# Can we Treat Congenital Blood Disorders by Transplantation of Stem Cells, Gene Therapy to the Fetus?

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2019

A thesis submitted for the degree of Doctor of Philosophy

**Declaration**

I, Panicos Shangaris confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

**Abstract**

Congenital diseases such as blood disorders are responsible for over a third of all pediatric hospital admissions. *In utero* transplantation (IUT) could cure affected fetuses but so far in humans, successful IUT has been limited to fetuses with severe immunologic defects, due to the maternal immune system and a functionally developed fetal immune system. I hypothesised that using autologous fetal cells could overcome the barriers to engraftment. Previous studies show that autologous haematopoietic progenitors can be easily derived from amniotic fluid (AF), and they can engraft long term into fetal sheep.

In normal mice, I demonstrated that IUT of mouse AFSC results in successful haematopoietic engraftment in immune-competent mice. Congenic AFSCs appear to have a significant advantage over allogenic AFSCs. This was seen both by their end haematopoietic potential and the immune response of the host.

Expansion of haematopoietic stem cells (HSC) has been a complicated and demanding process. To achieve adequate numbers for autologous stem cells for IUT, HSCs need to be expanded efficiently. I expanded and compared AFSCs, fetal liver stem cells and bone marrow stem cells. Culturing and expanding fetal and adult stem cells in embryonic stem cell conditions maintained their haematopoietic potential.

Using a humanised mouse model of thalassaemia, in which heterozygous animals are affected by anaemia, splenomegaly and extramedullary haematopoiesis, I explored whether *in utero* gene therapy (IUGT) to the fetal HSC compartment using a vector carrying the corrected beta-globin gene might cure the disease before birth. IUGT resulted in phenotypic normalisation with increased levels of beta-globin and associated downregulation of gamma globin, consistent with a switch from fetal to adult human haemoglobin, confirming successful prenatal correction of the genetic defect.

**Impact Statement**

Cell-free fetal DNA is detectable in the mother's circulation from the fifth week of pregnancy, which makes it possible in the future, to detect any gene disorder, such as thalassaemia, much earlier than currently feasible (Byrou et al., 2018; Hudecova and Chiu, 2017; Saba et al., 2017). Earlier detection would give parents more time to consider their options and clinicians to consider early interventions. Currently, the two main alternatives are 1) termination of pregnancy or 2) careful monitoring and delivery followed by postnatal treatment where appropriate. This thesis addresses a potential third option; in-utero treatment of the genetic disorder by delivering the corrected version of a cell or protein. In the last two decades, studies in animal models have shown promising results, where gene therapy or stem cell delivery to the fetus in utero corrected hereditary disorders (Hartman et al., 2018; Loukogeorgakis et al., 2019; Loukogeorgakis and De Coppi, 2016; Shangaris et al., 2017, 2018, 2019; Shaw et al., 2015; Witt et al., 2018).

The results from this thesis demonstrate that amniotic fluid stem cells have haematopoietic potential and can be used in an *in-utero* therapy setting. The culture of amniotic fluid stem cells was optimised, and the cells maintained their hematopoietic potential *in vitro* for up to 7 days (Loukogeorgakis et al., 2019). The developing fetus can mount an immune response to non-self (allogenic) stem cells but not to self (congenic) amniotic fluid stem cells. This is a fundamentally important principle for the development of clinical trials of in utero therapies for severe congenital disease such as haemoglobinopathies, and skeletal dysplasias. This important element in the pathway towards prenatal therapy for blood disorders has been disseminated to the scientific community with two publications in Stem Cells and Stem Cells and Development (Loukogeorgakis et al., 2019; Shangaris et al., 2018). The results were also presented to many nationals and international conferences, such as the Society of Reproductive investigation, European Society of Gene and Cell Therapy, Gynaecological Visiting Society and Blair Bell Academic Meeting and International Society of Stem Cell

Research. The second study in this thesis demonstrates the successful application of in utero gene therapy to the treatment of heterozygous beta-thalassemia, using a unique humanised mouse model in which the developmental haemoglobin switch occurs just as in humans, due to insertion of a “switching cassette”. Injection of a GLOBE lentivector intra-hepatically into fetal mice, resulted in long-term clinical improvement, as evidenced by a reduction in anaemia, extramedullary haematopoiesis, hepatosplenomegaly, and improvement in cardiac function.

Furthermore, the results also demonstrate the utility of non-invasive MRI as a tool to assess clinical status of individuals with beta-thalassemia with respect to cardiac function and organ damage/organomegaly. The results of the study were presented in international conferences, and it received more than five awards. The final results were disseminated to the scientific community via a publication in Scientific Reports Journal(Shangaris et al., 2019). A presentation to lay, members of the UK Thalassemia society, resulted in securing further funding to test a new superior and more efficient lentivector.

This treatment would have a huge impact, especially in those countries such as the Middle East and the Far East where termination of pregnancy may not be available, blood transfusions are prohibitively expensive, and where thalassaemia is most prevalent.

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**Acknowledgements**

My thirst for science and guidance of my primary supervisor Professor Anna David, pushed me to apply and secure funding by UCLH Charities, Wellcome Trust and SPARKS, to start my PhD. I am extremely grateful for her guidance, encouragement, mentorship and the faith she showed me during my science training. I cannot thank enough my secondary supervisor, Professor Paolo De Coppi, a passionate clinician-scientist, who accommodated me in his laboratory, providing support, both spiritual and in experimental design, for me to complete the laboratory work and finalise my thesis.

I would also like to acknowledge my wife Katerina Stamati for her strength for putting up with me; during my laboratory work, and especially during the writing of my thesis and also for her help in proofreading my work before submission.

My sincere appreciation to my friend and colleague Dr Stavros Loukogeorgakis, for his help with the experimental design, technical support and endless skype discussions, troubleshooting and encouragement. I would like to especially thank Dr Mike Blundell and Simon Waddington for their guidance, training, suggestions and feedback on my project. I would like to thank the previous PhD student, Dr Steven Shaw, who handed over to me such an amazing project, gave me preliminary data to support my research training fellowship application and taught me the essential laboratory techniques to start.

It has been a great pleasure to work at the UCL's Institute for Women's Health and the Institute of Child Health with the Stem Cells and Regenerative Medicine team. Special thanks to Dr Angela Poulter and Dr Sioban SenGupta for their support during my PhD.

My special acknowledgements and gratitude also go to the team at the University of Alabama at Birmingham, USA; To Dr Tom Ryan and his post-doctoral fellow Dr Shanrun Liu for hosting me in their department for six weeks providing training and sending over to UCL the humanised animal model of thalassaemia.

My gratitude also goes to Dr Mike Antoniou at Kings College London who provided the GLOBE vector and his PhD student Christina Flouri for her hard work with the animal colony and the additional animal experiments.

I thankfully acknowledge and express my gratitude to my friends and collaborators Caterina Pipino, Panagiotis Maghsoudlou, Luca Urbani, Fanos Georgiades, Durrgah Ramachandra, Eleni Antoniadou, Simon Eaton, their precious support, collaboration, encouragement and discussions. It was a great pleasure working with all of them.

I cannot think of any words to thank the incredible man at the flow cytometry facility Ayad Eddaoudi and his support team Stephanie Canning and Anna Rose for their support and advise during the endless flow cytometry analysis and sorting of my cells.

I would like to thank the team at the UCL's Advanced Biomedical Imaging Center, Dr Daniel Stuckey and Laurence Jackson for their help with the MRI as well as Professor John Porter's team, Elina Vlachodimitropoulou at the Research Department of Haematology for their help with the control MRI experiments.

I would also like to take this opportunity to thank all the staff at the Western Laboratory BSU, for taking excellent care of the experimental animals, particularly Debby Mustafa, Katy Mendoza, Carly Tagg, Ailsa Greppi and Kyle O'Sallivan.

During this Ph.D., I had an opportunity to supervise several wonderful students in the lab and really enjoyed the experience. These included Lakshita Shah, Sindhu Subramaniam, Eleni Petra and Nahla Bakhamis, I am thankful to them for their hard work.

I would like to thank my funding bodies: UCLH Charities, The Wellcome Trust, Sparks, UK Thalassaemia Society and the UCLH Charities Prenatal Therapy Fund for their support throughout the research time and conferences.

Most importantly I would like to thank my family, my parents, Yiannoula and Yiannakis, my brother Alexis, my parents in law Alecos and Ino, my sister in law, Ioanna, brothers in law Yiannis and Yiannis, my god daughter Melia, god son James, my grandparents Alexis and late grandmother Maroulla, my friends Andros and Pavlina for their unconditional love, guidance and motivation. I am very lucky to have this strong family circle around me; they have been pillars of support in my life journey.

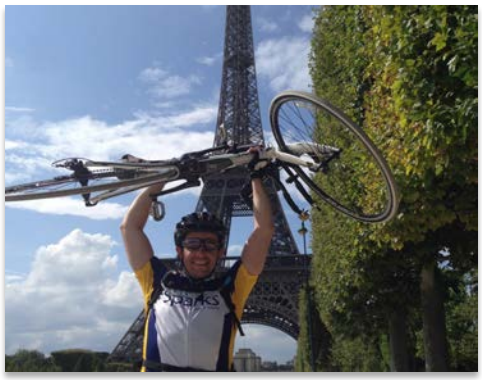
My PhD time was an incredible journey with many obstacles and incredible moments in the laboratory, conferences, and social gatherings. During my PhD, I got married to an

incredible woman, Dr Katerina Stamati, I had the opportunity to take part in a charity ride from London to Paris, raising money for SPARKS, I did the London Triathlon for the UCLH Prenatal Therapy fund and became member in two of the committees at the Society of Reproductive Investigation. I met many people and formed friendships and collaborations which will last for a lifetime.

With my wife Dr Katerina Stamati
at her PhD graduation (UCL, July 2015)

Cycling in San Francisco with my Supervisor
Professor Anna David and Katerina (April 2014)

With Dr Stavros Loukogeorgakis
at the ASH conference in San Franscisco (December 2014)



Completing the London to Paris
charity ride for SPARKS (August 2014)

**Prizes & Awards**

Giorgio Pardi Junior Scientist Award March 2017, Society of Reproductive Investigation (SRI), “In Utero Gene Therapy (IUGT) Using GLOBE Lentiviral Vector Phenotypically Corrects the Heterozygous Humanized Mouse Model and Its Progress Can Be Monitored Using MRI Techniques”

Best Oral Presentation in the Fetal Medicine Category, March 2017, The British Maternal & Fetal Medicine Society (BMFMS), “In Utero Gene Therapy (IUGT) Using GLOBE Lentiviral Vector Phenotypically Corrects the Heterozygous Humanized Mouse Model and Its Progress Can Be Monitored Using MRI Techniques”

Joe Leigh Simpson Award for the best oral presentation, Washington, DC, July 2015 International Society for Prenatal Diagnosis, “Correction of haemoglobin levels in a humanized mouse model of thalassemia after fetal gene therapy”

Award for Best new investigator Poster Presentation, Society of Reproductive Investigation (SRI) San Francisco, California, March 2015, “Correction of haemoglobin levels in a humanized mouse model of thalassemia after fetal gene therapy”

Best presentation by a Trainee, Spring Meeting 2015, The Neonatal Society, “Correction of haemoglobin levels in a humanized mouse model of thalassemia after fetal gene therapy”

Vandenberghe-Storz Award & Travel Award September 2014, International Fetal Medicine and Surgery Society (IFMSS), “Correction of haemoglobin levels in a humanized mouse model of thalassemia after fetal gene therapy”

Best PhD/MD Poster Presentation, May 2014, UCL Institute for Women's Health, "Congenic Amniotic Fluid Stem Cells Show Stable Long-Term Engraftment in the Haematopoietic System after In Utero Transplantation"

President's Presenter Award, March 2014, Society of Gynecologic Investigation (SGI), "Congenic Amniotic Fluid Stem Cells Show Stable Long-Term Engraftment in the Haematopoietic System after In Utero Transplantation"

Award for Best new investigator Poster Presentation, March 2013, SGI**, "**Second and third trimester Human Amniotic Fluid Stem Cells engraft after in Utero Transplantation in immunocompetent Mice."

**Funding**

- UK Thalassemia Society, £39000, February 2015
- Wellcome Trust, SPARKS Research Training Fellowship, £181,169, July 2012
- Bogue Travel Fellowship, £1548, December 2011
- UCLH Charities Clinical R&D Committee Entry-Level Fellowship, £89417, February 2011

**Publications**

**Shangaris, P**, Loukogeorgakis, SP, Subramaniam, S, Flouri, C, Jackson, LH, Wang, W, Blundell, MP, Liu, S, Eaton, S, Bakhamis, N, Ramachandra, DL, Maghsoudlou, P, Urbani, L, Waddington, SN, Eddaoudi, A, Archer, J, Antoniou, MN, Stuckey, DJ, Schmidt, M, Thrasher, AJ, Ryan, TM, Coppi, PD & David, AL 2019, 'In Utero Gene Therapy (IUGT) Using GLOBE Lentiviral Vector Phenotypically Corrects the Heterozygous Humanised Mouse Model and Its Progress Can Be Monitored Using MRI Techniques', Scientific Reports, vol. 9, no. 1. 12 August 2019, doi.org/10.1038/s41598-019-48078-4

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**Conference Presentations (Oral & Poster)**

**March 2017: Society of Reproductive Investigation, Oral Presentation** "In Utero Gene Therapy (IUGT) Using GLOBE Lentiviral Vector Phenotypically Corrects the Heterozygous Humanized Mouse Model and Its Progress Can Be Monitored Using MRI Techniques"

**March 2017, Amsterdam, The Netherlands, Oral Presentation, The British Maternal & Fetal Medicine Society** "In Utero Gene Therapy (IUGT) Using GLOBE Lentiviral Vector Phenotypically Corrects the Heterozygous Humanized Mouse Model and Its Progress Can Be Monitored Using MRI Techniques"

**March 2016, Montreal, Canada, Poster Presentation, Society of Reproductive Investigation,** "Expansion of Fetal and Adult Hematopoietic Progenitors Using Modified Embryonic Stem Cell Conditions."

**October 2015, London, UK, Oral Presentation, Second International Conference on In Utero Transplantation and Fetal Gene Therapy,** "Correction of haemoglobin levels in a humanized mouse model of thalassemia after fetal gene therapy"

**July 2015 Washington DC, USA, Oral Presentation, International Society for Prenatal Diagnosis,** "Correction of haemoglobin levels in a humanized mouse model of thalassemia after fetal gene therapy"

**March 2015, London, UK, Oral Presentation, The Neonatal Society** "Correction of haemoglobin levels in a humanized mouse model of thalassemia after fetal gene therapy"

**March 2015, San Francisco, USA, Poster Presentation, Society of Reproductive investigation** Correction of Hemoglobin Levels in a Heterozygous Humanized Mouse Model of Thalassemia after Fetal Gene Therapy

**January 2015, London, UK Blair Bell Academic Society, Annual Meeting, Oral Presentation,** Correction of Hemoglobin Levels in a Heterozygous Humanized Mouse Model of Thalassemia after Fetal Gene Therapy

**December 2014, San Francisco, USA, Poster Presentation American Society of Hematology** Correction of Hemoglobin Levels in a Heterozygous Humanized Mouse Model of Thalassemia after Fetal Gene Therapy

**September 2014, Cape Cod, USA Oral Presentation International Fetal Medicine and Surgery Society:** "Correction of hemoglobin levels in a humanized mouse model of thalassemia after Fetal Gene Therapy"

**April 2014, San Francisco, USA, Oral Presentation First International Conference on In Utero Transplantation and Fetal Gene Therapy** "Congenic Amniotic Fluid Stem Cells Show Stable Long-Term Engraftment in the Haematopoietic System after In Utero Transplantation"

**March 2014, Florence, Italy, Oral Presentation, Society of Gynaecologic investigation:** "Congenic Amniotic Fluid Stem Cells Show Stable Long-Term Engraftment in the Haematopoietic System after In Utero Transplantation"

**September 2013, London, UK, Gynaecological Visiting Society:** Oral Presentation "Amniotic fluid stem cells engraft the hematopoietic system following in utero transplantation"

**April 2013, London, UK, Poster Presentation, British Society for Gene and Cell:** "Amniotic fluid stem cells engraft the hematopoietic system following in utero transplantation"

**Abbreviations (selective)**

| Abbreviation | Meaning |
|---|---|
| **18SrRNA** | 18S Ribosomal Ribonucleic Acid |
| **AF** | Amniotic Fluid |
| **AFSC** | Amniotic Fluid Stem Cells |
| **ANGPTL** | Angiopoietin Like Protein |
| **B382** | CA Mice, Thalassaemia Heterozygote, α2α1/α2/α1, $\gamma^{HPFH}\beta/\gamma\beta^{A}$ |
| **B383** | HbA control, Humanised Non-Thalassaemia Control, α2α1/α2/α1, $\gamma\beta^{A}/\gamma\beta^{A}$ |
| **BM** | Bone Marrow |
| **CA** | Cooley's Anaemia |
| **CFU** | Colony Forming Unit |
| **CFU-GEMM** | Colony Forming Unit Granulocyte Erythrocyte Monocyte |
| **CFU-GM** | Colony Forming Unit Granulocyte Monocyte |
| **CVS** | Chorionic Villus Sampling |
| **DAPI** | Dimethyl Sulfoxide |
| **DNA** | Deoxyribonucleic Acid |
| **EDTA** | Ethylenediaminetetraacetic Acid |
| **ESC** | Embryonic Stem Cells |
| **FACS** | Fluorescence Activated Cell Sorting |
| **FBS** | Fetal Bovine Serum |
| **FITC** | Fluorescein Isothiocyanate |
| **FL** | Fetal Liver |
| **FoxP3** | Forkhead/winged-helix transcription factor |
| **GFP** | Green Fluorescent Protein |
| **HIV** | Human Immunodeficiency Virus |
| **HLA** | Human Leukocyte Antigen |
| **HPLC** | High Performance Liquid Chromatography |
| **HS** | Hypersensitive Site |
| **HSC** | Haematopoietic Stem Cells |
| **IL** | Interleukin |
| **iTregs** | Induced Regulatory T Cells |
| **IUGT** | *In Utero* Gene Therapy |
| **IUT** | *In utero* Transplantation |
| **KI** | Knock In |
| **KO** | Knock Out |
| **LCR** | Locus Control Region |
| **MACS** | Magnetic Activated Cell Sorting |
| **mAF** | Mouse Amniotic Fluid |
| **mBM** | Mouse Bone Marrow |
| **MEFs** | Mouse Embryonic Fibroblast |
| **MEL** | Mouse Erythroid Leukaemia |
| **mFL** | Mouse Fetal Liver |
| **MHC** | Major Histocompatibility Complex |
| **mRNA** | Messenger Ribonucleic Acid |

| | |
|---|---|
| **NK** | Natural Killer Cells |
| **nTregs** | Naturally Regulatory Cells |
| **PB** | Peripheral Blood |
| **PBS** | Phosphate Buffered Saline |
| **PCR** | Polymerase Chain Reaction |
| **PE** | Phycoerythrin |
| **PFA** | Paraformaldehyde |
| **RBC** | Red Blood Cells |
| **RNA** | Ribonucleic acid |
| **rRNA** | Ribosomal Ribonucleic Acid |
| **SCA** | Sickle Cell Anaemia |
| **SCD** | Sickle Cell Disease |
| **SCF** | Stem Cell Factor |
| **SCID** | Severe Combined Immunodeficiency |
| **SD** | Standard Deviation |
| **SEM** | Standard Error of the Mean |
| **TGF-β** | Transforming Factor Beta |
| **UAB** | University of Alabama at Birmingham |
| **UCL** | University College London |
| **UCLH** | University College London Hospital |
| **α** | Alpha |
| **β** | Beta |
| **γ** | Gamma |
| **δ** | Delta |
| **ε** | Epsilon |
| **ζ** | Zeta |
| **ψ** | Psi |

# Chapter 1 Introduction

## 1.1 Introduction

The development of non-invasive prenatal diagnosis using circulating fetal DNA from the mother's blood allows clinicians to detect congenital fetal diseases as early as ten weeks of gestation (Lewis et al., 2014; Twiss et al., 2014). Cell-free fetal DNA is detectable in the mother's circulation from the fifth week of pregnancy, which could make it possible in the future, to detect any gene disorder, such as thalassaemia, much earlier (Byrou et al., 2018; Hudecova and Chiu, 2017; Saba et al., 2017). Early detection gives parents time to consider their options at an earlier stage during pregnancy. Currently, the two main options the couple has are termination of pregnancy or careful monitoring of pregnancy and delivery followed by postnatal treatment where appropriate. What if there was a third option available, an *in-utero* approach to treat the genetic disorder by delivering the corrected version of a cell or protein. In the last two decades, studies in animal models have shown promising results, where gene therapy or stem cell delivery *in utero* to the fetus cured hereditary disorders (Hartman et al., 2018; Loukogeorgakis and De Coppi, 2016; Witt et al., 2018). In addition, certain structural anomalies can be prevented using gene therapy approaches, while in some non-genetic conditions the expression of a specific protein at the right time of gestation may improve future pathology. Direct gene delivery using vectors carrying the correct receptor, or autologous transplantation of corrected stem cells derived from the fetus might help realise these objectives (Hartman et al., 2018; Witt et al., 2018).

In my thesis, I studied the progress in the field of perinatal gene/ stem cell therapy, the barriers into clinical translation and the earliest motions of therapy in to practice. I performed experiments on mouse model of stem cell transplantation using amniotic fluid stem cells (AFSCs) and gene therapy on a humanised model of beta thalassaemia.

## 1.2 The Concept of Perinatal Therapy

Perinatal therapy can be applied to the fetus prenatally, using three different ways: a. gene therapy to the fetal stem cell niche, b. *ex-vivo* gene therapy to the fetal stem cells prior to autologous transplantation of the cells back to the donor and c. delivery of healthy cells from a matched donor (allogenic transplantation) (Boelig et al., 2016; MacKenzie et al., 2002; Mehta et al., 2011, 2012; Shaw et al., 2016). One of the benefits of perinatal treatment lies in the potential to limit organ damage through early intervention. Also, applying a therapy to the fetus, where stem cell proliferation is high, results in a higher number of transduced cells, which leads to a better therapeutic effect (Flake, 2004). The fetus also offers a size advantage, allowing a higher vector-to-target cell ratio. Certain organs that are challenging to target after birth may be more easily accessible in the fetus due to their developmental stages or relative immaturity (Hartman et al., 2018; Loukogeorgakis and Flake, 2014; McClain and Flake, 2016; Peebles et al., 2004). The fetal epidermis is an example of this, as it undergoes remodelling by programmed cell death and is replaced by mature keratinocytes. The epidermis forms a thick barrier to gene transfer following birth (Polakowska et al., 1994) but could be better targeted *in utero* (Endo et al., 2012).

One of the main obstacles of postnatal gene therapy is the development of an immune response against the transgenic protein or the vector (Manno et al., 2006). This is of particular importance when gene therapy aims to correct a genetic disease, which completely lacks a gene product. It is also possible that some patients may have pre-existing antibodies to the viral vector that will inhibit long-term expression of the transgenic protein; this will limit therapeutic efficacy and prevent repeated vector administration. For example, pre-existing neutralising antibodies against adeno-associated virus (AAV) serotype 2 have been shown to interfere with AAV2 vector-mediated factor IX (FIX) gene transfer to the liver (Manno et al., 2006; Mattar et al., 2011; Nathwani et al., 2006, 2011). Delivering foreign protein to the fetus

can provide an advantage of immune tolerance during fetal life, a concept first suggested more than 60 years ago (Billingham et al., 1953; Billingham and Brent, 1956). Induction of tolerance relies on the introduction and expression of the foreign protein early in gestation before the immune system is fully developed. The protein needs to remain at a detectable level within the fetus and presented to the thymus at the correct time (Burt, 2013; Peranteau et al., 2002, 2015; Sabatino et al., 2007; Waddington et al., 2003).

## 1.3 Stem Cells

Stem cells are cells which are capable of renewing themselves, via cell duplications and can be induced to any kind of cell or tissue specific. They exist in the body during early life and growth. In many tissues and organs, stem cells serve as a repair mechanism which replaces the damaged or dying cells. When stem cells divide, they can either remain as stem cells or differentiate to a terminal cell such as red blood cell or bone cell(Watt and Driskell, 2010).

## 1.4 Stem Cell Hierarchy

Stem cells are named according to their tissue of origin or repopulation potential. For example a totipotent stem cell can form a whole organism, a pluripotent stem cells can give rise to the three germ layers ectoderm, mesoderm and endoderm(Watt and Driskell, 2010). Pluripotent stem cells can be found as embryonic stem cells or the man made induced pluripotent stem cell (iPSs). Multipotent or tissue specific stem cells are usually found around the tissue of origin such as mesenchymal stem cells, they can give rise to bone, haematopoietic stem cells, can give rise to blood(Tavian and Péault, 2005a) as seen in Figure 1-1.

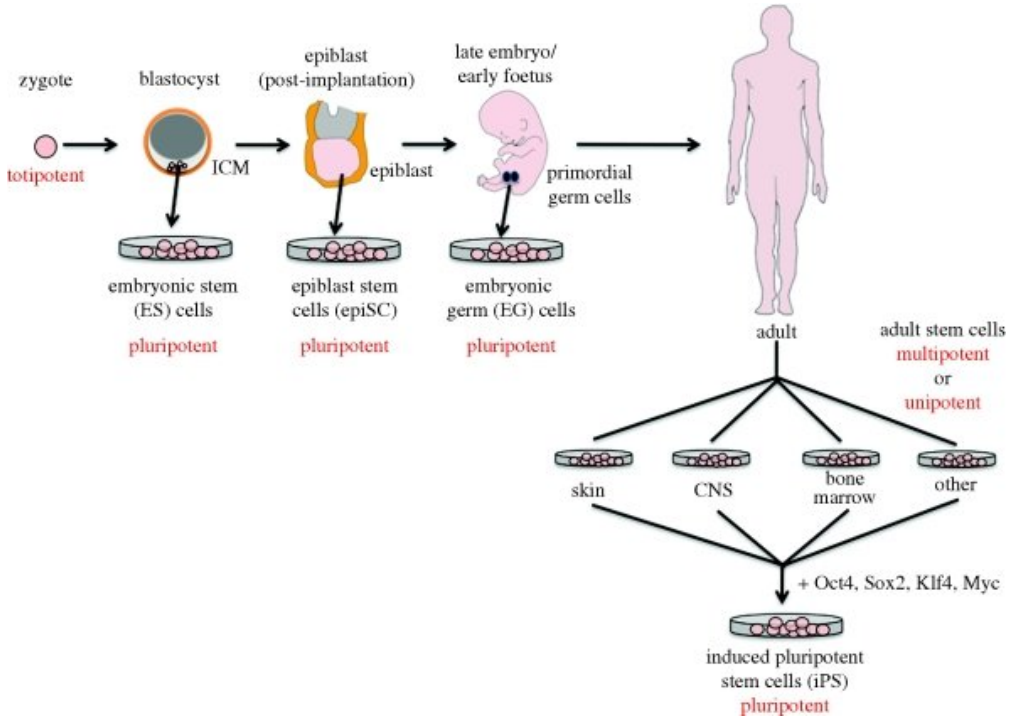



**Figure 1-1 Origin of stem cells. Cells are described as pluripotent if they can form all the cell types of the adult organism. If, in addition, they can form the extraembryonic tissues of the embryo, they are described as totipotent. Multipotent stem cells have the ability to form all the differentiated cell types of a given tissue. In some cases, a tissue contains only one differentiated lin- eage and the stem cells that maintain the lineage are described as unipotent (Figure from F Watt et al 2010, used as per license CC BY 2.0)(Watt and Driskell, 2010)**

## 1.5 Selecting the right cells for Therapy

When selecting stem cells for a potential therapy we need to take in to consideration the abilities of the stem cells(Tiwari et al., 2018).

- **Plasticity:** To be able to differentiate into the designated tissue such as blood cell or muscle

- **Homing:** Their ability to reach the site of damage or host's stem cell niche for example bone marrow stem cells transplanted in a patient affected with leukemia need to reach the bone marrow
- **Engraftment :** to be able to become part of the host's niche without rejection (Tiwari et al., 2018)

## 1.6 Haematopoietic Stem cells

Haematopoietic stem cells (HSCs), in adult life, are produced in the adult bone marrow. HSCs progenitors are responsible for their constant renewal and turnover. These progenitors have multilineage differentiation potential. During fetal life in humans and mice the HSC progenitors develop initially in the yolk sac, followed by progenitors in the aorta gonads and mesonephros. The HSCs progenitors then move to the fetal liver which is the main haematopoietic organ in humans and mice before the colonization of the bone marrow, which is the final destination of the HSC progenitors, as shown in Figure 1-2 (Charbord et al., 1996; Ivanovs et al., 2017; Mazo et al., 2011; Tavian and Péault, 2005a). Some of the HSC progenitors also move to the thymus and spleen for T cell and B cell differentiation(Cumano and Godin, 2007).

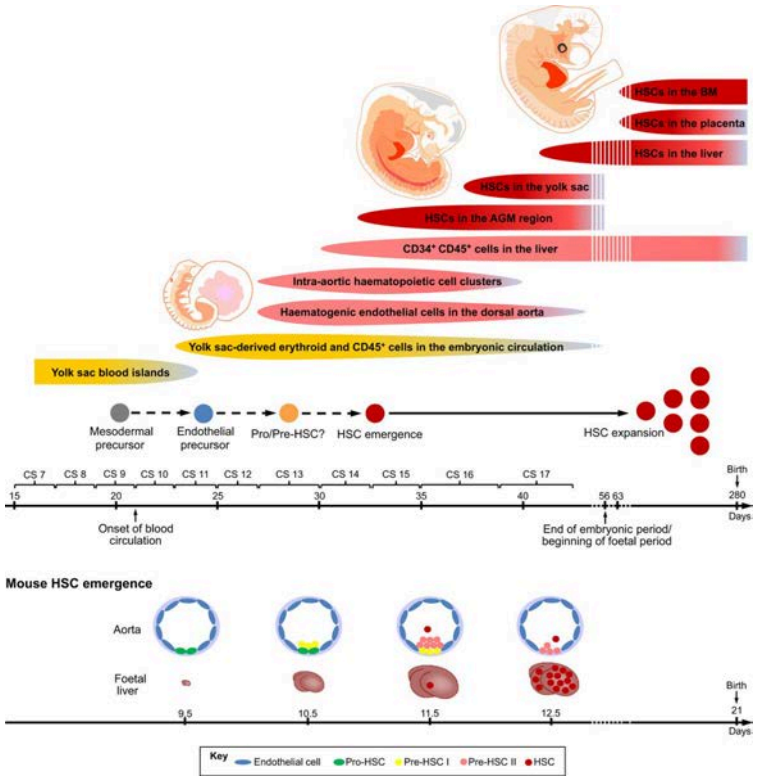



**Figure 1-2, Adapted from Andrejs Ivanovs et al. Development 2017;144:2323-2337, Chronology of human haematopoietic development. The haematopoietic lineage derives from a mesodermal precursor (grey) through the intermediate of the haematogenic endothelium before expanding. For the sake of comparison, the step-wise emergence of mouse HSCs through immature haematopoietic is shown at the bottom of the figure. The existence of similar precursors in human haematopoietic development has not been functionally shown, which is indicated by a question mark for human pro/pre-HSC. Red, bona fide HSCs; light red, haematopoietic lineages which may or may not be related to HSC development; yellow, yolk sac haematopoietic differentiation. Fading of coloured bubbles to blue represents extinction of the process. White striped lines represent a change in time scale (omission of several days for the mouse or weeks for the human).**

## 1.7 Fetal Liver Stem cells

As described above, during development, in mice and humans, HSCs originate in the dorsal aorta, from where they migrate into the FL at E11 (mice), which makes the FL the main site of hematopoiesis until near delivery. At E16, HSCs migrate from the FL to the BM, where they reside during postnatal life. In the FL, HSCs undergo multiple rounds of symmetrical self-renewing cell divisions to give rise to the pool of stem cells required for the lifetime of the

organisms. These long-term repopulating stem cells (LT)-HSCs, that re- populate the hematopoietic system long-term, are quiescent, dividing only every 140–180 days in mouse(Manesia et al., 2015). Quiescent LT-HSCs can respond very rapidly to stress or damage, and quickly exit from quiescence to re- generate the blood system (Manesia et al., 2015) (Cheng et al. 2000). There are phenotypic differences and functional differences between adult BM and FL LT-HSCs such as faster expansion kinetics when grafted in adult mice for the FL HSC, compared with HSCs from BM of 8-week-old mice(Bowie et al., 2007). In addition, FL HSCs undergo significantly more symmetrical self-renewing cell divisions compared with 8-week-old adult BM HSCs (Manesia et al., 2015).

### 1.8 Amniotic Fluid stem cells

In the last few years the hematopoietic potential of C-Kit+ (CD117+) / Lineage- (Lin-) AFSC has been explored(Ditadi et al., 2009). Mouse AFSC can be found in about 1% of mouse and human amniotic fluid(De Coppi, Bartsch, et al., 2007; Ditadi et al., 2009). Specifically, from each mouse fetus an estimate of 10000 to 50000 AFSC can be isolated, using the CD117 marker, at around E13 (Loukogeorgakis et al., 2019). The AFSC express high levels of haematopoietic marker CD45 (96.8%) and also other haematopoietic markers such as Sca-1 (31.3%) and CD34 (9.6%). Gene array analysis shows that a majority of fresh AFSC express haematopoietic genes (64.3%). Single cell qRT-PCR analysis shows that the majority of fresh AFSC (75%) express the key hematopoietic regulator Lmo2, with lower levels of expression of Gata1 (45%) and Gata2 (Loukogeorgakis et al., 2019).

Freshly isolated AFSC have a phenotype similar to fetal liver hematopoietic stem cells (FL-HSC) and can engraft after postnatal transplantation into adult immunocompromised hosts generating multilineage hematopoietic engraftment(Loukogeorgakis et al., 2019). Similarly, freshly isolated CD34+ sheep AFSC can be genetically modified overnight and are able to

engraft after IUT in the hematopoietic system in an autologous setting (Shaw et al., 2015). AFSC have several potential advantages that may be salutary for IUT. They can be easily derived from routine amniocentesis which is commonly performed for prenatal diagnosis of genetic disorders, and early second trimester collection would allow expansion in time for autologous treatment of the target disease using a stem cell/gene therapy approach. Their fetal source and high proliferative capacity *in vivo* may allow them to more effectively compete with endogenous FL-HSC resulting in higher levels of engraftment compared to adult-derived cells. Moreover, they can be efficiently genetically modified allowing the potential for an autologous cell-gene therapy or gene editing approach(Ramachandra et al., 2014). However, their therapeutic utility has been so far limited because their hematopoietic potential was lost after *in vitro* expansion using “mesenchymal-type” culture protocols (culture in adherence with serum rich media). This was demonstrated by the paper by Loukogeorgakis and colleagues for the first time that expansion of AFSC on feeder layers of mitotically inactivated embryonic fibroblasts using “embryonic-type” culture media maintains their hematopoietic characteristics, and results in highly efficient engraftment in a congenic murine model of IUT. Conversely, no engraftment was seen in the allogenic setting suggesting inadequate presentation of donor antigen in the developing/fetal thymus. These findings support the potential use of *in vitro*-expanded, autologous AFSC for the treatment of hematopoietic disorders(Loukogeorgakis et al., 2019).

## 1.9 Bone marrow stem cells

Bone marrow formation arbitrarily marks the end of the human embryonic period. The initial CD34−CD45+ haematopoietic cells that invade the cartilaginous bone include mostly CD68+ monocytes/macrophages possibly participating in chondrolysis. This is followed by colonisation with CD34+CD45+ progenitors and HSCs (Ivanovs et al., 2017). The cells in the

bone marrow are capable of generating the complete blood cell system(Ng and Alexander, 2017). They can give rise to large numbers of differentiated cells over extended periods of time. Once they reach their optimal number postnatally they become quiescent. In the adult they can leave the bone marrow and return through homing mechanisms. In the mouse transplantation of a small number is capable of repopulating the whole bone marrow(Martinez-Agosto et al., 2007).

