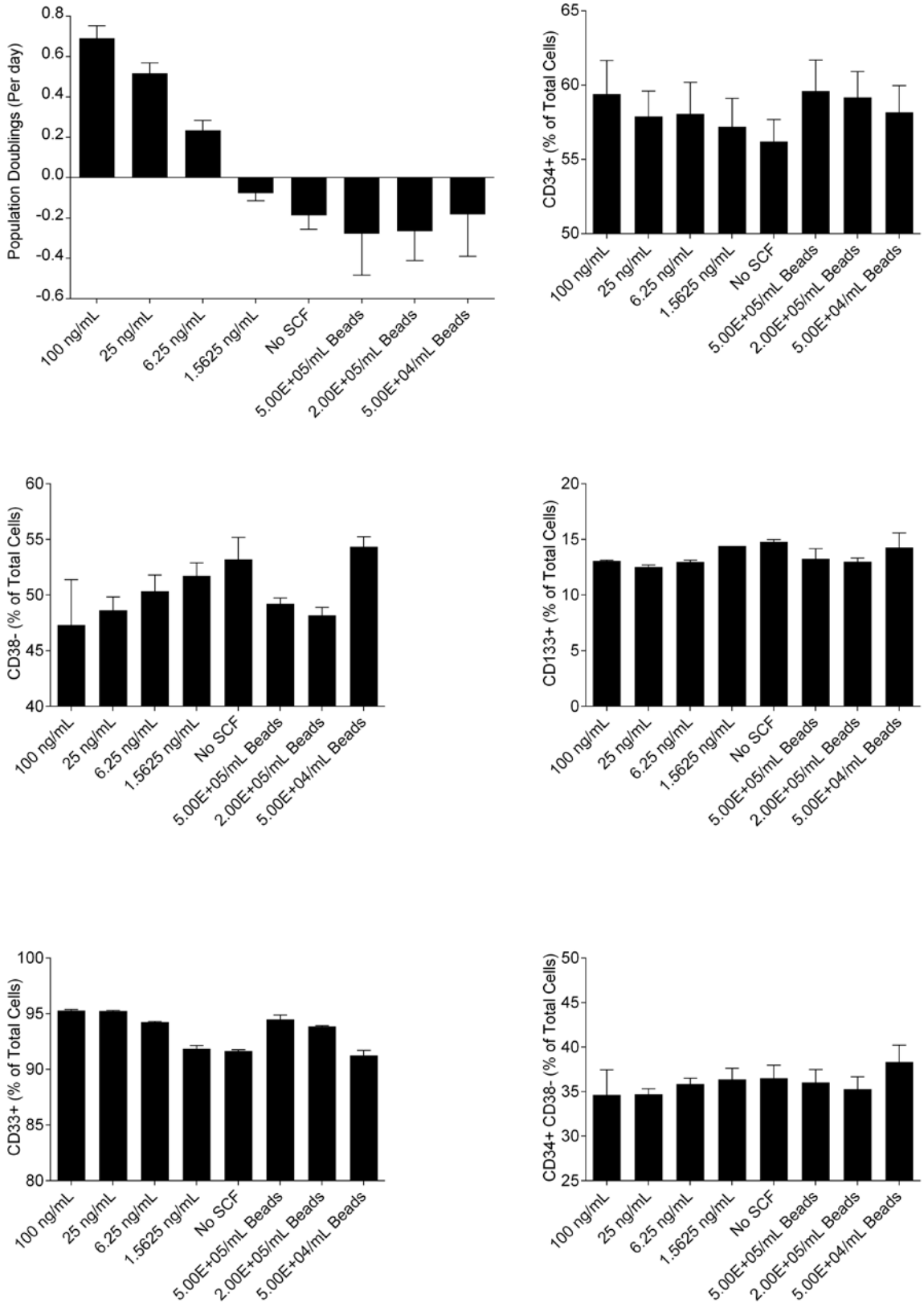


Figure 51. Expansion and phenotype of cryopreserved CD34+ cells with low concentrations of iSCF. No expansion of CD34+ cells was achieved with iSCF however stem cell phenotype was maintained in conjunction with high levels of sSCF (n=3 ±S.D).

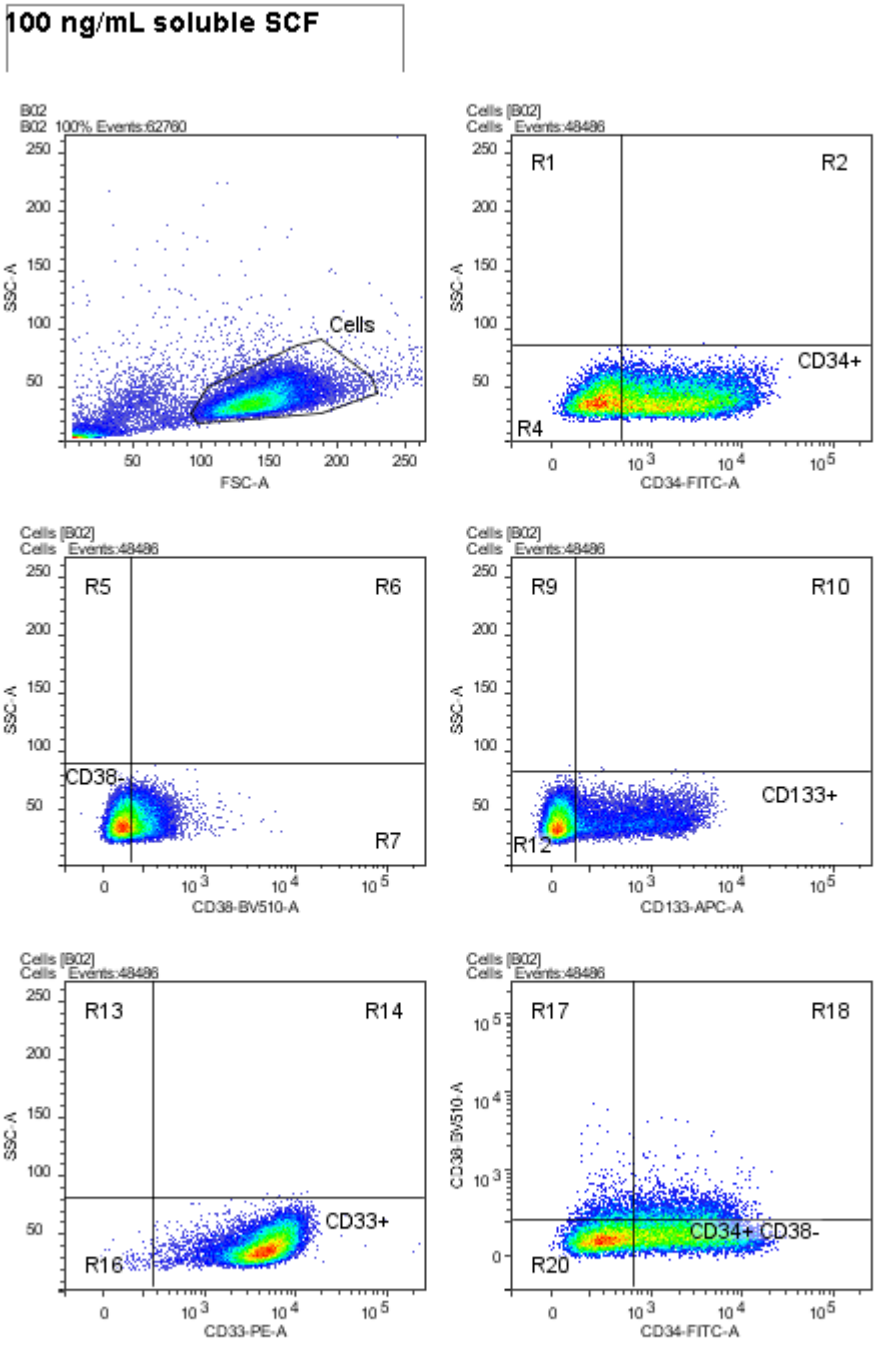


Figure 52. Exemplar flow cytometry plots and gating strategy for CD34+ cells incubated with soluble SCF at 100 ng/mL concentration.

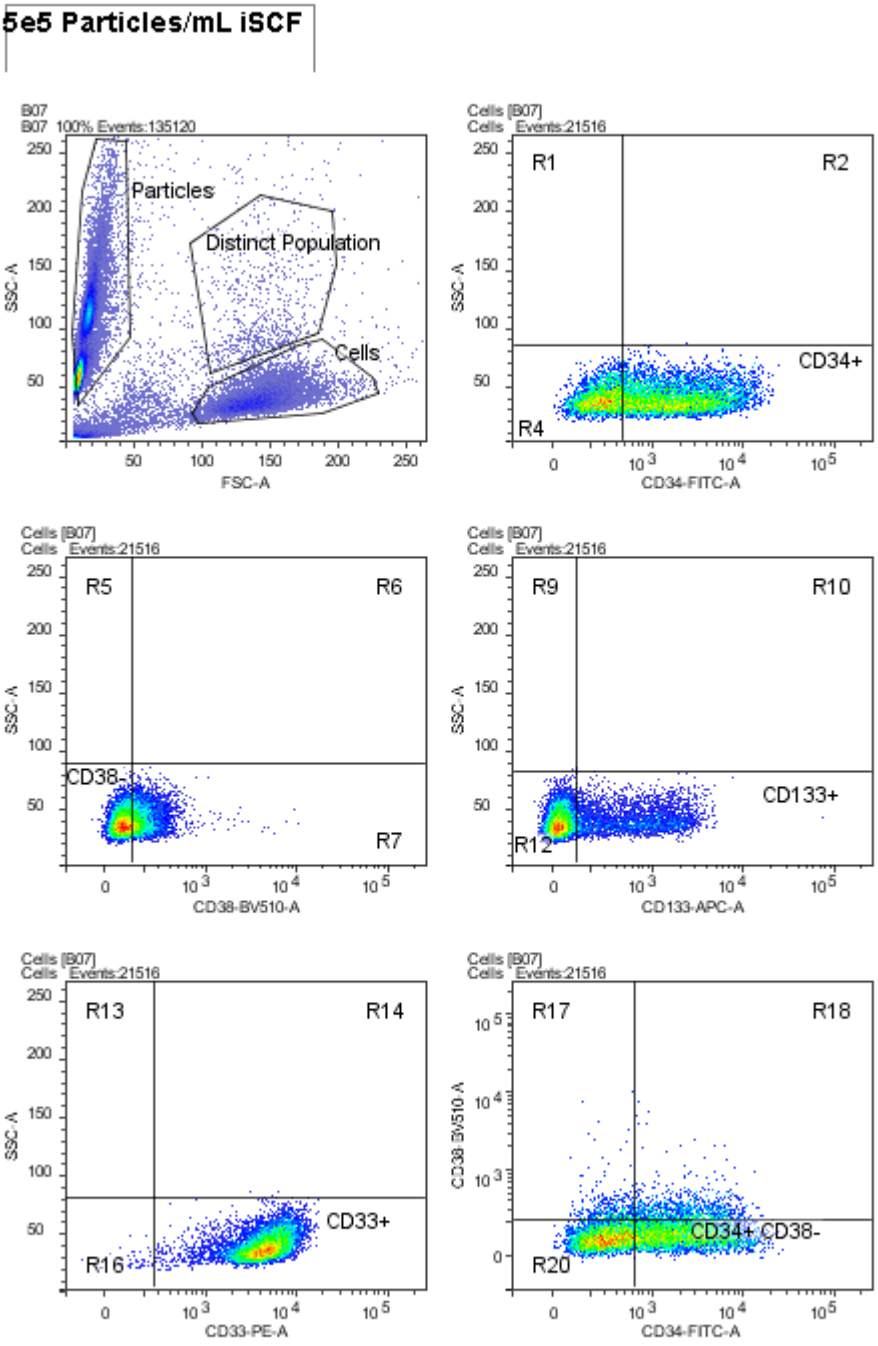


Figure 53. Exemplar flow cytometry plots and gating strategy for CD34+ cells incubated with immobilised SCF at $5x10^5$ particles per mL.