### 1.10 Rationale for *In utero* Stem cell transplantation

Fetal Intervention was first described in 1982 by Harrison et al for its treatment of congenital hydronephrosis and it was expanded to take care of additional anatomical anomalies like resection of sacrococcygeal teratomas, resection of congenital lung malformations, and myelomeningocele repair. Fetal surgical therapy was powerful for correction of anatomic flaws, but its range of curable ailments remains limited. Ever since that time, minimally invasive methods have expanded tremendously, allowing, as an instance, for its harmless cannulation of bronchial vein through ultrasound guidance. Even though patients affected by monogenic diseases can be treated using post-natal stem cell transplantation, the various morbidities associated with postnatal therapy makes prenatal therapy a necessary therapeutic niche. Post-natal bone marrow transplantation is associated with complications connected with myeloablative pre-conditioning, Graft versus host disease (GVHD), graft rejection, and it is also limited to the availability of HLA-matched donors. *In utero* hematopoietic cell transplantation (IUHCT) can be a unique approach as the growing fetus may form donor-specific tolerance and avoid lots of the complications related to post-natal stem cell transplantation. In some specific diseases which cause fetal hydrops as well as *in utero* death, prenatal therapy can improve survival in addition to prevent irreparable damage before birth. Advances in fetal imaging and molecular diagnostics, haven't only improved the power to

detect diseases early enough, they also have made it feasible to safely direct stem cells or gene therapy vectors to precise anatomic sites within the early embryo.

Various groups have published on preclinical studies on *in utero* therapy. Sheep is a well-used animal model. It was the first large animal model to demonstrate successful engraftment after *in utero* allogenic and xenogenic stem cell transplantation (Porada et al., 2004). Because of its similarities in physiology and developmental characteristics with humans it has been extensively studied. In contrast to other large animal models it also gives birth to one or two offspring each time. Fetal sheep also has the advantage of being similar in weight to human fetuses which makes testing of instruments made for humans possible (Ward et al., 2006).

### 1.11 Selecting the right disease for perinatal therapy

Similar to any novel therapeutic intervention, the risks that perinatal gene or stem cell therapy carries are not well known yet, therefore careful consideration must be given before selecting diseases for first-in-human trials (Götherström et al., 2014; Hartman et al., 2018; Kreger et al., 2016; Spencer et al., 2017; Witt et al., 2018). The National Institutes for Health Recombinant DNA Advisory Committee has provided guidelines related to *in utero* vector delivery for the treatment of genetic diseases (U.S. National Institutes of Health. Recombinant DNA Advisory Committee, 2000). In summary, the recommendations were that the use of such an approach should be limited to diseases that:

- have serious morbidity and mortality risks for the fetus either *in utero* or following birth
- lack effective postnatal therapy, or there is a poor outcome based on current postnatal therapies
- are not linked with severe abnormalities that are not corrected by the transferred gene
- can be definitively diagnosed *in utero* and have a well-defined genotype/phenotype relationship, and

- have an animal model for *in utero* gene transfer that recapitulates the human disease or disorder.

Achieving all this with a single direct fetal vector injection is challenging. Another approach that has been used successfully in fetal life or adult life is to combine stem cell transplantation (SCT) with gene therapy (Aiuti, 2002; Cavazzana-Calvo et al., 2010; Di Bartolomeo et al., 2008; Gaspar et al., 2006). The UK Gene Therapy Advisory Committee (GTAC) considered such an *in-utero* stem cell gene therapy (IUSCGT) approach in their broader judgments about fetal gene therapy. The New and Emerging Technologies subgroup of GTAC found that the use of genetically modified stem cells in stem cell transplantation to the fetus was a possibility, stating "such *ex vivo* modification would be unlikely to carry with it any higher risk to the germ line than the trials of postnatal somatic gene therapy which have already been approved"(Sue Eckstein, 2003). A list of diseases that could benefit and may be used for such an approach is listed in Table 1-1. Preclinical studies of direct fetal gene transfer are encouraging. Fetal application of gene therapy in mouse models of congenital disease such as haemophilia A (Lipshutz et al., 1999) and B (Waddington et al., 2004), congenital blindness (Dejneka et al., 2004), Crigler-Najjar type 1 syndrome (Seppen et al., 2003) and Pompe disease (glycogen storage disease type II) (Dejneka et al., 2004; Lipshutz et al., 1999; Rucker et al., 2004; Seppen et al., 2003; Waddington et al., 2004) have shown phenotypic correction of the condition. More recently the application of perinatal gene therapy has broadened with positive results in previously untreatable conditions. For structural anomalies, transient transduction of the periderm via intra-amniotic delivery of adenoviral vector encoding transforming growth factor β3 (TGFβ3) prevents cleft palate in a mouse model of disease (Wu et al., 2013). For obstetric conditions that affect the fetus, maternal uterine artery injection of adenovirus containing the vascular endothelial growth factor (VEGF) gene improves fetal growth in growth restricted sheep (Carr et al., 2014).

| Disease | Therapeutic gene product | Target cells/organ | Age at onset | Incidence | Life expectancy |
|---|---|---|---|---|---|
| **Cystic fibrosis** | cystic fibrosis transmembrane conductance regulator | airway & intestinal epithelial cells | *In utero* | 1:4,000 | Mid-thirties |
| **Duchenne muscular dystrophy** | dystrophin | myocytes | 2 years | 1:4,500 | 25 years |
| **Lysosomal storage disease: Gaucher** | glucocerebrosidase | hepatocytes | 9-11 years | 1:9,000 overall | < 2 years |
| **Lysosomal storage diseases in general** | lysosomes | Protein deficiency | At birth | 1:5,000 | Very severely affected patients may die within the first five or 10-years of life |
| **Leukocyte adhesion deficiency** | Leucocyte integrin CD18 on chromosome 21 | deficiency of the β-2 integrin subunit | Neonatal period | 1:100,000 | <1 year |
| **Osteogenesis imperfect** | Col1a1 | Collagen | *In utero* | 1:20,000 | variable |
| **Sickle cell disease** | α-globin | erythrocyte precursors | *In utero* | 4:100 (in Africa) | Homozygous patients survive on average to 53 years (men), 58 years (women) |
| **Spinal muscular atrophy** | survival motor neuron protein | motor neurons | *In utero* | 1:10,000 | 2 years |
| **Tay-Sachs disease** | Hexosaminidase A | Neuronal cells | At birth | 1:30 in Ashkenazi Jewish population | <5 years |
| **Urea cycle defects: ornithine transcarbamalase deficiency** | ornithine transcarbamylase | hepatocytes | 2 days | 1:30,000 overall | 2 days (severe neonatal onset) |
| **Haemophilias: A** | human VIII clotting factor | hepatocytes | 1 year | 1:6,000 | Adulthood with treatment |
| **Haemophilias: B** | human factor IX clotting factor | hepatocytes | 1 year | 1:20,000 | Adulthood with treatment |
| **Haemophilias: Factor VII deficiency** | human factor VII clotting factor | hepatocytes | *In utero* | 1:500,000 | Adulthood with treatment |
| **Thalassaemias: $\alpha^{o}$-thalassaemia** | $\alpha$-globin | erythrocyte precursors | *In utero* | 1:2,700 | Lethal |
| **Thalassaemias: β-thalassaemia** | β-globin | erythrocyte precursors | First 2 years of life | 1:100000 | For thalassaemia major affected patients die before the age of 20 if untreated |
| **SCID: X linked** | $\gamma c$ cytokine receptor | haematopoietic precursor cells | birth | 1:1,000,000 | < 6 months if no bone marrow transplant |
| **SCID: Adenosine deaminase deficiency** | adenosine deaminase | haematopoietic precursor cells | birth | 1:1,000,000 | < 6 months if no bone marrow transplant |
| **Epidermolysis bullosa** | type VII collagen | keratinocytes | birth | 1:40,000 | Adulthood |
| **Severe fetal growth restriction** | vascular endothelial growth factor | uterine arteries | *In utero* | 1:100 | Adulthood if individual survives the neonatal period |
| **Congenital diaphragmatic hernia** | lung growth factors | alveoli | *In utero* | 1:2,200 | Adulthood if individual survives neonatal surgery |
| **Cleft lip and palate** | transforming growth factor β3 | palatal shelf medial edge epithelium | *In utero* | 1:500 | Adulthood |

**Table 1-1 Examples of candidate diseases for perinatal gene/ stem cell therapy**

### 1.12 Selecting the right vector for perinatal gene therapy

Vectors are the means used to carry the therapeutic gene into cells. The ideal vector for perinatal gene therapy should be able to produce long-term regulated and therapeutic expression of the transferred gene via a single and efficient gene delivery method and it should be safe for the mother and fetus, to allow integration into clinical practice. In addition, there are other essential vector characteristics depending on the intended application, described in Table 1-2. For example a vector carrying the β globin gene should deliver and express the gene only to erythroid specific cells and lineages.

| Characteristic | Reason |
|---|---|
| Highly efficient, regulated transgene expression | Provide therapeutic protein expression |
| Length of time of transgene expression to suit disease | Example (1) Long term transgene expression for a monogenic disorder requires protein expression to last the lifetime of the individual eg haemophilias<br>Example (2) Transient transgene expression for a developmental or obstetric disorder requires protein expression at a critical window of fetal development or gestation eg fetal growth restriction |
| Specific tropism to target organ | Avoid systemic gene transfer |
| Large carrying capacity | Accommodate therapeutic gene and any required regulatory elements |
| No toxicity | Safe for mother, fetus and future progeny |
| No immunogenicity | Avoid generating a fetal immune response |
| No mutagenic properties | Safe for fetus and future progeny |
| High Concentration | To allow as many cells to be infected as possible |
| Reproducibility of production | Able to be reproduced in various laboratories and under GMP /GLP conditions |
| Ability to Transduce dividing and non-dividing cells | Homogenous transduction of specific stem cell population |

**Table 1-2 Characteristics of the ideal vector for prenatal gene therapy**

The most frequently used vectors in pre-clinical gene therapy studies in the fetus have been adenovirus and adeno-associated virus, lentivirus and retrovirus vectors. Less is known about the effect of other viral vectors and non-viral vectors prenatally. A summary of vectors is provided in Table 1-3.

| Vector | DNA | Efficiency | Tropism | Advantages | Disadvantages | Prenatal Considerations |
|---|---|---|---|---|---|---|
| **Non-viral DNA** | no limit | + | Limited | Low toxicity<br>Low immunogenicity | Low transduction efficiency | Expression may not last through gestation |
| **Adenovirus** | 7.5kb | +++ | depends on serotype | Can grow to high titre.<br>Highly efficient gene transfer | Short term expression & immunogenic | Expression may not last through gestation |
| **Helper-dependent Adenovirus** | 35kb | +++ | Broad | Low immunogenicity, high capacity, long-term expression in quiescent cells | Inefficient production | Insufficient vector concentration |
| **Adeno-associated virus** | 4.7 kb generally | ++ | Depends on sub-type | Long term expression<br>Low immunogenicity<br>Very high titre | Liver toxicity in adult trials due to anti-capsid T cells | Some subtypes associated with miscarriage.<br>Low level integration into active genes so theoretical mutagenesis risk. |
| **Gamma retrovirus** | 10kb | + | Depends on pseudotyping | Long term gene transfer | Potential for insertional mutagenesis.<br>Infect dividing cells only. | Risk of germ-line transmission and insertional mutagenesis.<br>Virus inactivated by amniotic fluid |
| **Lentivirus** | 10kb | ++ | Depends on pseudotyping | Long term gene transfer<br>Infects dividing and non-dividing cells | Potential for insertional mutagenesis | Risk of germ-line transmission and insertional mutagenesis |
| **Non-integrating lentivirus** | 10kb | ++ | Depends on pseudotyping | Insertional mutagenesis unlikely | Short term expression | Rapidly dividing fetal cells may result in long term low transgenic protein expression |
| **Foamy virus (type of retrovirus)** | 10kb | ++ | Broad | Long term expression.<br>Not associated with disease in humans. | Potential for insertional mutagenesis | Less likely to integrate into transcriptionally active regions when compared to classical retroviral vectors, such as lentiviruses |
| **Adeno-retro hybrids** | 7.5kb | ++ | As per adenovirus | Highly efficient gene transfer with long term expression | Potential for insertional mutagenesis | Risk of insertional mutagenesis |
| **Herpes simplex** | 40-50kb | ++ | Broad: CNS | Retro-axonal transduction<br>Infects non-dividing cells | Mainly infect sensory neurons. | Expression can be altered from short term to latent by alteration of viral promoter |
| **Vaccinia** | 25kb | + | Broad | Infects dividing and non-dividing cells | Elicits immune responses when used as a vaccine | Not used in prenatal therapy to date |

**Table 1-3 Types of vector and considerations in relation to perinatal gene therapy application**

## 1.13 Non-viral vectors

Non-viral vectors are an appealing choice as they are thought to be safer and can be used to transfer large fragments of genetic material. Some fetal studies have shown promising results. Intrahepatic injection of the cationic polymer polyethylenimine (PEI) in murine fetuses at a late stage in pregnancy enhanced gene transfer to the liver when compared to delivery of naked DNA. Results were encouraging, with marker gene expression 40-fold higher per milligram of protein in fetuses compared with adults (Gharwan et al., 2003). Low-level gene transfer to the

airway epithelium of sheep fetuses was also achieved using guanidium-cholesterol cationic liposomes delivered into the trachea in mid-gestation (Luton et al., 2004).

Unfortunately, current non-viral systems have low transduction efficiency and short expression time, which limit their usefulness. Manipulation of non-viral vector particles can overcome some of the issues with their use. For example, altering the chemical structure of carbon bonds within cationic liposome-DNA complexes improves their transfection efficiency and reduces their toxicity *in vivo* (Fletcher et al., 2006). Other developments include artificial chromosomes and Epstein—Barr-virus-based plasmids. DNA introduced as plasmid molecules remains episomal and will be lost following cell division, which during fetal growth is fast and could be a particular disadvantage. However transient gene transfer may be used for managing developmental conditions that require therapy for a relatively short time, for example in cases of patent ductus arteriosus, as shown in a sheep study by Mason et al. (Mason et al., 1999).

### 1.14 Adenovirus

Adenoviruses are useful for proof-of-principle studies in gene therapy because they achieve highly efficient gene transfer in several fetal tissues, which can vary depending on the route of administration (David et al., 2003). While they do not have a tropism for the liver, these vectors tend to specifically target liver tissue after intravenous (IV) delivery. However, gene expression is usually transient, as the vector does not integrate into the host genome, and it is rapidly diluted by the continuous cellular proliferation that takes place in the fetus. Although these vectors are very immunogenic in adults, their use in the fetus can lead to extended gene expression and immune tolerance to the transgene and even to the vector. It should be noted that immune responses to adenovirus have also been reported after fetal application, even in early gestation (David et al., 2003; Lipshutz et al., 2000; Waddington et al., 2003). To reduce the immunogenicity and toxicity of the vector, adenoviral coding sequences can be removed

to generate 'gutless vectors'. The creation of novel hybrid vectors that combine the high infectivity of adenoviruses with the permanent nature of integrative vectors (eg. retroviruses and lentiviruses) will be useful in the fetus (Crystal, 2014; Joyeux et al., 2014). Adenoviral vectors have also been used for the treatment of fetal growth restriction (FGR) in a sheep model of FGR, with good outcomes (Carr et al., 2014).

### 1.15 Adeno-associated virus vectors (AAV)

AAV vectors are thought to be less toxic and immunogenic than early-generation adenovirus vectors; however, an immune response has also been observed after fetal intramuscular injection of AAV. Long-term transgene expression can be achieved after muscular, peritoneal or amniotic injection into the fetal mouse and rat (Bouchard et al., 2003; Lipshutz et al., 2001; Mitchell et al., 2000; Mühle et al., 2006; Waddington et al., 2003). AAV vectors slowly integrate into the genome and are therefore likely to be quickly diluted by the increasing tissue mass that takes place during fetal growth. Wild-type viruses mainly integrate at a specific location on human chromosome (which doesn't have a functional role), reducing the theoretical risk of insertional mutagenesis (Garrett et al., 2003).

Replication deficient adenoviral vectors (rAAV) have been found to have a therapeutic benefit, improving vision, for some inherited degenerative conditions that compromise photoreceptor by genetically modifying cells in the retina (Hellström and Harvey, 2011). Indeed there were some promising outcomes in a Leber's congenital amaurosis (LCA) clinical trial using rAAV vectors encoding RPE65 (Al-Saikhan, 2013; Hellström and Harvey, 2011; High and Aubourg, 2011).

AAVs can cross the adult BBB and achieve widespread CNS transduction after systemic gene delivery to the CNS in marmosets. Systemically delivered rAAVrh.10 can transduce the CNS efficiently and its transgene expression can be limited in the periphery by endogenous miRNAs

in adult marmosets (Yang et al., 2014).

### 1.16 Retrovirus and Lentivirus

Retroviruses and the closely related lentiviruses can integrate permanently into the genome, thus offering the possibility of permanent gene delivery. Moloney leukaemia retrovirus (MLV) was the first vector used to test the distribution of neuronal clones throughout the developing cerebral cortex(Tarantal, O'Rourke, et al., 2001). MLV has since been used in several fetal studies of gene therapy, with long-term expression in rats, sheep and non-human primates after intraperitoneal (IP) and intrahepatic delivery. In order for retroviruses to be successful for gene transfer they need dividing cells, which makes them ideal for use in fetal rather than adult tissues where there is rapid cell division. One limitation for *in vivo* human application is that human serum can almost completely inactivate some retroviral particles. However, increased resistance to serum inactivation can be achieved by pseudotyping, a process that replaces the natural envelope of the retrovirus with one from another virus. A significant issue with *in utero* administration is that AF also has a mild inhibitory effect on retrovirus infection. This was probably the cause for the poor gene transfer seen following intra-amniotic application of retroviruses in fetal sheep and non-human primates (Burguete, 1999; Porada et al., 1998; Schoenhard and Hatzopoulos, 2010).

Lentiviruses such as those similar to HIV can also infect non-dividing cells, although with some lentiviruses gene transfer to the liver is improved by cell cycling. Pseudotyping improves lentivirus stability and allows vector titres to be improved by ultracentrifugation. Viral envelope changes can preferentially enhance gene transfer to different target tissues. For example, intramuscular and intrahepatic injection of an HIV vector pseudotyped with vesicular stomatitis virus protein G (VSVG) envelope in murine fetuses preferentially transduced the fetal liver, whereas pseudotyping with mokola or ebola envelope proteins efficiently transduced

myocytes. Lentivirus vectors integrate into the genome randomly, thus in theory, are able to cause insertional mutagenesis (Cavazzana-Calvo et al., 2010; Joyeux et al., 2014; Levasseur et al., 2003).

### 1.17 Gene editing approaches

The first approach that was developed to edit genes was zinc finger nuclease (ZFN)–mediated repair. ZFNs are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain. Zinc finger domains are engineered to target desired DNA sequences within complex genomes and can be used in various ways. Heterozygous individuals with dominant mutations can benefit by disabling the mutation by producing double-strand breaks (DSBs) in the DNA. Alternatively, the sequence of an allele can be rewritten by invoking the homologous recombination machinery to repair the DSB using the supplied DNA fragment as a template. ZFN-encoding plasmids can also be used to transiently express ZFNs to target a DSB to a specific gene locus in human cells providing a targeted gene editing approach (Kim and Kim, 2014). This approach has been since enhanced with the use of transcription activator-like effector nucleases (TALENs) (Bedell et al., 2012; Gaj et al., 2013). TALENs use DNA-recognition modules that recognize single base pairs, linked to the same FokI-derived cleavage domain that is used in ZFNs (Gaj et al., 2013; Hwang et al., 2013). Natural TALE proteins have several different modules for each of the four base pairs. However, a code has now been developed based on the most common modules, allowing simple and effective assembly of new binding domains (Boch et al., 2009; Cermak et al., 2011; Christian et al., 2010; Moscou, 2009). Reports of successful applications to genomic targets are increasingly reported (Bedell et al., 2012; Beumer et al., 2013; de Souza, 2011; Hartman et al., 2018; Hwang et al., 2013; Xiao et al., 2013) .

RNA-guided engineered nucleases (RGENs) derived from the bacterial Clustered Regularly

Interspaced Short Palindromic Repeat (CRISPR)-Cas (CRISPR-associated) system are now available. In bacteria, the CRISPR system provides acquired immunity against invading foreign DNA via RNA-guided DNA cleavage. 'Spacers', which are small segments of foreign DNA are integrated within the CRISPR genomic loci and transcribed and processed into short CRISPR RNA (crRNA). These crRNAs anneal to transactivating crRNAs (tracrRNAs) and direct sequence-specific cleavage and silencing of pathogenic DNA by Cas proteins. CRISPR/Cas-mediated genome editing was successfully demonstrated in zebrafish, bacterial cells, human haematopoietic stem cells (HSCs) and human induced pluripotent stem cells (iPSCs) (Bak et al., 2018; Gaj et al., 2013; Kim and Kim, 2014; Lyu et al., 2018).

It is likely that ongoing advances in these gene-editing approaches may be used in human disease (Xie et al., 2014) to induce site-specific DNA cleavage in the genome and repair, through endogenous mechanisms, giving high-precision genome editing (Humbert et al., 2018).

### 1.18 Perinatal gene therapy delivery

For gene therapy to the fetus to be useful clinically, developments in vector technology must also be followed by improvements in minimally invasive methods of delivering vectors to the fetus. Usually invasive surgical techniques such as maternal laparotomy or hysterotomy were used to access the fetus in small- and even large-animal models. However, minimally invasive techniques, for example ultrasound-guided injection, or even fetoscopy, are more likely to be used clinically to deliver gene therapy to the fetus as these techniques are associated with lower rates of morbidity and mortality. Ultrasound-guided techniques can be used to deliver gene therapy to the placenta or amniotic fluid, while interventional radiology can be used to access the uterine vessels (Boelig et al., 2016; David et al., 2003; Endoh et al., 2002; Foust et al., 2009; Lipshutz et al., 2001; MacKenzie et al., 2002; Rahim et al., 2011).

In order to reach the clinic, a gradual progression from small animal disease models, such as the mouse, followed by studies in larger animals, for example sheep or rabbits, to test the feasibility and safety of the delivery method will be necessary. A final test of safety might be required, using non-human primates before establishing a clinical trial of fetal gene therapy (Hartman et al., 2018; Poletti et al., 2018).

Rodent models have numerous advantages including a shorter gestation of around 20-22 days, the higher number of litters, the ease of maintaining the colonies due to the small physical size and their relatively low cost. A further advantage is that the mouse genome is well defined with many transgenic models available that mimic human monogenetic disorders (Rosenthal and Brown, 2007). Current improvements in high frequency ultrasound and in technical expertise have made it possible to more accurately perform procedures on the fetal mouse (Carlon et al., 2012; Coutelle et al., 2005; Endo et al., 2012).

Sheep serve as a well-established animal model of human fetal physiology and are easy to breed and maintain (Barry and Anthony, 2008; Morrison, 2008). Additional benefits of using sheep include a consistent gestation period and the similarities of fetal development and the immune system to humans (Porada et al., 2004). Ultrasound-guided injection techniques have been adapted from fetal medicine practice in pregnant sheep and new methods have been developed to deliver gene therapy.

Non-human primate research is not widely used for ethical and cost-related issues; maintenance and breeding are costly, which do not justify animal use for the development of novel injection techniques. However, techniques developed in sheep have been tested in non-human primates in the US. These include the use of ultrasound guidance to deliver gene therapy into the amniotic cavity or for direct injection of the lung and liver parenchyma (Tarantal, Lee, et al., 2001). In order to progress to human, the appropriate time frames for the use of different application routes still need to be determined, and technical feasibility, fetal

physiology and the development of the fetal and adult immune system need to be considered (Davis and Brodin, 2018; Mold and McCune, 2012). For example, in the human fetus, the immune system develops from 12 to 14 weeks of gestation, when significant increases in circulating T lymphocytes can be seen. Therefore, if gene therapy needs to be delivered before this occurs, the routes of application that can be safely used will be limited. Non-human primate research may help identify some of these obstacles before human clinical application (Aluvihare et al., 2005; Burt, 2013; Mold and McCune, 2012).

In clinical fetal medicine practice, there are rare complications associated with cordocentesis (approximately 1% of cases) and include emergency preterm delivery, cord rupture, spasm, tamponade from a haematoma or excessive bleeding, volume overload and chorioamnionitis (Lindenburg et al., 2014). Liley was the first to describe an IP approach (LILEY, 1963) which is sometimes used in clinical practice when early gestation blood transfusion is required but intra-umbilical vein transfusion carries more risk for the fetus (Geaghan, 2012).

### 1.19 Primary Immune Deficiencies

Primary immune deficiencies arise from inherited mutations in genes required for the production, function or survival of specific leukocytes such as T, B or NK lymphocytes, neutrophils and antigen-presenting cells, or caused by cytotoxic metabolites. Leukocytes originate from pluripotent HSCs in the bone marrow (BM), and therefore allogenic bone marrow transplantation (BMT) from a healthy donor into an affected patient can restore the immune system. Successful BMT has been achieved in deficiencies such as Wiskott-Aldrich Syndrome (WAS), Chronic Granulomatous Disease (CGD) and Adenosine Deaminase Deficiency, where there is toxicity rather than a defect of the proliferation gene and in X-linked Severe Combined Immunodeficiency (SCID). Except for X-SCID all the other primary immune deficiencies require extra therapeutic steps such as pre-transplant conditioning, marrow cytoreduction to “make

space" in the marrow for the transplanted HSC and immune ablation to prevent rejection of the donor HSC. These strategies carry risks for the patient, and in some cases, a haploidentical donor is not available. Gene therapy was therefore developed for the treatment of some patients.

Post-natal gene therapy using *in-vitro* transduced autologous HSCs with subsequent transplantation into the same patient has been used successfully in adenosine deaminase deficient SCID (Aiuti, 2002; Gaspar et al., 2006), X-linked SCID (Hacein-Bey-Abina et al., 2002) and CGD (Ott et al., 2006). Despite encouraging results, four out of twenty-six subjects subsequently developed a T cell leukemia-like condition, which may have been related to integration of the retroviral vector near a suspected proto-oncogene (Hacein-Bey-Abina et al., 2003). Newer approaches to decrease this risk have used lentivirus vectors that have been studied in non-human primates (Hanawa et al., 2004).

Prenatal diagnosis of the primary immune deficiencies is available where the gene mutation is known. For example, it has been applied in families that have been identified to be at risk of these conditions, such as those harbouring mutations in both of the recombination activating genes RAG1 and RAG2 that are involved in SCID syndromes (Tabori et al., 2004; Touzot et al., 2014).

### 1.20 Congenital blood disorders

Targeting inherited blood disorders for fetal application of gene therapy appears a feasible task, as the fetal circulation can be reached safely from approximately mid-gestation during its circulation through the umbilical vein (UV), at the placental cord insertion or the intrahepatic UV. If the fetus needs to be reached at an earlier time during gestation, for example before the development of the immune system, this can be achieved through the peritoneal cavity as this is already used clinically, with success, to transfuse anaemic fetuses (Fox and Saade, 2012;

Lindenburg et al., 2014).

Congenital blood disorders have high prevalence in some populations, with prenatal screening and diagnostic services possible in many countries. For some conditions, the disease could be corrected by the presence of the correct therapeutic proteins in the blood; these can be secreted functionally from various tissues, with the actual site of production not critical, providing that therapeutic plasma levels can be achieved. Therefore, ectopic sites of protein production such as the liver and muscle can be targeted (Aronovich et al., 2007; MacKenzie et al., 2002; Mattar et al., 2011).

### 1.21 Lentiviral-mediated globin gene transfer

Lentiviruses (LV), belong to the group of HIV-1 based retroviruses. They are able to transduce and integrate more efficiently in various dividing and non-dividing cells compared to other viruses. In addition, they have several advantages over γ-retroviral vectors (Moi and Sadelain, 2008). These properties make them ideal for use in clinical gene therapy. Third generation LV vectors (devoid of gag, pol and env HIV genes) can transfer large fragments containing LCR with high titres and fidelity due to the presence of RRE elements (Han et al., 2007; Montiel-Equihua et al., 2012). LVs are commonly pseudotyped using the envelope from the vesicular stomatitis virus (VSV- G), which extends their tropism and allows infection of virtually all mammalian cell types mediating endocytic entry without the need for accessory proteins (Cronin et al., 2005). They can be made replication defective (SIN-LV) and have the capability to transduce non-dividing cells including quiescent cells (Miyoshi et al., 1998; Moreau-Gaudry et al., 2001; Ravot et al., 2002; Zufferey et al., 1998)

A breakthrough study by May et al., (May et al., 2000) showed that gene transfer using a LV that contained proximal and distal regulatory elements with a large segment of the LCR (TNS9) could correct β-thalassaemia in mice, with an average increase in Hb by 3-4g/dL per vector

copy and improvement of haematological indices. Similar studies have been published using other thalassaemia mouse models but with variable results (Breda et al., 2012; Dong et al., 2013; May et al., 2000; Negre et al., 2011; Rivella et al., 2003).

### 1.22 Haemoglobin

Haemoglobin (Hb) is an iron-containing metalloprotein found exclusively in red blood cells/Erythrocytes. Hb is the main oxygen carrier, which delivers oxygen from the lungs to the tissues in the body and carries carbon dioxide from the tissues back to the lungs. Hb makes up 95% of the RBC, with over 300 million Hb molecules. Hb is a heterotetrameric protein composed of two α-like (141 amino acids) and two β-like globin (146 amino acid) chains; each chain is associated with a single prosthetic haem group containing an iron atom held in a heterocyclic porphyrin ring. The two α and two β-chains form helices, which are connected by short non-helical regions and are stabilized by hydrogen bonds (Schechter, 2008). Thus, the four chains carry four haem groups that bind to four oxygen molecules. The iron groups in the Hb molecules render the colour red to the blood (Weatherall et al., 2006).

There are different isoforms of human Hb that are approximately 64 KDa in molecular weight but differ in chain composition. In adult humans, HbA or adult Hb consists of two α and two β-globin chains. HbA is produced in erythroid cells arising from HSCs in the bone marrow. However, in early development, the first embryonic types are expressed in the yolk sac, and then the fetal haemoglobins (HbF) are expressed in the liver and the spleen. Most forms of embryonic and fetal Hb are composed of two α- chains and two γ-chains. The HbF has a greater oxygen affinity in order to permit efficient gas exchange between the mother and the baby's blood supply via the placenta. The γ chains of HbF are gradually replaced by β-chains as the infant grows and by 6 months post-birth, all the γ-chains are replaced (Sankaran and Orkin, 2013).

The gene coding regions of the α-like chains (*HBA*) and β-like chains (*HBB*) are located in separate clusters, on chromosome 16 and chromosome 11 respectively. There are five active genes in the β locus region, each with its own unique promoter and a 3-exon 2-intron structure. They are arranged in the order of their temporal activation during development as seen in Figure 1-3 (Higgs et al., 2012; Ho and Thein, 2000; Weatherall, 2001).

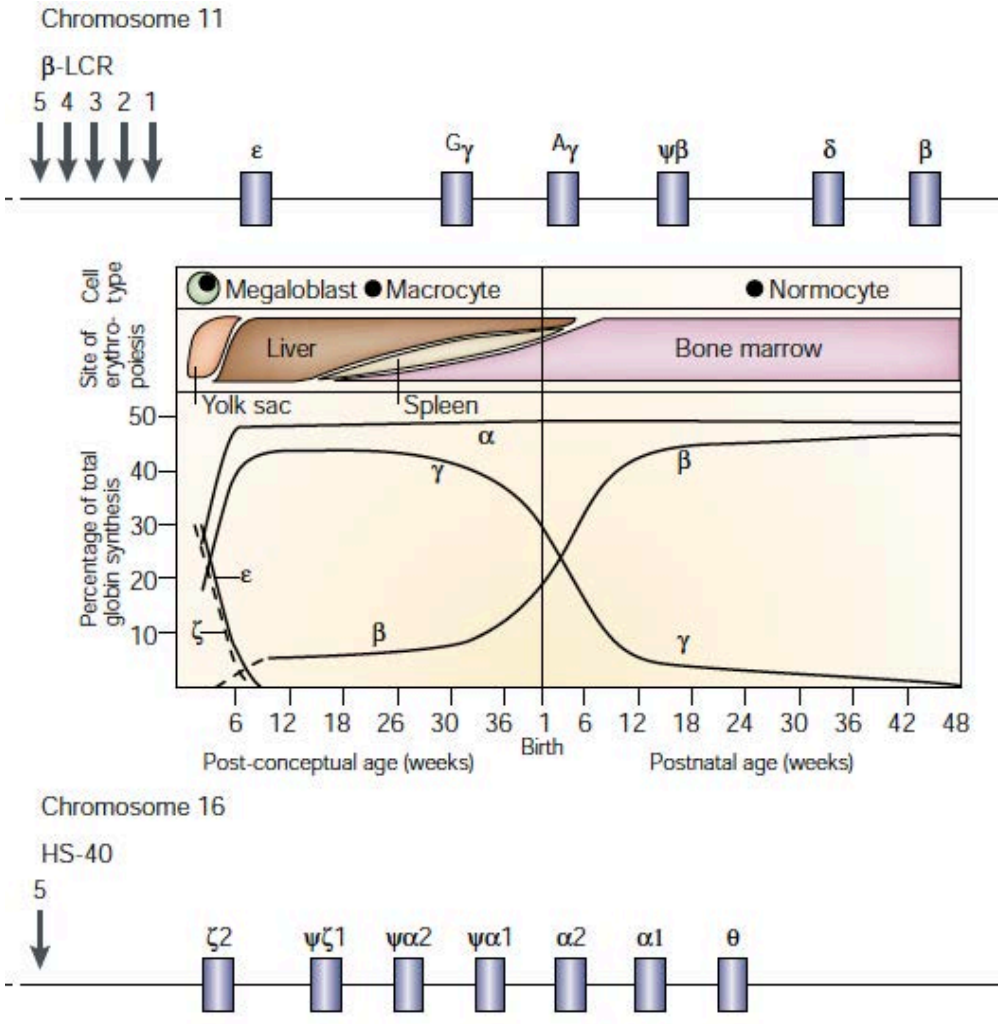



**Figure 1-3 The α-globin gene cluster on chromosome 16 and the β-globin gene cluster on chromosome 11. Haemoglobin Switching during human development: γ-globin is expressed during the fetal stages and is down regulated post-natally. Fetal Hb (HbF) is a tetramer of two α and two γ-globin chains (α2γ2). Hb switch from γ to β-globin chain occurs just after birth. Postnatal Hb (HbA) is a tetramer of two α and two β-globin chains**

**(α2β2). The vertical arrows indicate the location of DNaseI hypersensitive sites that are thought to be involved in globin gene regulation. A megaloblast is a large red cell precursor, a macrocyte is a large red cell and a normocyte is a normal sized red cell, LCR: locus control region. Figure adapted from Weatherall et al (Higgs et al., 2012; Weatherall, 2001).**

### 1.23 Haemoglobinopathies

Haemoglobinopathies are a group of genetic disorders, which result in abnormal Hb production; they are the most common disorders worldwide with 500,000 affected births every year (Modell and Darlison, 2008; Sii-Felice et al., 2018; Weatherall et al., 2006).

Diseases associated with Hb abnormalities can be divided into two main groups: 1) the structural Hb variants caused by mutations that affect the structure of Hb, for example sickle-cell anaemia (SCA), and 2) the functional mutants that reduce the number of normal functional globin chains leading to an imbalance between α and β chains, for example in the thalassaemias.

The prevalence of haemoglobinopathies is higher in sub-Saharan Africa, South-East Asia and the Mediterranean, with a link to the presence of such mutations with resistance to malaria, which provides a survival advantage to affected individuals. The highest carrier frequency of thalassaemias is found in Cyprus (14%), Sardinia (10.3%) and Southeast Asia (Weatherall et al., 2010). Current estimates suggest that approximately 275,000 babies are born with SCA and 300,000 babies are born with thalassaemia every year. The improvement and access to medical care has prolonged the survival of these patients; however, long term morbidity and mortality are still associated with these diseases (Giordano et al., 2014; Weatherall, 2013).

"Thalassaemia" derived from Greek for thalassa (sea) and haema (blood) refers to disorders related to impaired production of α and β-globin chains of Hb. Clinically, the range of symptoms varies from minimal to severe or even fatal *in utero* and early childhood. The thalassaemias are the most prevalent of the blood disorders and are split in two main

categories: α-thalassaemia (α- chains are defective) and β-thalassaemia (β-chains are defective) (Modell Bernadette and Darlison Matthew, 2008; Weatherall et al., 2006).

β-thalassaemia or Cooley's Anaemia (CA) is an autosomal recessive disorder, which manifests in three ways depending on the severity of the disease: minor, intermedia and major (Table 1-4). The disease is characterised by an inability ($\beta^0$) or reduced ability ($\beta^+$) to synthesise normal levels of the β-globin chains. The β-chain deficit leads to intracellular accumulation of the additional α-globin chains. These α-globin homotetramers cause membrane damage in RBCs, haemolysis and ineffective erythropoiesis (Higgs et al., 2012; Rivella, 2009; Shinar et al., 1989; Shinar and Rachmilewitz, 1990). The haematological characteristics of β-thalassaemia are RBC microcytosis (small-sized), hypochromia (pale-coloured), anisopoikilocytosis (abnormally shaped) and premature death of haematopoietic progenitors and RBCs (Rund and Rachmilewitz, 2005a).

The low levels of Hb and RBCs lead to anaemia, which if left untreated in homozygous and compound heterozygous conditions can lead to death during early childhood (Rachmilewitz and Giardina, 2011; Rund and Rachmilewitz, 2005b).

Most long-term symptoms of β-thalassaemia occur as a consequence of intrinsic compensatory mechanisms. Anaemia and hypoxia increase erythropoietin production, which leads to ineffective bone marrow (BM) development (Fibach and Rachmilewitz, 2017). Attempts to increase RBC production by the BM results in skeletal abnormalities such as long limbs and bowed chest (Higgs et al., 2012). Increased haemolysis leads to splenomegaly (enlarged spleen, the site of RBC destruction) and extra-medullary haematopoiesis.
Furthermore, the increase in intestinal absorption and iron storage, while replacing lost RBCs lead to iron overload and eventually chronic liver disease, such as cirrhosis, endocrine deficiencies and cardiac failure (Borgna-Pignatti et al., 2005; Rund and Rachmilewitz, 2005a).

Over 200-point mutations have been linked to β- thalassaemia, as well as a number of deletions affecting the coding region, promoter region and regulatory regions, which make β-thalassaemia a genetically heterogeneous disease. The location of the mutation can affect β-globin chain production, and this can interfere with transcription initiation and termination, RNA splicing, nuclear export and/or translation (Thein 2005). Heterozygous individuals usually have no symptoms as they have one copy of the functional gene, although there are some rare mutated dominant alleles, which lead to unstable β-globin chain production that interfere with regular tetramer formation and erythropoiesis (dominant negative) (Ho and Thein, 2000; Thein, 2005, 2013).

| β-thalassaemia types | Genotype | Phenotype |
|---|---|---|
| **Minor** | Heterozygous<br>$\beta^+/\boldsymbol{\beta}$, $\beta^0/\boldsymbol{\beta}$ | Asymptomatic carrier |
| **Intermedia** | Homozygous $\beta^+/\beta^+$<br>Compound Heterozygous<br>$\beta^+/\beta^+$, $\beta^0/\beta^+$ | Intermediate anaemia |
| **Major** | Homozygous $\beta^+/\beta^+$ $\beta^0/\beta^0$<br>Compound Heterozygous<br>$\beta^+/\beta^+$, $\beta^0/\beta^+$ | Severe anaemia<br>Transfusion dependent |

**Table 1-4 Classification of β-Thalassaemia types: β refers to normal wild type allele; $\beta^0$ refers to mutations that abolish β-globin chain production; $\beta^+$ and $\beta^+$ refer to two different mutations that reduce β-globin chain production.**

Mutations in other regulatory genes can change the expected phenotype. For instance, mutations, which stop the repression of γ-globin following birth, can reduce the severity of symptoms in β-thalassaemia major. The γ-globin chains will associate with excess α-chains forming functional fetal HbF effectively substituting with HbA in adults (Thein, 2013).

Treatment of hemoglobinopathies holds challenges for gene therapy since the vector is required to carry a large cargo of the globin gene and its regulatory elements, so as to ensure high levels of expression of β-/γ- globin genes for therapeutic correction. In β-thalassaemia,

the tissue and developmental-specific expression of the individual globin genes is governed by interactions between the upstream β-globin locus control region (β-LCR) and the globin promoters (Quek and Thein, 2007). Amelioration or even cure of mouse models of human sickle cell disease (Pawliuk et al., 2001) and β-thalassaemia major (Persons et al., 2003; Puthenveetil et al., 2004; Rivella et al., 2003) has been achieved using lentivirus vectors containing complex regulatory sequences from the LCR region. Yolk sac vessel injection of this optimized lentiviral vector into mid-gestation fetal mice resulted in human alpha-globin gene expression in the liver, spleen, and peripheral blood in newborn mice, with expression that peaked at 3–4 months and reached 20% in some recipients (Han et al., 2007). Expression declined at 7 months of age (normal life-span 2-3 years) possibly due to insufficient HSC transduction or the late stage of mouse gestation at which the vector was introduced.

Recent advances in vector design have improved gene transfer for these diseases. The ubiquitous chromatin opening element (UCOE) augmented Spleen Focus Forming Virus (SFFV) promoter/enhancer provides lentivirus vectors with a natural tropism for the haematopoietic system (Antoniou et al., 2003; Williams et al., 2005) resulting in reproducible and stable function in bone marrow stem and all differentiated, peripheral haematopoietic cell lineages (Zhang et al., 2007). Encouraging results from the first successful gene therapy in a patient with hemoglobin E-β-thalassaemia in a French trial has opened up gene therapy as a potential treatment option for patients with β-hemoglobinopathies (Cavazzana-Calvo et al., 2010). Trials with different versions of the β-globin and γ-globin genes in self-inactivating lentivirus vectors are beginning in Italy and four centres in the United States to treat thalassaemia or sickle cell disease.

Recently human β-thalassaemia iPSCs were generated from AF cells using a single excisable lentiviral stem cell cassette vector. AF cells from the prenatal diagnosis of a β-thalassaemia patient were reprogrammed by expression of the four human reprogramming

factors Oct4, KLF4, SOX2 and c-MYC using a doxycycline lentiviral system and demonstrated teratoma formation (Fan et al., 2012). This type of cell manipulation may provide clinicians with corrected autologous patient-specific iPS cells to use in a combination IUSCGT approach for the treatment of thalassaemia (Fan et al., 2012).

### 1.24 β-globin locus regulation

The β-like globin genes are arranged at the genomic locus in the order of their temporal expression during development. During the embryonic stages (up to eight weeks of gestation), the yolk sac is the site of erythropoiesis and embryonic haemoglobin composed of α-like ζ-chains combined with ε chains (ζ2ε2) are synthesized (Weatherall, 2001). The first important switch during development happens when haematopoiesis switches to the fetal liver, with the simultaneous synthesis of HbF comprised of two α and two γ chains (α2γ2) and down-regulation of ζ and ε chains (Sankaran et al., 2010, 2011; Weatherall, 2001).

The second and final shift occurs when haematopoiesis shifts from the fetal liver to the BM and leads to perinatal silencing of HbF and parallel induction in the synthesis of the adult form of haemoglobin, HbA (α2β2). At two years of age, HbA represents ~99% of the total haemoglobin being produced (Sankaran et al., 2010; Weatherall, 2001).

### 1.25 Humanised CA (B382) mouse model

It should be noted that mice do not have any fetal globin equivalent and in contrast to humans, there is only one Hb switch during their development. In mice, four genes are expressed from the β-globin locus- εY, βh1, βmaj and βmin as seen in Figure 1-4. The embryonic z and y chains are synthesized by the primitive erythroid cells in the yolk sac from βh1 and εY genes respectively. At E12, complete erythropoiesis switches to the mouse fetal

liver with the synthesis of adult βmaj and βmin globin chains, which continues into adulthood (Kingsley et al., 2006; Mazo et al., 2011; Whitelaw et al., 1990). The absence of a γ-like fetal globin results in the prenatal death of βmaj and βmin globin KO mice. Humans born with thalassaemia are healthy at birth because of the presence of high HbF levels in newborn circulating erythrocytes. Therefore, similar to humans, the fetal globin equivalent expressed from E12 to the initial stages of post-natal life in mice provides a window to test new therapies.

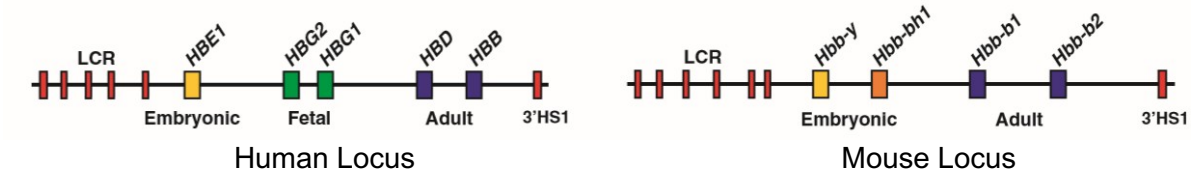



**Figure 1-4 Organization of the Human and Mouse β-globin loci with the linked globin genes (HBE-ε, HBD-δ, HBG-γ, HBB/Hbb-β, Hbb-y- εy), upstream LCR and downstream 3'Hypersensitive1 region. Mice do not have Fetal γ-globin equivalent and have only one Hb switch (from embryonic to adult) during development adapted from (Sankaran et al., 2010)**

A new mouse model of β thalassaemia (CA mice) was developed, which more closely mimics human disease. CA mice were engineered by targeted gene replacement of both adult mouse β-globin genes in a C57 BL/6J mouse with a delayed switching human $\gamma^{HPFH}$ to $\beta^{0}$- globin gene cassette as seen in (Huo et al., 2017; Huo, McConnell and Ryan, 2009; Huo, McConnell, Liu, et al., 2009) (Figure 1-5) adapted from Huo et al 2017. The human γ-globin gene promoter contained the Greek-type hereditary persistence of fetal haemoglobin (HPFH) mutation (Gelinas et al., 1985) to postpone the fetal-to-adult haemoglobin switch, and the human β-globin gene contained a splice donor site mutation to generate a non-functional $\beta^{0}$-thalassaemia allele.. Heterozygous $\gamma^{HPFH}\beta^{0}$ Knock In (KI) mice generated by crossing chimeric mice with WT C57BL/6J exhibit thalassaemia intermedia. Homozygous $\gamma^{HPFH}\beta^{0}$ KI CA mice were generated by breeding two heterozygous KI mice. These CA mice were bred

with human α-globin KI mice to generate humanised homozygous CA mice (α2α1/α2α1,$\gamma^{HPFH}\beta^0/\gamma\beta^A$). The human $\gamma^{HPFH}\beta^0$ cassette delays the human fetal-to adult globin gene switch in KI mice and mimics the postnatal haemoglobin- switch pattern observed in humans. The homozygous CA mice have severe β-thalassaemia phenotype, are paler in colour and survical up to 17 days solely on human HbF. The 17-day life span corresponds to 1.5-2 years of age for a patient with untreated β-thalassaemia major. As with human patients, regular blood transfusions, or BM transplant can save the CA mice from lethal anaemia (Huo et al., 2010, 2017; Huo, McConnell and Ryan, 2009; Huo, McConnell, Liu, et al., 2009).

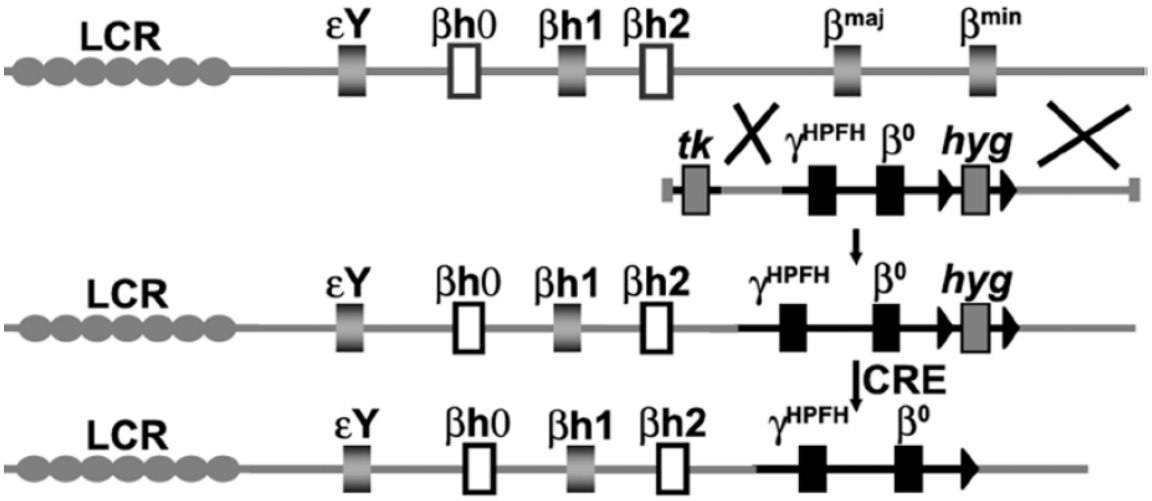



**Figure 1-5 Knock-in of a delayed human fetal-to-adult globin gene switching cassette into the mouse β- globin locus.**
**Gene targeting strategy to replace mouse β-globin genes with human globin genes in ES cells. The unmodified murine β-globin locus is shown at the top with the locus control region (LCR) upstream of the functional primitive embryonic (εY and βh1) and definitive adult (βmaj and βmin) globin genes and pseudogenes (βh0 and βh2). The human $\gamma^{HPFH}\beta^0$ targeting vector is designed to replace 16kb of mouse sequence containing the adult globin genes with 8.7kb of human sequence containing mutant human fetal ($\gamma^{HPFH}$) and adult ($\beta^0$) globin genes and a 2kb hygromycin (hyg) marker gene flanked by LoxP sequences (black triangles). The herpes simplex virus thymidine kinase (tk) gene is included upstream for negative selection. Homologous recombination, denoted by the crossed lines, occurs within the 5' and 3' mouse homology regions (grey lines) to generate a $\gamma^{HPFH}\beta^0$hyg KI allele in the mouse β-globin locus. After breeding to CMV-Cre transgenic mice the marker gene is deleted leaving a single LoxP sequence downstream of the $\gamma^{HPFH}\beta^0$ KI allele, figure adapted from Huo et al 2017 (Huo et al., 2010, 2017; Huo, McConnell and Ryan, 2009; Huo, McConnell, Liu, et al., 2009)**

### 1.26 Current Therapies for β-thalassaemia

There are two main treatments available for β-thalassaemia major patients: blood transfusions or HSC transplantation (HSCT). Blood transfusions need to be administered throughout life, starting from 6-18 months after birth, in order to improve anaemia. Iron chelation treatment is combined with blood transfusions, with the frequent administration of subcutaneous or oral iron chelators (e.g. Deferiprone), to minimise iron overload and organ damage (Borgna-Pignatti et al., 2005; Cao and Galanello, 2010). Patients that suffer from thalassaemia intermedia are less dependent on transfusions and iron chelation, but may require intermittent administration or at a later stage in their life. Individuals that carry the minor phenotype are asymptomatic and do not require transfusions.

The combined use of blood transfusions and iron chelation treatments are effective in prolonging the life expectancy of the severely affected patients but are only palliative. However, the high dependence on blood donations means it is an expensive treatment and a big healthcare burden to countries with high incidence. A one-off curative treatment would be beneficial both in terms of cost and quality of life for the patient.

Currently, the only available treatment that cures the disease is allogenic HSCT. HSCT is usually reserved for young patients with minimal organ damage. The use of HSCT has shown 90% survival and 80% thalassaemia-free survival, (Angelucci, 2010) but it is only available in approximately 30% of cases because of the unavailability of HLA-matched donors. The procedure can lead to adverse immunological reactions such as graft-versus-host disease or graft rejection (Lucarelli et al., 2002).

Therefore, both transfusion therapy and HSCT carry risks and are expensive in the long-term. This makes β- thalassaemia an ideal target for a number of new curative measures such as cell and gene therapy, iPS cell therapy and chemotherapy. These rely on the principle of

restoring the function of the patient's own HSCs and reduce the need to have access to suitable donors (Higgs et al., 2012).

### 1.27 Gene therapy for β-thalassaemia

Haemoglobinopathies were some of the first disorders for which genetic treatments were envisaged. However, the complexities of the expression and regulation of the globin genes make this challenging. It is worth noting that some patients have hereditary persistence of HbF, which results in milder disease phenotype and better survival (Perrine et al., 2005, 2010). This was a significant realisation, which suggested that the induction of γ-globin expression in adult patients might help long-term prognosis. Indeed, certain medicinal measures that promote HbF have improved clinical outcomes (Makis et al., 2016; Musallam et al., 2013; Vrontaki et al., 2016). Additional studies on γ-globin silencing can help the design of targeted interventions for its reactivation following birth.

Direct repair or editing of the defective β-globin gene would be ideal but it is not yet possible in HSCs. Gene addition using viral vectors and chromosomal integration of the provirus containing the corrected β-globin gene is the preferred approach. The requirements to achieve successful therapeutic gene therapy for β-thalassaemia are:

- High efficiency gene transfer into HSCs and HSC engraftment
- High/therapeutic levels of globin gene expression
- Regulated, erythroid, lineage-specific and developmental stage-specific expression of the transgene
- Viral Integration into the host genome devoid of replication competence and safe expression with little or no risk of insertional oncogenesis/mutagenesis
- Consistent expression independent of the site of integration

### 1.28 Use of Manipulated Stem Cells for Perinatal Therapy

The use of gene therapy at the initial stages of gestation before the immune system has matured could, in theory, minimise the need for marrow conditioning or the search for an HLA-matched donor. Prenatal treatment using HSC transplantation has been attempted in various immunodeficiencies and hemoglobinopathies. This was done through IP transfer of paternal or maternal haematopoietic cells or fetal liver (Muench, 2005). However, the most successefyl outcomes were seen in cases of X-linked SCID, (Flake et al., 1996; Westgren et al., 2002) where no immune response to the transplanted cells could be mounted.

Stem cells from several fetal tissues such as the blood, liver, AF and the placenta have been identified that could be used as an autologous cell source for gene transfer (Shaw et al., 2011). Accessing stem cells from the fetal liver or blood sampling at an early gestational age carry a significant risk of miscarriage (Orlandi et al., 1990; Schoeberlein et al., 2004). However, pluripotent stem cells can now be easily derived from fetal samples collected at amniocentesis (De Coppi, Bartsch, et al., 2007) or chorionic villus sampling (Lee et al., 2010; Parolini et al., 2008, 2010; Portmann-lanz et al., 2006), procedures that carry low risk to the fetus . hAF stem cells can differentiate into multiple cell types and can be transduced easily without changing their characteristics (Bollini et al., 2011; De Coppi, Bartsch, et al., 2007; Ditadi et al., 2009; Spinelli et al., 2013). *In vivo* experiments in sheep showed good fetal survival following autologous AF mesenchymal stem cell (MSC) transplantation. AF MSCs were obtained using ultrasound-guided amniocentesis, subsequently expanded and transduced before they were injected IP into the donor fetus. Extensive cell migration and engraftment were reported, specifically in the liver, heart, muscle, placenta, umbilical cord and adrenal gland (Shaw et al., 2016). A recent work done tested the functional haematopoietic potential of AF-derived stem cells, transduced with $GFP^+$, in sheep, before and after autologous IUSCT. AF was collected

from first trimester sheep using ultrasound-guided amniocentesis or at post mortem examination. Sheep $CD34^+$ AF or adult BM cells were selected and transduced with an HIV lentivirus vector containing eGFP. Transduced fresh or frozen $CD34^+$ AF, or BM cells were injected intravenously into NOD-SCID-gamma (NSG) mice. $GFP^+$ cells were detected in the haematopoietic organs and peripheral blood of NSG mice primary and secondary recipients three months later. Autologous IUT was performed in fetal sheep using ultrasound-guided IP injection of fresh transduced $GFP^+CD34^+$AF cells. $GFP^+$ cells were detected in the peripheral blood of injected lambs up to 6 months following birth. In addition, three months after secondary transplantation of BM from autologous IUT lambs into NSG mice, $GFP^+$ cells were detected in haematopoietic organs. These results are encouraging for future clinical translation of an *in utero* approach for the treatment of congenital haematopoietic diseases, as they demonstrate that autologous IUT of CD34+AF cells is successful in a large animal model (Shaw et al., 2015).

### 1.29 Considerations for Translation of Perinatal Gene Therapy to Human Application

As with any new treatment modality preclinical testing in animal models is a necessary step before clinical translation is realized. When choosing the most appropriate animal model researchers need to consider different parameters including the gestational development of the target organ and its resemblance to the human, the type of placentation, size and number of fetuses, lifespan, parturition, and fetal and maternal immune response.

In addition, toxicology studies are necessary to ensure safety; animals such as pregnant rabbits are ideal. Historically pregnant rabbits are used for reproductive toxicology testing, with good datasets available, which makes them widely accepted by regulators. Several guidelines and regulations will need to be considered when planning preclinical study protocols, for example, those described by the Committee for Medicinal Products for Human Use (CHMP)

of the European Medicines Agency. Such guidelines can contain suggestions on non-clinical testing for inadvertent germline transmission of gene transfer vectors (Agency, 2007; EU, 2006) or on the requirement for non-clinical studies before first clinical use of gene therapy medicinal products (Schneider et al., 2010).

### 1.30 Phase I trials

Phase I human trials are likely to face hurdles because of difficulties in testing pregnant women where toxicological studies are usually contraindicated. Thus, when human application becomes possible, extensive un-biased parental counselling and informed consent is paramount because of the uncertainties about the efficacy and long-term safety of prenatal gene therapy which may not become evident until much later in the individual's life. This can be difficult when the decision to participate in a fetal gene therapy trial will occur close to the time of prenatal diagnosis of the condition. Because the risks involve the mother, fetus and future progeny, parents will also be required to consent their offspring and themselves to lifelong follow up.

One criticism levelled at fetal gene therapy is a belief that couples pregnant with an affected child would be unlikely to proceed with prenatal therapy and would opt for termination instead. This concern is not solely applicable to perinatal gene therapy, but can also be raised for any fetal treatment such as fetal surgery and *in utero* stem cell transplantation. The general public have been concerned that ethical discussion about issues such as gene therapy, cloning and the Human Genome Project are falling behind the technology. There is almost no research in this area, and the views of the general public and patient groups will need to be solicited as the technology is being translated into the clinic. In this regard, the EVERREST consortium that is translating maternal perinatal gene therapy into the clinic for treatment of FGR is conducting a detailed bioethics investigation with stakeholders, patients and the public to

gauge their opinion (Spencer et al., 2017).

In June 2007, the first gene therapy clinical trial for a transfusion dependent β- thalassaemia patient was done using a SIN-LV based β-globin vector in France. The transduction efficiency was reported to be 30%. The patient became transfusion independent with a stable Hb of 8.5-9g/dL by two years after gene transfer. Clonal expansion at the HMGA2 locus in erythroid cells was observed suggesting insertional activation in the cells (Cavazzana-Calvo et al., 2010). Following that, the reported outcome in twenty two patients with transfusion-dependent β-thalassaemia is positive with reduction or complete elimination of the need for blood transfusions and no adverse effects to the drug product (Thompson et al., 2018).

A more recent trial for an *in-utero* treatment of α-thalassaemia is currently recruiting at UCSF in San Francisco and BOOSTB4 trial is recruiting in Europe for the treatment of osteogenesis imperfecta (Witt et al., 2018). While investigators are carefully weighing the benefit/risk ratio, studies are ongoing to develop better vectors, stem cell transplantation systems and protocols with respect to safety and efficacy.

### 1.31 Conclusion

Perinatal therapy offers the potential for clinicians not only to diagnose but also to treat inherited genetic disease. Fetal application may prove better than application in the adult for treatment, or even prevention of early onset genetic disorders such as CNS and liver disorders. Gene transfer to the developing fetus targets rapidly expanding stem cell populations that are inaccessible after birth. Integrating vector systems give permanent gene transfer. In animal models of congenital disease, the functionally immature fetal immune system does not respond to the product of the introduced gene, and therefore immune tolerance can be induced. For the treatment to be acceptable, it must be safe for both mother and fetus, and preferably avoid

germline transmission. Recent developments in the understanding of genetic disease, vector design, and minimally invasive delivery techniques have brought fetal gene therapy closer to clinical practice. However more research needs to be done to answer the questions below before it can be introduced as a therapy. Which vectors can provide long-term regulated gene expression preferably for the lifetime of the individual? What is the best route of administration and the optimum gestational age to target gene therapy for specific diseases? Is an *ex vino* approach better? How can informed consent best be obtained from the couple who are embarking on gene therapy treatment for their fetus?

## 1.32 Hypotheses

This thesis explores the following hypotheses

- Amniotic fluid stem cells can be isolated from the mouse fetus.
- Amniotic fluid stem cells show haematopoietic properties similar to fetal liver and bone marrow HSCs and produce haemoglobin *in vitro*.
- Amniotic fluid stem cells can be transduced using a lentiviral vector.
- Amniotic fluid stem cells can be expanded in a modified embryonic stem cell media.
- Amniotic fluid stem cells engraft after *in utero* transplantation.
- Amniotic fluid stem cells, when transplanted *in utero*, can rescue the homozygous mouse model of beta thalassemia.
- *In utero* gene therapy to the fetal haematopoietic compartment can rescue the homozygous mouse model of beta thalassemia.
- *In utero* gene therapy in a mouse model of beta-thalassemia results in a phenotypic correction.
- IUGT results in normalisation of haemoglobin, red blood cell indices, reduction of extramedullary haematopoiesis and iron overloading.
- Disease after IUGT can be monitored using MRI techniques.
- MRI techniques can be used to monitor cardiac function in a mouse model of beta-thalassemia.
- MRI techniques can be used to assess iron overload in a mouse model of beta-thalassemia.

# Chapter 2 Materials and Methods

## 2.1 Mouse amniotic fluid stem cells

### 2.1.1 Animal Care

All the mice used were sourced from either Harlan Company or the University of Alabama and were housed in a single cage after plugging. Vaginal plugs were checked by technicians and the first day of gestation was considered as the day after the plug was found. The dams were given wet food after surgery and closely monitored for the first seven days following the procedure. The animal experiments were done according to the project license PPL No: 70/7408 and Animals [Scientific Procedures] Act 1986 and the NC3Rs ARRIVE guidelines 2013.

### 2.1.2 Isolation of mouse amniotic fluid stem cells

To mimic autologous IUT, cKit+/lin-, congenic mAFSC were isolated at E13.5 from B6.SJL-Ptprca Pepcb /BoyJ dams (CD45.1; MHC class I: H2-$K^{b+}$). For allogenic IUT, mAFSC were isolated from fetuses of BALB/cJ ($CD45.2^{+}$; MHC class I: H2-$K^{d+}$) dams. Time-mated dams were culled in the UCL Biological Services Unit (Western Labs) using a schedule 1 method at E13.5. After confirming death, the abdomen of the mouse was cleaned and carefully opened with sterile scissors and the uterine horns were removed intact into a sterile petri dish. The uterus was irrigated with Phosphate Buffered Saline (PBS) to remove maternal blood. Using sterile scissors, each amniotic sac was carefully dissected from the surrounding uterus and washed with PBS. The amniotic sac was then punctured using fine sterile scissors and the AF (about 50µl) was collected into a 20ml falcon tube after passing through a 50µl nylon filter (Figure 2-1). Only clear mAF was collected. Obviously blood stained mAF was discarded to exclude maternal or placental contamination as described (Ditadi et al., 2009). The clear mAF from all fetuses from up to three dams was pooled. Flow Cytometry Mouse Lyse Buffer (FC003, R&D Systems USA) was used to remove any remaining red blood cells and to

prevent any fetal or maternal blood contamination. The cells were then washed with PBS three times and counted. *In utero* transplantation (IUT) was performed at E13.5 into C57BL/6 (CD45.2/H-2K$^{D}$) dams time mated with C57BL/6 (CD45.2/H-2K$^{D}$) male mice. There were at least three different dams transplanted in three biologically independent experiments in each group.


**Figure 2-1 Mouse Amniotic Fluid Isolation from individual Fetuses. Figure is demonstrating isolation of mAF from an individual fetal mouse**

### 2.1.3 Mouse amniotic fluid lineage depletion

#### 2.1.3.1 Magnetic Labelling

The mouse cells were lineage-depleted by using the lineage depletion kit (mouse, 130-090-858, Miltenyi Biotec, Germany). The cell number was determined by using a manual cell counter. The cells were centrifuged at *300xg* for 5 minutes. The supernatant was pipetted off

completely and the pellet was resuspended in 40µl of *SB* buffer for a maximum of $10^7$ cells, then 10µl of Biotin Antibody Cocktail was added. The solution was mixed well and incubated at 4°C for 10 mins, then 30µl of *SB* buffer and 20µl of Anti-Biotin Microbeads per $10^7$ cells were added. The cells were incubated for 15 minutes at 4°C. An *SB* buffer (1ml) was added before centrifugation at *300xg* for 5 minutes. The supernatant was then removed and the pellet was resuspended in 500µl of *SB* buffer.

#### 2.1.3.2 Magnetic Separation

The magnetic column was placed on the *QuadroMACS* separator. The column was rinsed with 500µl of *SB* buffer. The cells, which were resuspended in 500µl in *SB* buffer, were then applied to the column. The cells were allowed to pass through and collected in a 15ml Falcon tube. These cells represent the enriched lineage negative cells. The column was then washed 3 times with 500µl of *SB* buffer and the effluent was collected. The cells were then centrifuged at *300xg* for 5 minutes and the pellet was resuspended in an appropriate amount of *SB* buffer or culture medium accordingly.

### 2.1.4 Mouse amniotic fluid CD117 selection (MACS)

#### 2.1.4.1 Magnetic Labelling

The mouse cells were $CD117^+$ selected by using the CD117 Microbead Kit (Mouse, 130-091-224, Miltenyi Biotec, Germany). The cell number was determined by using a manual cell counter. The cells were centrifuged at *300xg* for 5 minutes. The supernatant was pipetted off completely and the pellet was resuspended in 80µl of *SB* buffer for a maximum of $10^7$ cells, then 20µl of CD117 Microbeads per $10^7$ was added. The solution was mixed well and incubated at 4°C for 15 minutes. The cells were washed with 1ml of *SB* buffer and centrifuged at *300xg* for 5 minutes. The supernatant was removed, and the pellet was resuspended in 500µl of *SB* buffer.

#### 2.1.4.2 Magnetic Separation CD117 selection

The magnetic column was placed on the *QuadroMACS* separator. The column was rinsed with 500µl of *SB* buffer. The cell suspension was applied to the magnetic column. The cells (negative selection) were allowed to pass through and collected in a 15ml Falcon tube. The column was then washed 3 times with 500µl of *SB* buffer. The column was removed and placed in a 15ml Falcon tube, then 1ml of SB buffer was placed into the column. The magnetically labelled cells were then flushed out by firmly pushing the plunger into the column. The cells were then centrifuged at *300xg* for 5 minutes and the pellet was resuspended in an appropriate amount of *SB* buffer or culture medium accordingly.

## 2.2 Mouse Embryonic Fibroblasts

### 2.2.1 Culture of mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) were purchased (Cell Biolab, INC) and expanded in an MEF medium. The MEF medium was prepared by using 500ml of DMEM high glucose, GlutaMAX (Gibco®, UK), 10% fetal bovine serum (FBS, Gibco®, UK) inactivated at 65$^{o}$C for 1 hour and 1% pen/strep (Invitrogen, UK).

### 2.2.2 Inactivation of mouse embryonic fibroblasts

MEFs were inactivated by incubation with 5ml of MEF inactivation medium (195ml of MEF medium, 5ml of PBS and 2mg of Mitomycin C (Thermo Scientific, country) for 2 hours. The culture was then washed twice with PBS and then replaced with MEF medium.

### 2.2.3 Feeder preparation

The 24 well plates, which were used to culture mHSCs, were initially treated with 0.1% of sterile gelatine (G7041, Sigma, UK) for 45 minutes at 37$^{o}$C. After incubation, the plates were washed carefully using sterile PBS twice. The inactivated MEFs were trypsinised and transferred to gelatine coated plates at a concentration of 80,000 cells per cm$^2$ in the MEF medium. These were kept overnight to attach. The following day the medium was changed to

the *modified ES* expansion medium and mHSC or mAF HSCs placed on top of the MEFs at a concentration of 50,000 cells per $cm^2$.

## 2.3 Expansion and Characterisation of mouse haematopoietic stem cells

### 2.3.1 Fetal liver

Fetal liver (mFL) stem cells were used for comparison with mAF HSCs in the *in utero*, expansion and gene expression assays. These were isolated from E13.5 fetuses by the careful removal of the fetal liver in the abdominal cavity, mechanical dissociation of the fetal liver through a 40µm filter and lineage depletion by magnetic separation. The FL cells went through CD117 selection using either flow cytometry or magnetic selection, as described above. The cells were characterised using flow cytometry to demonstrate the specific cell populations used for the culture, expansion experiments.

### 2.3.2 Bone Marrow

Bone Marrow (mBM) stem cells were isolated from adult mouse bone marrow using published techniques (Amend et al., 2016; Madaan et al., 2014). The mouse was euthanised using the schedule 1 method. The mouse was positioned on a clean area under a sterile hood and all the external areas were sprayed with 70% ethanol for disinfection. An midline incision was made in the anterior abdominal wall, and the skin was gently opened to expose the abdomen and leg muscles. The hind leg was cut just above the pelvic/hip joint using sharp and sterile dissecting scissors, ensuring that the epiphysis remained intact without exposing its contents. After removing the hind leg, sterile scissors were used to make an incision just above the claws to remove the lower portion of the hind leg. The hind leg was then cut below the knee-joint through ligaments to remove the tibia, ensuring that the epiphysis remained intact. The femur was dissected from surrounding muscles and excess tissue was carefully pilled off using sterile forceps and scissors. Both ends of the femurs were carefully trimmed using sterile, sharp scissors to expose the interior marrow shaft. The contents of the bone marrow cavity were

flushed with 1ml of RPMI-1640 (Gibco® Thermo Fischer Scientific, USA) using a 1-ml insulin syringe with a 29G needle into a 50ml Falcon tube after passing through a 40μm filter. The flushing was repeated twice and the filter was washed with 2-3ml of RPMI-1640 (Gibco® Thermo Fischer Scientific, USA). The solution with the cells was then centrifuged at *300xg* for 5 minutes and the pellet was resuspended in an appropriate amount of *SB* buffer or culture medium accordingly. The mBM cells passed through lineage depletion with magnetic separation (Miltenyi Biotec, Germany) followed by CD117 and Sca1 selection using flow cytometry or magnetic separation, as described in the previous section. Lin-/Sca1+ cells were used for BM transplantation (Merianos et al., 2009; Veiby et al., 1996).

## 2.4 Culture Methods

### 2.4.1 Erythroid differentiation

Erythroid differentiation was performed by culturing cells in a concentration of 100,000 cells per 1.9cm$^2$, 52,631 cells/cm$^2$ in erythroid differentiation medium containing 50ml stem span (Stem Cell Technologies) 10ml FBS Dexamethasone (Sigma-Aldrich, UK) 1ml of 100% EtOH in 50ml of medium (20mcg/ml medium concentration) 10μl of erythropoietin (10μl/ml) (EPO) (Sigma-Aldrich, UK), 100μl of stem cell factor (1mg/ml) (SCF) (Sigma-Aldrich, UK). Cells were plated in the erythroid medium for a total of 5 days before flow cytometry analysis was conducted for the presence of adult haemoglobin using a specific adult haemoglobin antibody (Hb-PE, Abcam).

### 2.4.2 Semi- Solid Colony Forming Assays

The semi-solid assays were performed as per manufacturer's instructions (Stem Cell Technologies) by culturing $10^4$ – $10^5$ cells expressing the donor's marker (CD45.1$^+$ or H2-K$^{d+}$) per 35mm dish. The cells were isolated from the bone marrow of the transplant recipients or freshly isolated mAFSCs at E13.5. The cells were prepared, washed and counted and were

added to a 5ml tube containing 2.5ml of MethoCult (MethoCult$^{TM}$ GF M3434, Stem Cell Technologies, containing Methylcellulose in Iscove's MDM, fetal bovine serum, bovine serum albumin, recombinant human insulin, human transferrin (iron-saturated), 2-Mercaptoethanol, recombinant mouse stem cell factor (SCF), recombinant mouse interleukin 3 (IL-3), recombinant human interleukin 6 (IL-6), and, recombinant human erythropoietin (EPO)). The tube was vortexed and allowed to stand to allow bubbles to dissipate. The cells were then dispensed into 35mm culture dishes using a syringe and a blunt needle. The cells were incubated for 14 days in a humidified incubator at 37°C and 5% $CO_2$ (Figure 2-2) The colonies were then counted using an inverted microscope and gridded scoring dishes. The culture was liquefied using RPMI-1640 medium (GIBCO, UK) for further flow cytometry processing.

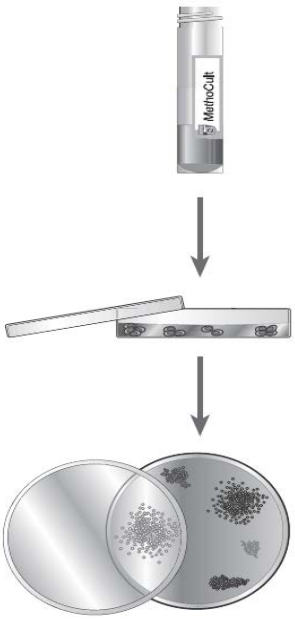



**Figure 2-2 Semisolid Culture using MethoCult. Cells are initially mixed with the semisolid medium before applied into the petri dish (Figure is taken from Stem Cell Technologies website)**

## 2.5 Mouse Erythroid Leukaemia (MEL) Cell Culture

MEL (Nishigaki et al., 2006) cells were grown either in 175cm$^2$ culture flasks (Falcon 353109) or 24-well culture plates (Falcon 351147) containing sufficient amounts of complete Dulbecco´s Modified Eagle Medium (DMEM, Sigma Aldrich, D6429) supplemented with 4500mg/L glucose, L-glutamine, 10µg/mL penicillin and streptomycin and 10% fetal bovine serum (PAA, USA). Cells were incubated at 37ºC, 5% CO2 in a CO2 Air Jacket Incubator (Thermo Scientific). Cells were split before reaching confluency (~80-90%), while still in logarithmic-phase growth. Cell stocks, resuspended in 900µl of FBS and 10% dimethylsulphoxide (DMSO; Sigma-Aldrich) (v/v) in a cryogenic vial were frozen in liquid nitrogen.