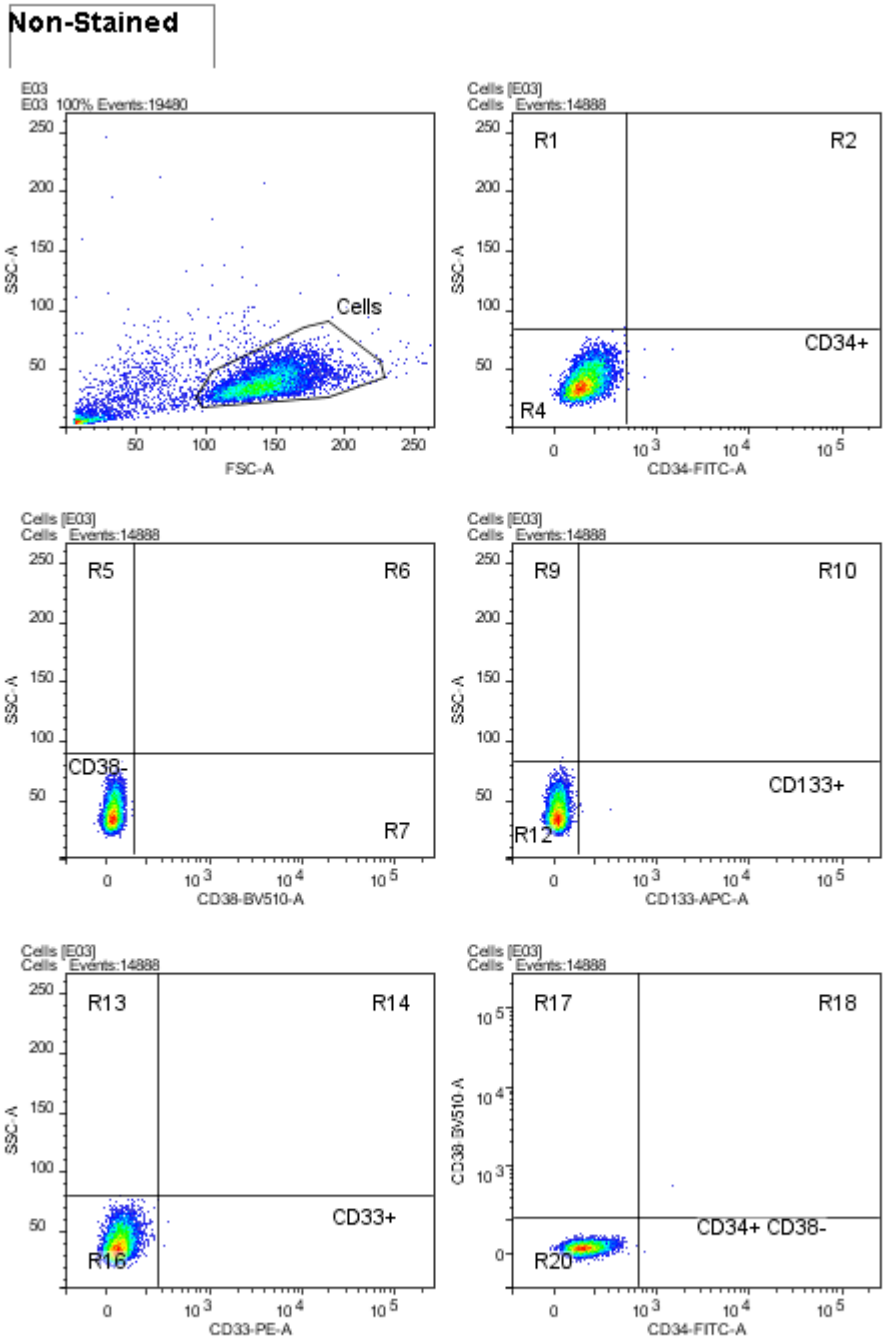


Figure 54. Example of a non-stained CD34 sample used to gate negative and positive samples.

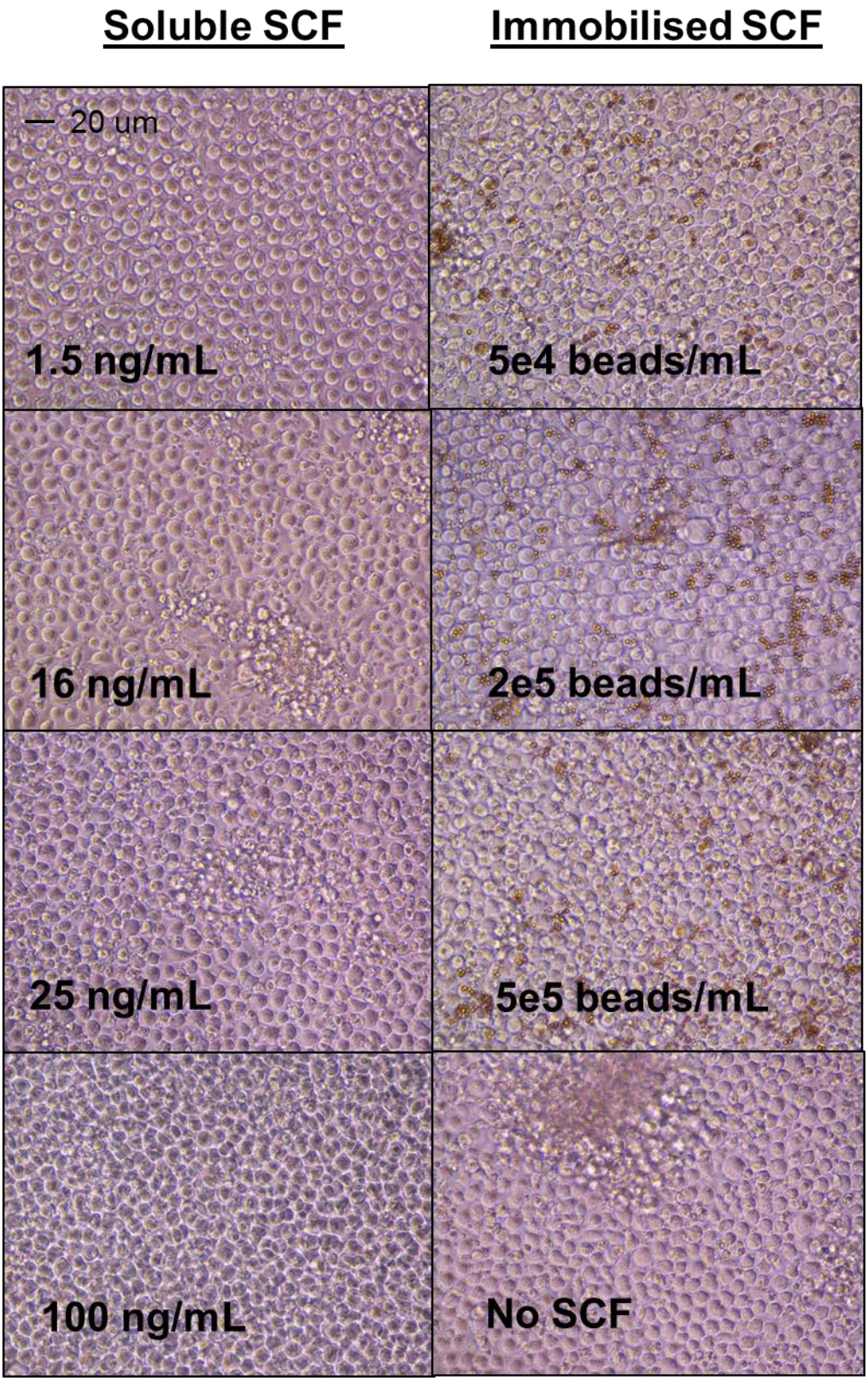


Figure 55. Representative photomicrographs of CD34+ cells cultured with various concentrations of soluble or immobilised SCF (40x magnification). Increasing cell densities are observed with increasing concentrations of soluble of immobilised SCF, where differentiated cell morphology is observed with low concentrations or no SCF.

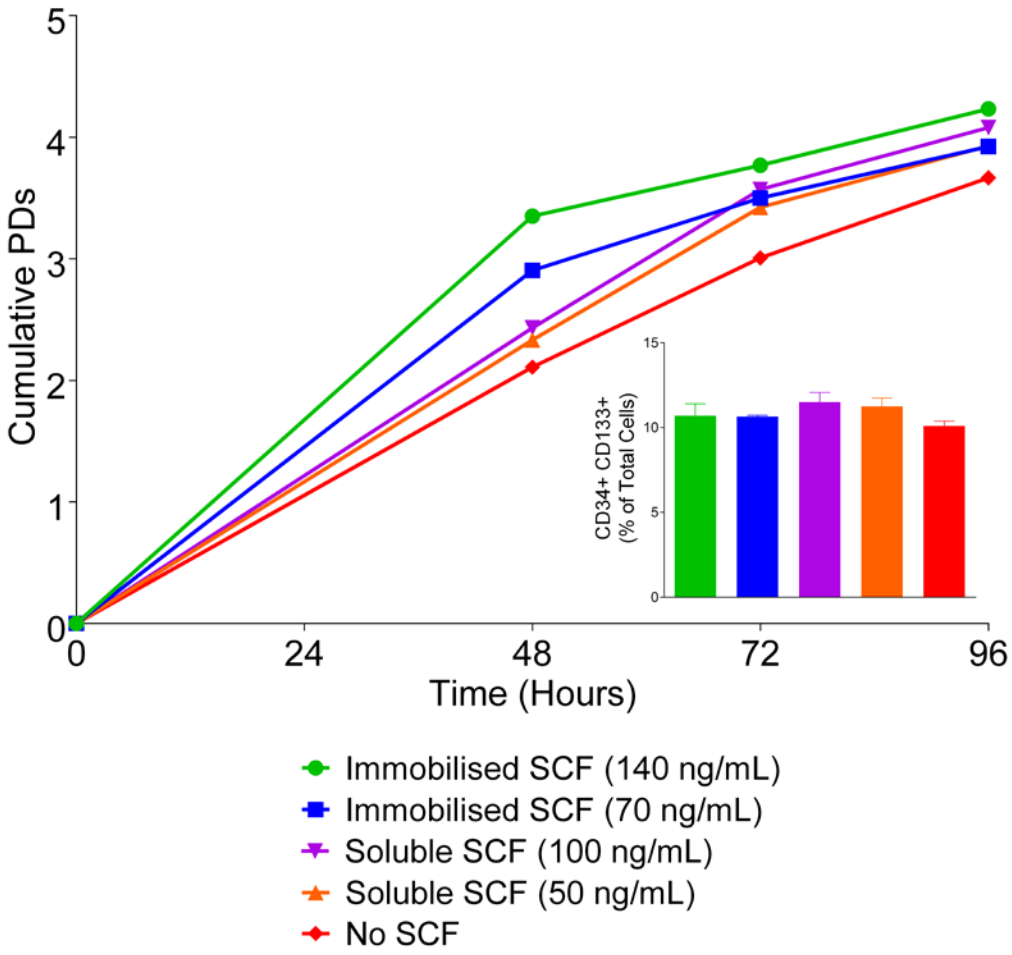


Figure 56. Growth curve of non-cryopreserved CD34+ cells and phenotype (at 96 hours) with high concentrations of iSCF. 4 population doublings were achieved over 96 hours with iSCF inducing the fastest cell growth at 24 hours (n=3±SD).

5.2.4 Particle Internalisation

In both to the CD34 expansion experiments, it was observed that there was a distinct population of cells when incubated with iSCF. The distinct population of cells were in parallel to the particle population on the y axis of FSC vs. SSC dot plots. This lead to the hypothesis that the particles could be internalised by CD34+ cells or it could be that the particles are bound tightly to the SCF receptor. Furthermore, particle internalisation or membrane binding could be unique to SCF and therefore other growth factors should be investigated.

To determine if particles were being internalised or membrane-bound, CD34+ cells were incubated with iSCF, iTPO and iFlt-3L for 72 hours or 30 seconds before analysis using flow cytometry with the hypothesis that if the distinct populations were not present following 30 seconds of incubation with iGFs then bead internalisation is likely.

Figure 57 shows the dot plots of CD34+ cells following incubation with three iGFs and an untreated control. The flow cytometry plots show distinct cell populations were present in both the 72 hour and 30 second incubation conditions for all three immobilised growth factors.

Internalisation of particles following 30 seconds of incubation is unlikely; the rate of endocytosis is typically in the range of minutes to hours [165]–[167]. Therefore it is likely that the particles bind tightly to the cell membrane of CD34+ cells.

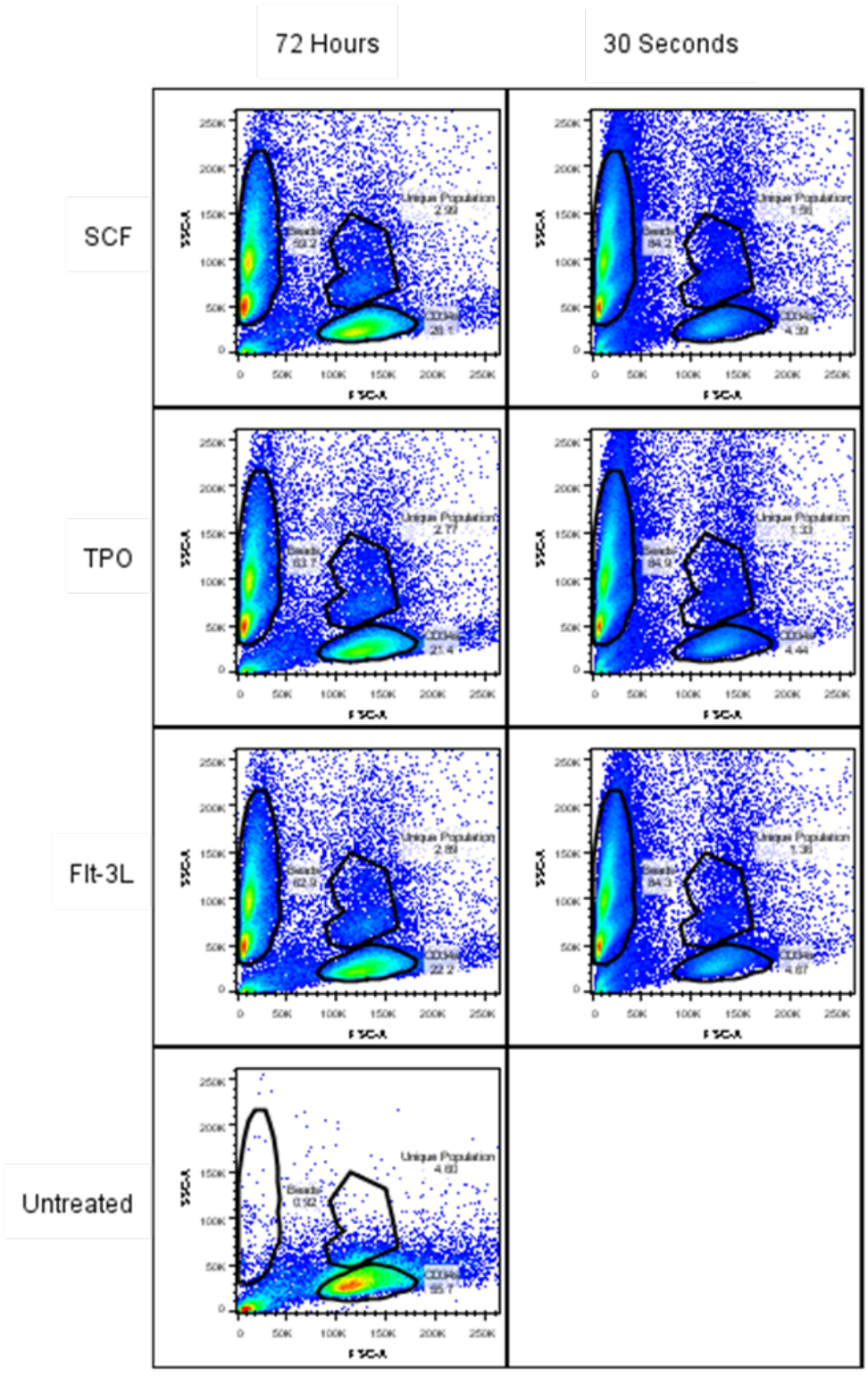


Figure 57. Flow cytometry dot plots showing unique populations of CD34+ cells incubated with iSCF for 30 seconds or 72 hours. Unique populations were present at both time points suggesting that particles were adhered to the cell surface membrane as opposed to being internalised.