## 2.6 DNA Extraction for Genotyping of the Humanised Mouse Colonies

Ear biopsies from mice were performed by the staff at Western Labs (ICH) unless otherwise specified. The biopsy procedure was performed under the project licence PPL No:70/7408.

About 2-3mm of tissue was taken and placed in a 1.5ml eppendorf tube. Then, 75µl NaOH (25mM) containing 0.2mM EDTA with a pH 12 was added (Table 2-1). The eppendorf was then vortexed and heated at 95°C for 10-15 minutes. The tissue was left to cool at room temperature. Once the solution was at room temperature, 75µl of 40mM Tris-HCl (Table 2-1), pH 5 was added. Then, 2 or 4µl of this solution was used to perform the PCR reaction.

| | Concentration | Volume | Final Concentration |
|---|---|---|---|
| **NAOH Solution** | | | |
| NAOH | 0.5M | 2.5ml | 25mM |
| EDTA $Na_2$ | 0.5M | 20ul | 0.2mM |
| $dH_2O$ | | 47.48ml | |
| Final Volume | | 50ml | (pH=12) |
| | | | |
| **Tris-HCl Buffer** | | | |
| Tris-HCl | 1.0M | 2ml | 40mM |
| pH8.0 | | 48ml | |
| $dH_2O$ | | 50ml | |
| Add HCl to decrease the pH to 5. | | | |

**Table 2-1 Solution for PCR reaction used in Genotyping, showing the solution, concentrations, volumes and final concentrations used**

## 2.7 PCR for Genotyping DNA

### Primers

Animals were genotyped using primers specific for mouse or human haemoglobin as per Table 2-4. The reactions were conducted in 0.2ml polypropylene PCR tubes (Corning, UK) in a Thermo Scientific thermal cycler using a MyRedTaq PCR mix (Bioline, UK).

### Template

The volume of the template in each reaction was 2µl. The reaction was prepared in a 96 well PCR plate and run in an eppendorf thermocycler.

### Annealing temperature and time

The optimal annealing temperature was determined based on the primer sequences and this was 2-5°C below the lower Tm of the pair. For the genotyping primers this was at 63°C.

| PCR MyTaq Red Mix Protocol (Bioline) | |
|---|---|
| Primer 1 (Mouse β KI Reverse) | 1 µl |
| Primer 2 (β KI Forward) | 1 µl |
| Primer 3 (B383 KI Reverse) | 1 µl |
| DNA | 2 µl |
| MyTaq Red Mix, 2X | 25µl |
| RNase free $H_2O$ | 20µl |
| Total volume | 50µl |

**Table 2-2 Genotyping PCR Mix shows the reagents/ solutions and the volumes used**

| Thermocycler PCR programme for genotyping using MyTaq Red Mix Protocol (Bioline, UK) | | | |
|---|---|---|---|
| **Step** | **Temperature** | **Time** | **Cycles** |
| Initial Denaturation | 95°C | 1 minute | 1 |
| Denaturation | 95°C | 15 seconds | 35 |
| Annealing | 63°C | 15 seconds | |
| Extension | 72°C | 10 seconds | |

**Table 2-3 Thermocycler Programme**

### 2.8 Gel Electrophoresis

The PCR product was run through agarose gel (2%) electrophoresis in a 1X Tris-Borate-EDTA (TBE) buffer. The agarose gel (2%) was made using 1xTBE plus SYBR DNA stain (1 in 10,000 dilution, Sigma Aldrich). For DNA visualisation, 100ng was loaded onto the gel and fragments resolved at 140V, 90mA for 45 minutes. Nucleic acid fragments were visualised on a UV transilluminator (UVP®, USA) and photographed under ultraviolet light employing the Bio-Doc-ItTM System (UVP®). Size markers of 100bp ladder (New England Biolabs, NEB®, USA) were used for determination of fragment sizes.

| Gene | Primers/probe | Melting (˚C) temperature |
|---|---|---|
| LRT Forward | TGT GTG CCC GTC TGT TGT GT | 59.4 |
| LRT Reverse | GAG TCC TGC GTC GAG AGA GC | 63.5 |
| LRT Probe | FAM -CAG TGG CGC CCG AAC AGG GA- TAM | 65.5 |
| mTitin forward | AAA ACG AGC AGT GAC GTG AGC | 59.8 |
| mTitin reverse | TTC AGT CAT GCT GCT AGC GC | 59.4 |
| mouse beta forward | GCT GCT GGT TGT CTA CCC TTG | 61.8 |
| human gamma forward | GTG GAA GAT GCT GGA GGA GAA | 60.3 |
| human beta forward | CGT GCT GGT CTG TGT GCT G | 61 |
| mouse alpha forward | AGC TGA AGC CCT GGA AAG GAT | 59.8 |
| human alpha forward | CAG ACT CAG AGA GAA CCC ACC AT | 62.4 |
| mouse beta reverse | CCC ATG ATA GCA GAG GCA GAG | 61.8 |
| Human gamma reverse | TGC CAA AGC TGT CAA AGA ACC T | 58.4 |
| human beta reverse | CTT GTG GGC CAG GGC ATT AG | 61.4 |
| mouse alpha reverse | GCC GTG GCT TAC ATC AAA GTG | 59.8 |
| human alpha reverse | GCC TCC GCA CCA TAC TCG | 60.5 |
| mouse beta probe | FAM -CCA GCG GTA CTT TGA TAG CTT TGG AGA CC –TAM | 68.1 |
| human gamma probe | FAM - AGG CTC CTG GTT GTC TAC CCA TGG ACC -TAM | 69.5 |
| human beta probe | FAM - CCC ATC ACT TTG GCA AAG AAT TCA CCC -TAM | 65 |
| mouse alpha probe | FAM -TGC TAG CTT CCC CAC CAC CAA GAC C -TAM | 67.9 |
| human alpha probe | FAM -TGC TGT CTC CTG CCG ACA AGA CCA A -TAM | 66.3 |
| mTitin probe | FAM - TGC ACG GAA GCG TCT CGT CTC AGT C -TAM | 67.9 |
| Mouse β KI reverse | GTCAGAAGCAAATGTGAGGAGCA | 62.5 |
| β KI forward | TTGAGCAATGTGGACAGAGAAGG | 62.4 |
| B383 KI reverse | GTTTAGCCAGGGACCGTTTCA | 62 |

**Table 2-4 Primers & Probes used for Genotyping and qPCR**

## 2.9 DNA Extraction

For DNA extraction, the Purelink Genomic DNA extraction kit (Invitrogen, ThermoFisher Scientific, USA) or Trizol Reagent (Invitrogen, ThermoFisher Scientific, USA) were used.

### 2.9.1 DNA extraction from cells

The cells were pelleted and resuspended in 300µl of PBS. (in the case of blood, a total of 200µl of blood was used instead of 300µl of PBS) A total of 20µl Proteinase K was added to the samples and further 20µl RNase A. The sample was vortexed and left at room temperature for 2 mins. Then, 200µl of Purelink Genomic lysis/binding buffer was then added, the samples was vortexed and incubated at 55$^{o}$C for 10 mins, for protein digestion. After 10 mins, 200µl of 100% ethanol was added and briefly vortexed to achieve a homogenous solution. The lysate was added to the Purelink Spin Column and the column was centrifuged for 1 minute at *10,000g.* The collection tube was discarded and replaced with a new one.

Then, 500µl of Wash Buffer 1 was added to the column and centrifuged at 10,000*g* for 1 minute. The collection tube was replaced with a new one and 500µl of Wash Buffer 2 was added and centrifuged. The spin column was placed in a sterile 1.5ml micro-centrifuge tube and 25-200µl of genomic elution buffer was added accordingly. The column was incubated for 1 minute and centrifuged for a further 1.5 minutes; the DNA was collected in the micro-centrifuge tube and stored at -20$^{o}$C.

#### 2.9.1.1 DNA Extraction For Tissues

At this point, <25mg of minced tissue was placed in a micro-centrifuge tube and 180µl of Purelink genomic digestion buffer and 20µl of proteinase K was added to each sample. The samples were then incubated at 55$^{o}$C on a heat block with regular mixing for 1-4 hours.

The tube was then centrifuged for 3 mins and the supernatant was placed in a new micro-centrifuge tube. Then, 20µl of RNase A was added and the sample vortexed and incubated for

2 mins at room temperature. The steps were then the same as previously described in the cell DNA extraction section above, by adding 200µl of Purelink Genomic lysis binding buffer.

## 2.10 RNA Extraction

Then, 350µl of lysis buffer RLY and 3.5µl β-Mercaptoethanol were added to the cell pellet or to ground tissue and the mixture was vortexed. The mixture was placed in an ISOLATE II filter and spun for 1 minute at *11,000g.* The filter was then discarded and 350µl, 70% ethanol was added to the homogenised lysate and mixed by pipetting.

Each Isolate II RNA Mini Column was placed in a collection tube. The lysate was placed on the column and centrifuged for 30 seconds. The collection tube was then replaced.

Then, 350µl of Membrane Desalting Buffer was loaded on the column and centrifuged for 1 minute at *11,000g.* The DNA digestion was performed on the column by applying 95µl of DNase I reaction mixture directly onto the centre of the silica membrane and incubated for 15 minutes. The membrane was washed with 200µl wash buffer RW1 and spun for 30 seconds at *11,000g.* After replacing the collection tube, the column was further washed with 600µl wash buffer RW2 and centrifuged for 30 seconds at *11,000g.* The flow-through was discarded and replaced. The column was then further washed with 250µl wash buffer RW2 and centrifuged for 2 mins at *11,000g.* The column was placed in a nuclease free 1.5ml micro-centrifuge tube. The RNA was eluted by adding 60µl RNase-free water and centrifuged at *11,000g.*

## 2.11 RNA and DNA Quantification

The concentarion of both RNA and DNA was measured by measuring the absorbance at 260nm ($A_{260}$) and 280nm ($A_{280}$) using a spectrophotometer.

The ratio of 260nm/280nm was determined to assess the level of purity in each sample.

## 2.12 Plasmid Preparation and Virus Production

### 2.12.1 Plasmid

The plasmids used, MA-821 (GLOBE) and MA-1047 (GLOBE-GFP), were kindly provided by Dr Michael Antoniou at Kings College London.

### 2.12.2 Transformation using Heat Shock

DH5α competent cells (18265-017, Invitrogen, Thermoscientific, UK) were used. The cells were thawed on ice and placed in 1.5ml microcentrifuge tubes on wet ice. The cells were gently mixed with the pipette tip and 50µl of cells aliquoted into a 1.5ml microcentrifuge tube. The unused cells were then re-frozen. Then 10ng of DNA (plasmid MA-821 or control MA-1047) was added to the cells and mixed gently. For the pUC19 control, 2.5µl (250 pg) of DNA was added to the cells and mixed gently. The tubes were incubated on ice for 30 mins. The tubes were placed in a pre-warmed 42°C water bath for 20 seconds and then on ice for 120 seconds. Following that, 950µl of LB (with no antibiotic added) medium was added and incubated in tubes for 1 hour at 37°C. Following incubation, 100µl of the resulting culture on LB plates was spread (with appropriate antibiotic added – usually Ampicillin or Kanamycin) and left to grow overnight. The colonies were picked 12-16 hours later.

**Mini Preparation of Plasmid MA-821 or MA-1047**

A single colony from the bacterial culture plate was picked and inoculated in a culture of 10ml LB medium containing Ampicillin. The culture was left to grow overnight at 37°C with vigorous shaking (~240rpm). The following day the cells were harvested by centrifugation at 3000 x *g* for 15 min at 4°C. The pelleted bacterial cells were resuspended in 250µl Buffer P1 (which had RNaseA added to it previously) containing lysozyme at a final concentration of 1 mg/ml. The cells were then incubated at 37°C for 10 min, and then 250µl Buffer P2, and gently mixed by inverting the tube 4–6 times. Then, 350µl Buffer N3 was added, and the tube was inverted immediately but gently 4–6 times. The solution was then centrifuged at ≥10,000 x *g* for 10 min. During centrifugation, a QIAprep Spin Column was placed in a 2ml collection tube. The supernatant was placed on the QIAprep Spin Column by pipetting and then centrifuged at ≥10,000 x *g* for 30–60s. The flow-through was discarded. The QIAprep Spin Column was washed by adding 0.5ml Buffer PB and centrifuged at ≥10,000 x *g* for 30–60s. The QIAprep Spin Column was washed by adding 0.75ml Buffer PE and centrifuged at ≥10,000 x *g* for 30–60s. The flow-through was discarded, and centrifuged at ≥10,000*g* for an additional 1 min to remove residual wash buffer**.** The QIAprep Spin Column was placed in a clean 1.5ml microcentrifuge tube. To elute DNA, 50µl Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the centre of each QIAprep Spin Column, allowed to stand for 1 min, then centrifuged for 1 min.

### 2.12.3 Restriction enzyme digestion

To confirm that the amplified DNA was identical to the original, a restriction enzyme digestion was performed using selected restriction enzymes, such as EcoRI. The enzymes were selected after loading the MA821 sequence on Vector NTI Software.

The Restriction Enzyme reaction mix was prepared as shown in Table 2-5 - the incubation time was dependant on the enzyme used. The reaction mix was then mixed with a Loading Buffer and ran to an agarose gel.

| Restriction Enzyme Reaction Mix | |
|---|---|
| | Quantity |
| Enzyme: (BamH I, Hind II) | 1µl |
| 10X Buffer M | 2µl |
| Substrate DNA from preparation | ≤1µg |
| Sterile Water | To 20µl |
| Incubation Temperature (incubation time minimum 60 mins) | BamHI: 30°C, HindIII:37°C |

**Table 2-5 Restriction Enzyme Reaction Mix**

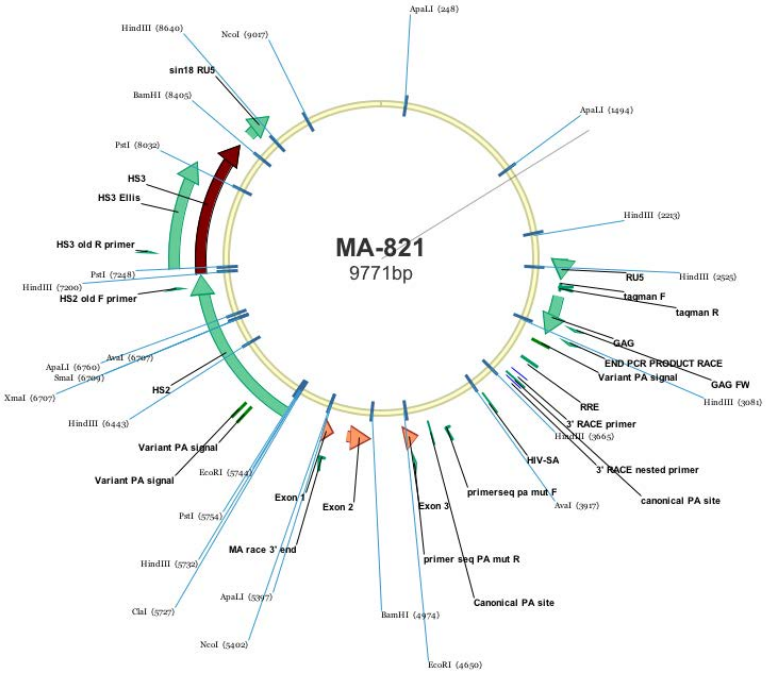



**Figure 2-3 Map of MA-821 with Restriction Enzymes. The figure was created with VectorNTI software**

### 2.12.4 Sequencing

The plasmids were also sent for sequencing (to a commercial independent provider, Eurofins, Germany) which confirmed that they were identical to the original product.

### 2.12.5 293T Transfection and virus production

The GLOBE (MA-821 and MA-1047) Lentiviral vectors were prodiced using standard methods (Blomer et al., 1996) by transient transfection of HEK293T cells through a three-plasmid vector system (Transfer vector - 50µg, pMDG (Env) - 17µg, pCMVΔ8.74 (Gag/Pol) - 32µg). A total of 20x106 293T cells were seeded in 175cm$^2$ flasks (Falcon 353109) containing complete DMEM (Sigma Aldrich D6429), 24-48h prior to cell transfection, aiming for the cells to be >90% confluent.

A DNA mix was made by adding to a 250ml stericup, 6mL per 175cm$^2$ flask of Optimem® (PAA) together with 50µg of the vector construct, 17.5µg of pMD.G2 envelope plasmid and 32.5µg pCMVΔ8.74 packaging plasmid.

In addition, in a different stericup, 1µL of 10mM PEI was added to every 6mL of OptiMEM per flask. Then, the DNA and PEI solutions (PEI; Sigma-Aldrich, 10mM stock) were mixed at 1:1 and incubated for 20 mins at room temperature to allow complexes to form.

Then, cell media was removed from flasks containing 293T cells and 12ml of the PEI and DNA complex per flask were added. The cells were then incubated at 37˚C, 5% $CO_2$ for 3 hours. After incubation the media was then changed with 15mLs of complete DMEM (DMEM + antibiotics + 10% FCS). 24 hours later the media was changed with 15mL of fresh complete DMEM per flask. Then, 48 and 72 hours later the supernatant was collected and spun at 1,500rpm for 10 mins. The supernatant was carefully removed without dislodging the pellet and filter through a 0.22µm filter. Then, the supernatant was spun at 23,000rpm for 2 hours at 4˚C in the ultracentrifuge.

The supernatant was then poured off carefully and the tubes were inverted on paper towel for 2 mins. Then, 50µl of RPMI (Sigma-Aldrich R0883) (per flask of supernatant) was added and incubated on ice for 20 mins, flicking the tube to aid resuspension of the virus.

The virus pellets were resuspended well, pooled and mixed together. The final viral stocks were snap-frozen on dry ice as single-use aliquots before storage at -80ºC. Lentiviral titration was conducted by qPCR. The viral titres were nearly $10^7$ viral particles/ml.

### 2.13 Vector Copy Number Determination

Viral titers were quantified by determination of integrated viral copy number (VCN) in cell genomic DNA or unintegrated proviral vector genomes in the cytoplasm by a TaqMan® real-time qPCR method. The qPCR reactions were set-up in 96-well reaction plates (MicrAmpTM Optical; Applied Biosystems, USA) and analysed using Butler´s late reverse transcriptase (LRT) primer/probe set (Ingham et al., 2001) ().

The endogenous *titin* was used to determine absolute numbers of genomes present in each qPCR reaction using a primer/probe set. *Titin*, also called connectin, is a protein kinase involved in the structural assembly of microfilaments in muscle and during chromosome segregation and cell division in non-muscle cells. Standard curves were generated using serial dilutions ($10^6$ copies to $10^1$ copies) of the GLOBE and the *Titin* plasmids (PGK plasmid) (Charrier et al., 2005) to determine copy number per cell [VCN per Cell=Number of Copies of GLOBEx2/Number of Copies of Titin].

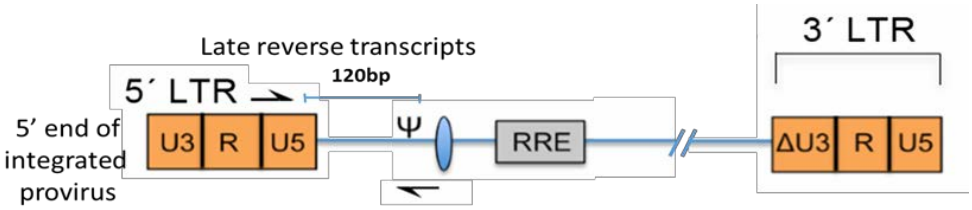



**Figure 2-4 Schematic representation of the Late RT primers used to quantify integrated viral forms. The LRT primers amplify the region of the HIV vector backbone between the right 5´LTR sequence and the 5´end of the gag gene, such that, only integrated DNA or provirus forms that have completed the two template switches of reverse transcription are detected.**

## 2.14 High Performance Liquid Chromatography (HPLC)

Red cells (20µl) were lysed in 500µl 0.1M 2-mercaptoethanol/0.1M HCl and diluted to 1ml with 500µl 50% aqueous acetonitrile. After centrifugation (13,000rpm x 5 mins), samples were transferred to HPLC autosampler vials and analysed on a Grace Vydac 214TP C4 column (250 x 4.6mm), using a gradient of solution A (water/acetonitrile/trifluoroacetic acid/heptafluorobutyric acid, 700:300:0.7:0.1) and solution B (water/acetonitrile/trifluoroacetic acid/heptafluorobutyric acid, 450:550:0.5:0.1). Proteins were detected using a Jasco MD-1510 detector and the area of globin chains quantified at 220nm. The HPLC was performed with the help of Dr. Simon Eaton at UCL's Institute of Child Health.

## 2.15 *In utero* Stem cell Transplantation

The methodology used for IUT in mice was as previously described (Merianos and Tiblad, 2009) with minor modifications. The pregnant dam was anesthetised by using vaporised isofluorane-Vet 5% in oxygen (VetSolutions Ltd, UK), in an anaesthetic chamber. The surgical area was dressed using sterile drapes. The skin hair of the mouse was previously removed using hair removal cream (Veet, UK) around the lower abdomen, Figure 2-5.

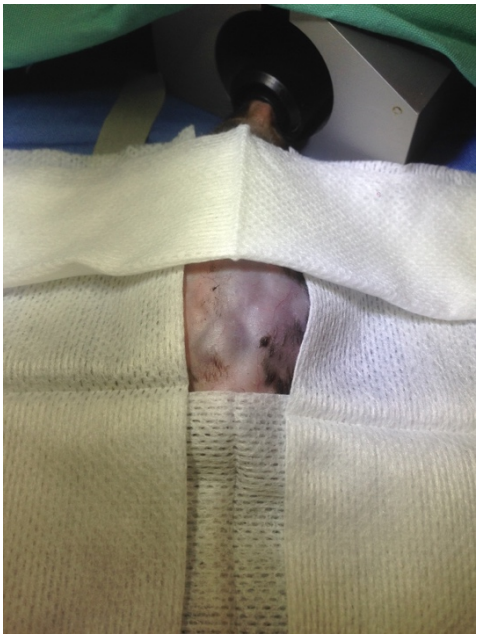


**Figure 2-5 Representative Photo of Mouse Abdomen post hair removal [Western Labs, March 2013]**

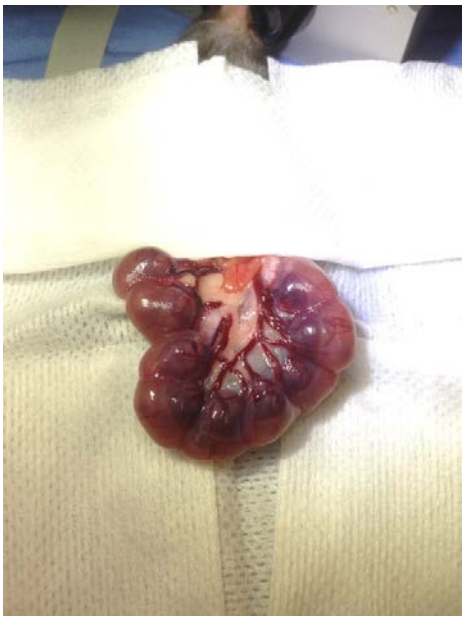


**Figure 2-6 Exposed Mouse uterus prior to IUT**

The dams were placed on sterile surgical drapes and their abdomen was disinfected using ChoraPrep® cutaneous solution (2% Chlorhexidine Gluconate (w/v) and 70% Isopropyl alcohol (v/v); CareFusion Corporation, UK). A 1-2cm longitudinal midline laparotomy incision was carried out in layers to expose the pelvic cavity and the pregnant uterus. The bicornuate uterus was exteriorised and the fetuses were counted, Figure 2-6. The liver of each embryo was identified through the transparent, at this gestational age, uterine wall and 20µl of the prepared cells were delivered intraperitoneally beneath the liver of each embryo, using a 33 gauge needle attached to a digital or manual syringe (Hamilton, Switzerland, Figure 2-7). The uterus was kept wet with a sterile gauze embedded in warm sterile PBS. The uterus was repositioned in the abdomen, which was closed with continuous suturing for the peritoneum and interrupted suture for the skin using absorbable vicryl 6-0 suture (Ethicon Inc., USA). A total of 0.2mL local anaesthetic (Bupivacaine 0.25%W/V solution for injection, Taro Pharmaceuticals, UK) was infiltrated around the incision. The dams were transferred to a warm cage (28$^{o}$C) for initial recovery and then to a regular cage. The dams were checked daily and delivered the recipient mice 6-7 days post-IUT (E20-21). The dam was transferred into a controlled temperature heating chamber (27-28$^{o}$C) overnight Figure 2-8. All the operated mice were given wet food for the first day and monitored regularly for any signs of infection, especially during the first seven days.


**Figure 2-7 Hamilton Digital Syringe used in IUT**

**Figure 2-8 Post-operative recovering chamber**

## 2.16 Post-natal Period and Cross Fostering

The newborn pups were kept with their mothers for the first 3 weeks after birth, and those that survived were later placed into separate cages according to gender. Some were cross fostered and were placed in a cage with a CD1 dam, that had been time-mated and plugged one day before the treated dam. The cross fostering was performed at day 1 post-natally.

## 2.17 Post Mortem Examination

### 2.17.1 Blood Collection

Blood was collected under terminal anaesthesia, induced by injecting 0.8ml of 1.25% Tribromoethanol (Avertin Sigma- Aldrich, UK) solution. Then, 0.5-1ml of blood was collected by cardiac puncture using a preheparinised 26G syringe in 0.5ml EDTA tubes.

### 2.17.2 Tissue Isolation

The animals were culled and the thoracic and abdominal cavity exposed by a midline incision and the spleen, kidneys, heart, lungs and liver identified. The organs were removed using sterile forceps and scissors and placed in 1.5ml Safe Lock microtubes (Eppendorf, UK) for RNA/DNA isolation. Small amounts of tissue were put in 15ml falcon tubes with PBS for flow cytometry analysis. In addition, tissue sections were also sent to central diagnostic services in 4% PFA for histology analysis. For bone marrow collection, the legs were separated from the body and the muscle tissue removed from the femur and tibia. After separating the femur and tibia, the epiphysis was cut off on both ends and placed in 1.5ml Safe Lock microtubes (Eppendorf, UK). All tissue was quickly frozen in liquid nitrogen and stored at -80°C.

### 2.17.3 Weight

Animal and spleen weight was also measured using a high precision balance (Kern, UK).

### 2.17.4 Hb Calculation with Hemacue

Peripheral blood was collected from anesthetised mice using a heparinised syringe into BD micro trainer EDTA collection tubes (Fisher Scientific). Red blood cell counts and Haematocrit values were measured. Hb concentrations were determined by a Hemocue Hb 201+ system. Full blood counts and Serum Protein Electrophoresis (SPE) analysis for all the samples were performed by the Central Diagnostics facility at the University of Cambridge.

## 2.18 Immunohistochemistry and Immunofluorescence

Bone marrow, spleen and liver from treated and untreated animals were fixed in 10% formalin and sent to a histology facility at the University of Cambridge where they were decalcified, embedded in paraffin, sectioned and laid onto glass slides. These sections were immunostained for human β-globin antibody (LifeSpan Biosciences, UK) using the R and D systems staining protocol with minor modifications.

The paraffin-embedded sections were immersed in Histoclear (Fisher Scientific HS-200) and gradually rehydrated using ethanol, deionised water and PBS (wash buffer). An antigen retrieval (CT S013) step was employed to maximise specific signals. This was followed by incubating the sections in blocking buffer (1% horse serum in PBS) for 30- 60 mins at room temperature and an additional AffinityPure Fab anti-Mouse IgG (H+L) (Abcam) blocking step (1hour at RT) to reduce background caused by endogenous mouse IgG and Fc receptors. Post blocking, the tissue sections were incubated with a primary antibody (mouse anti-human β-globin, LSB, UK) diluted (1:100) in an incubation buffer (1% BSA, 1% normal donkey serum, 0.3% triton- X-100 in PBS) overnight at 2-8˚C. Following 3x15 min washes in wash buffer, the

sections were incubated with a goat anti-mouse secondary antibody (conjugated to Alexa 488; Life Technologies) for 30-60 min at room temperature. After the washes (3x15 min in PBS), the sections were mounted in anti-fade mounting media containing DAPI nuclear counterstain (Fluoroshield, Abcam). Among the controls were a 'no primary antibody' negative control, a positive HbA (α2α1/α2α1, hγβ$^{A}$/hγβ$^{A}$) Humanised Non-Thalassaemia Controls and an untreated heterozygous CA control (mβ/h$^{HPFH}$γβ0).

### 2.19 Flow Cytometry

#### 2.19.1 Blood staining for flow cytometry

The sampled blood was added to 5ml RBC Lysis Buffer in conical tubes (15ml, Falcon, Bd Biosiences) and part of it sent to the haematology laboratory for analysis. It was then centrifuged at 300*g* for 5 mins. The lysate was aspirated, resuspended in 100µL SB buffer. Then, 1µl of the conjugated antibody was added and incubated at 4°C for 15 mins then the lysate was washed with 1-2ml of SB buffer and spun for 5 min at 300xg. The supernatant was discarded. The cell pellet was transferred to a flow cytometry tube (5mL, BD Biosciences) and then resuspended with 500µL of *SB* Buffer. The sample was then analysed using flow cytometry analyser LSR II.

#### 2.19.2 Analysis LSRII

Analysis of flow cytometry samples was performed using the LSRII (BD, UK) and ARIA II (BD, UK) flow analyser and FACSDiva software. The raw files were then transferred to a shared drive before downloading to a personal computer for detailed analysis using FlowJo V10 software.

## 2.20 *In vitro* MLR

The *in vitro* MLR assay was performed as published (Mold et al., 2008), in three different animals of each group in triplicate. For the proliferation assays, splenocytes from recipients of congenic and allogenic transplants were labelled with the dye carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) by incubating cells in CFSE (1μM, Invitrogen) in 1ml PBS at 37°C for 10 mins, followed by three washes in RPMI with 10% FBS.

Then 1ml of medium containing labelled cells were added to 96 well U-bottom plates (Figure 2-9) at a concentration of 1,000,000 cells/ml in RPMI culture media [10% FBS, 2 mM L-Glu, 100 U/mL penicillin/streptomycin (Invitrogen Life Sciences)]. For both groups, allogenic and congenic MLRs, cells from uninjected (naïve) BALBc and CD45.1 animals, unlabelled splenocytes/lymphocytes, were irradiated (6,000 rads from a cesium source, =60Gy x 33 sec= 1,980 sec) and added to the wells for a final ratio of 1:1.

The CFSE-labelled lymphocytes from the transplant recipients were incubated for five days (120 hours) with the unlabelled irradiated naïve splenocytes/lymphocytes. The cells were washed and prepared for flow cytometry analysis by incubation with antibodies: CD3, CD4, CD8 (Miltenyi Biotec, Germany). The viability dye, 7-Amino-Actinomycin D (7AAD) (Sigma-Aldrich, UK) was used to exclude dead cells from the analysis.

**Figure 2-9 96 well u-bottom culture plate used in MLR culture**

## 2.21 Real Time PCR

### 2.21.1 Reverse Transcription

The first strand of cDNA for use in Real-time qPCR studies was synthesised using the Bioline Sensifast cDNA synthesis kit (BIO-65053). The 5X TransAmp Buffer provided in the kit included a unique blend of anchored oligo-dT primers and random hexamers to ensure unbiased 3' and 5' coverage for enhanced reporting of all the regions of the RNA and generated representative cDNA pools of the transcriptomes of the samples in comparison. The combined priming strategies enhanced data quality and accuracy. The buffer also contained reverse transcriptase enhancers that reduced RNA secondary structures and increased efficiency and sensitivity. Then, 500ng of total RNA from each sample was used for the reaction. A Mastermix was prepared by mixing the required amounts of the 5X

transamp buffer, RT enzyme and deionised water on ice. A 'no RT' control was included in the experimental design and an RT-PCR reaction was conducted in a Thermo Scientific thermal cycler.

### 2.21.2 qPCR using Taqman® probes

Taqman® oligonucleotide probes have a covalently attached fluorophore at the 5' end and a quencher at the 3' end. As the qPCR proceeds, 5'-3' exonuclease activity of the Taq polymerase degrades the probe and releases the fluorophore from the quencher. The use of a probe in the Taqman® method lends additional specificity to the reaction (Livak, Flood et al. 1995). It ensures that the fluorescent signal generated during qPCR is derived, only from the amplification of the target sequence making it more robust, sensitive and quantitative in comparison to the SYBR green method. Real time RT-qPCR was conducted using the Bioline SensiFAST Probe Hi-ROX Kit (BIO-82002). The Hi-ROX mix contains optimised concentration of MgCl2 (3mM) and was premixed with the passive reference ROX (5-carboxy-X-rhodamine, succinymidyl ester; λmax=610nm) for optional detection wherever necessary. The 18S ribosomal RNA was used as an internal control in all samples. Each reaction was duplexed with the target and 18S primers in the same mix to ensure data accuracy. The probes for the two sets of primers were differentially and fluorescently labelled. The target probes were FAM-TAMRA conjugated and the 18S probe was VIC-TAMRA conjugated. FAM (6-carboxyfluorescein; λmax=518nm) and VIC® (λmax=554nm) are the fluorophores and TAMRA (tetramethylrhodamine; λmax=582nm) is the quencher. The target genes included human α, β, γ globin and Mouse α and β globin chains. The qPCR reactions were performed in triplicate in 96-well optical plates (Microamp®, Applied Biosystems, N8010560). 'No template controls' were included for all primers sets.

The reactions were run on the Applied Biosystems Step-One Plus Real Time PCR system machine at the ICH, UCL and analysed using the LifeTechnologies Step- One software.

### 2.21.3 Determination of RNA expression levels by qPCR

Expression levels of mouse α, β and human α, β and γ-globin chains (target genes) were assessed in the blood and bone marrow samples of treated and untreated mice. Then, 5μl of cDNA (diluted 1:50) was added to 15μl of Master Mix containing the Hi- ROX mix, primers, probe and deionised water. Relative quantification of each target gene studied was calculated by the ΔΔCt method.

In the ΔΔCt method, the treated/test (T) samples (GLOBE) were compared to the calibrator (C) sample (untreated CA or wild-type). The gene of interest/target (globin genes) in both the test and calibrator sample were adjusted in relation to their respective normaliser gene (18S housekeeping/internal control). The resulting ΔΔCt value was used to determine the relative quantity (RQ) or fold difference in expression.

$Ct\ globin^{T} - Ct18S^{T} = \Delta Ct\ Test.$

$Ct\ globin^{C} - Ct18S^{C} = \Delta Ct\ Calibrator$

$\Delta Ct\ Test - \Delta Ct\ Calibrator = \Delta\Delta Ct$

Assuming PCR efficiencies of the target and internal control to be the same and close to 1, the quantity of target in the test (normalised to the internal control) relative to the calibrator is given by:

$Relative\ Quantity = 2^{-\Delta\Delta Ct}$

### 2.21.4 Primers and Probes

Primers already described in the literature (Huo et al, 2009) were used for the study. The oligonucleotides were custom made from Eurofins MWG operon, Germany. The lyophilised stock was re-suspended using DEPC-treated water (DNase/RNase-free) (Bioline, UK) to a concentration of 100nM. The 18S ribosomal RNA endogenous control primers and probes (VIC-TAMRA) were from Applied Biosystems (4308329). The 18S sequences were conserved in Human, Mouse, Rat, Xenopus, Yeast and Arabidopsis. The samples were stored at -20°C. Sequences of primers and probes used are listed in Table 2-4 Primers & Probes used for Genotyping and qPCR.

All primers had a melting temperature (Tm) of approximately 60˚C; the melting temperature of the probes was approximately 10˚C higher than that of the primers. Mouse-α, mouse-β and human α-globin primer and probe sets measured the RNA expression from both the alleles of their respective genes.