5.3 Discussion

Cell and gene therapies require defined combinations of growth factors for maintenance, expansion or for directed differentiation of stem cell and progenitor cells. The importance of co-presentation of growth factors has been reported in the literature [85]. Furthermore, co-presentation is seen in the stem cell niche which is linked to stem cell maintenance and or stem cell homing to the niche.

This chapter aimed to investigate immobilisation of multiple clinically relevant growth (SCF, TPO and Flt-3L) factors in a model, scalable culture system for the expansion of UCB derived HSCs. SCF, TPO and Flt-3L are commonly used in HSC expansion protocols and are known to have roles in stem cell maintenance and or proliferation [168]. SCF, TPO and Flt-3L were shown to retain functionality after immobilisation, demonstrating that the immobilisation technique is universally applicable for a variety of haematopoietic growth factors. Whilst SCF was shown to have a reduced biological response in comparison to the soluble equivalent, it may be that immobilised SCF role is restricted to phenotypic maintenance when immobilised, by influencing downstream signalling[120], [159], [169]. Therefore a balance between proliferation and cell phenotype may need to be considered.

Co-presentation of multiple iGFs on a single particle was demonstrated where certain growth factors bound preferentially over others. Binding preferences were later shown to be correlated with the number of residual lysine amino acids in the proteins amino acid sequence. This finding allows the surface concentration of multiple GFs to be manipulated by compensating for lysine number. This method for manipulating surface concentration could be used in conjunction with other methods described in previous chapters (i.e. PEG

molar excess) in order to fine tune the presentation of growth factors depending on the desired cell phenotype/dose.

Immobilising multiple GFs has highlighted a limitation of the immobilisation technique – in that unreacted biotin competitively binds with biotinylated GFs effectively coating the particles and blocking GF immobilisation. This issue is magnified when multiple GFs are mixed together, since more unreacted biotin molecules are present, reducing the overall surface concentration of immobilised GFs. To overcome this, the immobilisation reaction could be reversed, so that the particles are biotinylated followed by the addition of GFs which would immobilise via the terminal NHS group (see Figure 58). However, the immobilisation efficiency would be limited by the rate of hydrolysis of the NHS group in the reverse reaction[170]. Another solution would be to purify the biotinylated GFs from unreacted biotin in chromatography columns (following Biotinylation step of the immobilisation reaction) this would improve the efficiency of the immobilisation process but would add increased costs and time to the immobilisation process.

This chapter has also demonstrated that particles bind tightly to the membrane of CD34+ cells. Membrane binding of iGFs could pose a problem when cells are required to be separated in from the particles for example in the envisaged manufacturing process (described in the succeeding chapter). However, these results were only shown in static cultures and it is likely that the shear stress (within a stirred tank bioreactor) would disengage the particles from the cell surface following brief interactions in agitated culture systems. This is why agitation during cell selections involving MACS particles is not recommended along with T-cell activation with Dynabeads™. Particles could be released with trypsinisation if permanent binding occurred in agitated systems, but this would be an

undesirable process step as this could affect the downstream cell phenotype and health and would also affect the function of the iGFs following release [171], [172].

In conclusion, this chapter has demonstrated the use of multiple immobilised growth factors for cell culture applications. In final proof of concept experiments the technology has been shown suitable for the expansion of umbilical cord blood derived HSCs by immobilisation of SCF. Taking the findings of the current chapter and those from previous chapters, the immobilisation technique is applicable for the expansion of UCB derived HSCs (and other cell culture applications) in standard scalable stirred tank bioreactors with the potential to significantly reduce the costs of growth factor required throughout the culture period. However, this chapter has highlighted further optimisation of the immobilisation technology is required to improve efficiency.

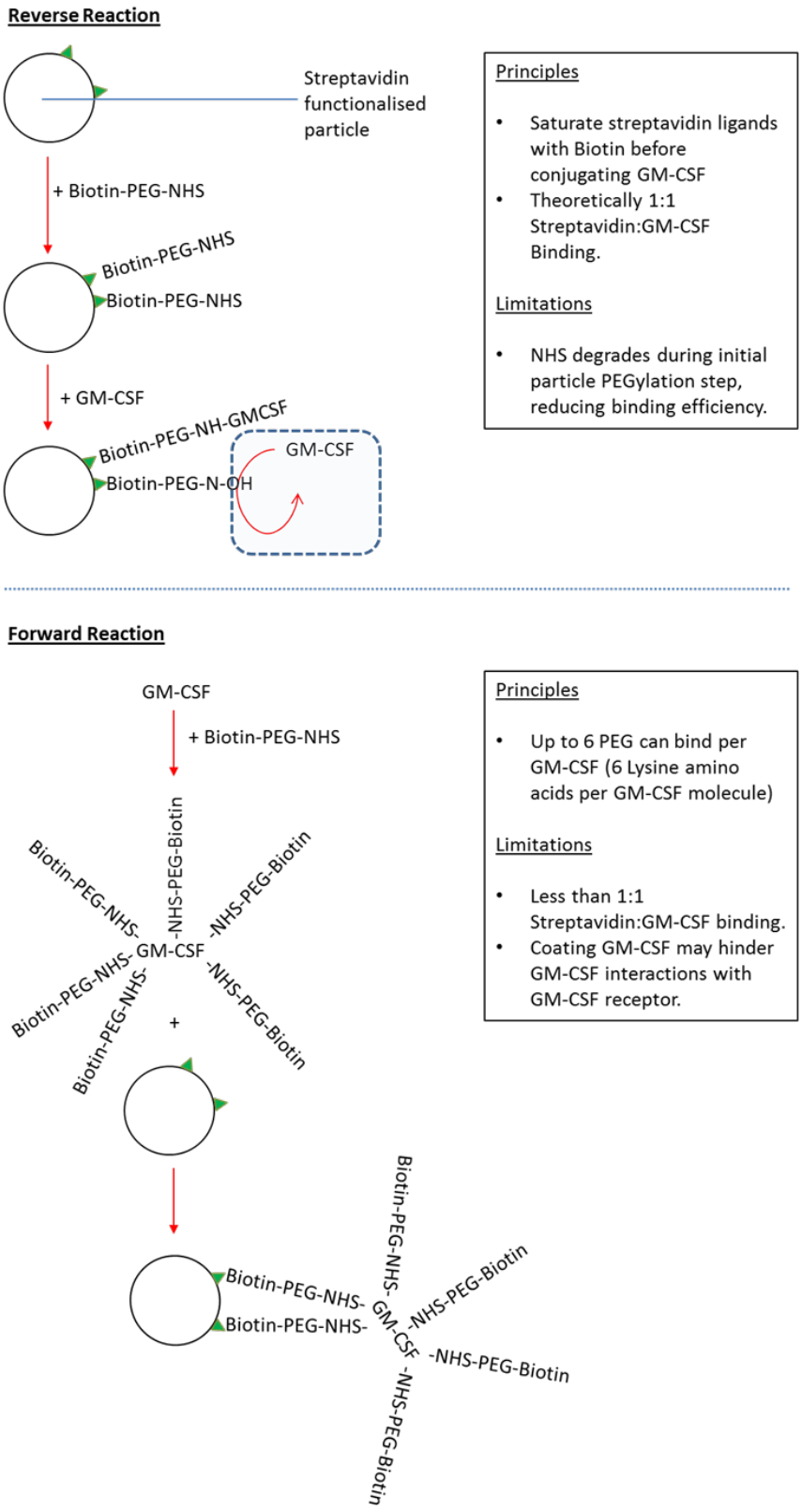


Figure 58. A schematic of the Streptavidin immobilisation method using the standard 'forward' method and proposed 'reverse' method which may increase NHS susceptibility to hydrolysis.

6 Final Discussion and Future Work

This thesis set out to address the issues associated with the use of soluble growth factors used in the manufacture of cell and gene therapies. High costs and lack of cellular phenotypic controls are some of the issues involved in the use of soluble growth factors.

The experimental results in this thesis have shown that immobilised growth factors can replace soluble growth factors in scalable cell manufacturing processes. They have shown increased potency and distinct functional effects (i.e. increased signalling times) in comparison to their soluble equivalents. Being tethered to magnetic particles has allowed the growth factors to be manipulated independently from the media bulk and thus recycled during media changes. Remarkably it was shown that using immobilised growth factors - as little as 1.5% of the soluble growth factor requirements were required in an example culture system over a 192 hour experiment.

The need to produce high doses of HSCs and the consequent need for scalable manufacturing systems was highlighted as early as the 1980's where protocols to expand HSCs in 5L stirred tank bioreactors were described[173]. In this protocol a combination of six growth factors were used with four media passages which equated to over £4000 worth of growth factor for a single product assuming a cost of 15.4 pence per nanogram of growth factor (see Table 9). If we take the results from this thesis that approximately 1.5% of the total soluble growth factor is required then for this particular protocol the costs of growth factor would have been reduced to ~£60. This could be further decreased with a manufacturing process that involves transient exposure.

Table 12. Comparison of growth factor costs in an example stirred tank manufacturing process as described by Collins et. Al, 1998 and potential costs savings using immobilised growth factors, assuming 1.5% of the total soluble growth factor is required [173].

Soluble growth factor process

Total Volume (L)	GF	GF Conc (ng/mL)	Total GF (ng/mL)	Number of media exchanges	Total GF over culture period (ng)	Total cost (£)
5	IL-3	5	2.50E+04	4	1.00E+05	154
5	IL-6	50	2.50E+05	4	1.00E+06	1540
5	SCF	50	2.50E+05	4	1.00E+06	1540
5	GM-CSF	2	1.00E+04	4	4.00E+04	61.6
5	G-CSF	1.5	7.50E+03	4	3.00E+04	46.2
5	EPO	28	1.40E+05	4	5.60E+05	862.4
						4204.2

Immobilised growth factor process

						63.063

HSCs have been reported to be sensitive to the shear stress in agitated systems and CD marker expression can be altered[168], [174]. Such culture conditions were most likely optimised for cell expansion and not phenotypic maintenance. Simple DoE experiments could be conducted to elucidate the optimal agitation conditions required to achieve a balance between the desired phenotype and level of cell expansion required. Furthermore, it would be interesting to compare the effects of soluble and immobilised growth factors in agitated systems and their effects on phenotypic maintenance since it is reported that immobilised growth factors can improve control of cell phenotype[175].

The lack of on-line monitoring tools in scalable cell therapy manufacturing platforms (particularly) has been identified as a major problem for the field going forward[45], [94]. Effective media feeding strategies that maintain consistent levels of media supplements and reduce the inhibitory paracrine signalling factors or toxic metabolic by-products is necessary for the production of consistent cell based products with good viability and the desirable phenotypic profile and also reduced the waste associated with over-supplementation in standard batch-feeding regimes. Strategies to monitor cytokine levels on-line in perfusion systems have been described with the ability to automatically adjust media feeding rates to maintain consistent levels of cytokines[93]. Such systems are likely to require significant modifications to standard culture platforms and will require significant development of equipment and algorithms for individual culture protocols. In this thesis, the growth factor immobilisation method was shown to maintain stability up to 192 hours which negates the need for such on-line measurement systems and highlights that the technology is transferrable to standard agitated systems without further modifications.

Despite the promising results, there is further work and questions relating to the immobilisation method that should be addressed in future work. In the selected immobilisation method, the orientation of the immobilised protein cannot be controlled. Incorrect orientation could mean that the growth factors epitope is not accessible to the receptor and therefore is essentially inactive. However, experimental results have shown that the immobilised growth factors are more potent than soluble, despite a proportion of the growth factors being immobilised in the incorrect orientation. Therefore this is not problematic from a functional point of view, but it does decrease the manufacturing efficacy.

Additionally, Immobilisation efficiencies are further decreased because biotinylated growth factors are not purified from non-biotinylated growth factors after the initial PEGylation stage. The result is that non-reacted PEG molecules competitively bind for pegylated growth factors. A solution to this could be to introduce molecular weight cut off chromatography columns to separate PEGylated proteins from unreacted PEG molecules. However, the recovery of protein is likely to be impacted and this would add further processing steps which increases manufacturing times, cost and may introduce further variability into the process.

The PEGylation site within growth factors could affect the proteins activity and or folding[176]. Although generally lysine residues are not involved in the folding of the tertiary structure, such amino acid groups in proximity to the active site or binding domain could significantly affect the proteins activity. Furthermore, PEG molecules bind 2-3 water molecules per subunit, meaning PEGylated proteins effectively swell in size and can significantly impact spatial presentation and steric hindrance[176]. No impact was seen

from PEGylating any of the growth factors investigated in this thesis however it is important to note some proteins may not be suitable for the immobilisation method.

Edman degradation is a technique which could be employed to investigate the common sites for conjugation however this is usually only amenable to small peptides[177]. MALDI-TOF Mass Spectroscopy may be more suited to determine the site and degree of PEGylation per growth factor molecule. Whilst X-ray laser diffraction could be employed to determine the protein structure differences before and after PEGylation.