## 2.22 Iron Staining

Tissue samples from the transgenic mice and age-matched controls were analysed histologically. Small sections of the heart, liver and spleen were embedded in paraffin wax, cut and mounted onto glass slides. The tissue sections were stained with SIGMA-ALDRICH ACCUSTAIN IRONSTAIN (Procedure No. HT20, Germany) with minor modifications. The principle of this protocol is based on Prussian blue reaction, where the reaction of ionic iron with acid ferrocyanide results in a blue colour. Deparaffinising the tissue sections was performed using Histoclear and hydrated to deionised water. The tissues were then placed in a working iron solution (prepared by mixing an equal volume of a potassium ferrocyanide solution [catalogue no. HT20-1] and a hydrochloric acid solution [catalogue no. HT20-2]) for 30 mins, then rinsed in deionised water. After that, the iron was stained with the working

pararosaniline solution (prepared by adding 1ml of the paraosaniline solution [catalogue no. HT20-3] to 50ml of distilled water) for 15 mins and then rinsed in deionised water. Finally, the tissues were rapidly dehydrated in alcohol, cleared with Histoclear and mounted for analysis.

## 2.23 MRI *in Vivo* (performed by Dr Laurence Jackson, 2017)

### 2.23.1 *In vivo* MRI

Imaging was performed at 5 months of age using a 9.4T MR system (Agilent Technologies, Santa Clara, USA) at UCL CABI equipped with 1000mT/m gradient inserts and a 39mm volume resonator RF coil (RAPID Biomedical, Rimpar, Germany). A small animal physiological monitoring system (SA Instruments, Stony Brook, NY) was used to record the ECG trace, respiration rate and internal temperature. Animals were anesthetised under a mixture of isoflurane and oxygen with physiological measurements used to maintain depth of anaesthesia.

### 2.23.2 Spleen volume

Spleen volume data were acquired in control and non-loaded thalassaemia mice using a respiration gated multislice gradient echo axial sequence (GEMS) with in-plane resolution = 156μm, slice thickness = 0.5mm, flip angle = 20°, TE = 3.2ms. In iron loaded mice, due to ultra-short T2/T2* decay in the spleen, a GEMS protocol was impractical. In this case, a respiration gated T1-weighted spin-echo multislice sequence was used (SEMS) with in-plane resolution = 234μm, slice thickness = 1mm, TE = 2.5ms, TR = 600ms. In both cases, the number of slices was adapted to the cover the whole spleen volume. Image data was reconstructed offline and spleen tissue was identified by drawing contours on individual 2D slices and propagated through the plane to measure organ volume. The ratio of spleen volume to total animal mass was used as a quantitative and animal-independent value for spleen size.

### 2.23.3 Relaxometry

To fully characterise the magnetic properties of tissues T1, T2 and T2* mapping protocols was performed. To measure T1 relaxation, a cardiac gated look locker sequence was used with a minimum TE of 2.8ms followed by 30 echo times separated by the RR interval. The slice was orientated to cover the mid-papillary level short axis of the heart and a portion of liver allowing for both tissues to be quantified during a single scan with an in-plane resolution of 300μm and a slice thickness of 1.5mm. This resulted in an image sequence where a signal could be modelled by Eq. 1, where $S_0$ initial signal intensity, TI is the time following the look locker inversion pulse, $B$ is a fitted parameter to account for imperfect inversion, $C$ is a fitted offset to account for image noise and $T1^*$ is the apparent T1 under the influence of look locker saturation, which is corrected by the look locker correction factor (Eq. 2).

$$S(TI) = S_0(1 - Be^{-\frac{TI}{T1^*}}) + C \quad \textbf{Eq. 1}$$

$$T1 = (B - 1)\, T1^* \quad \textbf{Eq. 2}$$

T2 relaxation was measured using a cardiac and respiratory gated spin echo sequence, where a respiratory gate triggering acquisition of one phase encoding line per slice per R-wave until the next respiration and then repeated at 8 echo times ranging from 2.7 – 20ms. Typical respiration and cardiac rates allowed for 7 slices at 1.5mm with 300μm in plane resolution covering 6 slices of the heart and liver with one through the spleen. The T2 signal decay could then be modelled from the image series using Eq. 3, where $TE$ is echo time.

$$S(TE) = S_0 e^{\frac{-TE}{T2}} + C \quad \textbf{Eq. 3}$$

T2* decay was then measured using two multi gradient echo sequences with 15 echo times in the range of 0.9 – 14.9ms at 1ms intervals. These images had a 234μm in plane resolution and 1.5mm slices, one slice was placed in the same orientation as the T1 look locker and the

second acquisition was orientated in the spleen. The signal intensities were then modelled as T2* decay using Eq. 3 with T2* substituted for T2 (Stuckey et al., 2014).

Tissue regions were segmented, and models fitted to mean ROI values at each echo time. Model fitting was performed in the Matlab (2014b, The Mathworks, Inc., Natick, USA) using a minimisation function based on the Nelder-Mead Simplex method (Lagarias et al., 1998). Results are presented as mean value ± standard error and significance values were calculated by a one-way analysis of variance corrected for multiple comparisons (*p <0.05).

### 2.23.4 Cardiac function

Cardiac function was assessed with a gradient echo cine MRI sequence with a temporal resolution of 5.2ms, and in plane spatial resolution of 117μm and a slice thickness of 1mm. The left ventricular blood pool was segmented at systole and diastole using Segment v1.8 R0462 (Heiberg et al., 2010) and the corresponding volumes used to calculate left ventricular ejection fraction (EF), stroke volume (SV) and end systolic/diastolic volumes (ESV/EDV) (Stuckey et al., 2012).

## 2.24 Imaging

### 2.24.1 Confocal

Cells and Cell colonies were visualised using the Zeiss AX10 fluorescence microscope. Cells immunostained with human β-globin antibody were visualised using confocal microscopy (Leica SPE Confocal microscope). The confocal images were processed using the Fiji software (Open Source).

### 2.24.2 Slide Scanner

All bright field stained tissue slides were visualised using a Zeiss AxioScan Z1 slide scanner and analysed using the Zeiss Blue 2012 software (Germany).

## 2.25 Statistical Analysis

All the haematological indices were compared using one-way ANOVA with post hoc multiple comparison. Sample size calculation was performed before the experiments. To achieve a statistically meaningful result, for each group the rescue of n=6 homozygous mutant mice was attempted - 25% of each litter, giving a total pup number of n=24. On average there were 6 pups per litter, giving n=4 dams per group. Allowing an extra 50% for pregnancy losses, 8 dams per group were aimed towards. This had to be recalculated since no homozygotes were rescued. At least six mice pups in each group were aimed for, born by more than three different dams, in order to have more than three independent biological replicates. For the qPCR data, statistical significance was determined by a One-Way ANOVA and post hoc multiple comparison tests. Graph Pad Prism Version 7.0a for MAC, Graph Pad Software, LA Jolla California, USA was used for statistical analyses and graph drawing. Flow Jo 10.4.2 Software (USA) was used for the flow cytometric data analysis.

# Chapter 3 Expansion of Fetal and Adult Haematopoietic Progenitors using Modified Embryonic Stem Cell Conditions and *In utero* Transplantation of Congenic vs Allogenic Amniotic Fluid Stem cells

## 3.1 Introduction

In humans, success with *in utero* transplantation (IUT) using allogenic haematopoietic stem cells (HSCs) has been limited to fetuses with severe immunologic defects where there is an active lack of immune response to the transplanted allogenic cells, and where genetically healthy cells have a proliferative advantage (Witt et al., 2018). Mesenchymal stem cells (MSCs) appear to be less immunogenic than their haematopoietic counterparts (O'Donoghue and Fisk, 2004); they engraft following IUT in human fetuses with osteogenesis imperfecta in an allogenic setting (Götherström et al., 2014; Le Blanc et al., 2005; Tiblad and Westgren, 2008) and reduce the fracture rate (mouse model) (Guillot et al., 2008). However, attempts to treat congenital haematological diseases such as sickle cell disease (Westgren et al., 1996) using *in utero* HSC transplantation have been unsuccessful, despite having suitably matched donors. Studies in mice suggest that the immune barrier to allogenic *in utero* HSC transplantation may be stronger than previously thought (Peranteau et al., 2006). Even though induction of tolerance for allogenic cells could be a promising approach (Peranteau et al., 2015), the transplantation of autologous progenitor cells, which have been corrected for congenital disease, could avoid the fetal immune barrier. Another option, to avoid a maternal antibody response, is to transplant maternal BM HSCs to the fetus. This was attempted at UCSF and fetuses with alpha thalassaemia survived to term. The centre is currently recruiting patients for this clinical trial for *in utero* therapy for alpha thalassaemia ClinicalTrials.gov - identifier (NCT number): NCT02986698 (Kreger et al., 2016).

Expansion of haematopoietic progenitors is important for transplantation as engraftment success is related to the cell number delivered. This is particularly an issue for autologous *in utero* approaches as only a small number of cells are available in the fetus (Csaszar et al., 2012; Dolznig et al., 2005). Amniotic fluid (AF) is a source of ckit+ stem cell-like progenitors with haematopoietic potential that is characterised by long-term self-renewal and the ability to differentiate into all three lineages (De Coppi, Callegari, et al., 2007; Ditadi et al., 2009; Fauza, 2004; Loukogeorgakis et al., 2019; Prusa et al., 2003). AF can be collected using a clinically safe ultrasound-guided method currently used for the prenatal diagnosis of congenital diseases. AFSCs from mice and humans can be expanded without feeder layers (Piccoli et al., 2012) that are not tumorigenic and retain long telomeres and a normal karyotype for over 250 population doublings (De Coppi, Bartsch, et al., 2007). Their potential for gene transfer; prenatal and postnatal therapy, is enormous(Grisafi et al., 2008).

Maintenance of cell potency and characteristics is the aim of expansion cultures, but success has been previously limited by rapid differentiation (Holmes et al., 2012). Various groups used expansion systems using bone marrow stromal cells with cytokines such as SCF, TPO FGF-1 and achieved an expansion from fivefold to sixtyfold. The culture time of these expansion systems is between two to four weeks(Amsellem et al., 2003; Butler et al., 2012; Hammoud et al., 2011; Isern et al., 2013; Kawano et al., 2003; Khoury et al., 2010; Yamaguchi et al., 2001). Culturing mAF while keeping their haematopoietic properties has been a challenge. Human cord HSC can also be expanded without any feeder layers (Bhatia et al., 1997; Gammaitoni et al., 2003; Kollet et al., 1999; Piacibello et al., 2011), in cytokines even though these culture systems have less expansion potential and differentiation of the expanded cells. Attempts to culture mAF HSC without any feeders were unsuccessful and not described in the thesis. I then used an expansion system using MEFs, which was more successful(Yuan et al., 2004). The culture system was a modified ESC system as described in the methods chapter(Lee et al., 2009).

## 3.2 Hypothesis

My hypotheses in this chapter are:

- Amniotic fluid stem cells can be isolated from the mouse fetus.
- Amniotic fluid stem cells show haematopoietic properties similar to fetal liver and bone marrow HSCs and produce haemoglobin *in vitro*.
- Amniotic fluid stem cells can be transduced using a lentiviral vector.
- Amniotic fluid stem cells can be expanded in a modified embryonic stem cell media.
- Amniotic fluid stem cells engraft after *in utero* transplantation.
- Amniotic fluid stem cells, when transplanted *in utero*, can rescue the homozygous mouse model of beta thalassemia.

Initially, in this chapter, I studied multipotency after the expansion of fetal and adult stem cell sources, and efficiency of gene transfer with the aim of developing an efficient expansion method, which can provide high cell numbers for *in utero* stem cell and gene therapies.

I hypothesised that such cells could be used as an autologous source of cells in the treatment of congenital haematological disease (Ramachandra et al., 2014). I then studied the engraftment and the immune response to AFSCs after congenic or allogenic IUT in mice using the intraperitoneal route of injection. This is a clinically relevant delivery method that has been safely used in the first trimester for IUT in humans (Touraine, 2000).

## 3.3 Mouse amniotic fluid stem cells have haematopoietic potential and produce haemoglobin *in vitro*

To demonstrate if AFSCs could be used as an autologous source of cells in the treatment of congenital haematological disease, I first isolated AF cells in mice and shown that they expressed haematological markers, as shown in Figure 3-2.

AF was isolated from pups of time-mated dams at E13.5, as described in the methods section. The fluid was then processed and stem cells were selected using lineage depletion and the CD117 marker, then characterised using flow cytometry. AFSCs were compared with mouse fetal liver from E13.5 fetal mice and adult mBM HSCs. The mFL cells were used to provide a gestational age-matched haematopoietic stem cell comparison, and mouse bone marrow was used as the gold standard for HSC. As shown in Figure 3-1, mAF had a mean 1.87% of CD117

positive stem cells (range 0.43% to 2.5 %, n=3). After purification using magnetic separation, the isolated stem cells contained a mean total of 82.4% CD117 positive cells (range 78% to 85%, n=3).

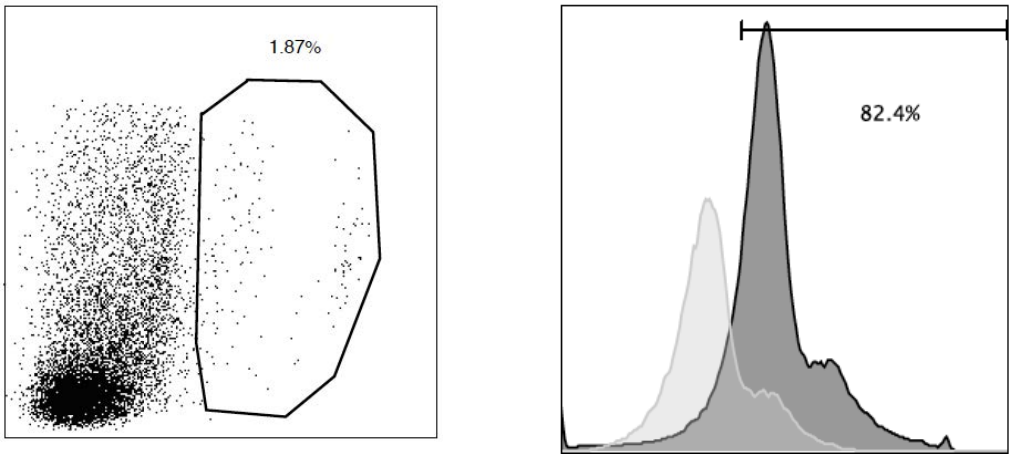



**Figure 3-1 Isolation of Amniotic Fluid Stem Cells. A) Percentage of mAF HSC selected using CD117 antibody; B) Purity of the population after MACS selection.**

I then characterised the mAF HSCs. Characterisation (purity) of freshly isolated cells using flow cytometry (FACS) are shown in Figure 3-2 for CD117, CD45 and Sca1. Differences in Sca1 and CD117 markers were found, but there were no differences in the CD45 marker across different groups: FL: CD117 (100 ± 0.0), CD45 (83.85 ± 2.45), Sca1(65.20 ± 4.4) (BM: CD117 (73.10 ± 2.30), CD45 (86.80 ± 0.90), Sca1(69.30 ± 3.5), AFSC: CD117 (95.25 ± 0.95), CD45 (85.5 ± 2.55), Sca1(86.95 ± 1.65). There was no difference in CD45 expression between the groups, n=6, p=61. Table 3-1 shows the results of the statistical analysis when comparing groups.

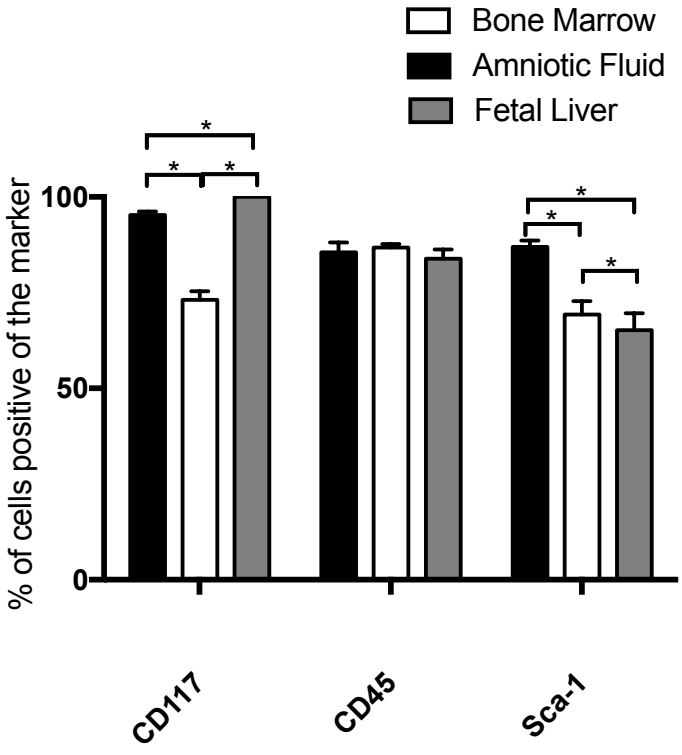



**Figure 3-2 Characterisation of Fresh Mouse Amniotic Fluid Stem Cells. Anti-CD117, CD45 and Sca-1 antibodies were used to characterise and compare fresh haematopoietic progenitors from mAF, BM and FL (*p<0.05 ).**

| Comparison of different groups using ANOVA | Mean Diff. | Ind. P Value |
|---|---|---|
| CD117 | | |
| mBM vs. mAF | -22.2 | <0.001 |
| mFL vs. mAF | 4.75 | 0.05 |
| mFL vs. mBM | 26.9 | <0.001 |
| CD45 | | |
| mBM vs. mAF | 1.25 | 0.54 |
| mFL vs. mAF | -1.7 | 0.41 |
| mFL vs. mBM | -2.95 | 0.18 |
| Sca-1 | | |
| mBM vs. mAF | -17.7 | <0.001 |
| mFL vs. mAF | -21.8 | <0.001 |
| mFL vs. mBM | -4.1 | 0.08 |

**Table 3-1 Results of statistical analysis to compare the groups of haematopoietic progenitors.**

To determine if mAFSCs could differentiate into all three haematopoietic lineages, fresh AFSCs were cultured in methocult M3434, as described in the methods section. The plates were then analysed and imaged using a bright light microscope. Colonies from all three haematopoietic lineages were identified, as shown in Figure 3-3, after 14 days of culture. The representative image shows colony-forming units.

CFU-GM: This is a colony forming unit-granulocyte, macrophage, including colony-forming unit-granulocyte (CFU-G) and colony-forming unit-macrophage (CFU-M). Colonies usually contain 30 to thousands of granulocytes (CFU-G), macrophages (CFU-M) or both cell types (CFU-GM).

CFU-GEMM: Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte. Because of their primitive nature, CFU-GEMM tends to produce large colonies of >500 cells containing erythroblasts and recognisable cells of at least two other lineages. Colonies derived from CFU-GEMM have a highly dense core with an indistinct border between the core and peripheral cells. Erythroblast clusters are visible at the periphery of the colony.

BFU-E: Burst-forming unit-erythroid. Colonies derived from BFU-E are made up of erythroid clusters. Each cluster contains a group of cells that are tiny, irregular in shape, difficult to distinguish and appear fused. BFU-E do not usually have a dense core.

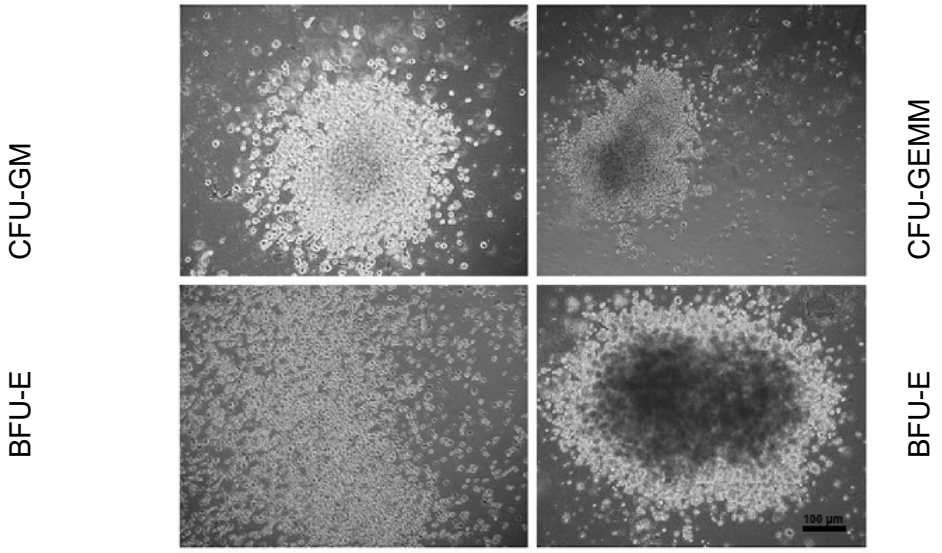



**Figure 3-3 Fresh mAF HSCs in methocult after 14 days. The panel shows the formation of all three haematopoietic colonies, colony-forming unit-granulocyte(CFU-GM), colony-forming unit-granulocyte(CFU-GEMM) and burst-forming unit-erythroid(BFU-E).**

mAF HSCs were then cultured in a terminal erythroid differentiation medium for five days to determine if they could produce haemoglobin and were therefore functional see Figure 3-4 below. In the AFSCs group, there was 66.05±5.66% of haemoglobin compared to 58.83±6.44% in the BM positive control cells and 0.59±0.22%, n=4 in the mouse embryonic fibroblast negative control cells, see Figure 3-5.

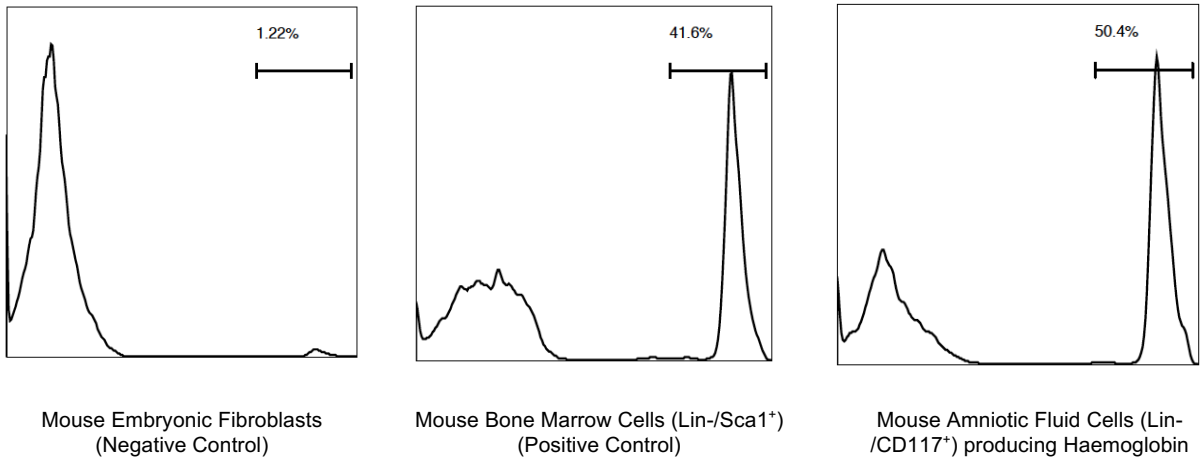



**Figure 3-4 Representative Flow Cytometry Histograms showing the presence of Adult Hb. The first histogram (left) shows the negative control used, mouse embryonic fibroblasts, the middle shows the production of haemoglobin from bone marrow HSC as the positive control, and on the right, haemoglobin production from mouse mAF HSCs.**

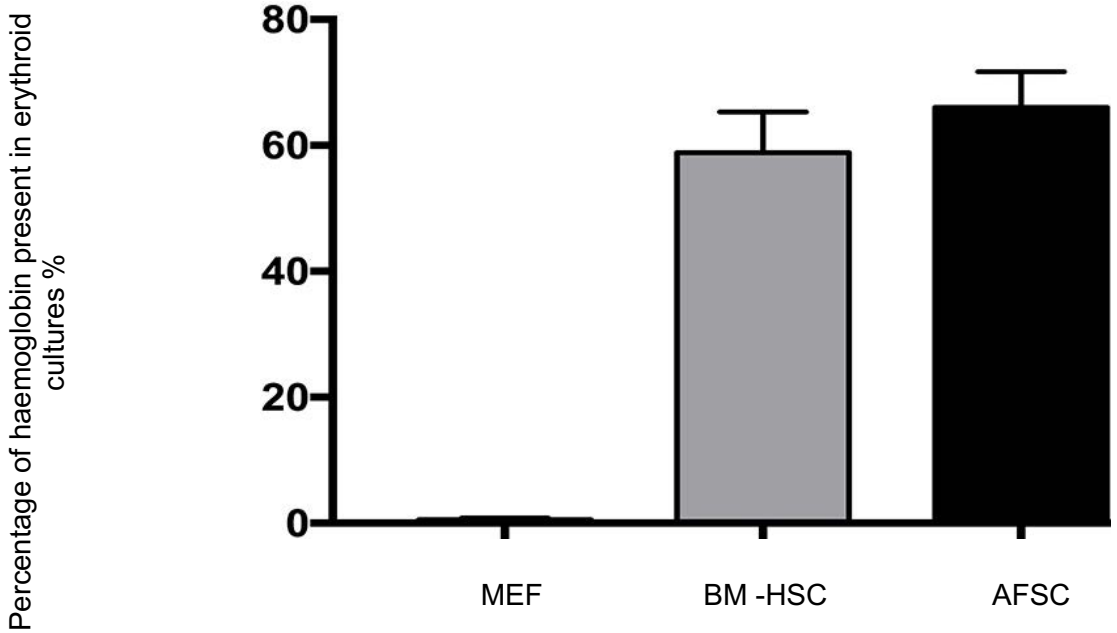



**Figure 3-5 Quantitative Haemoglobin Production in erythroid cultures in mMEF, mBM and mAF HSCs. The haemoglobin production, the mAF HSCs, were comparable to the mBM HSCs.**

In summary, I demonstrate that mAF HSCs have haematopoietic potential *in vitro*. This can be seen by the erythroid definition in semi-solid medium and the haemoglobin production in the erythroid, terminal differentiation medium.

### 3.4 Comparison of HSCs from Amniotic Fluid, Fetal Liver and Bone Marrow

I isolated mouse haematopoietic stem cells as described in the methods section, from mAF at E13.5, mFL at E13.5 and adult mBM (average mouse age=3 months).

The cells were isolated using flow cytometry assisted sorting (FACS) or magnetic assisted cell sorting (MACS). Characterisation of the cells was initially done to confirm multipotency. The cells were then transduced using various methods to identify the best technique giving the highest transduction efficiency. The cells were also expanded using a modified embryonic stem cell medium (Piccoli et al., 2012; Rota et al., 2011). The fold expansion was studied as well as the genetic profile of the cells before and after culture using a mouse haematopoietic PCR array (QIAGEN) (not described in this thesis) as published(Loukogeorgakis et al., 2019). The study design is presented diagrammatically in Figure 3-6.

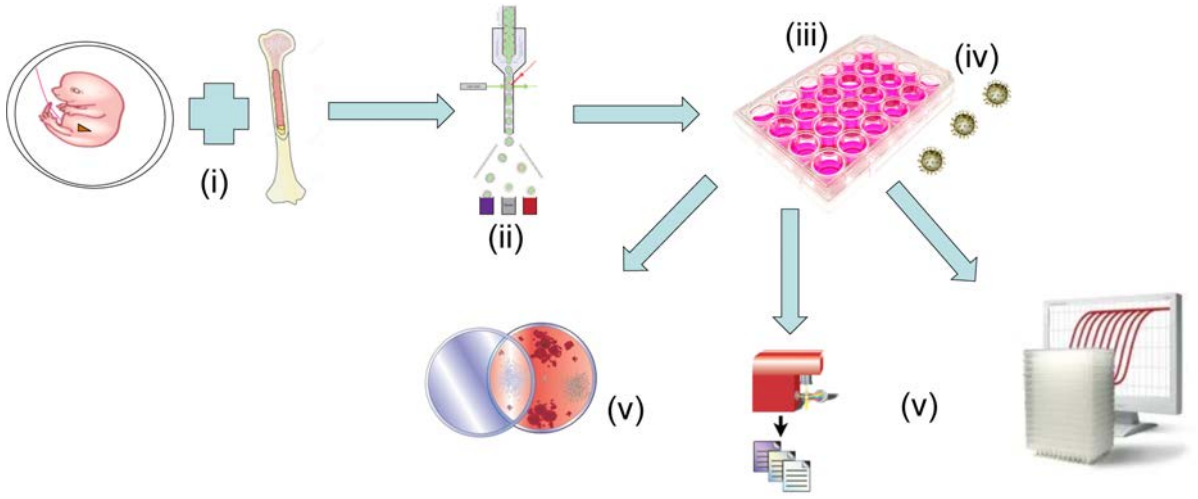



**Figure 3-6 Study design: (i) Cells were isolated from mBM (adult) , mouse fetal liver and AF at E13.5, (ii) Selection for CD117(fetal) and +/SCA1+ (adult) using FACS (iii) Culture in a pretreated 24 well plate containing inactivated mouse embryonic fibroblasts, in a modified embryonic stem cell medium (iv) Separately, freshly isolated cells were transduced overnight using a GFP lentiviral vector (MOI:50) in a cytokine cocktail with or without rapamycin and cultured as above (v) Cells were analysed after 7 days in culture using flow cytometry, PCR array or differentiated in a semi-solid medium**

### 3.5 Characterisation of Haematopoietic Progenitors

mAF was isolated as described in the methods section. Lineage depletion was performed to remove all terminally differentiated cells. The cells were then characterised using flow cytometry. In the representative figure below the lineage, the negative portion of the mAF contained 13% of CD117$^{+}$ cells (see Figure 3-7 & Figure 3-8).

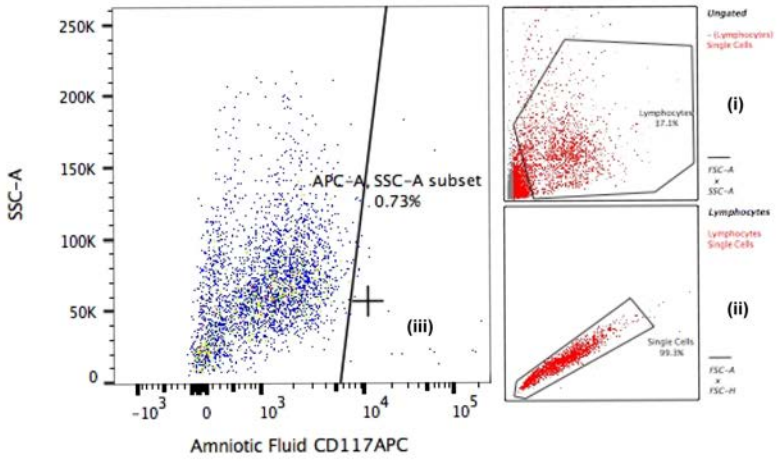



**Figure 3-7 Gating Strategy in a representative flow cytometry profile of mAF cells stained with anti-CD117. The figure is demonstrating a negative control sample used for identifying Lineage negative, CD117 positive cells in the mAF, i) sample selection, ii) only single cells were selected and analysed, iii) gate for APC CD117$^{+}$ cells, dead cells were excluded from analysis (gating strategy for dead cells not shown in the graph)**

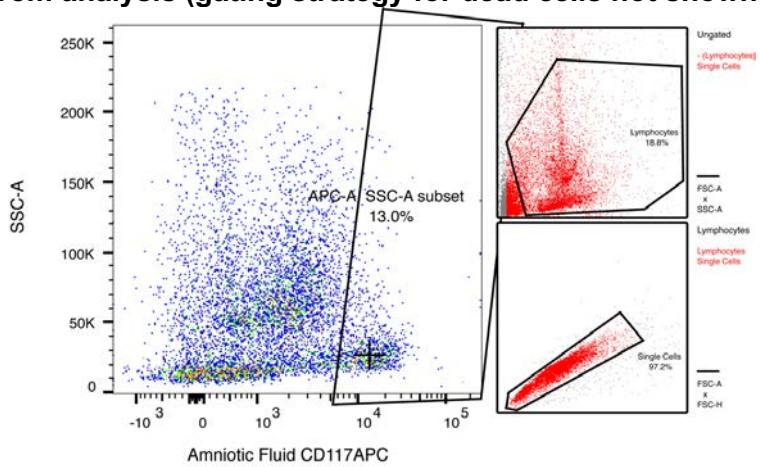



**Figure 3-8 Lineage negative, APC CD117 positive cells in the mAF, representative figure, only single cells were characterised and dead cells were excluded from analysis, similar to the previous figure the cell population was initially selected then doublets were excluded, and the gate was placed similarly to Figure 3-7**

Sorting and Characterization of mFL stem cells using CD117 antibody. The cells were lineage depleted before selection. The percentage of CD117 positive cells was 19.4%.

Similarly, the percentage of cells positive for CD117 in the mBM in a representative sample was 31.9%

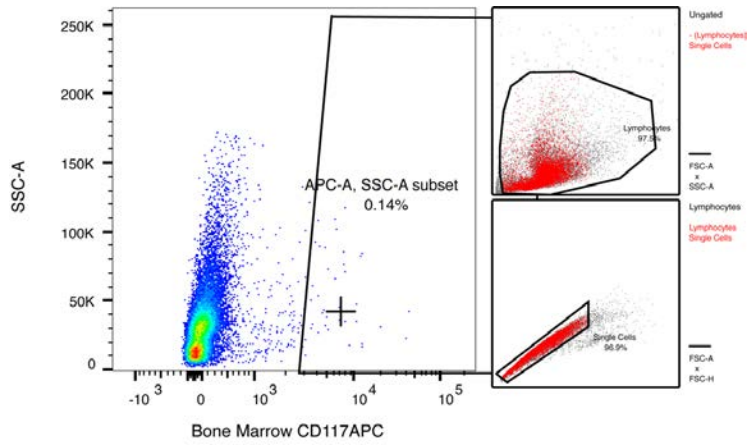



**Figure 3-9 mBM Negative control sample for gating strategy of the Lineage negative, CD117 positive cells in the BM, only single cells were analysed, and dead cells were excluded from the analysis**

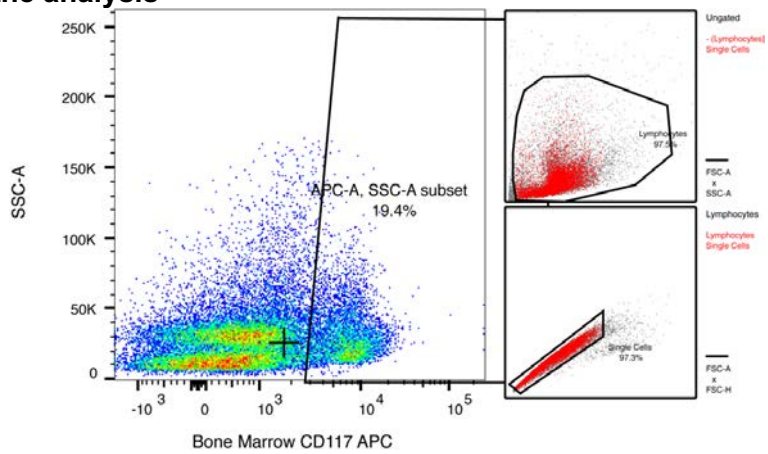



**Figure 3-10 mBM HSC selection gating strategy using negative control in Figure 3-9. These are Lineage negative, CD117 positive cells in the BM, only single cells were analysed, and dead cells were excluded from the analysis**

The fetal liver had the highest percentage of CD117+ cells and in a representative sample that was 31.9%, as seen in Figure 3-12.

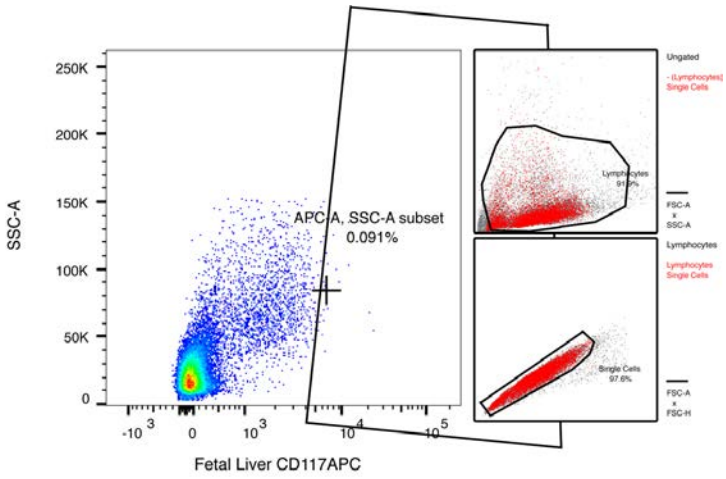



**Figure 3-11 mFL Negative control used to apply the gates for the Lineage negative, CD117 positive cells in the mFL, (HSCs) only single cells were analysed, and dead cells were excluded from the analysis**

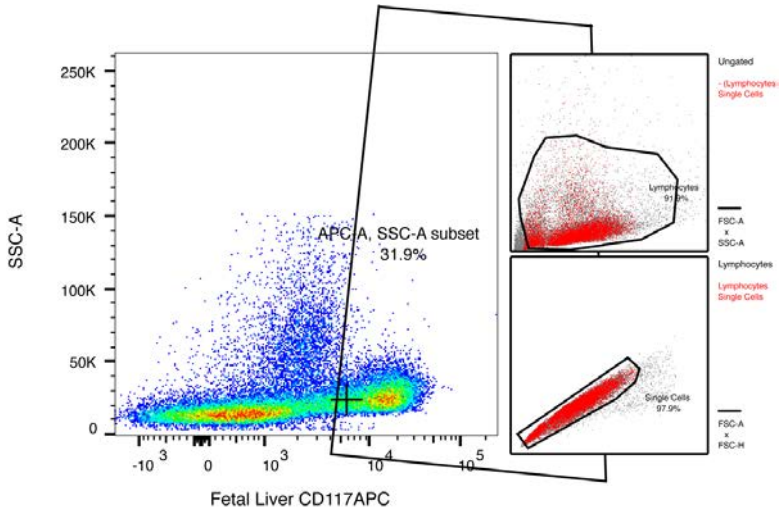



**Figure 3-12 mFL HSCs selection, these are Lineage negative, CD117 positive cells in the FL, only single cells were analysed, and dead cells were excluded from the analysis**

## 3.6 Production of Haematopoietic Colonies

When cultured in semi-solid medium mAFSCs for two weeks, they produced haematopoietic colonies from all three haematopoietic lineages (Ditadi et al., 2009)(CFU-GM, CFU-GEMM, BFU-E, n=3). This was demonstrated in the previous chapter.