The rationale for incorporating a PEG linker within the conjugation bond was to enable the spatial presentation of different growth factor molecules to be tailored depending on 1) their required surface concentration and 2) molecular weight to prevent steric hindrance. The schematic shown in Figure 59 shows how tailoring PEG linker lengths to growth factors has the potential to control surface concentrations and spatial presentation to enable receptor binding. The schematic also shows how PEG length will be a balance between optimal surface concentration and correct spatial presentation to allow growth factor – receptor binding particularly in relation to the growth factor size. A simple screening experiment using a functional assay could be used to investigate the effects of PEG length on the functional activity of individual growth factors.

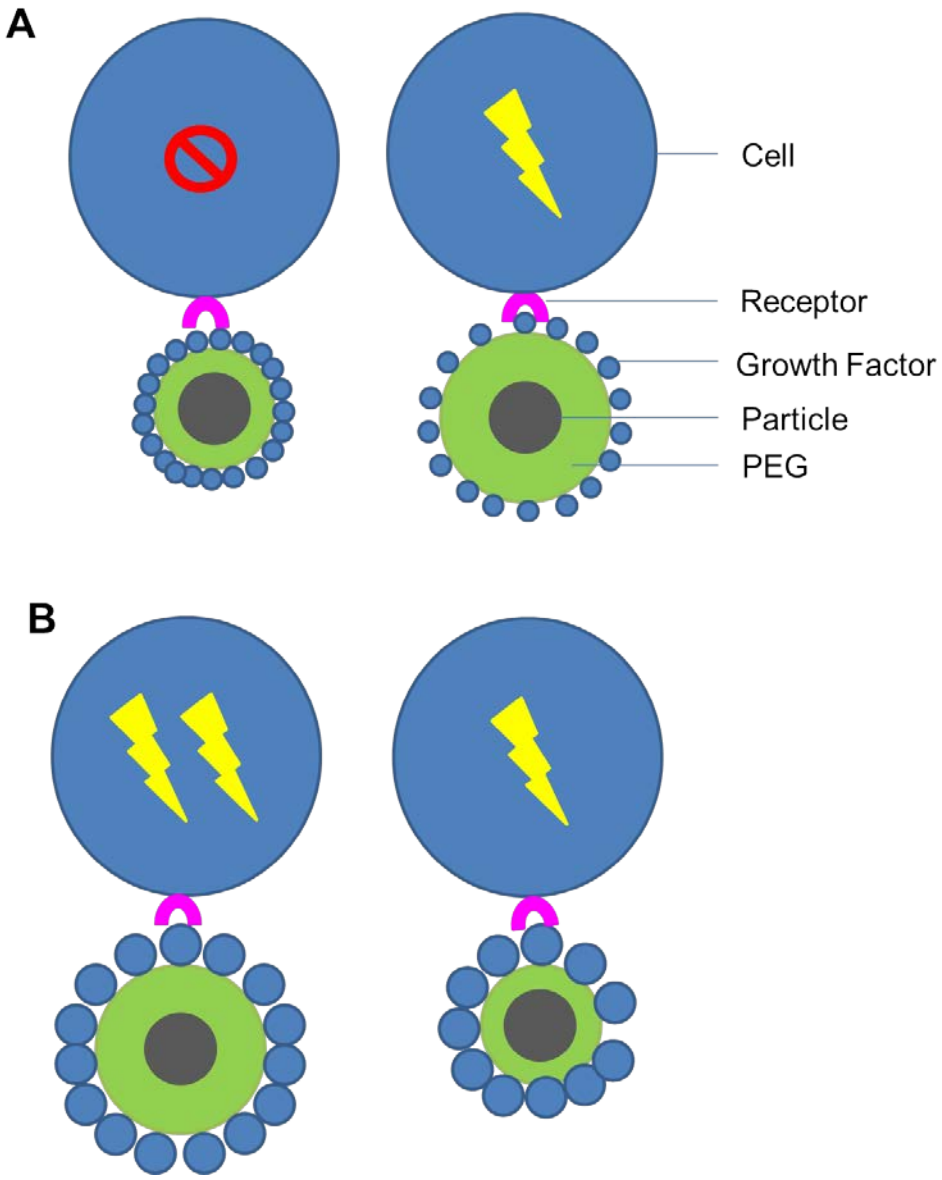


Figure 59. The effect of PEG length on spatial presentation and surface concentration of immobilised growth factors. A) Shows that at a fixed surface concentration smaller PEG molecules will cause steric hindrance between growth factor and receptor binding and by increasing peg length the spatial presentation is optimised allowing binding and cell signalling B) For larger growth factor molecules, a larger PEG length may increase surface concentration of immobilised growth factor and smaller PEG lengths will limit surface concentration by steric hindrance.

In addition to the growth factors highlighted in this thesis that are fundamental for HSC expansion (SCF, TPO, Flt-3l) immobilisation of delta-1 ligand (notch) has been shown to successfully expand HSCs[51], [86]. Immobilising a combination of such growth factors and notch ligands has the potential to recapitulate the stem cell niche – maintaining stem cell

phenotype throughout cell expansion. Combinations of such factors could be introduced individually or co-presented on particles. It is easy to envisage the multiple applications of the immobilisation technology for different cell manufacturing processes. For example, immobilisation of VCAM-1 and ICAM-4 adhesion molecules could recapitulate the erythroblast islands *in vitro* which are fundamental for enucleation in the process of erythropoiesis[178], [179].

To conclude, the novel growth factor immobilisation method described herein has demonstrated many functional, financial and process benefits in comparison to standard soluble growth factors routinely used in cell therapy manufacturing processes. The unique immobilisation chemistry can be tailored to individual growth factors (and combinations of growth factors) in order to optimise surface concentration and spatial presentation for the desired functional effects. Once immobilised onto the particles, they can be manipulated with external magnetic fields, allowing temporal exposure and recycling of the growth factors which otherwise would be (almost) impossible with soluble growth factors. Immobilised growth factors were shown to be significantly more potent and stable and can increase the duration of downstream signalling responses with transient exposure. Overall, the immobilisation method has the potential to be transferred to all scalable cell therapy manufacturing protocols and may have significant impact by reducing cost and improving process control.

7 References

[1] H. L. Greenwood, H. Thorsteinsdottir, G. Perry, J. Renihan, P. A. Singer, and A. S. Daar, "Regenerative medicine: new opportunities for developing countries," *Int. J. Biotechnol.*, vol. 8, no. 1/2, p. 60, 2006.

[2] C. Mason and P. Dunnill, "A brief definition of regenerative medicine," *Regen. Med.*, vol. 3, no. 1, pp. 1–5, Jan. 2008.

[3] L. G. LAJTHA, "Stem Cell Concepts," *Differentiation*, vol. 14, no. 1–3, pp. 23–33, Sep. 1979.

[4] N. Malik, "Allogeneic Versus Autologous Stem-Cell Therapy," *BioPharm Int.*, vol. 25, no. 7, 2012.

[5] T. Wirth, N. Parker, and S. Ylä-Herttuala, "History of gene therapy," *Gene*, vol. 525, no. 2, pp. 162–169, Aug. 2013.

[6] D. Wang and G. Gao, "State-of-the-art human gene therapy: part I. Gene delivery technologies.," *Discov. Med.*, vol. 18, no. 97, pp. 67–77.

[7] J. Dalal, K. Gandy, and J. Domen, "Role of mesenchymal stem cell therapy in Crohn's disease," *Pediatr. Res.*, vol. 71, no. 4–2, pp. 445–451, Apr. 2012.

[8] V. Karantalis and J. M. Hare, "Use of mesenchymal stem cells for therapy of cardiac disease.," *Circ. Res.*, vol. 116, no. 8, pp. 1413–30, Apr. 2015.

[9] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.," *Cell*, vol. 126, no. 4, pp. 663–76, Aug. 2006.

[10] S. Nori, Y. Okada, S. Nishimura, T. Sasaki, G. Itakura, Y. Kobayashi, F. Renault-Mihara, A. Shimizu, I. Koya, R. Yoshida, J. Kudoh, M. Koike, Y. Uchiyama, E. Ikeda, Y. Toyama, M. Nakamura, and H. Okano, "Long-Term Safety Issues of iPSC-Based Cell Therapy in a Spinal Cord Injury Model: Oncogenic Transformation with Epithelial-Mesenchymal Transition," *Stem Cell Reports*, vol. 4, no. 3, pp. 360–373, Mar. 2015.

[11] L. Gattinoni, E. Lugli, Y. Ji, Z. Pos, C. M. Paulos, M. F. Quigley, J. R. Almeida, E. Gostick, Z. Yu, C. Carpenito, E. Wang, D. C. Douek, D. A. Price, C. H. June, F. M. Marincola, M. Roederer, and N. P. Restifo, "A human memory T cell subset with stem cell-like properties.," *Nat. Med.*, vol. 17, no. 10, pp. 1290–7, Sep. 2011.

[12] C. S. Hinrichs, Z. A. Borman, L. Cassard, L. Gattinoni, R. Spolski, Z. Yu, L. Sanchez-Perez, P. Muranski, S. J. Kern, C. Logun, D. C. Palmer, Y. Ji, R. N. Reger, W. J. Leonard, R. L. Danner, S. A. Rosenberg, and N. P. Restifo, "Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior

antitumor immunity," *Proc. Natl. Acad. Sci.*, vol. 106, no. 41, pp. 17469–17474, Oct. 2009.

[13] E. C. Morris, A. Tsallios, G. M. Bendle, S.-A. Xue, and H. J. Stauss, "A critical role of T cell antigen receptor-transduced MHC class I-restricted helper T cells in tumor protection.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 22, pp. 7934–9, May 2005.

[14] S.-K. Tey, "Adoptive T-cell therapy: adverse events and safety switches," *Clin. Transl. Immunol.*, vol. 3, no. 6, p. e17, Jun. 2014.

[15] M. H. Kershaw, J. A. Westwood, and P. K. Darcy, "Gene-engineered T cells for cancer therapy," *Nat. Rev. Cancer*, vol. 13, no. 8, pp. 525–541, Jul. 2013.

[16] H. Sutherland, C. Eaves, A. Eaves, W. Dragowska, and P. Lansdorp, "Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro," *Blood*, vol. 74, no. 5, 1989.

[17] M. Bhatia, J. C. Wang, U. Kapp, D. Bonnet, and J. E. Dick, "Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 94, no. 10, pp. 5320–5, May 1997.

[18] J. R. Passweg, H. Baldomero, P. Bader, C. Bonini, R. F. Duarte, C. Dufour, A. Gennery, N. Kröger, J. Kuball, F. Lanza, S. Montoto, A. Nagler, J. A. Snowden, J. Styczynski, and M. Mohty, "Use of haploidentical stem cell transplantation continues to increase: the 2015 European Society for Blood and Marrow Transplant activity survey report," *Bone Marrow Transplant.*, Mar. 2017.

[19] J.-A. Ribeil, S. Hacein-Bey-Abina, E. Payen, A. Magnani, M. Semeraro, E. Magrin, L. Caccavelli, B. Neven, P. Bourget, W. El Nemer, P. Bartolucci, L. Weber, H. Puy, J.-F. Meritet, D. Grevent, Y. Beuzard, S. Chrétien, T. Lefebvre, R. W. Ross, O. Negre, G. Veres, L. Sandler, S. Soni, M. de Montalembert, S. Blanche, P. Leboulch, and M. Cavazzana, "Gene Therapy in a Patient with Sickle Cell Disease," *N. Engl. J. Med.*, vol. 376, no. 9, pp. 848–855, Mar. 2017.

[20] A. Aiuti, F. Cattaneo, S. Galimberti, U. Benninghoff, B. Cassani, L. Callegaro, S. Scaramuzza, G. Andolfi, M. Mirolo, I. Brigida, A. Tabucchi, F. Carlucci, M. Eibl, M. Aker, S. Slavin, H. Al-Mousa, A. Al Ghonaium, A. Ferster, A. Duppenthaler, L. Notarangelo, U. Wintergerst, R. H. Buckley, M. Bregni, S. Marktel, M. G. Valsecchi, P. Rossi, F. Ciceri, R. Miniero, C. Bordignon, and M.-G. Roncarolo, "Gene Therapy for Immunodeficiency Due to Adenosine Deaminase Deficiency," *N. Engl. J. Med.*, vol. 360, no. 5, pp. 447–458, Jan. 2009.

[21] L. Robb, "Cytokine receptors and hematopoietic differentiation.," *Oncogene*, vol. 26, no. 47, pp. 6715–23, Oct. 2007.

[22] P. Qiu, E. F. Simonds, S. C. Bendall, K. D. Gibbs, R. V Bruggner, M. D. Linderman, K. Sachs, G. P. Nolan, and S. K. Plevritis, "Extracting a cellular hierarchy from high-

dimensional cytometry data with SPADE," *Nat. Biotechnol.*, vol. 29, no. 10, pp. 886–891, Oct. 2011.

[23] C. I. Civin, L. C. Strauss, C. Brovall, M. J. Fackler, J. F. Schwartz, and J. H. Shaper, "Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells.," *J. Immunol.*, vol. 133, no. 1, pp. 157–65, Jul. 1984.

[24] B. Giebel and M. Punzel, "Lineage development of hematopoietic stem and progenitor cells," *Biol. Chem.*, vol. 389, no. 7, pp. 813–24, Jan. 2008.

[25] L. W. Terstappen, S. Huang, M. Safford, P. M. Lansdorp, and M. R. Loken, "Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells.," *Blood*, vol. 77, no. 6, pp. 1218–27, Mar. 1991.

[26] Q. L. Hao, F. T. Thiemann, D. Petersen, E. M. Smogorzewska, and G. M. Crooks, "Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population.," *Blood*, vol. 88, no. 9, pp. 3306–13, Nov. 1996.

[27] A. Larochelle, J. Vormoor, H. Hanenberg, J. C. Y. Wang, M. Bhatia, T. Lapidot, T. Moritz, B. Murdoch, X. L. Xiao, I. Kato, D. A. WIlliams, and J. E. Dick, "Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: Implications for gene therapy," *Nat. Med.*, vol. 2, no. 12, pp. 1329–1337, Dec. 1996.

[28] F. Notta, S. Doulatov, E. Laurenti, A. Poeppl, I. Jurisica, and J. E. Dick, "Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment.," *Science*, vol. 333, no. 6039, pp. 218–21, Jul. 2011.

[29] M. J. Kiel, O. H. Yilmaz, T. Iwashita, O. H. Yilmaz, C. Terhorst, and S. J. Morrison, "SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells.," *Cell*, vol. 121, no. 7, pp. 1109–21, Jul. 2005.

[30] M. B. Kastan, E. Schlaffer, J. E. Russo, O. M. Colvin, C. I. Civin, and J. Hilton, "Direct demonstration of elevated aldehyde dehydrogenase in human hematopoietic progenitor cells.," *Blood*, vol. 75, no. 10, pp. 1947–50, May 1990.

[31] D. A. Hess, L. Wirthlin, T. P. Craft, P. E. Herrbrich, S. A. Hohm, R. Lahey, W. C. Eades, M. H. Creer, and J. A. Nolta, "Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells," vol. 107, no. 5, pp. 2162–2169, 2006.

[32] C. M. Kelley, T. Ikeda, J. Koipally, N. Avitahl, L. Wu, K. Georgopoulos, and B. a Morgan, "Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors.," *Curr. Biol.*, vol. 8, no. 9, pp. 508–15, May 1998.

[33] E. P. L. M. de Grouw, M. H. G. P. Raaijmakers, J. B. Boezeman, B. A. van der Reijden, L. T. F. van de Locht, T. J. M. de Witte, J. H. Jansen, and R. A. P. Raymakers, "Preferential

expression of a high number of ATP binding cassette transporters in both normal and leukemic CD34+CD38− cells," *Leukemia*, vol. 20, no. 4, pp. 750–754, Apr. 2006.

[34] D. A. Hess, T. E. Meyerrose, L. Wirthlin, T. P. Craft, P. E. Herrbrich, M. H. Creer, and J. A. Nolta, "Functional characterization of highly purified human hematopoietic repopulating cells isolated according to aldehyde dehydrogenase activity," *Blood*, vol. 104, pp. 1648–1655, 2004.

[35] Leonard l. Zon, Ed., *Hematopoiesis: A Developmental Approach*. Oxford University Press, 2001.

[36] S. H. Orkin and L. I. Zon, "Hematopoiesis: an evolving paradigm for stem cell biology.," *Cell*, vol. 132, no. 4, pp. 631–44, Feb. 2008.

[37] S. J. Morrison, A. C. Spradling, P. Charnay, D. K. Burns, L. F. Parada, W. G. Tong, J. Ross, J. Haug, T. Johnson, J. Q. Feng, and et al., "Stem cells and niches: mechanisms that promote stem cell maintenance throughout life.," *Cell*, vol. 132, no. 4, pp. 598–611, Feb. 2008.

[38] H. K. A. Mikkola and S. H. Orkin, "The journey of developing hematopoietic stem cells," *Development*, vol. 133, no. 19, pp. 3733–3744, Oct. 2006.

[39] S. J. Morrison and D. T. Scadden, "The bone marrow niche for haematopoietic stem cells.," *Nature*, vol. 505, no. 7483, pp. 327–34, 2014.

[40] K. a Moore and I. R. Lemischka, "Stem cells and their niches.," *Science*, vol. 311, no. 5769, pp. 1880–5, Mar. 2006.

[41] A. Wilson and A. Trumpp, "Bone-marrow haematopoietic-stem-cell niches.," *Nat. Rev. Immunol.*, vol. 6, no. 2, pp. 93–106, Feb. 2006.

[42] C. Nombela-Arrieta, G. Pivarnik, B. Winkel, K. J. Canty, B. Harley, J. E. Mahoney, S.-Y. Park, J. Lu, A. Protopopov, and L. E. Silberstein, "Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment," *Nat. Cell Biol.*, vol. 15, no. 5, pp. 533–543, Apr. 2013.

[43] W. Bensinger, J. F. DiPersio, and J. M. McCarty, "Improving stem cell mobilization strategies: future directions," *Bone Marrow Transplant.*, vol. 43, no. 3, pp. 181–195, Feb. 2009.

[44] S. Civriz Bozdag, E. Tekgunduz, and F. Altuntas, "The current status in hematopoietic stem cell mobilization," *J. Clin. Apher.*, vol. 30, no. 5, pp. 273–280, Oct. 2015.

[45] E. Csaszar, D. C. Kirouac, M. Yu, W. Wang, W. Qiao, M. P. Cooke, A. E. Boitano, C. Ito, and P. W. Zandstra, "Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling.," *Cell Stem Cell*, vol. 10, no. 2, pp. 218–29, Feb. 2012.

[46] A. Dahlberg and F. Milano, “Cord blood transplantation: rewind to fast forward,” *Bone Marrow Transplant.*, Dec. 2016.

[47] J. N. Barker, C. E. Byam, N. A. Kernan, S. S. Lee, R. M. Hawke, K. A. Doshi, D. S. Wells, G. Heller, E. B. Papadopoulos, A. Scaradavou, J. W. Young, and M. R. M. van den Brink, “Availability of Cord Blood Extends Allogeneic Hematopoietic Stem Cell Transplant Access to Racial and Ethnic Minorities,” *Biol. Blood Marrow Transplant.*, vol. 16, no. 11, pp. 1541–1548, Nov. 2010.

[48] D. Allan, T. Petraszko, H. Elmoazzen, and S. Smith, “A review of factors influencing the banking of collected umbilical cord blood units.,” *Stem Cells Int.*, vol. 2013, p. 463031, 2013.

[49] Y.-H. Lee, J. Y. Kim, Y.-C. Mun, and H. H. Koo, “A proposal for improvement in the utilization rate of banked cord blood.,” *Blood Res.*, vol. 48, no. 1, pp. 5–7, Mar. 2013.

[50] A. Sideri, N. Neokleous, P. Brunet De La Grange, B. Guerton, M.-C. Le Bousse Kerdilles, G. Uzan, C. Peste-Tsilimidos, and E. Gluckman, “An overview of the progress on double umbilical cord blood transplantation.,” *Haematologica*, vol. 96, no. 8, pp. 1213–20, Aug. 2011.

[51] a. Dahlberg, C. Delaney, and I. D. Bernstein, “Ex vivo expansion of human hematopoietic stem and progenitor cells,” *Blood*, vol. 117, no. 23, pp. 6083–6090, 2011.

[52] Y. Kong, Y. Hu, Y. Song, Y.-T. Wang, Z.-F. Jiang, H.-K. Deng, and X. J. Huang, “Increased Levels of Reactive Oxygen Species and Exhaustion of the Quiescent CD34+ Cells May Operate in Poor Graft Function after Allogeneic Hematopoietic Stem Cell Transplantation,” *Blood*, vol. 126, no. 23, 2015.

[53] Y. Kong, Y. Song, Y. Hu, M.-M. Shi, Y.-T. Wang, Y. Wang, X.-H. Zhang, L.-P. Xu, K.-Y. Liu, H.-K. Deng, and X.-J. Huang, “Increased reactive oxygen species and exhaustion of quiescent CD34-positive bone marrow cells may contribute to poor graft function after allotransplants,” *Oncotarget*, vol. 7, no. 21, pp. 30892–906, Nov. 2014.

[54] P. R. T. Archibald, A. Chandra, D. Thomas, G. Morley, T. Lekishvili, A. Devonshire, and D. J. Williams, “Comparability of scalable, automated hMSC culture using manual and automated process steps,” *Biochem. Eng. J.*, vol. 108, pp. 69–83, Apr. 2016.

[55] C. Lehmann, S. Scaramuzza, K. Reck, M. Essl, R. J. Hernandez, S. La Seta Catamancio, C. Bovolenta, A. Aiuti, and I. C. D. Johnston, “Automated Generation of Genetically Modified Human CD34+ Cells in a Functionally Closed System,” *Mol. Ther.*, vol. 23, p. S185, May 2015.

[56] I. C. D. Johnston, “Automated Magnetic Isolation and Lentiviral Vector Modification of Human CD34+ Cells in a Functionally Closed System,” *Mol. Ther.*, vol. 22, pp. S283–S284, May 2014.

[57] U. Mock, L. Nickolay, B. Philip, G. W.-K. Cheung, H. Zhan, I. C. D. Johnston, A. D. Kaiser, K. Peggs, M. Pule, A. J. Thrasher, and W. Qasim, "Automated manufacturing of chimeric antigen receptor T cells for adoptive immunotherapy using CliniMACS Prodigy," *Cytotherapy*, vol. 18, no. 8, pp. 1002–1011, Aug. 2016.

[58] Y. Y. Lipsitz, N. E. Timmins, and P. W. Zandstra, "Quality cell therapy manufacturing by design," *Nat. Biotechnol.*, vol. 34, no. 4, pp. 393–400, Apr. 2016.

[59] M. a Walasek, R. van Os, and G. de Haan, "Hematopoietic stem cell expansion: challenges and opportunities.," *Ann. N. Y. Acad. Sci.*, vol. 1266, pp. 138–50, Aug. 2012.

[60] H. Hentze, P. L. Soong, S. T. Wang, B. W. Phillips, T. C. Putti, and N. R. Dunn, "Teratoma formation by human embryonic stem cells: Evaluation of essential parameters for future safety studies," *Stem Cell Res.*, vol. 2, no. 3, pp. 198–210, May 2009.

[61] D. Thirabanjasak, K. Tantiwongse, and P. S. Thorner, "Angiomyeloproliferative Lesions Following Autologous Stem Cell Therapy," *J. Am. Soc. Nephrol.*, vol. 21, no. 7, pp. 1218–1222, Jul. 2010.

[62] P. W. Marks, C. M. Witten, and R. M. Califf, "Clarifying Stem-Cell Therapy's Benefits and Risks," *N. Engl. J. Med.*, p. NEJMp1613723, Nov. 2016.

[63] R. Levi-Montalcini and S. Cohen, "In vitro and In vivo Effects of Nerve Growth Stimulating Agent Isolated from Snake Venom," *Proc Natl Acad Sci USA*, vol. 42, no. 9, pp. 695–699, 1956.

[64] S. Cohen and R. Levi-Montalcini, "Purification and Properties of a Nerve Growth-promoting Factor Isolated from Mouse Sarcoma 180 *," *Proc Natl Acad Sci USA*, vol. 46, no. 3, pp. 302–311, 1960.

[65] S. Cohen and G. Elliott, "The stimulation of epidermal keratinization by a protein isolated from the submaxillary gland of the mouse," *J. Invest. Dermatol.*, vol. 40, pp. 1–5, 1963.

[66] A. Isaacs and J. Lindenmann, "Virus Interference. I. The interferon," *R. Soc. Publ.*, vol. 147, no. 927, pp. 258–267, 1957.

[67] R. Harrison and M. Greenman, "Observations of the Living Developing Nerve Fiber," *Anat. Rec.*, vol. 1, no. 5, pp. 116–128, 1907.

[68] R. Harrison, "The outgrowth of the nerve fiber as a mode of protoplasmic movement," *J. Exp. Zool.*, vol. 9, no. 4787–846, 1910.

[69] A. Carrel, "On the Permanent Life of Tissues Outside of the Organism," *J. Exp. Med.*, vol. 15, no. 5, pp. 516–528, 1912.

[70] A. Carrel, “The Culture of Whole Organs,” *J. Exp. Med.*, vol. 65, no. 4, pp. 515–526, 1937.

[71] H. Temin, R. Pierson, and N. Dulak, “THE ROLE OF SERUM IN THE CONTROL OF MULTIPLICATION OF AVIAN AND MAMMALIAN CELLS IN CULTURE,” in *Growth Nutrition and Metabolism of Cells in Culture*, 1972, pp. 49–81.

[72] D. Barnes and G. Sato, “Serum-free cell culture: a unifying approach.,” *Cell*, vol. 22, no. 3, pp. 649–655, 1980.

[73] J. Taipale and J. Keski-Oja, “Growth factors in the extracellular matrix,” *FASEB*, vol. 11, pp. 51–59, 1997.

[74] P. Tayalia and D. J. Mooney, “Controlled Growth Factor Delivery for Tissue Engineering,” *Adv. Mater.*, vol. 21, pp. 3269–3285, 2009.

[75] V. C. Broudy, “Stem cell factor and hematopoiesis.,” *Blood*, vol. 90, no. 4, pp. 1345–64, Aug. 1997.

[76] H. J. McKenna, K. L. Stocking, R. E. Miller, K. Brasel, T. De Smedt, E. Maraskovsky, C. R. Maliszewski, D. H. Lynch, J. Smith, B. Pulendran, E. R. Roux, M. Teepe, S. D. Lyman, and J. J. Peschon, “Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells.,” *Blood*, vol. 95, no. 11, pp. 3489–97, Jun. 2000.

[77] E. Edelman, M. Nugent, and M. Karnovsky, “Perivascular and intravenous administration of basic fibroblast growth factor : Vascular and solid organ deposition,” *PNAS*, vol. 90, pp. 1513–1517, 1993.

[78] L. Naldini, L. Tamagnone, E. Vigna, M. Sachs, G. Hartmann, W. Birchmeier, Y. Daikuhara, H. Tsubouchi, F. Blasi, and P. M. Comoglio, “Extracellular proteolytic cleavage by urokinase is required for activation of hepatocyte growth factor / scatter factor,” *EMBO*, vol. 11, no. 13, pp. 4825–4833, 1992.