Also, mAFSC were capable of haemoglobin production after culture in a terminal erythroid differentiation medium for five days. This was also demonstrated in the previous chapter. In Figure 3-13 below, light microscope images can be seen demonstrating HSC from mASFC and mBM in the erythroid differentiation medium.

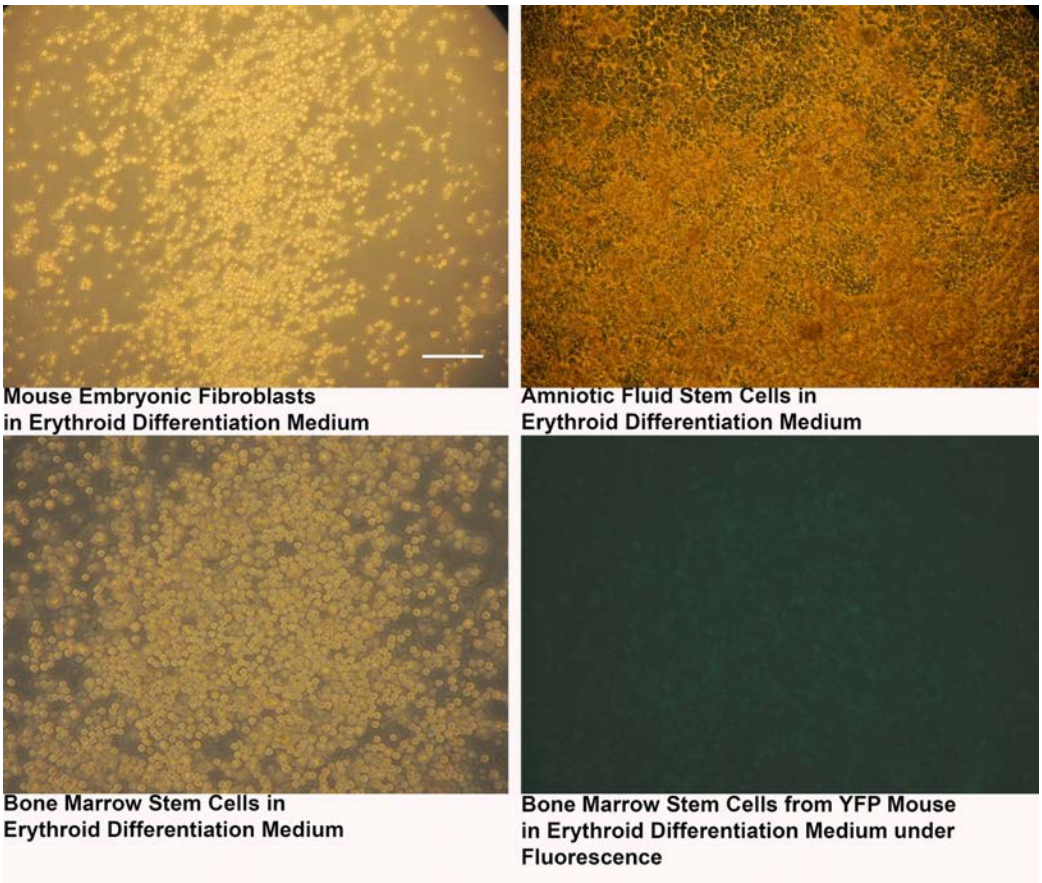


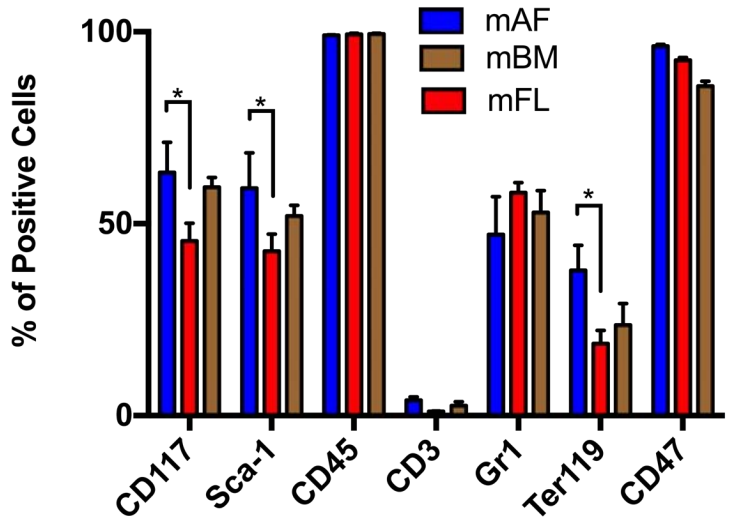

**Figure 3-13 Erythroid culture of mBM, mAFSC and MEFs showing progenitors on Day 2 in the erythroid differentiation medium. The BM progenitors were isolated from YFP mouse, which emits yellow fluorescent light**

In conclusion, I demonstrated that the percentage of CD117 in the cells is much higher when the cells are lineage depleted before stem cell selection. The cells do produce haemoglobin when cultured in an erythroid differentiation medium. The cells were then cultured on mouse embryonic fibroblasts in an embryonic stem cells medium as described in the methods section. I compared the gene expression of the cells before and after culture and directly compared the cells with mBM and mFL. The medium was later modified to include angiopoietin. I then tested gene transfer to mAFSC comparing different methods and trying to identify the most efficient one.

Characterisation of expanded cells after seven days (n=6, in six biologically independent experiments) culture on MEFs showed some differences in mAF. All the analysis can be seen in Figure 3-14. Especially in the expression of CD117, mAF was superior to mFL ($p=0.02$) but similar to mBM ($p=0.82$). This was also true in the case of the Sca1 stem cell marker

(p=0.0296). Also, the expression of the erythroid marker was higher in the mAF group (p=0.0093).

Interestingly, very high expression was seen in all three groups in the CD47 marker. This marker can be upregulated on mHSCs and progenitors just before and during their migratory phase. The level of expression determines the probability that they are engulfed *in vivo*(Jaiswal et al., 2009).




| | mAF (CD117) | | | mFL (CD117) | | | mBM (CD117) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | N | Mean | SD | N | Mean | SD | N |
| CD117 | 63.38 | 19.14 | 6 | 45.50 | 11.36 | 6 | 59.57 | 6.14 | 6 |
| Sca-1 | 59.28 | 22.52 | 6 | 42.83 | 10.89 | 6 | 52.05 | 6.76 | 6 |
| CD45 | 99.08 | 0.38 | 6 | 99.35 | 0.60 | 6 | 99.47 | 0.48 | 6 |
| CD3 | 4.07 | 2.05 | 6 | 1.09 | 0.38 | 6 | 2.59 | 2.49 | 6 |
| Gr1 | 47.23 | 24.09 | 6 | 58.15 | 6.27 | 6 | 52.92 | 14.05 | 6 |
| Ter119 | 37.90 | 15.76 | 6 | 18.81 | 8.31 | 6 | 23.65 | 13.63 | 6 |
| CD47 | 96.25 | 1.15 | 6 | 92.60 | 1.75 | 6 | 85.85 | 3.12 | 6 |

**Figure 3-14 Graph and table for characterisation of HSC after seven days in culture in a modified embryonic stem cell medium. * indicates a significant difference with p<0.05. The groups were compared using ANOVA with Tukey's multiple comparison test.**

### 3.7 Expansion Potential on Mouse Embryonic Fibroblasts

When the cells are cultured in the ESC medium, using leukaemia inhibitory factor (LIF), MEFs are used as feeder layers. This was shown before to enhance the number of mBM HSC and to suppress cell apoptosis(Yuan et al., 2004). To separate and characterise the cells from MEFs, I used flow cytometry. During flow cytometry, the feeders were separated using their size, and the stem cells were selected using the CD117 antibody. Figure 3-15 shows images under a light microscope of the culture of haematopoietic progenitors on MEFs in a modified ESC medium.

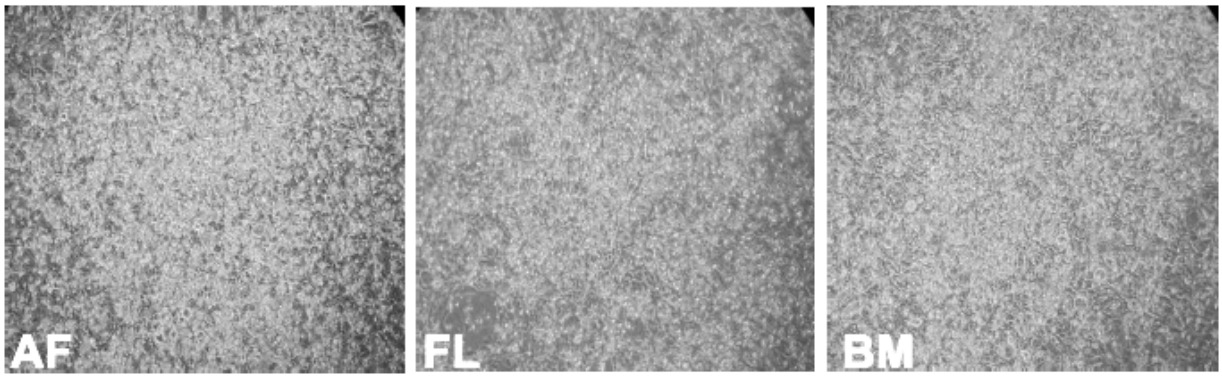



**Figure 3-15 HSCs in a modified embryonic stem cell medium, cultured on inactivated MEFs. From left to right, amniotic fluid, fetal liver, and bone marrow (magnification 20X)**

I then studied the expansion capability of the cells, comparing them to mBM and mFL. After seven days in the culture, there was an average 5.1-6.2-fold increase in the progenitor numbers with no difference between the groups, ($p=0.64$, Kruskal-Wallis test) as seen in Figure 3-23.

The expansion fold was calculated by counting the initial number of cells put in culture and then I used counting beads (Molecular Probes, Invitrogen, UK) to count the $CD117^{+}$ cells present, after seven days in culture as described in the methods section.

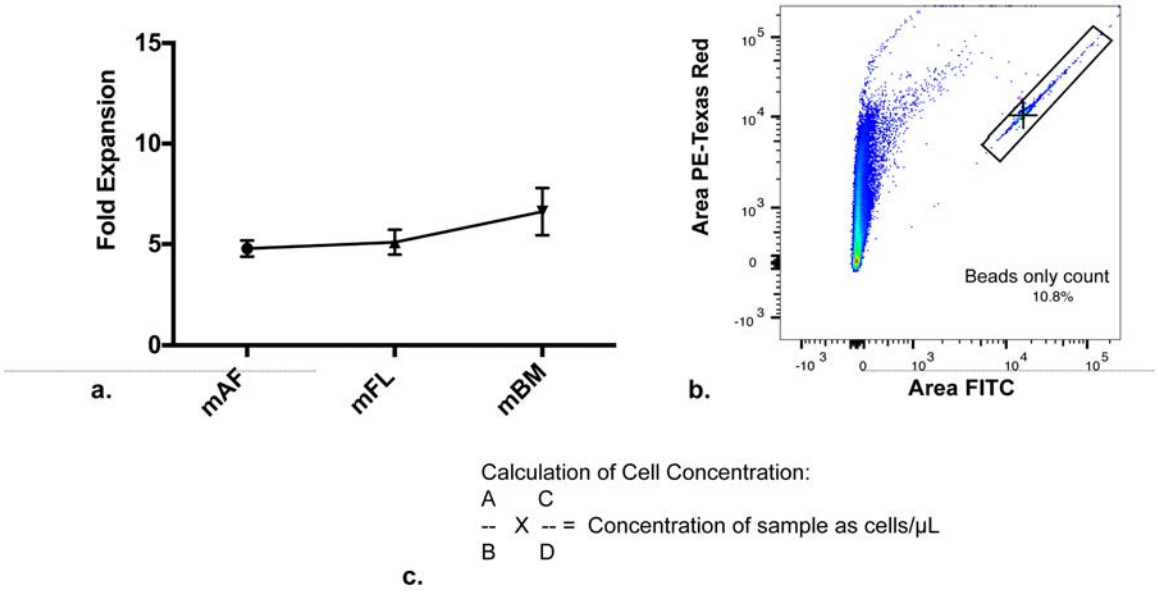



**Figure 3-16 Expansion potential of HSC progenitors in ESC modified medium. a. Expansion fold of CD117$^+$ cells; b. Cells were counted by flow cytometry using counting beads (Molecular Probes, Invitrogen, UK). Only cells expressing CD117$^+$ were used to plot the graph; c. the cell concentration was calculated A=number of cell events, B=number of bead events, C=assigned bead count of the lot (beads/50µL), D=volume of the sample (µL)**

## 3.8 Expansion Systems (these experiments were done together with Dr Miguel Calero-Garcia, PhD student, MIU, ICH)

To test the best and most efficient method of gene transfer into haematopoietic progenitors from mAFSCs, I transduced the cells with a lentiviral vector expressing EFSGFP. The transduction was performed using a cocktail of cytokines with and without rapamycin, spinfection and without spinfection. The rapamycin was used to test if mAF transduction can be augmented *in vitro* similarly to human cord CD34$^+$ cells as used by Wang et al. (Wang et al., 2014). For the transductions, MOIs of 50 was used after pilot experiments done to identify the ideal MOI.

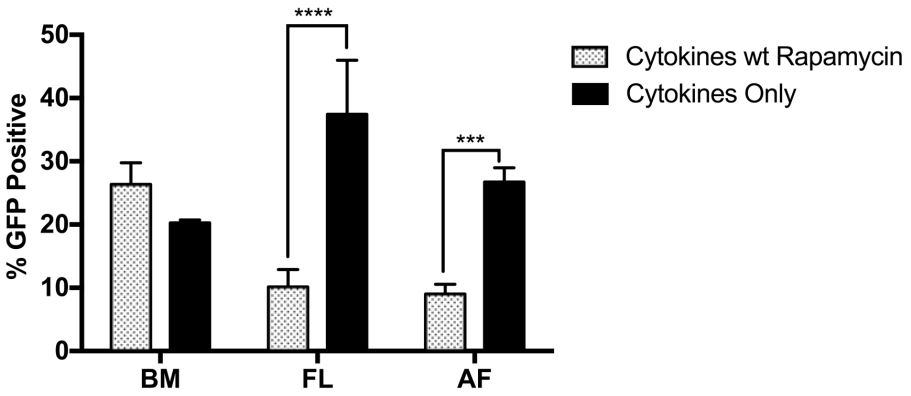
I used the following flow cytometry antibody panel, as shown in Table 3-2. The samples were all stained with the antibodies and FMOs were used for compensation. Compensation and analysis were performed on FlowJo V10.

| | **CD3** | **Gr-1** | **CD45** | **CD117** | **Sca-1** | **Ter-119** |
|---|---|---|---|---|---|---|
| GFP | FITC | FITC | FITC | | FITC | FITC |
| | PE | PE | PE | PE | PE | PE |
| | APC | APC | APC | APC | APC | APC |
| | VioBlue | VioBlue | VioBlue | | | VioBlue |
| | Biotin | Biotin | Biotin | Biotin | Biotin | Biotin |
| | | VioGreen | VioGreen | | | |
| | | PerCP | PerCP | | | |
| | PE-Vio770 | | | | | |
| | APC-Vio770 | | | | | |
| | | PerCP-Vio | PerCP-Vio | | | |

**Table 3-2 Flow cytometry panel used for the characterisation of expanded HSCs. Different colours were used to combine all antibodies. The antibodies were available in various colours, as seen in the table, and the panel was designed to avoid the need for substantial compensation.**

The transduction was performed with MOI 50 on 100000 cells. The cells were left in suspension in the different media (cytokines, cytokines with rapamycin) for approximately 24 hours. The cells were then transferred on the inactivated, with mitomycin C, MEFs in the modified embryonic stem cell medium. The cytokine cocktail was able to transduce the cells efficiently and maintain the stem cell markers but also to increase the differentiation markers.

The Cytokines only cocktail was superior to the mixture of rapamycin and cytokines (in transduction efficiency) for the fetal haematopoietic progenitors ($p=<0.005$) but not different for the adult bone marrow HSC ($p=0.25$). The rapamycin with the cytokines group seemed to be better at keeping the stemness (CD117+, Figure 3-18), probably since the cells stopped proliferating but unfortunately, the transduction efficiency was significantly lower as shown in Figure 3-17 below.




| | Cytokines wt Rapamycin | | | Cytokines Only | | |
|---|---|---|---|---|---|---|
| | Mean | SD | N | Mean | SD | N |
| BM | 26.33 | 3.44 | 3 | 20.27 | 0.47 | 3 |
| FL | 10.16 | 2.73 | 3 | 37.43 | 8.54 | 3 |
| AF | 9.00 | 1.57 | 3 | 26.71 | 2.26 | 3 |

**Figure 3-17 Transduction efficiency, comparison of cytokines medium versus cytokines with rapamycin. The transduction efficiency in the cytokines medium alone is higher than the medium containing cytokine and rapamycin when transducing fetal haematopoietic progenitors**

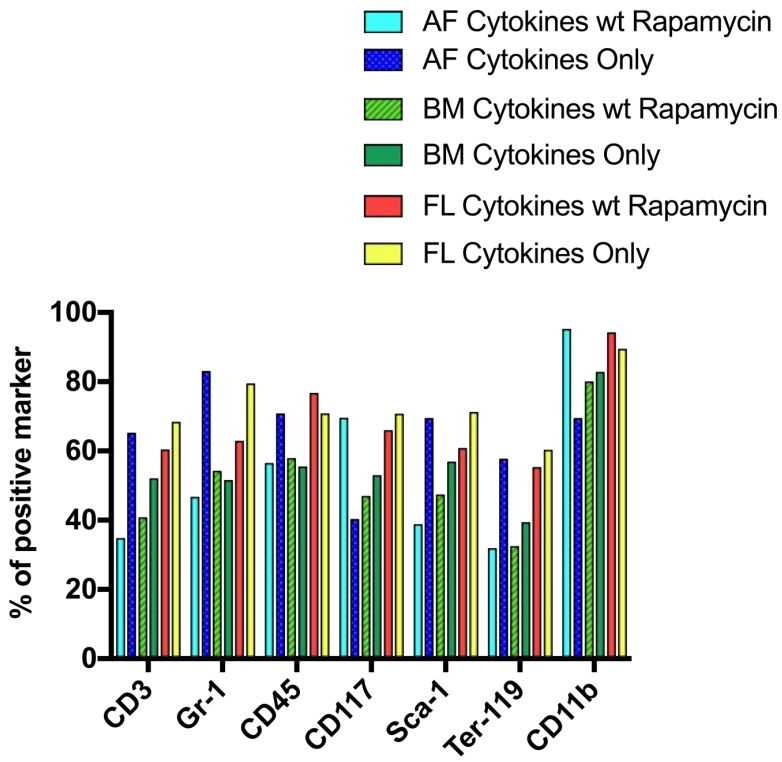



**Figure 3-18 Phenotype of cells after transduction and culture for seven days on MEFs (n=3)**

The figures below (Figure 3-19, Figure 3-20 and Figure 3-21) show the gating strategy used to determine transduction efficiency in the various groups. Representative figures are shown from AF analysis. DAPI was used to exclude dead cells from the analysis.

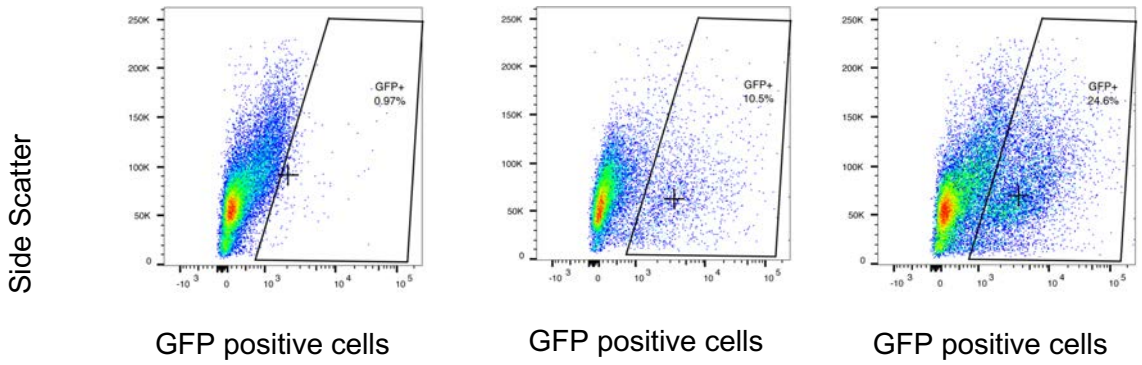



**Figure 3-19 Transduction Efficiency AF, negative control**

**Figure 3-20 Transduction Efficiency AF, Cytokines &Rapamycin**

**Figure 3-21 Transduction Efficiency AF, Cytokines only**

To determine the initial transduction efficiency cells were transduced with the same technique, but the analysis was performed before culturing them on MEFs (the cells were taken for flow cytometry analysis directly after 48 hours). The cells, positive for GFP, were much higher in the mAF(71.1%) versus mBM(54.9%). This might be due to some transduced cells being already in apoptosis or proliferating and once cultured on MEFS they became apoptotic after seven days.

To increase the expansion fold, another culture was performed using MEFs which I transduced with a virus expressing angiopoietin-like protein 3 (Angptl3, MOI 50)(Zhang et al., 2006; Zheng et al., 2011). The MEFs, after transduction, were genetically modified to produce Angtl3. The same modified ES medium was used. The MEFs were then inactivated and treated similarly to the MEFs used in the previous experiments. An initial concentration of 100000 cells was used in each well in a 24 well plate.

The HSCs which were expanded on the MEFs transduced with angptl3 had higher expansion fold in the mFL culture, $p<0.001$, $n=3$. There was no difference in the expansion of bone marrow. The amniotic fluid culture, unfortunately, failed since the cells retrieved were much less than the initial number of cells plated (Figure 3-22).

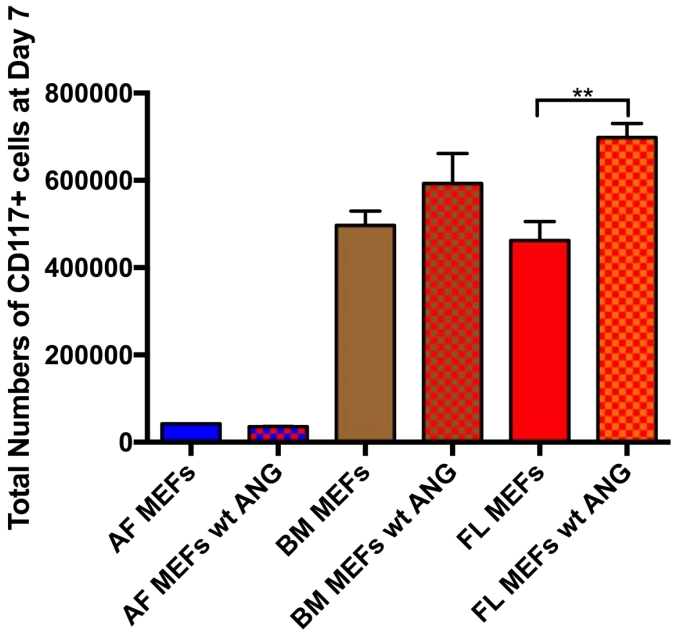



**Figure 3-22 Numerical expansion of HSC progenitors in a medium with and without Angiopoietin. Higher numerical expansion in the mFL culture in the medium with angiopoietin producing MEFs was found (ANOVA, Tukey's multiple comparison test, p=0.039). The mAF culture was unsuccessful, and no difference was seen in the mBM culture.**

The fold expansion was calculated based on the initial concentration of 100000 cells per well. There was an increase in the fetal liver stem cells, which were cultured on Angtl3 transduced MEFs (Figure 3-23).

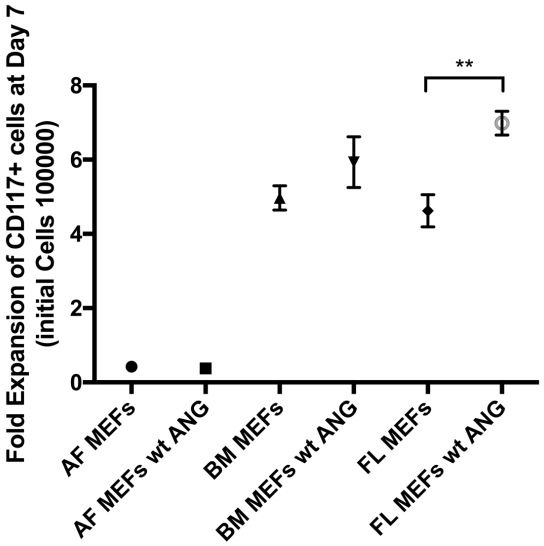
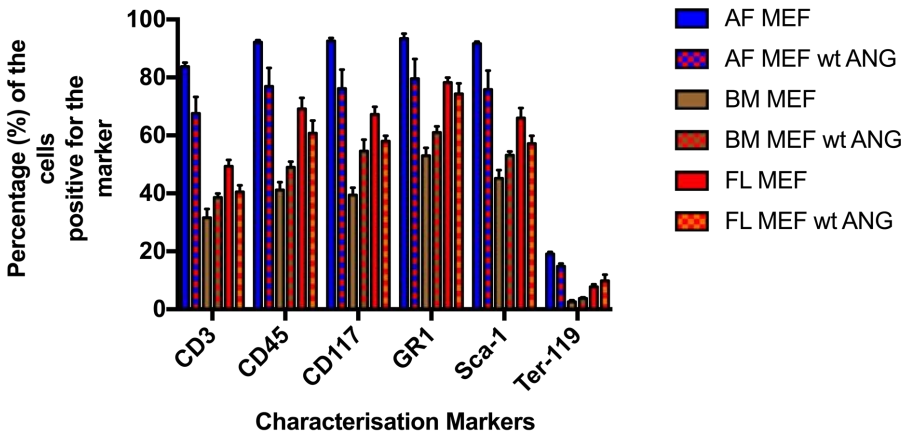



**Figure 3-23 Expansion fold of cells cultured in modified ESC medium on MEFs with and without angiopoietin producing MEFs. In the FL the expansion fold was higher in the angiopoietin producing MEFs (Tukey's multiple comparison test, **p=0.0039)**

The expression of stem cell markers (CD117, Sca1, CD45) was not different in the mFL culture on MEFs with angptl3 when compared to the MEFs only culture. Even though the mAF culture was unsuccessful, i.e. no increase in cell numbers, the culture of fetal progenitors (mFL) showed an increase in cell numbers (Figure 3-24, Table 3-3).




| | AF MEF | | | AF MEF wt ANG | | | BM MEF | | | BM MEF wt ANG | | | FL MEF | | | FL MEF wt ANG | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | N | Mean | SD | N | Mean | SD | N | Mean | SD | N | Mean | SD | N | Mean | SD | N |
| CD3 | 83.77 | 2.31 | 3 | 67.60 | 11.32 | 4 | 31.60 | 5.19 | 3 | 38.65 | 3.19 | 6 | 49.35 | 4.41 | 4 | 40.57 | 3.89 | 3 |
| CD45 | 92.23 | 1.15 | 3 | 76.95 | 12.64 | 4 | 41.20 | 4.60 | 3 | 49.00 | 4.82 | 6 | 69.23 | 7.46 | 4 | 60.80 | 7.49 | 3 |
| CD117 | 92.60 | 1.73 | 3 | 76.20 | 13.05 | 4 | 39.47 | 4.32 | 3 | 54.60 | 9.60 | 6 | 67.30 | 5.09 | 4 | 58.03 | 3.16 | 3 |
| GR1 | 93.43 | 2.89 | 3 | 79.60 | 13.51 | 4 | 53.03 | 4.56 | 3 | 61.02 | 5.11 | 6 | 78.30 | 3.32 | 4 | 74.33 | 6.35 | 3 |
| Sca-1 | 91.73 | 1.15 | 3 | 75.90 | 12.93 | 4 | 45.20 | 4.99 | 3 | 53.17 | 3.24 | 6 | 66.03 | 6.88 | 4 | 57.17 | 4.68 | 3 |
| Ter-119 | 19.13 | 1.15 | 3 | 14.85 | 1.91 | 4 | 2.66 | 0.82 | 3 | 3.75 | 1.00 | 6 | 7.82 | 1.65 | 4 | 9.88 | 3.66 | 3 |

**Figure 3-24 Graph and table for characterisation of cells after seven days in culture comparing MEFs and angiopoietin producing MEFs.**

In the mAF culture, there was a difference between MEFs and angiopoietin producing MEFs. The MEF only culture showed higher expression of stem cell markers, such as CD117 and Sca-1 (Table 3-3). Unfortunately, the stem cell numbers in mAF culture remained the same, which did not correspond to the results of the previous experiments, in which I saw a 6-7 fold increase in the cell numbers.

| ANOVA with Tukey's multiple comparisons test | Summary | Adjusted P Value |
|---|---|---|
| **CD3** | | |
| AF MEF vs. AF MEF wt ANG | * | 0.02 |
| BM MEF vs. BM MEF wt ANG | ns | 0.64 |
| FL MEF vs. FL MEF wt ANG | ns | 0.49 |
| **CD45** | | |
| AF MEF vs. AF MEF wt ANG | * | 0.03 |
| BM MEF vs. BM MEF wt ANG | ns | 0.53 |
| FL MEF vs. FL MEF wt ANG | ns | 0.53 |
| **CD117** | | |
| AF MEF vs. AF MEF wt ANG | * | 0.02 |
| BM MEF vs. BM MEF wt ANG | * | 0.02 |
| FL MEF vs. FL MEF wt ANG | ns | 0.42 |
| **GR1** | | |
| AF MEF vs. AF MEF wt ANG | ns | 0.07 |
| BM MEF vs. BM MEF wt ANG | ns | 0.51 |
| FL MEF vs. FL MEF wt ANG | ns | 0.97 |
| **Sca-1** | | |
| AF MEF vs. AF MEF wt ANG | * | 0.02 |
| BM MEF vs. BM MEF wt ANG | ns | 0.51 |
| FL MEF vs. FL MEF wt ANG | ns | 0.48 |
| **Ter-119** | | |
| AF MEF vs. AF MEF wt ANG | ns | 0.95 |
| BM MEF vs. BM MEF wt ANG | ns | 0.99 |
| FL MEF vs. FL MEF wt ANG | ns | 0.99 |

**Table 3-3 Statistical comparisons between the cultures, on MEFs and Angiopoietin producing MEFs. ANOVA was used with follow up Tukey test. This compares every means with every other mean and allows unequal sample sizes, which is the case in this analysis. The test compares the difference between means to the amount of scattering which gives the test more power to detect differences.**

## 3.9 *In utero* intraperitoneal transplantation of congenic mAF HSCs shows long-term multilineage engraftment in the haematopoietic system.

### 3.9.1 Study design

The study design is shown in Figure 3-25. To mimic autologous IUT, cKit+/lin-, congenic mAFSC were isolated at E13.5 from B6.SJL-Ptprca Pepcb /BoyJ dams (CD45.1; MHC class I: H2-$K^{b+}$). For allogenic IUT, mAFSC were isolated from fetuses of BALB/cJ ($CD45.2^{+}$; MHC class I: H2-$K^{d+}$) dams. These transplantation systems are well established in the literature (Delemarre et al., 2016; Peranteau et al., 2002; Waterstrat et al., 2010). Only clear AF was collected; blood stained AF was discarded to exclude maternal or placental contamination (Ditadi et al., 2009). The clear AF from all fetuses from up to three dams was pooled. Red blood cell lysis was used to remove any remaining red blood cells and to prevent any fetal or maternal blood contamination. The cells were then washed with PBS three times and counted. IUT was performed at E13.5 into C57BL/6 (CD45.2/H-$2K^{D}$) dams time-mated with C57BL/6 (CD45.2/H-$2K^{D}$) male mice. I used pups injected with PBS as negative controls and adult bone marrow cells as the gold standard control. The prepared mAFSCs or Congenic BMSC in 20µl (1x10^4 or 5x10^4 cells) of PBS were injected into the peritoneal cavity inferior to the liver of each embryo using a 33-gauge needle attached to a digital syringe (Hamilton, Switzerland). The pups injected with PBS received 20µl of PBS. All embryos of each litter were injected.

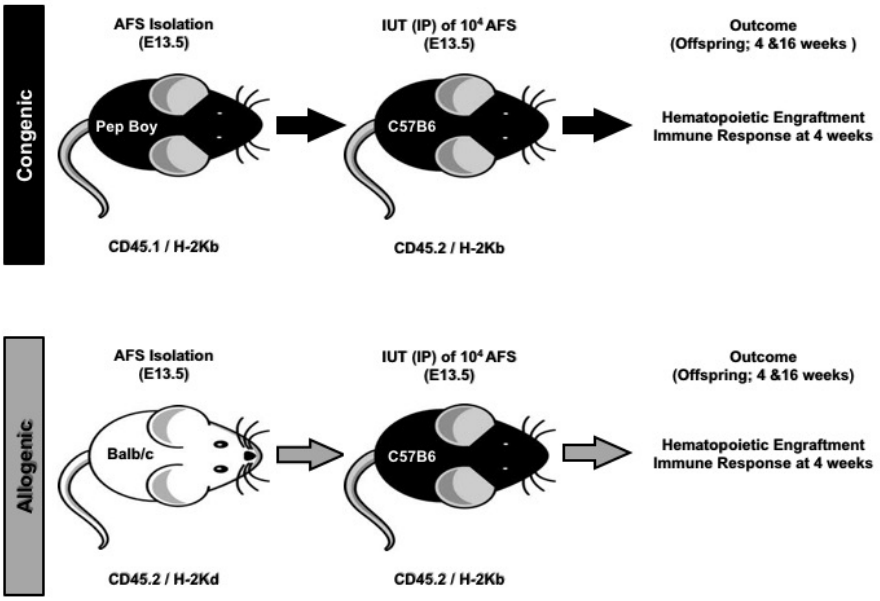



**Figure 3-25 Study Design**

Before starting the experiments, I performed a sample size calculation and determined that I needed at least three different dams transplanted in three biologically independent experiments in each group (see Methods section).

### 3.9.2 Experimental procedures and short-term outcome

All pups in each litter were successfully injected, and all dams survived the surgical procedure.

To assess the short term *in utero* impact of the experimental procedure, a group of animals were examined just before delivery.

To assess survival, pups were counted after birth on the day of cross-fostering (day P0 or day P1). Pups tend to deliver overnight and were detected the next morning. It was only possible to assess pup survival at cross-fostering on day P0 or day P1 since earlier assessment leads to maternal rejection and cannibalism. Pup survival from the injection procedure, until it was time cross-foster, was therefore used to assess the effect of the fetal stem cell therapy injection and surgical procedure. Pup loss was due to either maternal cannibalism seen when pups were cross-fostered, or due to *in utero* fetal demise post-IUT, before birth, which occurred in 30% of injected pups (evidence of miscarriage was seen

during the post-mortem analysis of the dams, which was performed after cross-fostering). Pup survival to cross-fostering was comparable between the two groups (18 pups out of 37, 48.6%: congenic, 17 out of 36, 47.2%: allogenic, Fisher's exact test, P-value >0.99) (Figure 3-26). There were no late pup deaths from cross-fostering up to the scheduled postmortem examination at 4 or 16 weeks.