[79] J. Taipale, K. Kolis, and J. Keski-ojasgli, “Release of Transforming Growth Factor-β1 from the Pericellular Matrix of Cultured Fibroblasts and Fibrosarcoma Cells by Plasmin and Thrombin,” *J. Biol. Chem.*, vol. 267, no. 35, pp. 25378–25384, 1992.

[80] A. Partenheimer, K. Schwarz, C. Wrocklage, E. Kölsch, and H. Kresse, “Proteoglycan form of colony-stimulating factor-1 (proteoglycan-100). Stimulation of activity by glycosaminoglycan removal and proteolytic processing.,” *J. Immunol.*, vol. 155, no. 12, pp. 5557–65, Dec. 1995.

[81] M. Ehrbar, V. G. Djonov, C. Schnell, S. a Tschanz, G. Martiny-Baron, U. Schenk, J. Wood, P. H. Burri, J. a Hubbell, and A. H. Zisch, “Cell-demanded liberation of VEGF121 from fibrin implants induces local and controlled blood vessel growth.,” *Circ. Res.*, vol. 94, no. 8, pp. 1124–32, Apr. 2004.

[82] M. Kasugat, “Protein-free cell culture on an artificial substrate with covalently immobilized insulin,” vol. 93, no. April, pp. 3598–3601, 1996.

[83] F. Aratsu, I. Harada, S. Yoshimura, C.-S. Cho, T. Akaike, and Y. Tagawa, “Dynamic chemotactic response of fibroblasts to local stimulation using EGF-immobilized microbeads.,” *Biomaterials*, vol. 35, no. 8, pp. 2471–6, Mar. 2014.

[84] a G. Guex, D. Hegemann, M. N. Giraud, H. T. Tevaearai, a M. Popa, R. M. Rossi, and G. Fortunato, “Covalent immobilisation of VEGF on plasma-coated electrospun scaffolds for tissue engineering applications.,” *Colloids Surf. B. Biointerfaces*, vol. 123, pp. 724–33, Nov. 2014.

[85] Y. Ito, “Covalently immobilized biosignal molecule materials for tissue engineering,” *Soft Matter*, vol. 4, no. 1, pp. 46–56, 2008.

[86] C. Delaney, B. Varnum-Finney, K. Aoyama, C. Brashem-Stein, and I. D. Bernstein, “Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells.,” *Blood*, vol. 106, no. 8, pp. 2693–9, Oct. 2005.

[87] T. Pompe, K. Salchert, K. Alberti, P. Zandstra, and C. Werner, “Immobilization of growth factors on solid supports for the modulation of stem cell fate.,” *Nat. Protoc.*, vol. 5, no. 6, pp. 1042–50, Jun. 2010.

[88] H. Markides, M. Rotherham, and a. J. El Haj, “Biocompatibility and Toxicity of Magnetic Nanoparticles in Regenerative Medicine,” *J. Nanomater.*, vol. 2012, pp. 1–11, 2012.

[89] Y. Li and R. J. Kurlander, “Comparison of anti-CD3 and anti-CD28-coated beads with soluble anti-CD3 for expanding human T cells: differing impact on CD8 T cell phenotype and responsiveness to restimulation.,” *J. Transl. Med.*, vol. 8, p. 104, Oct. 2010.

[90] H. Dai, Y. Wang, X. Lu, and W. Han, “Chimeric Antigen Receptors Modified T-Cells for Cancer Therapy,” *JNCI J Natl Cancer Inst*, vol. 108, no. 7, 2016.

[91] R. Archer and D. J. Williams, “Why tissue engineering needs process engineering,” *Nat. Biotechnol.*, vol. 23, no. 11, pp. 1353–1355, Nov. 2005.

[92] B. O. Palsson, S. H. Paek, R. M. Schwartz, M. Palsson, G. M. Lee, S. Silver, and S. G. Emerson, “Expansion of human bone marrow progenitor cells in a high cell density continuous perfusion system.,” *Biotechnology. (N. Y).*, vol. 11, no. 3, pp. 368–72, Mar. 1993.

[93] J. Caldwell, W. Wang, and P. W. Zandstra, “Proportional-Integral-Derivative (PID) Control of Secreted Factors for Blood Stem Cell Culture,” *PLoS One*, vol. 10, no. 9, p. e0137392, 2015.

[94] M. Lim, H. Ye, N. Panoskaltsis, E. M. Drakakis, X. Yue, A. E. G. Cass, A. Radomska, and A. Mantalaris, “Intelligent bioprocessing for haemotopoietic cell cultures using monitoring and design of experiments.,” *Biotechnol. Adv.*, vol. 25, no. 4, pp. 353–68, 2007.

[95] S. S. Ozturk, J. C. Thrift, J. D. Blackie, and D. Naveh, “Real-time monitoring and control of glucose and lactate concentrations in a mammalian cell perfusion reactor.,” *Biotechnol. Bioeng.*, vol. 53, no. 4, pp. 372–8, 1997.

[96] D. C. Kirouac and P. W. Zandstra, “The Systematic Production of Cells for Cell Therapies,” *Cell Stem Cell*, vol. 3, no. 4, pp. 369–381, 2008.

[97] E. Ratcliffe, R. J. Thomas, and D. J. Williams, “Current understanding and challenges in bioprocessing of stem cell-based therapies for regenerative medicine.,” *Br. Med. Bull.*, vol. 100, pp. 137–55, 2011.

[98] J. Rowley, E. Abraham, A. Campbell, H. Brandwein, and S. Oh, “Meeting lot-size challenges of manufacturing adherent cells for therapy,” *Bioprocess Int.*, vol. 10, no. SUPPL. 3, pp. 16–22, 2012.

[99] N. S. Hwang, S. Varghese, and J. Elisseeff, “Controlled differentiation of stem cells,” *Adv. Drug Deliv. Rev.*, vol. 60, no. 2, pp. 199–214, Jan. 2008.

[100] R. Peerani and P. W. Zandstra, “Enabling stem cell therapies through synthetic stem cell–niche engineering,” *J. Clin. Invest.*, vol. 120, no. 1, pp. 60–70, Jan. 2010.

[101] G. Hermanson, *Bioconjugation Techniques*. Elsevier Inc., 2008.

[102] S. Rameez, S. S. Mostafa, C. Miller, and A. A. Shukla, “High-throughput miniaturized bioreactors for cell culture process development: Reproducibility, scalability, and control,” *Biotechnol. Prog.*, vol. 30, no. 3, pp. 718–727, May 2014.

[103] C. M. Nelson and C. S. Chen, “Cell-cell signaling by direct contact increases cell proliferation via a PI3K-dependent signal,” *FEBS Lett.*, vol. 514, pp. 238–242, 2002.

[104] P. Eliasson and J.-I. Jönsson, “The hematopoietic stem cell niche: low in oxygen but a nice place to be.,” *J. Cell. Physiol.*, vol. 222, no. 1, pp. 17–22, 2010.

[105] Y. Berthois, J. a Katzenellenbogen, and B. S. Katzenellenbogen, “Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 83, no. 8, pp. 2496–2500, 1986.

[106] G. L. TRITSCH and G. E. MOORE, “Spontaneous decomposition of glutamine in cell culture media.,” *Exp. Cell Res.*, vol. 28, pp. 360–4, Nov. 1962.

[107] S. S. Ozturk and B. O. Palsson, “Chemical decomposition of glutamine in cell culture media: effect of media type, pH, and serum concentration,” *Biotechnol. Prog.*, vol. 6, no. 2, pp. 121–128, Mar. 1990.

[108] C. S. Sevier and C. a Kaiser, “Formation and transfer of disulphide bonds in living cells.,” *Nat. Rev. Mol. Cell Biol.*, vol. 3, no. 11, pp. 836–47, Nov. 2002.

[109] D. Delcassian, D. Depoil, D. Rudnicka, M. Liu, D. David, M. Dustin, and L. Dunlop, “Nanoscale ligand spacing influences receptor triggering in T cells and NK cells,” vol. 13, no. 11, pp. 5608–5614, 2013.

[110] A. N. L. Batista, J. M. Batista, V. S. Bolzani, M. Furlan, and E. W. Blanch, “Selective DMSO-induced conformational changes in proteins from Raman optical activity.,” *Phys. Chem. Chem. Phys.*, vol. 15, no. 46, pp. 20147–52, 2013.

[111] T. Arakawa, Y. Kita, and S. N. Timasheff, “Protein precipitation and denaturation by dimethyl sulfoxide,” *Biophys. Chem.*, vol. 131, no. 1–3, pp. 62–70, 2007.

[112] L. Gao, J. Zhuang, L. Nie, J. Zhang, Y. Zhang, N. Gu, T. Wang, J. Feng, D. Yang, S. Perrett, and X. Yan, “Intrinsic peroxidase-like activity of ferromagnetic nanoparticles.,” *Nat. Nanotechnol.*, vol. 2, no. 9, pp. 577–83, Sep. 2007.

[113] F. Jin and W. Krzyzanski, “Pharmacokinetic Model of Target-Mediated Disposition of Thrombopoietin,” *AAPS PharmSci*, vol. 6, no. 1, pp. 1–8, 2004.

[114] P. J. Tummino and R. A. Copeland, “Residence Time of Receptor-Ligand Complexes and Its Effect on Biological Function,” *Biochemistry*, vol. 47, no. 20, pp. 5481–5492, 2008.

[115] H. Shankaran, H. S. Wiley, and H. Resat, “Receptor downregulation and desensitization enhance the information processing ability of signalling receptors.,” *BMC Syst. Biol.*, vol. 1, p. 48, Jan. 2007.

[116] N. S. Yeesq and H. Langenl, “Mechanism of kit Ligand , Phorbol Ester , and Calcium-induced Down-regulation of c-kit Receptors in Mast Cells *,” no. 19, pp. 14189–14201, 1993.

[117] P. M. Comoglio, C. Boccaccio, and L. Trusolino, “Interactions between growth factor receptors and adhesion molecules: breaking the rules,” *Curr. Opin. Cell Biol.*, vol. 15, no. 5, pp. 565–571, Oct. 2003.

[118] G. J. Tortora and B. Derrickson, *Principles of Anatomy and Physiology.* John Wiley & Sons, 2011.

[119] C. Leclerc, C. Brose, C. Nouzé, F. Leonard, L. Majlessi, S. Becker, H. von Briesen, and R. Lo-Man, “Immobilized cytokines as biomaterials for manufacturing immune cell based vaccines,” *J. Biomed. Mater. Res. Part A*, vol. 86A, no. 4, pp. 1033–1040, Sep. 2008.

[120] K. Alberti, R. E. Davey, K. Onishi, S. George, K. Salchert, F. P. Seib, T. Pompe, A. Nagy, C. Werner, and P. W. Zandstra, “Functional immobilization of signaling proteins enables control of stem cell fate,” vol. 5, no. 7, 2008.

[121] A. Nur-E-Kamal, I. Ahmed, J. Kamal, A. N. Babu, M. Schindler, and S. Meiners, “Covalently attached FGF-2 to three-dimensional polyamide nanofibrillar surfaces demonstrates enhanced biological stability and activity,” *Mol. Cell. Biochem.*, vol. 309, no. 1–2, pp. 157–166, Feb. 2008.

[122] E. M. Pietras, M. R. Warr, and E. Passegué, “Cell cycle regulation in hematopoietic stem cells.,” *J. Cell Biol.*, vol. 195, no. 5, pp. 709–20, Nov. 2011.

[123] Y. Soen, A. Mori, T. D. Palmer, and P. O. Brown, “Exploring the regulation of human neural precursor cell differentiation using arrays of signaling microenvironments.,” *Mol. Syst. Biol.*, vol. 2, p. 37, 2006.

[124] M. Delaney and K. K. Ballen, “The role of HLA in umbilical cord blood transplantation,” *Best Pract. Res. Clin. Haematol.*, vol. 23, no. 2, pp. 179–187, Jun. 2010.

[125] J. E. Wagner and E. Gluckman, “Umbilical Cord Blood Transplantation: The First 20 Years,” *Semin. Hematol.*, vol. 47, no. 1, pp. 3–12, Jan. 2010.

[126] M. W. Naing, D. a Gibson, P. Hourd, S. G. Gomez, R. B. V Horton, J. Segal, and D. J. Williams, “Improving umbilical cord blood processing to increase total nucleated cell count yield and reduce cord input wastage by managing the consequences of input variation.,” *Cytotherapy*, vol. 17, no. 1, pp. 58–67, 2015.

[127] T. Peled, E. Landau, J. Mandel, E. Glukhman, N. R. Goudsmid, A. Nagler, and E. Fibach, “Linear polyamine copper chelator tetraethylenepentamine augments long-term ex vivo expansion of cord blood-derived CD34+ cells and increases their engraftment potential in NOD/SCID mice.,” *Exp. Hematol.*, vol. 32, no. 6, pp. 547–55, 2004.

[128] T. Peled, E. Landau, E. Prus, A. J. Treves, and E. Fibach, “Cellular copper content modulates differentiation and self-renewal in cultures of cord blood-derived CD34+ cells,” *Br. J. Haematol.*, vol. 116, no. 3, pp. 655–661, 2002.

[129] F. Milano, S. Heimfeld, I. B. Riffkin, I. Nicoud, F. R. Appelbaum, I. D. Bernstein, and C. Delaney, “Infusion of a Non HLA-Matched Off-the-Shelf Ex Vivo Expanded Cord Blood Progenitor Cell Product Following Myeloablative Cord Blood Transplantation Is Safe, Decreases the Time to Hematopoietic Recovery, and Results in Excellent Overall Survival,” *Blood*, vol. 124, no. 21, pp. 46–46, 2014.