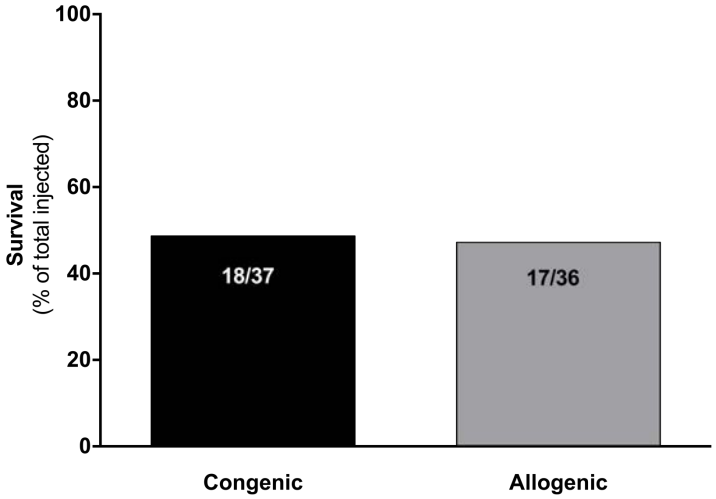



**Figure 3-26 Survival Rate to cross-fostering (%) post-IUT in congenic and allogenic groups. The survival was similar in congenic (48.7%) vs allogenic groups (47.2%)**

In conclusion, the total survival rate of cross-fostering and post-mortem was around 48%. The mortality rate from the IUT was 30%, and maternal cannibalism was 22%.

### 3.9.3 Postnatal pup growth after congenic and allogenic *in utero* AFSC injection

The postnatal growth of pups injected with allogenic or congenic AFSCs was compared to pups injected with PBS as controls by weighing animals at four weeks. As shown in Figure 3-27 below, pup weight showed no difference at four weeks after birth, which means that postnatal growth was not affected by the IUT.

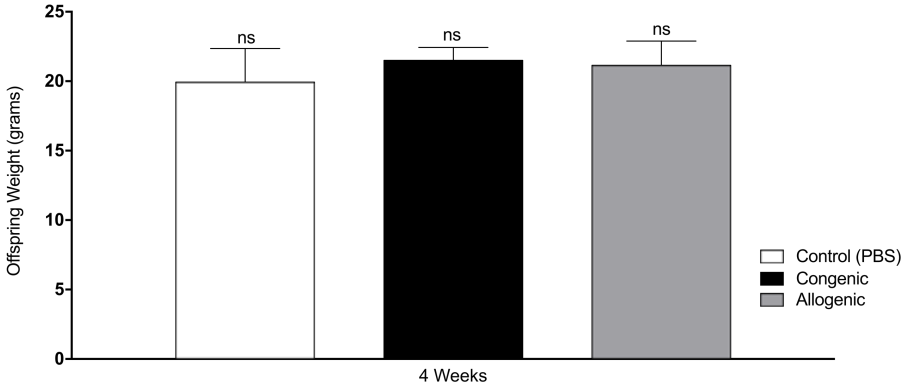



**Figure 3-27 Comparison of pup size between congenic, allogenic and PBS recipients at four weeks. No difference observed in the weight of offspring, p=0.91, Kruskal-Wallis test.**

### 3.9.4 Chimerism after *in utero* intraperitoneal transplantation

All congenic transplanted animals (18 out of 18) were chimeric (>1% of positive donor cells among the CD45+ cells population) compared to only 29% (5 out of 17) of the animals receiving allogenic donor cells (Figure 3-28).

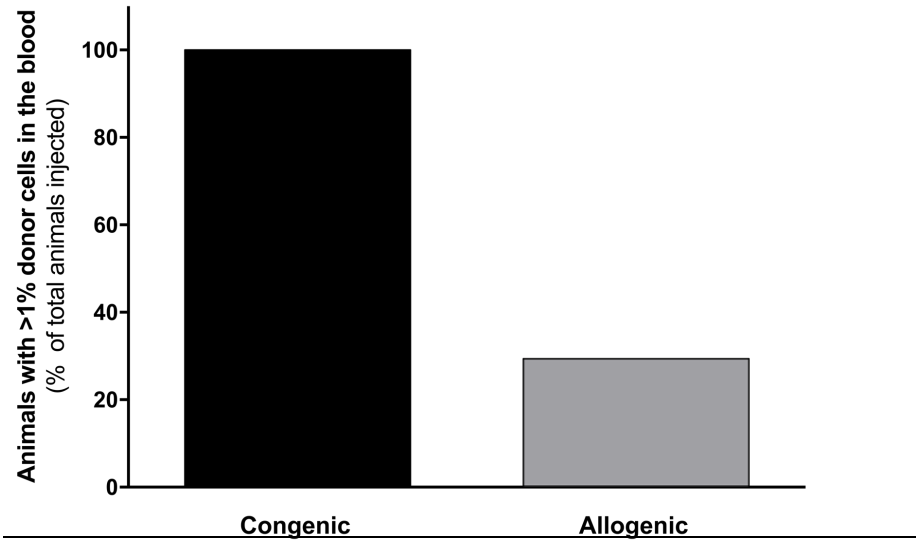



**Figure 3-28 Chimerism in recipient animals. All congenic animals (100%) were chimeric compared to allogenic (29%)**

### 3.10 Short and long-term analysis of recipients

Compared to the allogenic transplantation of AFSCs (Ckit+/Lin-) and Congenic Bone Marrow (Sca1+/Lin-), autologous transplantation of AFSCs resulted in far higher levels of engraftment in animals analysed at 4 or 16 weeks, (see Table 3-5 and Table 3-7), in the blood, bone

marrow and spleen (Figure 3-29 and Figure 3-30). Increasing the number of injected cells five-fold (5x10^4) did not improve the engraftment of allogenic AFSCs at four weeks; indeed, engraftment was lower (see Table 3-4).

| | **Congenic 10^4 AF cells** | | | **Allogenic 10^4 AF cells** | | | **Allogenic 50x10^4 AF cells** | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | N | Mean | SEM | N | Mean | SEM | N |
| **Blood** | 8.76 | 1.54 | 7 | 1.18 | 0.36 | 8 | 0.087 | 0.035 | 6 |
| **Bone Marrow** | 9.17 | 1.85 | 7 | 2.25 | 0.70 | 8 | 0.17 | 0.15 | 6 |
| **Spleen** | 5.17 | 0.88 | 7 | 0.94 | 0.23 | 8 | 0.35 | 0.23 | 6 |

**Table 3-4 Engraftment at four weeks**

| **Statistical analysis of engraftment at four weeks ANOVA with Tukey's multiple comparisons tests** | Mean diff. | 95.00% CI of diff. | Adjusted P Value |
|---|---|---|---|
| **Blood** | | | |
| Congenic 10^4 AF cells vs Allogenic 10^4 AF cells | 7.6 | 4.5 to 11 | *<0.0001 |
| Congenic 10^4 AF cells vs. Allogenic 50x10^4 cells | 8.7 | 5.4 to 12 | *<0.0001 |
| Allogenic 10^4 AF cells vs. Allogenic 50x10^4 cells | 1.1 | -2.1 to 4.3 | 0.6821 |
| **Bone Marrow** | | | |
| Congenic 10^4 AF cells vs Allogenic 10^4 AF cells | 6.9 | 3.9 to 10 | *<0.0001 |
| Congenic 10^4 AF cells vs. Allogenic 50x10^4 cells | 9 | 5.7 to 12 | *<0.0001 |
| Allogenic 10^4 AF cells vs. Allogenic 50x10^4 cells | 2.1 | -1.1 to 5.2 | 0.2610 |
| **Spleen** | | | |
| Congenic 10^4 AF cells vs Allogenic 10^4 AF cells | 4.2 | 1.2 to 7.3 | *0.0039 |
| Congenic 10^4 AF cells vs. Allogenic 50x10^4 cells | 4.8 | 1.6 to 8.1 | *0.0022 |
| Allogenic 10^4 AF cells vs. Allogenic 50x10^4 cells | 0.59 | -2.6 to 3.8 | 0.8948 |

**Table 3-5 Statistical Analysis at four weeks**

| | **Congenic 10^4 AF cells** | | | **Allogenic 10^4 AF cells** | | | **Congenic 10^4 Bone Marrow Cells** | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | N | Mean | SEM | N | Mean | SEM | N |
| **Blood** | 7.69 | 1.55 | 11 | 1.41 | 0.52 | 9 | 1.47 | 1.20 | 3 |
| **Bone Marrow** | 9.17 | 2.94 | 8 | 0.60 | 0.14 | 9 | 1.075 | 0.26 | 4 |
| **Spleen** | 3.69 | 0.78 | 8 | 0.57 | 0.17 | 9 | 1.12 | 0.09 | 3 |

**Table 3-6 Engraftment at 16 weeks**

| **Statistical analysis of engraftment at 16 weeks, ANOVA with Tukey's multiple comparisons tests** | Mean diff. | 95.00% CI of diff. | Adjusted P Value |
|---|---|---|---|
| **Blood** | | | |
| Congenic vs Allogenic | 6.3 | 2.1 to 10 | *0.0018 |
| Congenic vs. Congenic Bone Marrow | 6.2 | 0.18 to 12 | *0.0423 |
| Allogenic vs. Congenic Bone Marrow | -0.057 | -6.2 to 6.1 | 0.9997 |
| **Bone Marrow** | | | |
| Congenic vs. Allogenic | 8.6 | 4.1 to 13 | *<0.0001 |
| Congenic vs Congenic Bone Marrow | 8.1 | 2.4 to 14 | *0.0032 |
| Allogenic vs. Congenic Bone Marrow | -0.47 | -6 to 5.1 | 0.9778 |
| **Spleen** | | | |
| Congenic vs. Allogenic | 3.1 | -1.4 to 7.6 | 0.2277 |
| Congenic vs. Congenic Bone Marrow | 2.6 | -3.7 to 8.8 | 0.5894 |
| Allogenic vs. Congenic Bone Marrow | -0.55 | -6.7 to 5.6 | 0.9752 |

**Table 3-7 Statistical Analysis 16 weeks**

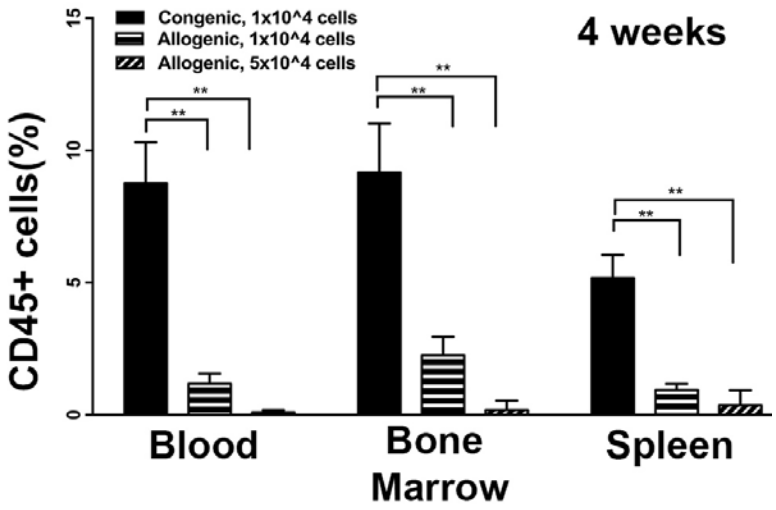



**Figure 3-29 Short-term analysis of stem cell recipients. Engraftment (measured as a percentage of donor cells in a total of CD45+ cells) at four weeks after birth in the congenic vs the allogenic group vs transplanting a five-fold higher number of cells. in the blood, bone marrow and spleen.**

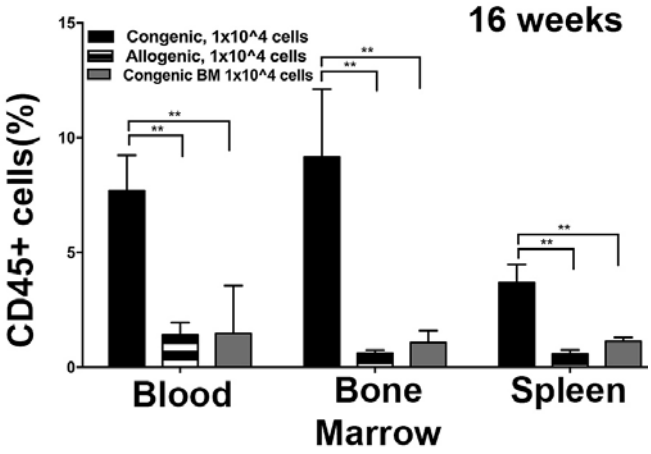



**Figure 3-30 Long-term analysis of stem cell recipients. Engraftment (measured as a percentage of donor cells in a total of CD45+ cells) at 16 weeks after birth in the congenic vs the allogenic group in the blood, bone marrow and spleen. Results are compared with Congenic Bone Marrow Transplantation.**

When engraftment was compared at four weeks, and then 16 weeks within groups (congenic four weeks, compared to congenic 16 weeks and allogenic four weeks compared to allogenic 16 weeks) there were no differences in the congenic group, which confirms stable engraftment of cells in this group (Table 3-9). In the allogenic group, the engraftment in the bone marrow was lower, which confirms that cells were being rejected (Table 3-8).

| ANOVA with Bonferroni's multiple comparisons tests | Mean diff. | 95.00% CI of diff. | Adjusted P Value |
|---|---|---|---|
| **Allogenic 4 weeks vs. Allogenic 16 weeks** | | | |
| Blood | -0.2283 | -1.647 to 1.19 | >0.9999 |
| Bone Marrow | 1.642 | 0.224 to 3.06 | *0.0182 |
| Spleen | 0.3616 | -1.057 to 1.78 | >0.9999 |

**Table 3-8 Comparison Allogenic 4 vs 16 weeks**

| ANOVA with Bonferroni's multiple comparisons tests | Mean diff. | 95.00% CI of diff. | Adjusted P Value |
|---|---|---|---|
| **Congenic 16 weeks vs Congenic 4 weeks** | | | |
| Blood | 0.014 | -6.7 to 6.8 | >0.9999 |
| Bone Marrow | 1.3 | -5.5 to 8 | >0.9999 |
| Spleen | -1.1 | -7.9 to 5.6 | >0.9999 |

**Table 3-9 Comparison congenic four vs 16 weeks**

In summary, in this section, I show that AFSCs can engraft the haematopoietic system in a mouse model of haematopoietic transplantation. Congenic cells are superior to allogenic, and even higher numbers of allogenic cells fail to achieve tolerance and successful engraftment.

## 3.11 Characterisation of donor cells in the blood and bone marrow in stem cell recipients

At 16 weeks, donor cells (CD45.1 and $H2K^d$) from the bone marrow were positive for all three blood lineages (% of lineage marker within CD45+ cells) (CD3, B220, CD11b, Gr1, Ter119) and no different from the host cell populations (CD45.2 and $H2K^b$) (n=3, p=0.99) (Figure 3-31). Because of the low number of donor cells in the allogenic recipients, both the characterisation of fresh cells and cultured cells was not possible. The cells were selected and cultured in a semi-solid medium (Methocult 3434, StemCell Technologies) for two weeks. The colonies formed from both congenic and allogenic recipients were characterised.

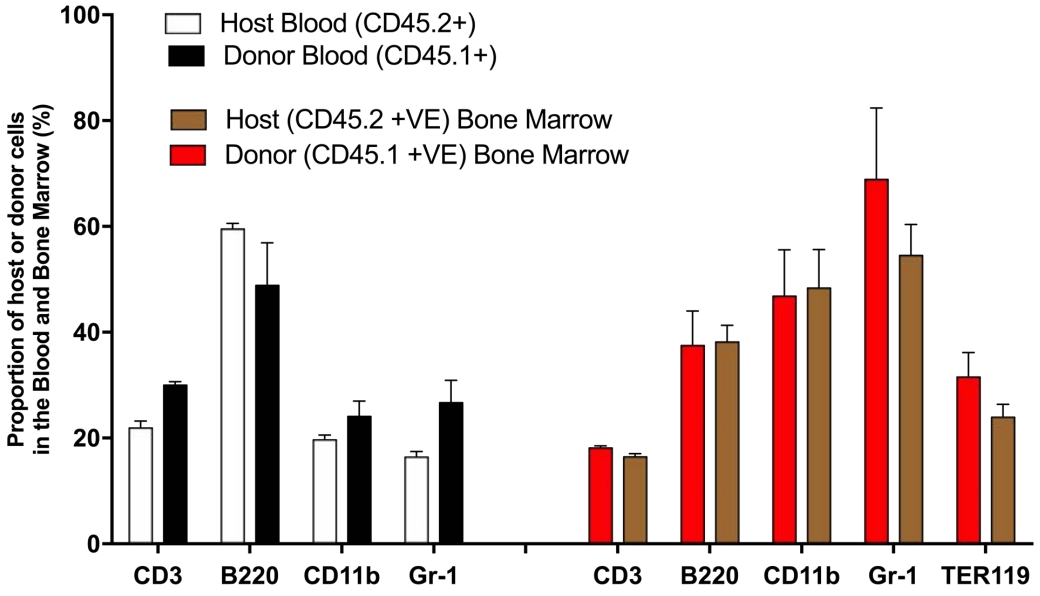
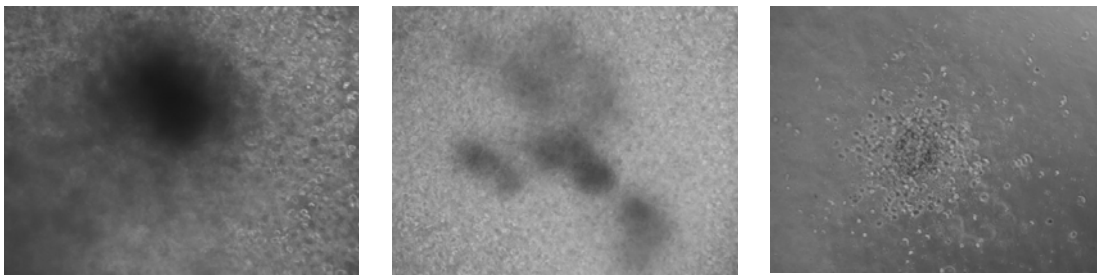



**Figure 3-31 Characterisation of congenic donor cells in the blood and bone marrow at 16 weeks. Donor cells (CD45.1 and $H2K^d$) from the bone marrow and blood at 16 weeks were positive for all three blood lineages (% of lineage marker within CD45+ cells) (CD3, B220, CD11b, Gr1, Ter119) and not different from the host cell populations (CD45.2 and $H2K^b$) (n=3, p=0.99).**

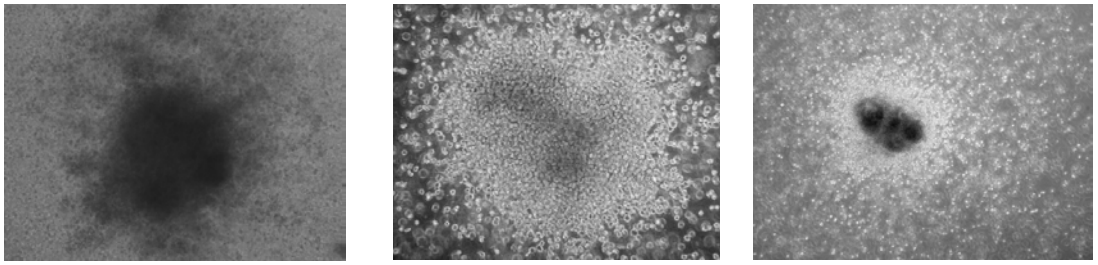
### 3.12 Congenic cells show higher levels of colony forming units than allogenic transplanted cells 16 weeks after IP *in utero* transplantation

Cells positive for donor markers from 16-week postnatal recipient BM were selected using FACS and cultured in a semi-solid medium for two weeks. The culture confirmed the presence of haematopoietic colonies from blood lineages (see Figure 3-32 and Figure 3-33).



CFU – GEMM (50X) CFU-GM (50X) BFU-E (50X)

**Figure 3-32 Congenic haematopoietic colonies (CD45.1 positive cells from BM of Congenic 16 week recipients two weeks in M3434) showing CFU-GM, which is a colony-forming unit-granulocyte, macrophage, CFU-GEMM (colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte) and a BFU-E (burst-forming unit-erythroid).**



CFU-GEMM (50X) CFU-GM (50X) BFU-E (50X)

**Figure 3-33 Allogenic Haematopoietic colonies (H2Kd positive cells from BM of Allogenic 16 week recipients two weeks in M3434) showing CFU-GM (colony-forming unit-granulocyte, macrophage), CFU-GEMM (colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte) and a BFU-E (burst-forming unit-erythroid).**

There was a higher number ($p<0.05$) of CFU-GM (colony-forming unit, granulocyte – monocyte) and CFU-GEMM (colony-forming unit, granulocyte, erythrocyte, monocyte, megakaryocyte) colonies generated from the BM of animals injected with congenic AFSCs when compared to the BM of animals injected with allogenic AFSCs (BFU-E 14.67 vs. 8.83

p=0.331, CFU-GM 50.00 vs. 22.17, p<0.05, CFU-GEMM 68.67 vs. 31.67, n=3, p<0.05) as shown in Figure 3-34 and Table 3-12.

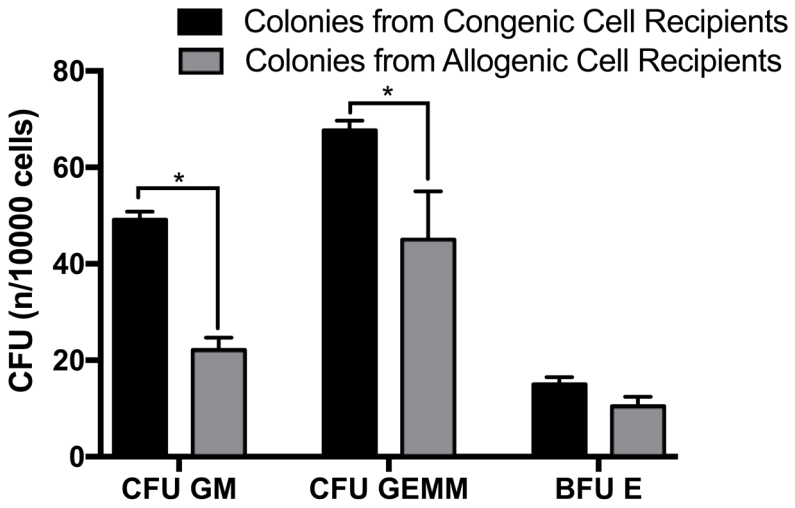



**Figure 3-34 Quantification of colonies after 14 days in methocult culture. Comparison of colonies derived from Allogenic vs Congenic recipients**

| ANOVA with Bonferroni's multiple comparison tests | Mean diff. | 95.00% CI of diff. | Congenic Mean | Allogenic Mean | Adjusted P Value |
|---|---|---|---|---|---|
| **Colonies from Congenic vs. Allogenic Cell Recipients** | | | | | |
| **CFU GM** | 27 | 11 to 43 | 49 | 22 | 0.0006 |
| **CFU GEMM** | 23 | 6.6 to 39 | 68 | 45 | 0.0036 |
| **BFU E** | 4.5 | -12 to 21 | 15 | 11 | >0.9999 |

**Table 3-10 Analysis of Colony Forming Units**

The colonies were liquefied using RPMI medium, and a flow cytometry analysis confirmed the presence of donor cells ($CD45.1^+$ and $H2K^{d+}$). The liquefied colonies were also analysed using differentiation markers: CD3, B220, CD11b, Gr1, and Ter-119. The results show the presence of donor cells and markers of terminal haematopoietic differentiation in the liquefied colonies (Figure 3-35).

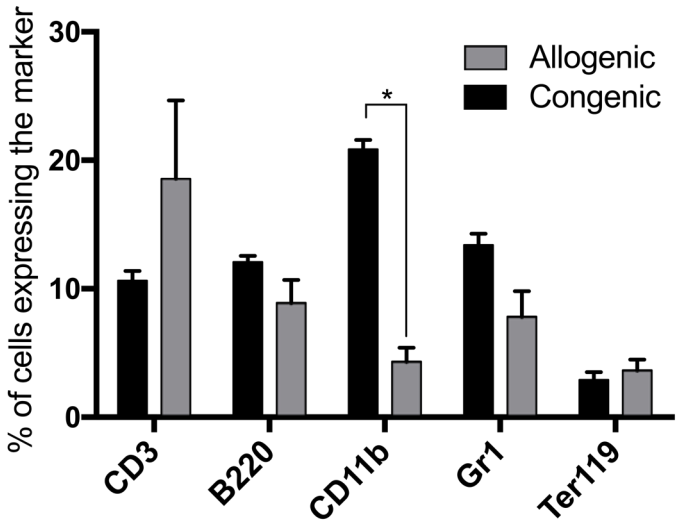



**Figure 3-35 Characterisation of Colony Forming Units after RPMI liquefaction and flow cytometric analysis showed expression of markers from all three haematopoietic colonies (CD3, B220, CD11b, Gr1, Ter119) The colony forming units were expressing similar markers from the three haematopoietic lineages, as seen in Table 3-11. The marker CD11b was the only marker which was different between the two groups as per Table 3-12**

| | Congenic | | | Allogenic | | |
|---|---|---|---|---|---|---|
| **Marker** | Mean | SEM | N | Mean | SEM | N |
| **CD3** | 10.62 | 0.75 | 6 | 18.57 | 6.08 | 6 |
| **B220** | 12.08 | 0.47 | 6 | 8.92 | 1.76 | 6 |
| **CD11b** | 20.85 | 0.73 | 6 | 4.32 | 1.08 | 6 |
| **Gr1** | 13.4 | 0.89 | 6 | 7.82 | 1.97 | 6 |
| **Ter119** | 2.915 | 0.59 | 6 | 3.65 | 0.82 | 6 |

**Table 3-11 Quantification of markers expressed by colony forming units**

| ANOVA with Sidak's multiple comparisons tests | Mean diff. | 95.00% CI of diff. | Adjusted P Value |
|---|---|---|---|
| **Allogenic - Congenic** | | | |
| **CD3** | 7.94 | -0.359 to 16.2 | 0.06 |
| **B220** | -3.16 | -11.5 to 5.14 | 0.85 |
| **CD11b** | -16.5 | -24.8 to -8.22 | *<0.0001 |
| **Gr1** | -5.57 | -13.9 to 2.73 | 0.34 |
| **Ter119** | 0.735 | -7.57 to 9.04 | 0.99 |

**Table 3-12 Analysis of colony forming units expressing the haematopoietic marker**

The liquefied colonies were also analysed for the presence of donor cells ($CD45.1^+$ and $H2K^{d+}$) (see. This confirms the presence of donor cells as well as the functional ability of those cells to form terminal haematopoietic cells.

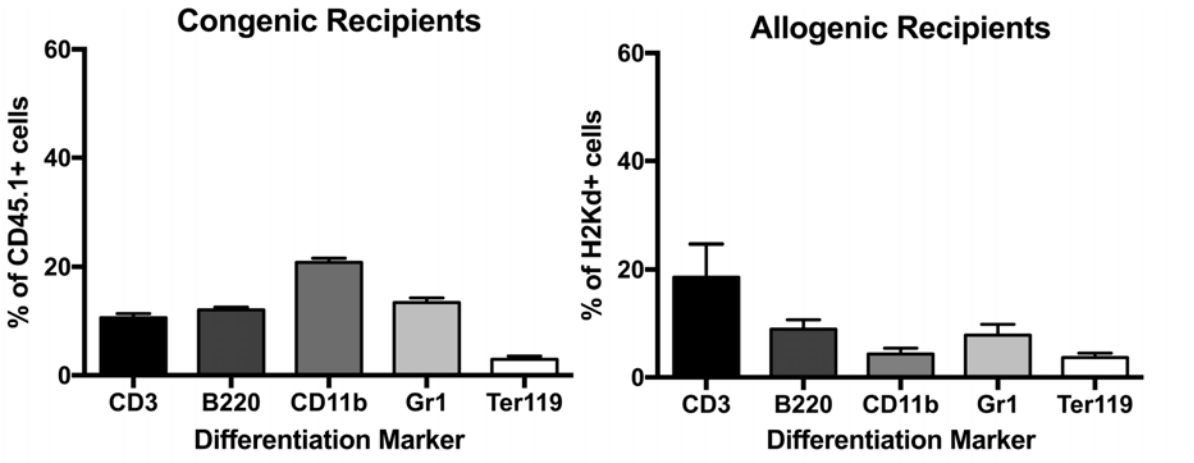



**Figure 3-36 Flow cytometric analysis of colony forming units (CFUs) derived from pooled colonies from congenic and allogenic recipients, showing the presence of donor markers (H2K-d, CD45.1) and expression of terminal differentiation markers.**

In summary, I have shown the presence of both donor cells and markers of terminal haematopoietic differentiation in the liquefied colonies confirming *in vitro* the plasticity of the stem cells, the engraftment potential and the fact that the recipients were true chimaeras.

## 3.13 An immune response is detectable after allogenic amniotic fluid stem cells IP *in utero* transplantation to immune competent mice

To investigate the immune response to transplantation, I studied CD4 and CD8 levels in congenic and allogenic transplanted pups at 16 weeks. I hypothesised that a robust immune response might be responsible for the lower levels of engraftment following allogenic transplantation. Due to the low level of engraftment in the allogenic transplanted group, the total number of $CD4^+$ and $CD8^+$ cells were analysed as a percentage of the total $CD45^+$ cell population.

The percentage of $CD4^+$ and $CD8^+$ cells per total $CD45^+$ cells was higher in the allogenic group compared to congenic and control groups (n=3, p=<0.001, ANOVA with Bonferroni's multiple comparisons test) as shown in Table 3-13, Figure 3-37, Figure 3-38 and Figure 3-39.

There were no differences between the congenic and control groups (n=3, P>0.999, Bonferroni's multiple comparisons test).

**Blood**

| | Control (PBS) | | | Congenic | | | Allogenic | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | N | Mean | SD | N | Mean | SD | N |
| CD4 | 13.57 | 2.50 | 3 | 15.70 | 4.62 | 3 | 59.33 | 8.91 | 3 |
| CD8 | 12.70 | 3.37 | 3 | 14.20 | 1.28 | 3 | 62.37 | 6.53 | 3 |

**Bone Marrow**

| | Control (PBS) | | | Congenic | | | Allogenic | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | N | Mean | SD | N | Mean | SD | N |
| CD4 | 20.50 | 2.46 | 3 | 23.43 | 8.57 | 3 | 65.67 | 2.31 | 3 |
| CD8 | 17.70 | 1.28 | 3 | 21.17 | 5.10 | 3 | 71.50 | 3.62 | 3 |

**Spleen**

| | Control (PBS) | | | Congenic | | | Allogenic | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | N | Mean | SD | N | Mean | SD | N |
| CD4 | 22.43 | 1.65 | 3 | 18.37 | 7.20 | 3 | 65.40 | 6.06 | 3 |
| CD8 | 19.17 | 2.23 | 3 | 22.23 | 7.33 | 3 | 74.97 | 4.91 | 3 |

**Table 3-13 The percentage of CD4+ and CD8+ cells per total CD45$^+$ cells in the blood, bone marrow and spleen**

The higher percentage of CD4$^+$ and CD8$^+$ T lymphocytes in the allogenic group suggests the presence of an immune response towards donor cells.

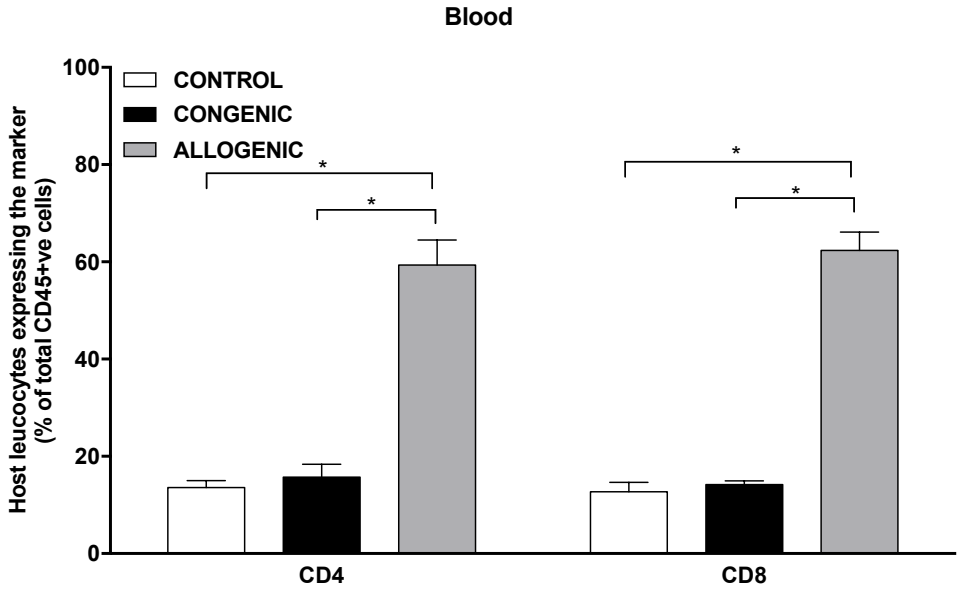



**Figure 3-37 Host leucocytes are expressing CD4/CD8 marker in the blood. Compared to control and congenic cell transplanted groups, there was a higher percentage of CD4 and CD8 cells per total CD45$^+$ count in the allogenic transplanted group. There was no difference between the congenic and control transplanted groups (n=3, P=0.99).**

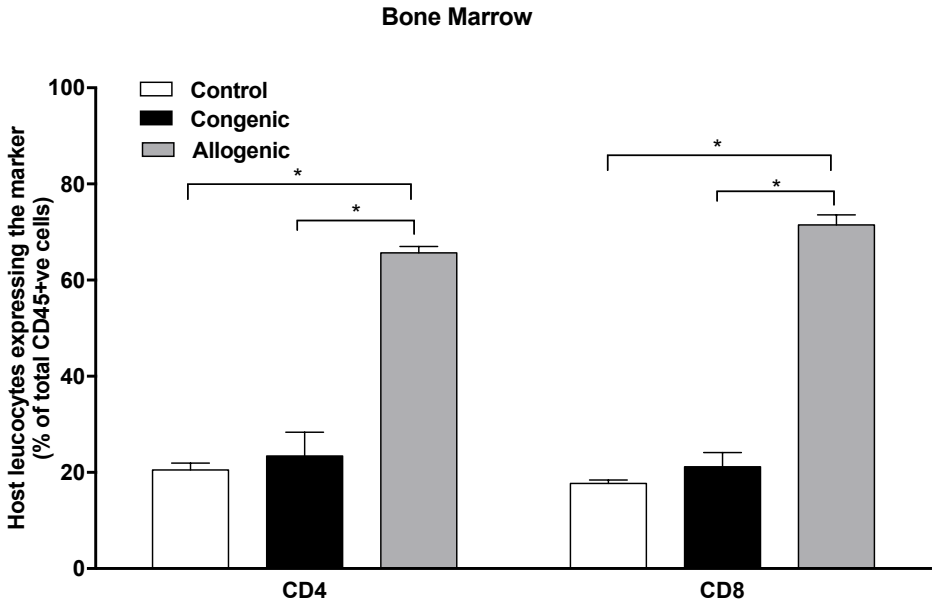



**Figure 3-38 Host leucocytes are expressing CD4/CD8 marker in the bone marrow. Compared to the control and congenic cell transplanted groups, there was a higher percentage of CD4 and CD8 cells per total CD45+ count in the allogenic transplanted group. There was no difference between the congenic and control transplanted groups (n=3, P=0.99).**

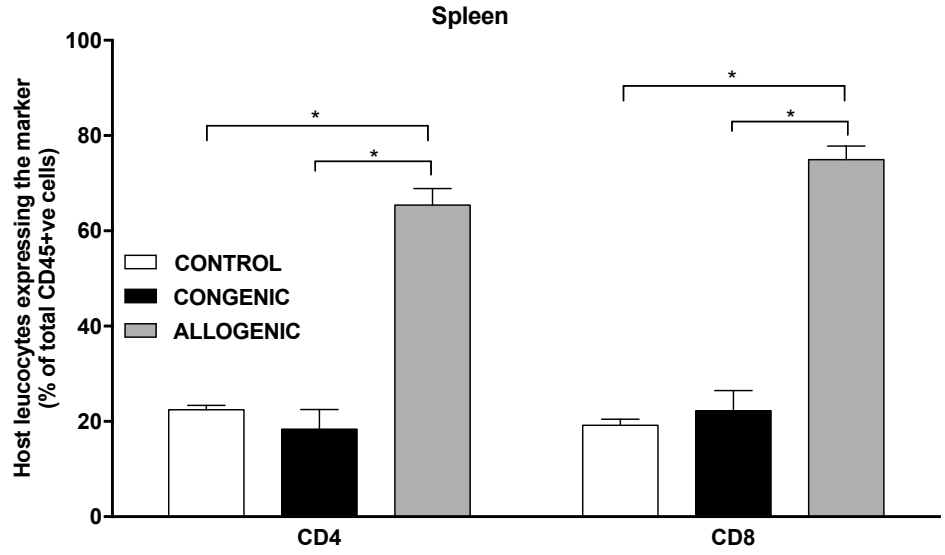



**Figure 3-39 Host leucocytes expressing CD4/CD8 marker in the spleen compared to control and congenic cell transplanted groups. There was a higher percentage of CD4 and CD8 cells per total CD45+ count in the allogenic transplanted group. There was no difference between the congenic and control transplanted groups (n=3, P=0.99).**

### 3.14 *In vitro* mixed lymphocyte reaction

To investigate the cellular immune response to transplantation, I performed T cell proliferation studies, using carboxyfluorescein diacetate succinimidyl ester (CFSE). Stained splenocytes from congenic, allogenic and controls were co-cultured in the presence of splenocytes from the corresponding donor (BALBc or BL6/CD45.1 splenocytes). The percentage of proliferating cells in each culture was measured using flow cytometry. As shown in the representative gating strategy in Figure 3-40, Figure 3-41 and Figure 3-42, the percentage of T-cell proliferation in the allogenic co-cultures was higher compared to congenic and control.