[130] D. M. Gerhardt, K. V Pajcini, T. D’altri, L. Tu, R. Jain, L. Xu, M. J. Chen, S. Rentschler, O. Shestova, G. B. Wertheim, J. W. Tobias, M. Kluk, A. W. Wood, J. C. Aster, P. A. Gimotty, J. A. Epstein, N. Speck, A. Bigas, and W. S. Pear, “The Notch1 transcriptional activation domain is required for development and reveals a novel role for Notch1 signaling in fetal hematopoietic stem cells.,” *Genes Dev.*, vol. 28, no. 6, pp. 576–93, Mar. 2014.

[131] T. Peled, S. Adi, I. Peleg, N. G. Rosenheimer, Y. Daniely, A. Nagler, E. Fibach, and A. Peled, “Nicotinamide Modulates Ex-Vivo Expansion of Cord Blood Derived CD34+ Cells Cultured with Cytokines and Promotes Their Homing and Engraftment in SCID Mice.,” *Blood*, vol. 108, no. 11, 2015.

[132] S. R. Narala, R. C. Allsopp, T. B. Wells, G. Zhang, P. Prasad, M. J. Coussens, D. J. Rossi, I. L. Weissman, and H. Vaziri, “SIRT1 acts as a nutrient-sensitive growth suppressor and its loss is associated with increased AMPK and telomerase activity.,” *Mol. Biol. Cell*, vol. 19, no. 3, pp. 1210–9, Mar. 2008.

[133] J. E. Wagner, C. Brunstein, D. McKenna, D. Sumstad, S. Maahs, M. Laughlin, M. S. Perry, A. E. Boitano, M. P. Cooke, and C. C. Bleul, “StemRegenin-1 (SR1) Expansion Culture Abrogates the Engraftment Barrier Associated with Umbilical Cord Blood Transplantation (UCBT),” *Blood*, vol. 124, no. 21, 2014.

[134] K. P. Singh, F. L. Casado, L. A. Opanashuk, and T. A. Gasiewicz, “The aryl hydrocarbon receptor has a normal function in the regulation of hematopoietic and other stem/progenitor cell populations,” *Biochem. Pharmacol.*, vol. 77, no. 4, pp. 577–587, Feb. 2009.

[135] B. Varnum-Finney, C. Brashem-Stein, and I. D. Bernstein, “Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability,” *Blood*, vol. 101, no. 5, pp. 1784–1789, 2003.

[136] P. W. Zandstra, E. Conneally, a L. Petzer, J. M. Piret, and C. J. Eaves, “Cytokine manipulation of primitive human hematopoietic cell self-renewal.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 94, no. 9, pp. 4698–703, May 1997.

[137] C. C. Hofmeister, J. Zhang, K. L. Knight, P. Le, and P. J. Stiff, “Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche,” *Bone Marrow Transpl.*, vol. 39, no. 1, pp. 11–23, 2007.

[138] H. Hassan and A. Zander, “Stem Cell Factor as a survival and growth factor in human normal and malignant hematopoiesis.,” *Acta Haematol*, vol. 95, pp. 257–262, 1996.

[139] M. B. Bowie, D. G. Kent, M. R. Copley, and C. J. Eaves, “Steel factor responsiveness regulates the high self-renewal phenotype of fetal hematopoietic stem cells.,” *Blood*, vol. 109, no. 11, pp. 5043–8, Jun. 2007.

[140] D. L. DiGiusto, R. Lee, J. Moon, K. Moss, T. O’Toole, a Voytovich, D. Webster, and J. J. Mule, “Hematopoietic potential of cryopreserved and ex vivo manipulated umbilical cord blood progenitor cells evaluated in vitro and in vivo.,” *Blood*, vol. 87, no. 4, pp. 1261–71, Feb. 1996.

[141] P. H. Shaw, D. Gilligan, X.-M. Wang, P. F. Thall, and S. J. Corey, “Ex vivo expansion of megakaryocyte precursors from umbilical cord blood CD34 cells in a closed liquid culture system.,” *Biol. Blood Marrow Transplant.*, vol. 9, no. 3, pp. 151–6, Mar. 2003.

[142] P. C. Collins, L. K. Nielsen, S. D. Patel, E. T. Papoutsakis, and W. M. Miller, “and Glycolysis in a Controlled , Stirred-Tank Bioreactor System,” pp. 466–472, 1998.

[143] Z. Du, H. Cai, Z. Ye, and W.-S. Tan, “Optimization of SCF feeding regimen for ex vivo expansion of cord blood hematopoietic stem cells.,” *J. Biotechnol.*, vol. 164, no. 2, pp. 211–9, Dec. 2012.

[144] L. Pierelli, G. Scambia, a Fattorossi, G. Bonanno, a Battaglia, C. Rumi, M. Marone, S. Mozzetti, S. Rutella, G. Menichella, V. Romeo, S. Mancuso, and G. Leone, “Functional, phenotypic and molecular characterization of cytokine low-responding circulating CD34+ haemopoietic progenitors.,” *Br. J. Haematol.*, vol. 102, no. 5, pp. 1139–50, Sep. 1998.

[145] a Rice, C. Flemming, J. Case, J. Stevenson, L. Gaudry, and M. Vowels, “Comparative study of the in vitro behavior of cord blood subpopulations after short-term cytokine exposure.,” *Bone Marrow Transplant.*, vol. 23, no. 3, pp. 211–20, Feb. 1999.

[146] I. D. Lewis, “Umbilical cord blood cells capable of engrafting in primary, secondary, and tertiary xenogeneic hosts are preserved after ex vivo culture in a noncontact system,” *Blood*, vol. 97, no. 11, pp. 3441–3449, Jun. 2001.

[147] E. Ratcliffe, K. E. Glen, V. L. Workman, a J. Stacey, and R. J. Thomas, “A novel automated bioreactor for scalable process optimisation of haematopoietic stem cell culture.,” *J. Biotechnol.*, vol. 161, no. 3, pp. 387–90, Oct. 2012.

[148] C. Zhang and H. Lodish, “Cytokines regulating hematopoietic stem cell function.,” vol. 15, no. 4, pp. 307–311, 2009.

[149] M. Kobayashi, T. Kato, H. Miyazaki, and M. Ogawa, “Thrombopoietin supports proliferation of human primitive hematopoietic cells in synergy with steel factor and/or interleukin-3,” pp. 429–436, 2013.

[150] C. Robin, K. Ottersbach, C. Durand, M. Peeters, L. Vanes, V. Tybulewicz, and E. Dzierzak, “An unexpected role for IL-3 in the embryonic development of hematopoietic stem cells.,” *Dev. Cell*, vol. 11, no. 2, pp. 171–80, Aug. 2006.

[151] A. Nitsche, I. Junghahn, S. Thulke, J. Aumann, A. Radonić, I. Fichtner, and W. S. M.D., “Interleukin-3 Promotes Proliferation and Differentiation of Human Hematopoietic Stem Cells but Reduces Their Repopulation Potential in NOD/SCID Mice,” *Stem Cells*, vol. 21, pp. 236–244, 2003.

[152] H. Toshio, T. Tetsuya, M. Tadashi, H. Masahiko, S. Sachiko, T. Bo, and K. Masaaki, Murakami Tadamitsu, “Interleukin 6 and its receptor in the immune response and hematopoiesis.,” *Int. J. Cell Cloning.*, vol. 8, pp. 155–167, 1990.

[153] H. H. Nandurkar, L. Robb, and C. G. Begley, “The role of IL-II in hematopoiesis as revealed by a targeted mutation of its receptor.,” *Stem Cells*, vol. 16 Suppl 2, no. suppl 2, pp. 53–65, Jan. 1998.

[154] N. Ahmed, M. A. Khokher, and H. T. Hassan, “Cytokine Induced Expansion of Human CD34+ stem/Progenitor and CD34+ CD41+ Early Megakaryocytic Marrow Cells Cultured on Normal Osteoblasts,” *Stem Cells*, vol. 17, no. 2, pp. 92–99, Mar. 1999.

[155] a J. Shah, E. M. Smogorzewska, C. Hannum, and G. M. Crooks, “Flt3 ligand induces proliferation of quiescent human bone marrow CD34+CD38- cells and maintains progenitor cells in vitro.,” *Blood*, vol. 87, no. 9, pp. 3563–70, May 1996.

[156] D. N. Haylock, M. J. Horsfall, T. L. Dowse, H. S. Ramshaw, S. Niutta, S. Protopsaltis, L. Peng, C. Burrell, I. Rappold, H. J. Buhring, and P. J. Simmons, “Increased recruitment of hematopoietic progenitor cells underlies the ex vivo expansion potential of FLT3 ligand.,” *Blood*, vol. 90, no. 6, pp. 2260–72, Sep. 1997.

[157] I. McNiece, D. Kubegov, P. Kerzic, E. J. Shpall, and S. Gross, “Increased expansion and differentiation of cord blood products using a two-step expansion culture.,” *Exp. Hematol.*, vol. 28, no. 10, pp. 1181–6, Oct. 2000.

[158] P. Kuhl and L. Griffith-Cima, “Tethered epidermal growth factor as a paradigm for growth factor-induced stimulation from the solid phase.,” *Nat. Med.*, vol. 2, no. 9, pp. 1022–7, 1996.

[159] E. J. Jervis, M. M. Guarna, J. G. Doheny, C. A. Haynes, and D. G. Kilburn, “Dynamic localization and persistent stimulation of factor-dependent cells by a stem cell factor / cellulose binding domain fusion protein,” *Biotechnol. Bioeng.*, vol. 91, no. 3, pp. 314–324, Aug. 2005.

[160] P. Li, S. Yuan, and J. Galipeau, “A Fusion Cytokine Coupling GMCSF to IL9 Induces Heterologous Receptor Clustering and STAT1 Hyperactivation through JAK2 Promiscuity,” *PLoS One*, vol. 8, no. 7, p. e69405, Jul. 2013.

[161] R. a Fox, M. Sigman, and K. I. M. Boekelheide, “Transmembrane Versus Soluble Stem Cell Factor Expression,” *Lab. Med.*, vol. 21, no. 4, 2000.

[162] E. J. Huang, K. H. Nocka, J. Buck, and P. Besmer, “Differential expression and processing of two cell associated forms of the kit-ligand: KL-1 and KL-2.,” *Mol. Biol. Cell*, vol. 3, no. 3, pp. 349–62, 1992.

[163] K. NAKAYAMA and K. NAKAYAMA, “Regulation of the cell cycle by SCF-type ubiquitin ligases,” *Semin. Cell Dev. Biol.*, vol. 16, no. 3, pp. 323–333, Jun. 2005.

[164] A. Silva, É. Silva-freitas, J. Carvalho, T. Pontes, R. Araújo-neto, K. Silva, A. Carriço, and E. Egito, “Magnetic Particles in Biotechnology : From Drug Targeting to Tissue Engineering.”

[165] M. R. K. Ali, S. R. Panikkanvalappil, and M. A. El-Sayed, “Enhancing the Efficiency of Gold Nanoparticles Treatment of Cancer by Increasing Their Rate of Endocytosis and Cell Accumulation Using Rifampicin,” *J. Am. Chem. Soc.*, vol. 136, no. 12, pp. 4464–4467, Mar. 2014.

[166] E. D. Gundelfinger, M. M. Kessels, and B. Qualmann, “Temporal and spatial coordination of exocytosis and endocytosis,” *Nat. Rev. Mol. Cell Biol.*, vol. 4, no. 2, pp. 127–139, Feb. 2003.

[167] D. Raucher and M. P. Sheetz, “Membrane expansion increases endocytosis rate during mitosis.,” *J. Cell Biol.*, vol. 144, no. 3, pp. 497–506, Feb. 1999.

[168] G. J. M. Cabrita, B. S. Ferreira, C. L. da Silva, R. Gonçalves, G. Almeida-Porada, and J. M. S. Cabral, “Hematopoietic stem cells: from the bone to the bioreactor.,” *Trends Biotechnol.*, vol. 21, no. 5, pp. 233–40, May 2003.

[169] V. H. Fan, A. Au, K. Tamama, R. Littrell, L. B. Richardson, J. W. Wright, A. Wells, and L. G. Griffith, “Tethered Epidermal Growth Factor Provides a Survival Advantage to Mesenchymal Stem Cells,” *Stem Cells*, vol. 25, no. 5, pp. 1241–1251, May 2007.

[170] S. S. Wong, *Chemistry of Protein Conjugation and Cross-Linking - Shan S. Wong - Google Books*. CRC Press LLC, 1991.

[171] S. Chen, E. C. So, S. E. Strome, and X. Zhang, “Impact of Detachment Methods on M2 Macrophage Phenotype and Function,” *J. Immunol. Methods*, vol. 426, pp. 56–61, Nov. 2015.

[172] H.-L. Huang, H.-W. Hsing, T.-C. Lai, Y.-W. Chen, T.-R. Lee, H.-T. Chan, P.-C. Lyu, C.-L. Wu, Y.-C. Lu, S.-T. Lin, C.-W. Lin, C.-H. Lai, H.-T. Chang, H.-C. Chou, and H.-L. Chan, “Trypsin-induced proteome alteration during cell subculture in mammalian cells,” *J. Biomed. Sci.*, vol. 17, 2010.

[173] P. C. Collins, L. K. Nielsen, S. D. Patel, E. T. Papoutsakis, and W. M. Miller, “Characterization of Hematopoietic Cell Expansion, Oxygen Uptake, and Glycolysis in a Controlled, Stirred-Tank Bioreactor System,” *Biotechnol. Prog.*, vol. 14, no. 3, pp. 466–472, Jun. 1998.

[174] L. K. Nielsen, “Bioreactors for Hematopoietic Cell Culture,” *Annu. Rev. Biomed. Eng.*, vol. 1, no. 1, pp. 129–152, Aug. 1999.

[175] R. Williams, *Surface modification of biomaterials : methods, analysis and applications*. Woodhead Pub, 2011.

[176] J. Kling, “PEGylation of Biologics,” *Bioprocess Int.*, 2013.

[177] F. M. Veronese, “Peptide and protein PEGylation: a review of problems and solutions,” *Biomaterials*, vol. 22, pp. 405–417, 2001.

[178] J. A. Chasis and N. Mohandas, “Erythroblastic islands: niches for erythropoiesis.,” *Blood*, vol. 112, no. 3, pp. 470–8, Aug. 2008.

[179] M. Hanspal and J. S. Hanspal, “The association of erythroblasts with macrophages promotes erythroid proliferation and maturation: a 30-kD heparin-binding protein is involved in this contact.,” *Blood*, vol. 84, no. 10, pp. 3494–504, Nov. 1994.

8 Appendix A – Optimisation of AlamarBlue Assay

8.1 Introduction

AlamarBlue assay is a non-toxic cell viability colourimetric assay, commonly used in dose-response assays. It is recommended that the culture conditions for the AlamarBlue assay are optimised. This section investigates cell seeding density, absorbance and fluorescence read-out, assay incubation time, and phenol red interference.

8.2 Results and Conclusions

Figure 60 shows the correlation between cell concentration and fluorescence intensity was highest with a four hour AlamarBlue incubation period (R^2 = 0.96). Correlation was at 0 hours and 48 hours where cell growth was limiting and AlamarBlue concentration was limiting respectively. In contrast, the optimal incubation period was one hour for an absorbance read-out (R^2 = 0.91). Sensitivity between cell concentrations was higher for fluorescence read-out in comparison to absorbance.

Figure 62 shows GM-CSF dose-response curves with and without phenol red media. Standard deviations were lower with phenol red media; however a sigmoidal relationship was achieved with phenol red free media and an increase in sensitivity was achieved for lower cell concentrations with phenol red free media.

The culture conditions taken forward for the AlamarBlue assay were; an incubation period of 4 hours, a read-out using fluorescence and phenol red free media to reduce interference.

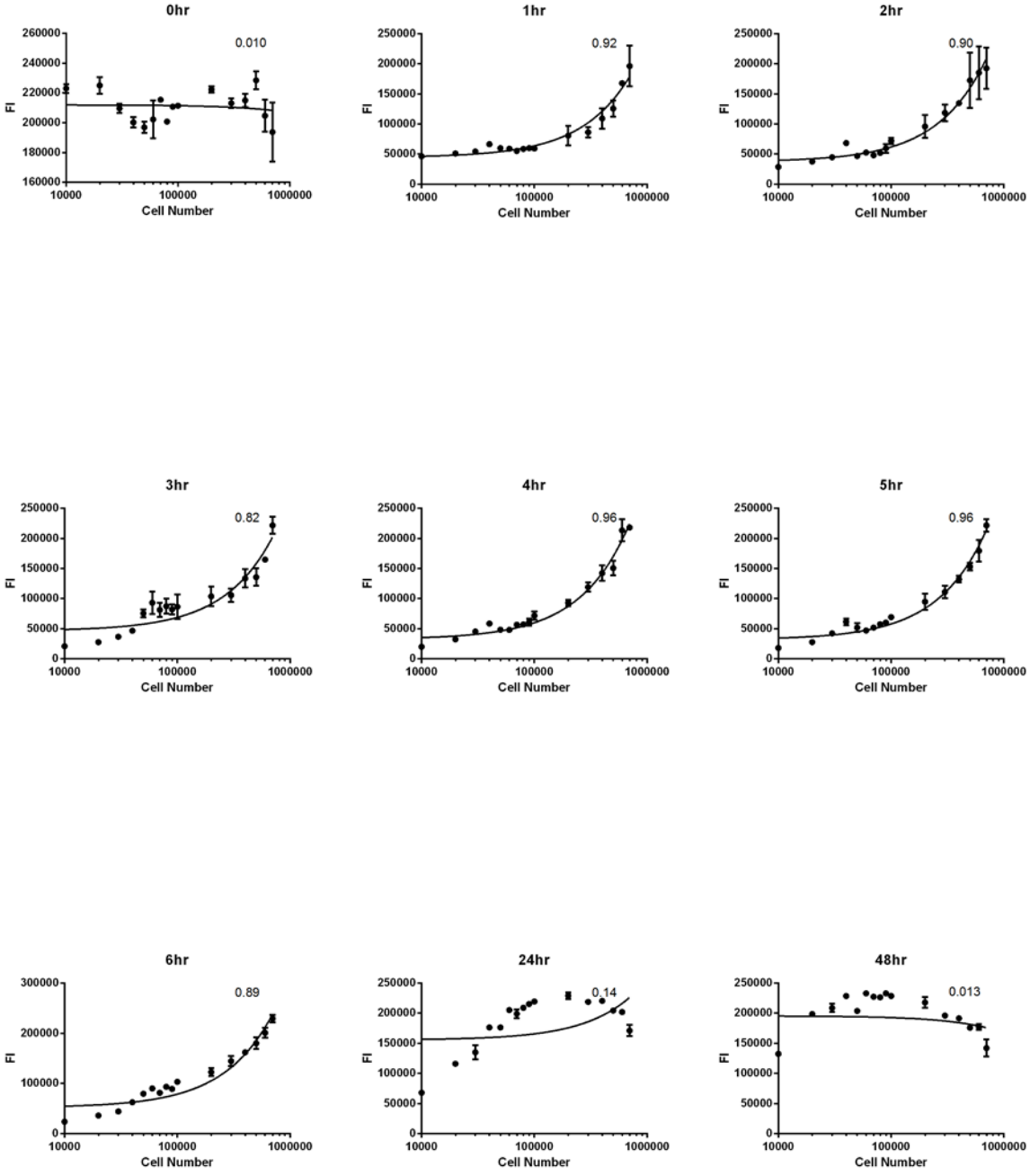


Figure 60. AlamarBlue assay read-out using Fluorescence. The effect of assay incubation time and cell concentration on fluorescence intensity. Data points show mean fluorescence intensity (n=3±S.D).

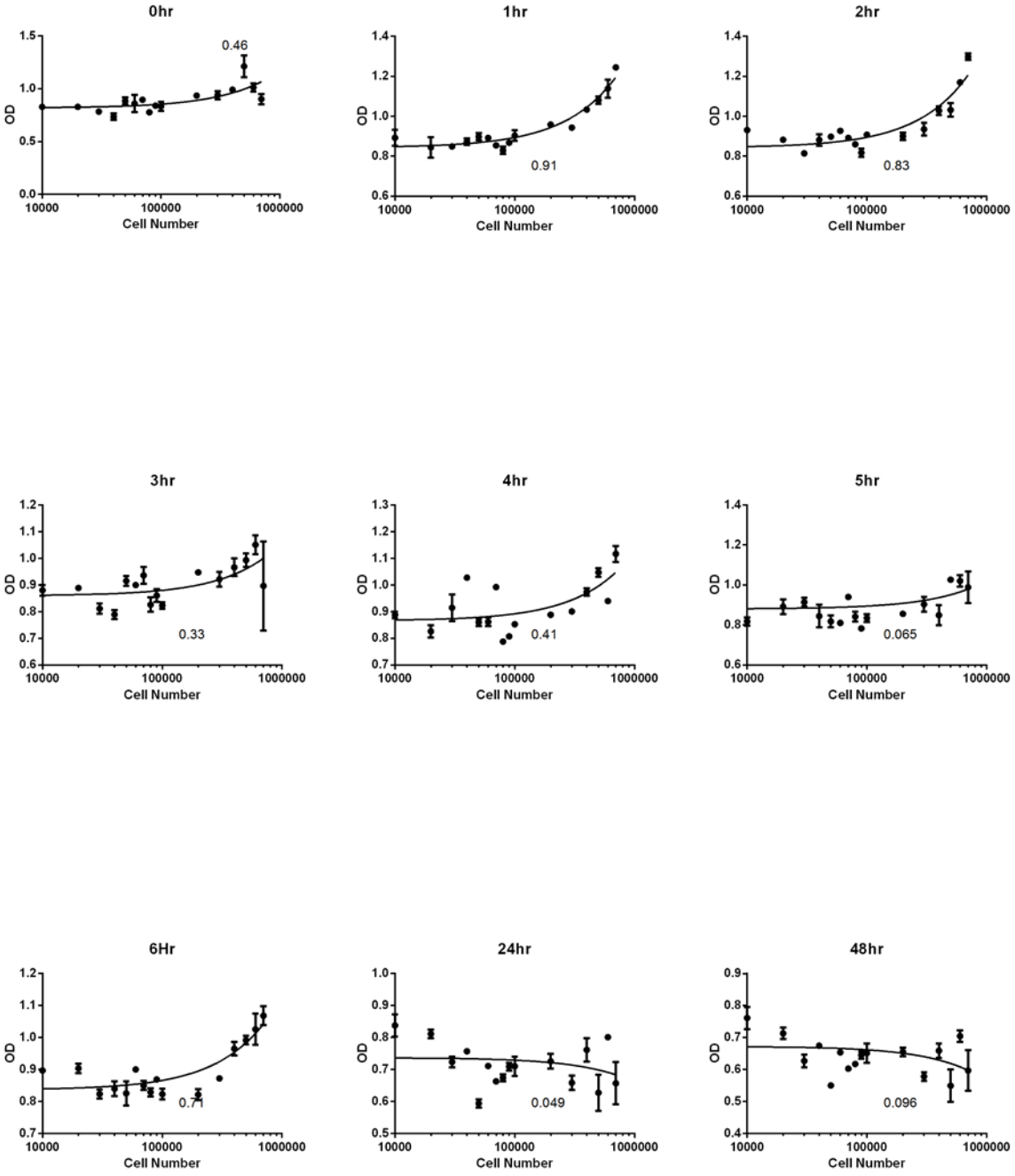


Figure 61. AlamarBlue assay read-out using Absorbance. The effect of assay incubation time and cell concentration on optical density. Data points show mean optical density (n=3±S.D).

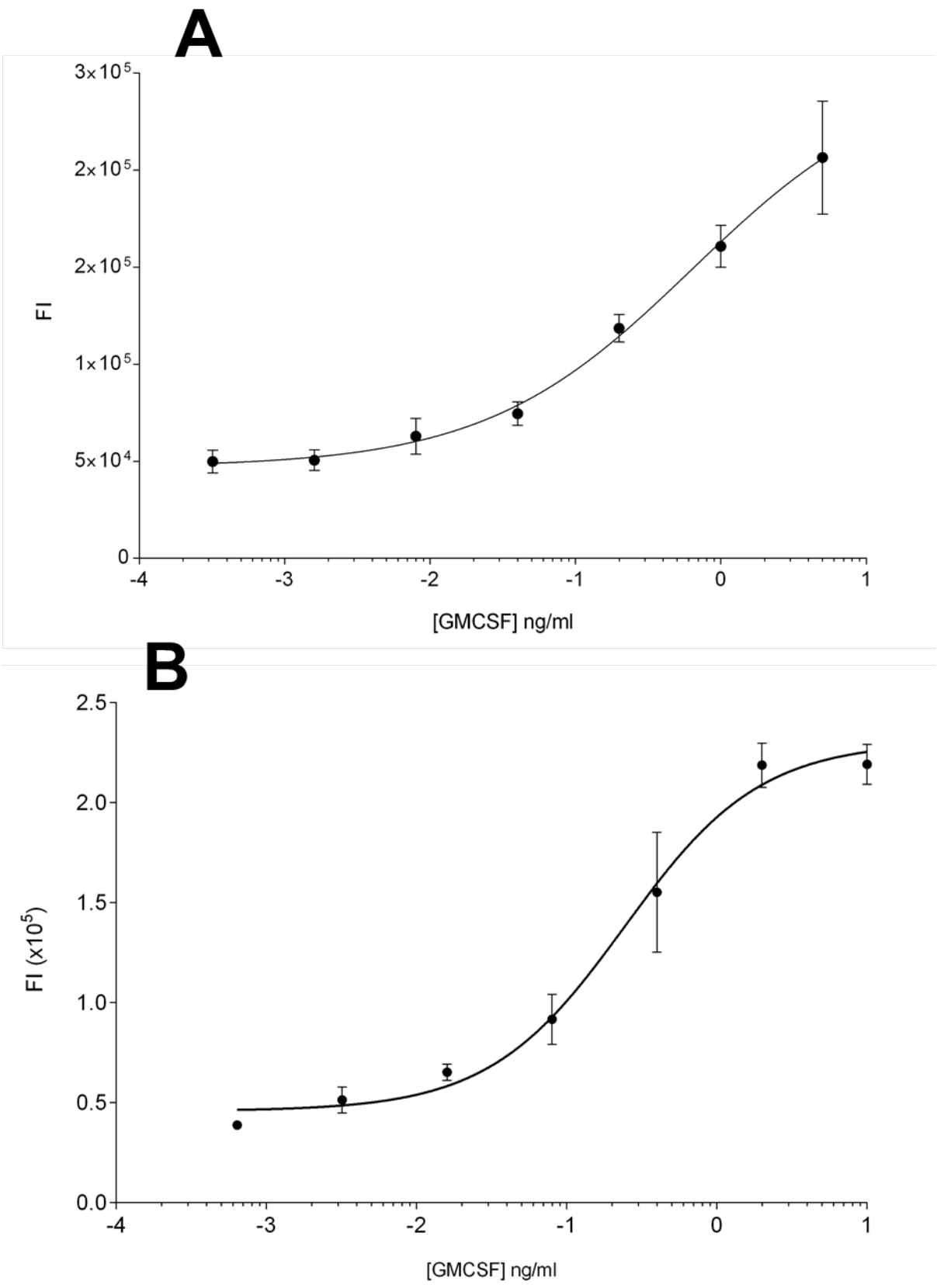


Figure 62. A GM-CSF dose-response assay in a GM-CSF dependent cell line, TF-1, using an AlamarBlue assay. A) Phenol red media B) phenol red free media. Data points show mean fluorescence intensity (n=3±S.D).