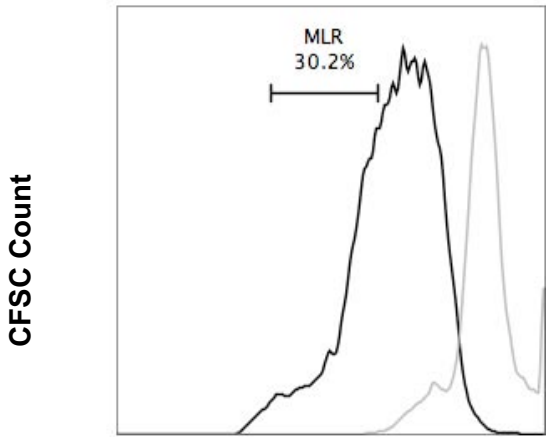



**Figure 3-40 MLR control CSFE proliferation assay**

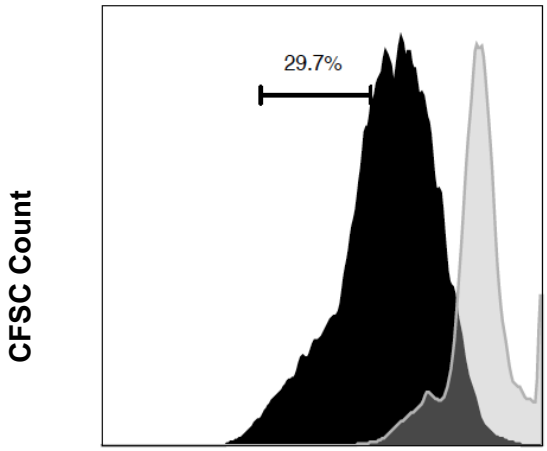



**Figure 3-41 MLR congenic CSFE proliferation assay**

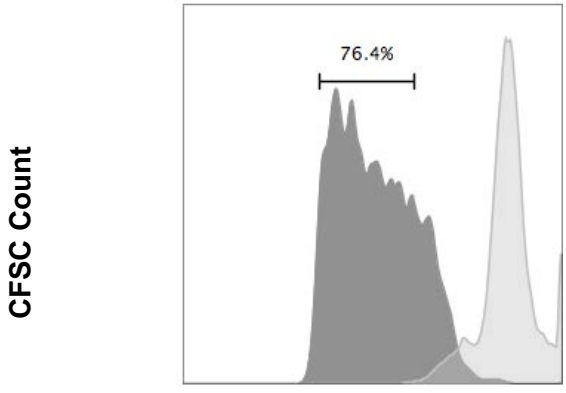



**Figure 3-42 MLR ellogenic CSFE proliferation assay**

The t-cell proliferation of recipient CSFE labelled splenocytes stimulated with inactivated splenocytes from the donor was higher in the allogenic group with no difference seen after stimulation in control transplanted and the congenic transplanted group (seen Table 3-14 and Figure 3-43). Statistical analysis was performed using ANOVA with Bonferroni's multiple comparisons tests (Table 3-15).

| | Control (PBS) | | | Congenic | | | Allogenic | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | N | Mean | SD | N | Mean | SD | N |
| CD4 | 46.07 | 2.80 | 3 | 48.57 | 3.66 | 3 | 64.53 | 5.59 | 6 |
| CD8 | 12.59 | 3.35 | 3 | 13.93 | 3.37 | 3 | 60.48 | 2.01 | 6 |

**Table 3-14 T-cell proliferation of recipient CSFE labelled splenocytes stimulated with inactivated splenocytes from the donor (%)**

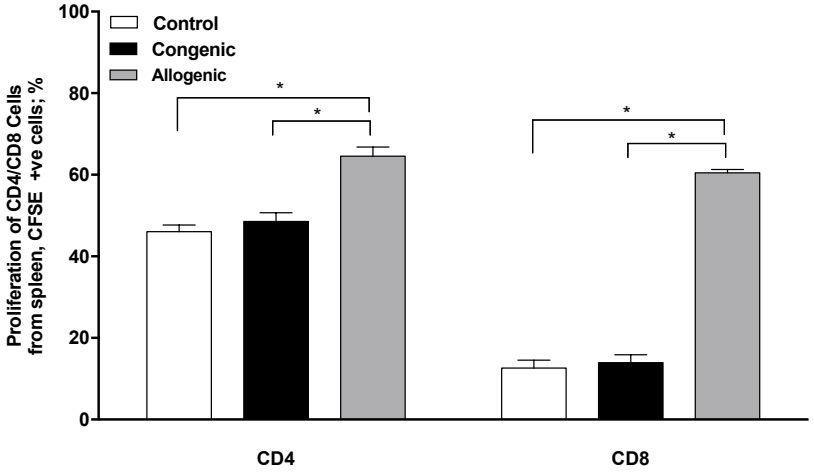



**Figure 3-43 *In Vitro* MLR, proliferating lymphocytes (spleen). There was a higher proliferation in the allogenic group compared to the control or congenic groups with no difference seen after stimulation in control transplanted and congenic transplanted group.**

| ANOVA with Bonferroni's multiple comparisons tests | Mean diff. | 95.00% CI diff. | Adjusted P Value |
|---|---|---|---|
| CD4 | | | |
| Control (PBS) vs. Congenic | -2.5 | -11.77 to 6.766 | >0.99 |
| Control (PBS) vs. Allogenic | -18.47 | -26.49 to -10.44 | *<0.0001 |
| Congenic vs. Allogenic | -15.97 | -23.99 to -7.942 | *<0.0001 |
| CD8 | | | |
| Control (PBS) vs. Congenic | -1.34 | -10.61 to 7.926 | >0.99 |
| Control (PBS) vs. Allogenic | -47.89 | -55.91 to -39.87 | *<0.0001 |

| | | | |
|---|---|---|---|
| Congenic vs. Allogenic | -46.55 | -54.57 to -38.53 | *<0.0001 |

**Table 3-15 MLR multiple comparisons, statistical analysis**

The higher proliferation rate of $CD4^+$ and $CD8^+$ T lymphocytes in the allogenic group confirmed the presence of an immune response towards donor cells. On the contrary, in the congenic group, T lymphocyte proliferation was similar to the uninjected control group, which indicates that congenic cells did not stimulate any antigen response.

### 3.15 Comparison of T-regulatory vs T-effector cell expression in MLR Cultures

In the peripheral lymph nodes, there was increased expression of Tregs in the congenic recipients stimulated with donor cells compared to other groups. This shows that there was a tolerogenic environment in the congenic group compared to the allogenic group. This can be seen by the fact that in the non-injected congenic group when stimulated with donor cells, we see increased expression of T-effectors. The T-effectors were reduced in the congenic group injected with CD45.1 donor cells (see Figure 3-44 and Table 3-16).

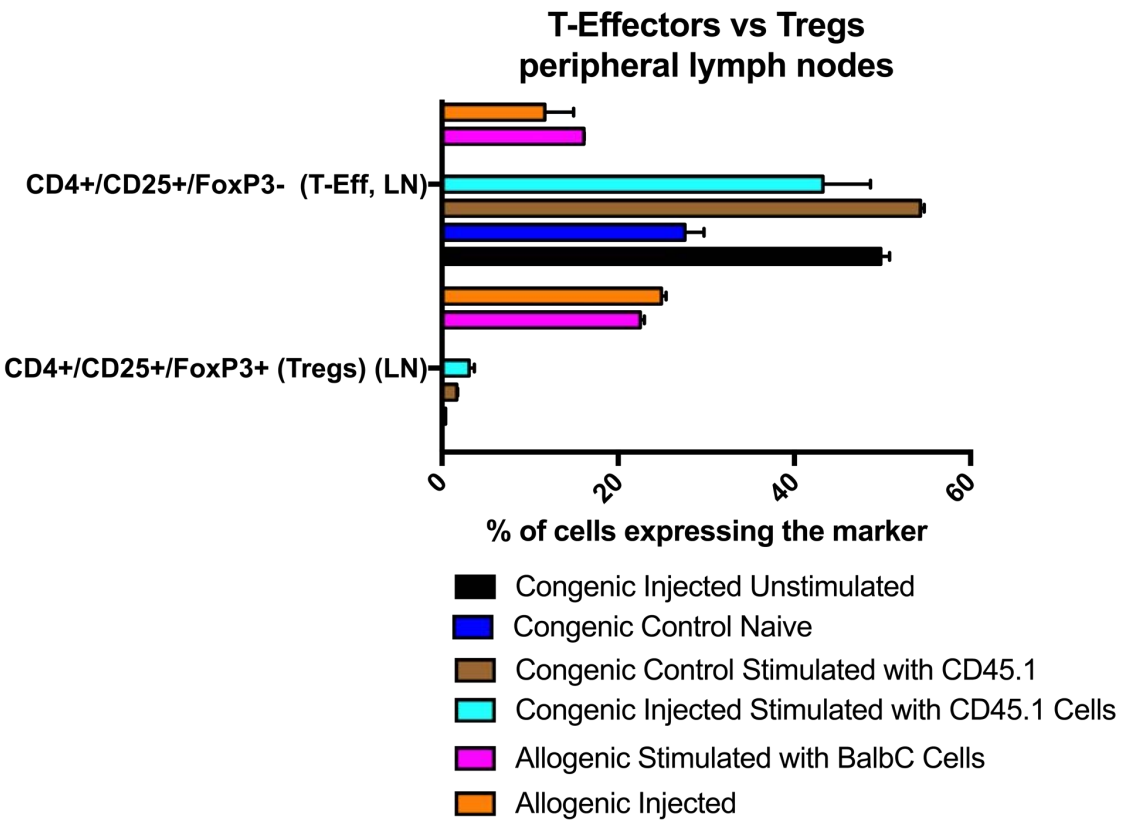



**Figure 3-44 T-Regulatory cells and T-Effector cells in MLR cultures from peripheral lymph nodes**

| ANOVA with Sidak's multiple comparisons tests | Mean diff. | 95.00% CI of diff. | Adjusted P Value |
|---|---|---|---|
| CD4+/CD25+/FoxP3- (T-Eff, LN) vs. CD4+/CD25+/FoxP3+ (Tregs) (LN) | | | |
| Congenic Injected Unstimulated | 50 | 43 to 57 | *<0.0001 |
| Congenic Control Naive | 27 | 20 to 34 | *<0.0001 |
| Congenic Control Stimulated with CD45.1 | 53 | 46 to 60 | *<0.0001 |
| Congenic Injected Stimulated with CD45.1 Cells | 40 | 33 to 47 | *<0.0001 |
| Allogenic Stimulated with BalbC Cells | -6.4 | -13 to 0.59 | 0.0884 |
| Allogenic Injected | -13 | -20 to -6.2 | *<0.0001 |

**Table 3-16 Statistical Analysis of T-Regulatory cells and T-Effector cells in MLR cultures from peripheral lymph nodes**

In the culture of splenocytes, the expression of Tregs in the congenic group injected with donor cells was also higher compared to the congenic naïve and congenic groups; uninjected stimulated with donor cells. Paradoxically the percentage of T-effector cells in the congenic recipient culture was also higher than the Tregs (see Table 3-17 and Figure 3-45).

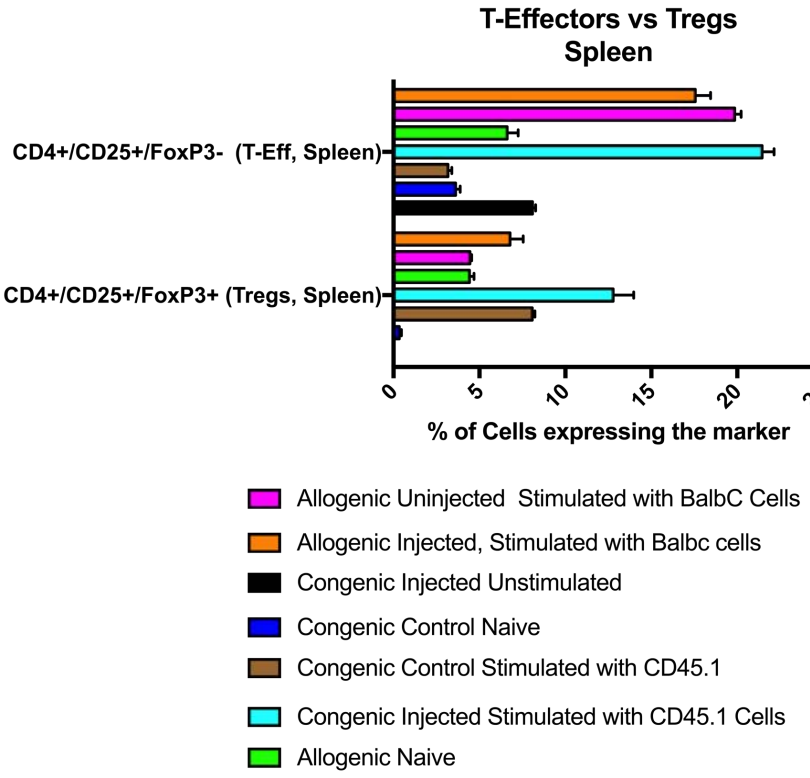



**Figure 3-45 T-Regulatory cells vs T-Effector cells in MLR cultures from splenocytes**

| ANOVA with Sidak's multiple comparisons tests | Mean diff. | 95.00% CI of diff. | Adjusted P Value |
|---|---|---|---|
| **CD4+/CD25+/FoxP3- (T-Eff, Spleen) - CD4+/CD25+/FoxP3+ (Tregs, Spleen)** | | | |
| **Congenic Injected Unstimulated** | 8.1 | 5.6 to 11 | *<0.0001 |
| **Congenic Control Naïve** | 3.3 | 0.82 to 5.8 | *0.0039 |
| **Congenic Control Stimulated with CD45.1** | -4.9 | -7.4 to -2.4 | *<0.0001 |
| **Congenic Injected Stimulated with CD45.1 Cells** | 8.7 | 6.2 to 11 | *<0.0001 |
| **Allogenic Naive** | 2.2 | -0.27 to 4.7 | 0.1047 |
| **Allogenic Uninjected Stimulated with BalbC Cells** | 15 | 13 to 18 | *<0.0001 |
| **Allogenic Injected, Stimulated with Balbc cells** | 11 | 8.3 to 13 | *<0.0001 |

**Table 3-17 Statistical analysis of T-Regulatory cells vs T-Effector cells in MLR cultures from splenocytes**

### 3.16 Tolerogenic Gene Expression in IUT at four weeks of gestation (the experiments in this section were performed together with Ms Eleni Petra, MSc student, ICH)

Measuring the relative expression of Foxp3 is an indirect way of analysing regulatory T cells as it is an intracellular marker for their activation. The relative gene expression of Foxp3 by qRT-PCR in the thymus was higher in the congenic compared to the allogenic chimeric animals at four weeks. Congenic vs. Allogenic Chimeric (1.0 vs 0.47, n=8, $p<0.05$), the Congenic vs Allogenic non-chimeric (1.0 vs 0.30, n=4, $p<0.05$), Congenic vs Control (1.0 vs 0.19, n=7, $p<0.0001$) and Allogenic chimeric vs Control animals (0.47 vs 0.19, n=8, $p<0.05$). These are shown in Figure 3-46 and Table 3-18.

In the spleen, for the congenic recipient animals, the relative expression of Foxp3 was increased ($p<0.05$) (4.94±1.12, n=4) while the allogenic recipient animals had a lower mean expression (0.05±0.015, n=5). In the congenic recipient animals, the relative expression of Foxp3 in the bone marrow increased ($p<0.05$) (1.71±0.25, n=4) compared to allogenic recipient animals (0.035±0.0086, n=5).

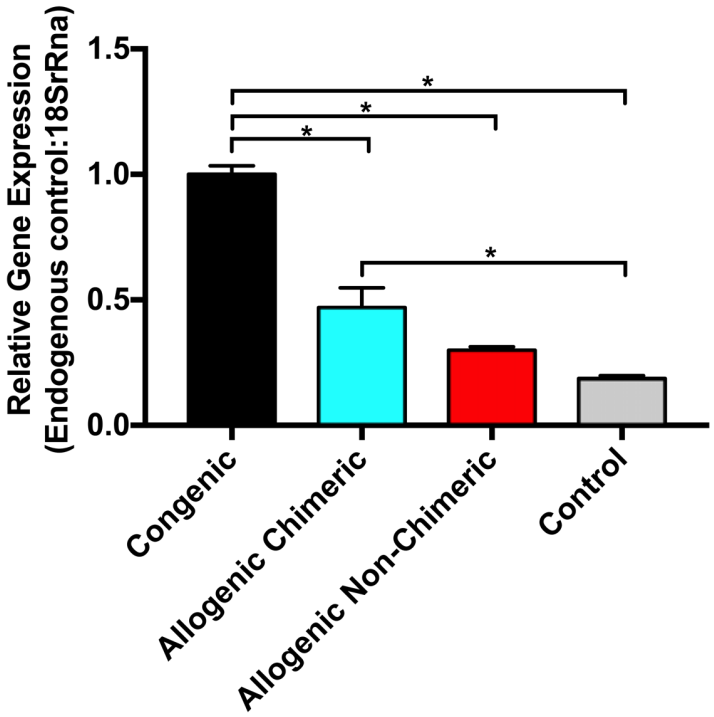



**Figure 3-46 Real-time PCR of cells Isolated from the thymus and expression of FoxP3. Congenic vs. Allogenic Chimeric (1.0 vs 0.47, n=8, p<0.05), the Congenic vs. Allogenic non-chimeric (1.0 vs 0.30, n=4, p<0.05), Congenic vs. Control (1.0 vs 0.19, n=7, p<0.0001) and Allogenic chimeric vs. Control animals (0.47 vs 0.19, n=8, p<0.05)**

| ANOVA with Tukey's multiple comparisons tests | Mean diff. | 95.00% CI of diff. | Adjusted P Value |
|---|---|---|---|
| | | | |
| **Congenic vs. Allogenic Chimeric** | 0.5315 | 0.3595 to 0.7034 | *<0.0001 |
| **Congenic vs. Allogenic Non-Chimeric** | 0.7013 | 0.4852 to 0.9173 | *<0.0001 |
| **Congenic vs. Control** | 0.8142 | 0.6353 to 0.9932 | *<0.0001 |
| **Allogenic Chimeric vs. Allogenic Non-Chimeric** | 0.1698 | -0.05682 to 0.3964 | 0.1943 |
| **Allogenic Chimeric vs. Control** | 0.2828 | 0.09126 to 0.4743 | *0.0022 |
| **Allogenic Non-Chimeric vs. Control** | 0.113 | -0.1189 to 0.3449 | 0.5490 |

**Table 3-18 Statistical analysis of real-time PCR results of cells isolated from the thymus for the expression of FoxP3**

I then studied the relative expression of the cytokine TGF-β in congenic, allogenic and control animals four weeks after IUSCT using real-time PCR. The role of TGF-β in nTreg and iTreg cells function is not well understood, as several contradictory observations exist (Liu et al., 2008). However, it seems to play a key role in Treg homeostasis as it may induce and maintain Foxp3 expression (Marie et al., 2005; Pyzik and Piccirillo, 2007).

Similar to Foxp3, relative gene expression of TGF-β by qRT-PCR in the thymus was higher in the congenic compared to the allogenic chimeric animals. Differences were seen in the Congenic vs. Control (0.90 vs 3.7, n=7, p<0.05), Allogenic Chimeric vs. Control (2.1 vs 0.90, n=8, p<0.05), Congenic vs. Allogenic Chimeric (3.7 vs 2.1, n=8, p=0.0025) and Congenic vs. Allogenic Non-Chimeric (3.7 vs 1.4, n=4, p<0.05), (see Figure 3-47 and Table 3-19).

In the spleen, in the congenic group (3.17± 0.53, n=4) the relative expression of TGF- β increased (p<0.05) vs the allogenic group (0.014±0.0065, n=5). In the congenic group, the relative expression of TGF- β in the bone marrow increased (9.46±0.84, n=4) vs the allogenic animals (0.76±0.28, n=5).

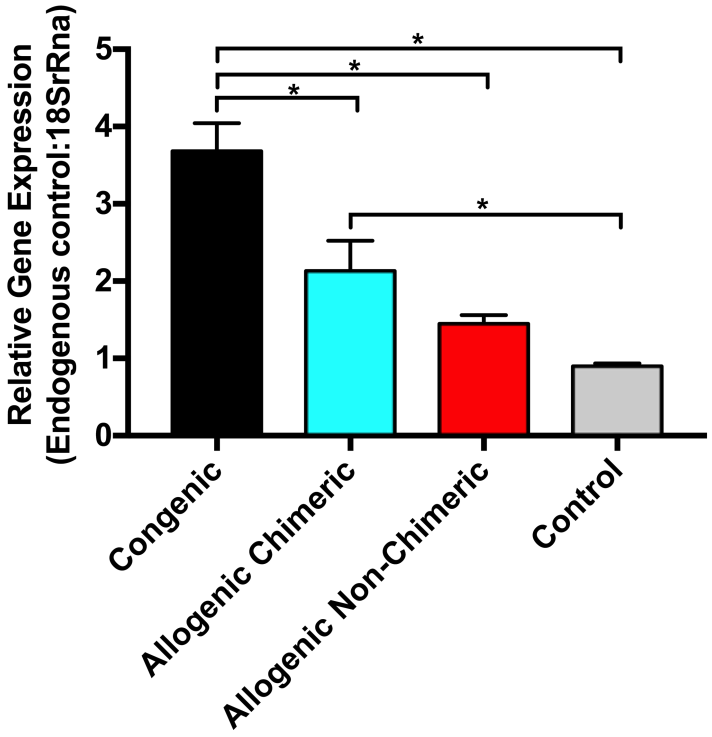



**Figure 3-47 Real-time PCR of cells isolated from the thymus, expression of TGF-b higher expression of TGF- β in the congenic compared to the allogenic animals. Differences were seen in the Congenic vs. Control (0.90 vs 3.7, n=7, p<0.05), Allogenic Chimeric vs. Control (2.1 vs 0.90, n=8, p<0.05), Congenic vs. Allogenic Chimeric (3.7 vs 2.1, n=8, p=0.0025) and Congenic vs. Allogenic Non-Chimeric (3.7 vs 1.4, n=4, p<0.05)**

| ANOVA with Tukey's multiple comparisons tests | Mean diff. | 95.00% CI of diff. | Adjusted P Value |
|---|---|---|---|
| **Congenic vs. Allogenic Chimeric** | 1.6 | 0.45 to 2.7 | *0.0033 |
| **Congenic vs. Allogenic Non-Chimeric** | 2.2 | 0.99 to 3.5 | *0.0002 |
| **Congenic vs. Control** | 2.8 | 1.7 to 3.9 | *<0.0001 |
| **Allogenic Chimeric vs. Allogenic Non-Chimeric** | 0.68 | -0.58 to 2 | 0.4702 |
| **Allogenic Chimeric vs. Control** | 1.2 | 0.1 to 2.4 | *0.0289 |
| **Allogenic Non-Chimeric vs. Control** | 0.55 | -0.72 to 1.8 | 0.6436 |

**Table 3-19 Statistical analysis of real-time PCR results of cells isolated from the thymus for the expression of TGF- β**

I also investigated IL10 expression. Studies from bone marrow transplantation in IL10-deficient mice have shown that Treg cells produce IL10 and that IL10 plays a key role in the Treg suppressor function (Hoffmann et al., 2002). Hara et al. (2001) demonstrated that IL10 is required for the functional activity of Treg cells following skin-allograft transplantation in rats. Therefore, I measured IL-10 expression by using real-time PCR. I found higher gene expression in the IL10 in the congenic group compared to allogenic and the control (12.64 vs 1.095 vs 1.66 vs1.10, n=5, p<0.05, Figure 3-48 and Table 3-20) which corresponds to higher engraftment of congenic cells and a higher expression of FoxP3, thus higher functional Tregs in the circulation.

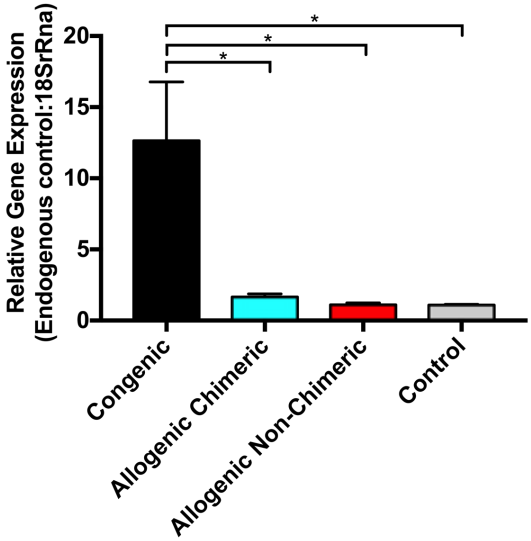



**Figure 3-48 Real-time PCR of cells isolated from the thymus, expression of IL10. There was higher IL10 gene expression in the congenic group compared to other groups and the control (12.64 vs 1.095 vs 1.66 vs1.10, n=5, p<0.05)**

| ANOVA with Tukey's multiple comparisons tests | Mean diff. | 95.00% CI of diff. | Adjusted P Value |
|---|---|---|---|
| **Congenic vs. Allogenic Chimeric** | 10.98 | 0.05749 to 21.91 | *0.0485 |
| **Congenic vs. Allogenic Non-Chimeric** | 11.54 | 2.307 to 20.77 | *0.0107 |
| **Congenic vs. Control** | 11.55 | 2.629 to 20.47 | *0.0080 |
| **Allogenic Chimeric vs. Allogenic Non-Chimeric** | 0.5581 | -10.62 to 11.74 | 0.9990 |
| **Allogenic Chimeric vs. Control** | 0.5663 | -10.36 to 11.49 | 0.9989 |
| **Allogenic Non-Chimeric vs. Control** | 0.008256 | -9.225 to 9.241 | >0.9999 |

**Table 3-20 Statistical analysis of real-time PCR results of cells isolated from the thymus for the expression of IL10**

The relative gene expression of FoxP3, TGF- β and IL-10 at four weeks was higher in the thymus, spleen and bone marrow of congenic compared to allogenic recipients, suggesting a higher acceptance of the graft and a lack of immune response in the congenic recipients. There was no difference in the gene expression between allogenic chimeric and allogenic non-chimeric recipients.

### 3.17 Histological Analyses

Tissues from all animals were fixed in 4% PFA and sent to the Cambridge Veterinary College Laboratory for processing and analysis. The tissue analyses included: liver, kidneys, lymph nodes, adrenals, heart, pancreas, intestine, spleen and lungs. The tissues were processed, and H&E slides were created and examined under the microscope. In congenic recipients, all tissues appeared normal in contrast to the allogenic recipients. Sections of the following tissues were examined: spleen, heart, liver, kidneys, bone marrow. In the spleen of the allogenic recipients, there were distinct prominent lymphoid follicles with abundant red pulp. This was consistent with extra medullary haematopoietis. The bone marrow cavity in the shaft and those in the head of the bone were filled with cells which were described as scattered megakaryocytes. Erythroid series cells were predominant with lower numbers of myeloid cells with maturation into the segmented neutrophil stages. This was diagnosed as mild erythroid hyperplasia.

### 3.18 Discussion

Culturing fetal and adult haematopoietic stem cells with and without gene transfer in embryonic stem cell conditions maintained a high expansion fold, multipotency and allowed efficient gene transfer.

CD117+ mAF HSCs isolated from mAF showed haematopoietic characteristics with an expression of haematopoietic markers and key haematopoietic regulators as seen in the $RT^2$ PCR array analysis published by Loukogeorgakis et al(Loukogeorgakis et al., 2019). When a cytokine cocktail was used in combination with gene transfer, the mAF HSCs were transduced with a transduction efficiency of 26%. Using a modified ESC medium, seven days of culture gives a 5.1-6.24-fold rise in the number of $CD117^+$ cells, not only in the mAF HSC but also in the mBM and mFL. mAF HSCs maintained a similar phenotype and exhibited significant haematopoietic potential equal to that of freshly- isolated mAF HSCs.

The MEF culture system is not as efficient as other systems such as the one used by Csaszar et al. where a closed culture system was used. Here the authors removed the inhibitory signals secreted by the expanding cells to achieve an 11 fold increase of HSCs (Csaszar et al., 2012). Alternatively, Zhang et al. (Zhang et al., 2008) used a cytokine cocktail containing stem cell factor (SCF), TPO, FGF-1, angiopoietin-like 5 and IGFBP2, in a serum-free medium to achieve an impressive 20 fold expansion of human cord-derived HSC. The system described in this chapter, though, is the only system describing the expansion of AF hemopoietic progenitors in the mouse in seven days.

In this chapter, I also have investigated the potential barriers to *in utero* engraftment of AFSCs in mice. Where possible, to ensure that the AF was free from contamination with maternal cells, fluid was collected using a well-documented technique (Ditadi et al., 2009). First, the uterus and then the amniotic sacs were carefully washed before the sterile puncture of the amniotic membrane, ensuring where possible that only AF was isolated from the amniotic sac. Contaminated samples were rejected, and the fluid was then subjected to red cell lysis to destroy any contaminating fetal or maternal red blood cells. In a previous study, GFP

expression was used to select donor amniotic fluid cells before transplantation (Ditadi et al., 2009). In this study, it was not possible to use the same technique because the experimental set up involved different mouse strains. Nevertheless, I believe that the collection technique is robust for selecting fetal cells. This is supported by the findings from the control group experiments in which maternal bone marrow-derived stem cells were found to have minimal engraftment.

When cultured in the semi-solid medium, the mouse AFSCs produced haematopoietic colonies from all three haematopoietic lineages and were capable of haemoglobin production when cultured in an erythroid differentiation medium, similar to bone marrow stem cells (Srivastava et al., 2006). Having demonstrated their haematopoietic potential *in vitro*, I used mouse AFSCs in an *in-utero* stem cell approach to test their ability to engraft long-term.

I found that allogenic transplantation *in utero* was associated with lower rates of engraftment at the 4 and 16 week time points when compared to autologous transplantation. This was surprising since an *in utero* approach is considered to be relatively more successful at achieving engraftment through fetal tolerance (Derderian et al., 2014; Fisher et al., 2012; Hayashi et al., 2003; Loukogeorgakis and Flake, 2014; Peranteau et al., 2006, 2015). Injecting higher numbers (5x) of allogenic AFSC cells did not achieve tolerance or higher engraftment.

I ensured that there was no maternal antibody effect postnatally by cross-fostering the pups to different dams after birth, thus avoiding passage of antibodies through lactation. This effect has previously been described in mice; indeed, the possibility that maternal antibodies passed across the placental barrier following allogenic IUT and before birth, cannot be ruled out. The lack of tolerance might also be due to the primitive nature of the ASFC and their inability to present in the thymus and achieve a 'self' identity (Bilate and Lafaille, 2012; Nijagal et al., 2013; Spence and Green, 2008).

Due to low engraftment seen in the allogenic transplanted group, I studied the immune reaction to *in utero* injection using the total number of $CD4^+$ and $CD8^+$ cells present at four weeks. The presence of an immune response was confirmed by performing *in vitro* MLR studies where

splenocytes (responders) isolated from the IUT recipient mice at four weeks were co-cultured with splenocytes from genetically identical naïve donors (stimulators). The higher proliferation rate of $CD4^+$ and $CD8^+$ T lymphocytes in the allogenic group confirmed the presence of an immune response towards donor cells. On the contrary, in the congenic group, the T lymphocyte proliferation was similar to the un-injected control, which indicates that the congenic cells did not lead to an antigenic response. However, the congenic recipients did have a tolerogenic response; here, FoxP3, TGF-β and IL10 gene expression were higher in comparison to the un-injected control and allogenic group, as measured by qPCR.

Multilineage long-term engraftment was confirmed in the recipient groups by the characterisation of cells expressing the donor markers using haematopoietic lineage markers such as CD3, B220, CD11b, Gr1 and Ter-119. I detected double positive CD45.1 and lineage-specific cells in bone marrow and blood, which is an indication that AFSCs can give rise to terminally differentiated cells and fully integrate into the haematopoietic system. The cells were also cultured in a semi-solid medium. They are thus confirming confirmed the multilineage engraftment by the presence of haematopoietic colonies from blood lineages, myeloid and erythroid. The haematopoietic colonies were also analysed using flow cytometry, which confirmed the expression of differentiation markers (Hayashi et al., 2003; Peranteau et al., 2006). The higher expression of FoxP3, TGF- β and IL-10, both centrally and peripherally in the congenic recipients suggests the possibility that congenic cells expressing marker CD45.1 lead to a more tolerant state towards transplanted cells. Studies have shown that increased FoxP3 expression is associated with self-tolerance as well as tolerance to a non-inherited antigen (Burt, 2013). Also, $CD4^+CD25^+FoxP3^+$ regulatory T cells are involved in transplantation tolerance, and they prevent rejection in solid organ transplantation when co-transplanted with the allograft (van der Net et al., 2016; Velásquez-Lopera et al., 2008).

All animals underwent a histopathological examination at the University of Cambridge by Dr Joy Archer (Queen's Vet School Hospital, Central Diagnostic Services). While in the congenic group, no abnormalities were detected, in the allogenic group, bone marrow erythroid

hyperplasia was evident. This could be due to the antigen immune response or early development of pancytopenia, a form of early, refractory anaemia or neoplasia (Chinen and Buckley, 2010; Jha et al., 2008).

For clinical translation, I have demonstrated that HSC derived from AF could be used for the treatment of congenital blood disorders. Since allogenic AFSCs appear to cause an immune response in mice, autologous (congenic) transplantation would be preferred. After combining gene therapy and subsequent correction and expansion of gene-corrected AFSCs, the cells could be given back to the same fetus with the aim of correcting haematological or other monogenic disease prior to birth (Loukogeorgakis and De Coppi, 2016; Loukogeorgakis and Flake, 2014; Ramachandra et al., 2014; Shaw et al., 2011). The multilineage engraftment observed suggests that these cells could reconstitute the haematopoietic system if given in sufficient numbers. AFSC expansion, while maintaining their haematopoietic potential, is an important hurdle to overcome to provide sufficient stem cells to cure haematopoietic disease. Also, the safety of gene transfer to AFSC for long-term transduction will need to be assessed. Transplantation of induced pluripotent stem cells from AF or other fetal sources, such as chorionic villi, if proven safe, could be another approach.

One limitation to the present study is that I do not demonstrate whether transplanted mAF HSCs can function *in vivo*; for example, to produce haemoglobin, further on in this thesis, I do attempt to correct a mouse model of beta thalassaemia.

In conclusion, I have shown successful haematopoietic engraftment in immune-competent mice using mAFSC. The production of haemoglobin *in vitro* shows that these cells are functional, and form terminal erythrocytes. Congenic AFSCs appear to have a significant engraftment advantage over allogenic AFSCs, which may be mediated by the reduced fetal immune response.

# Chapter 4 *In Utero* Stem Cell Transplantation of fresh and expanded haematopoietic progenitors into a humanised mouse model of thalassaemia

## 4.1 *In-utero* transplantation of beta-actin-Cre-YFP cells into a humanised mouse model of thalassaemia

To assess the *in-utero* therapy method in a mouse model of beta thalassaemia, I used the B382 model in similar study design to the experiment in the IUGT chapter. The experiments were initially performed by isolating mAF, and mBM stems cells from YFP mice as described in the methods chapter. A total 10^4-10^5 of either fresh or cultured mouse cells were injected into the intraperitoneal cavity of each fetus. The newborn pups were cross-fostered and sacrificed at 16 weeks of age.

## 4.2 Various types of haematopoietic progenitors

As discussed in the previous chapter, I isolated haematopoietic progenitors from amniotic fluid, bone marrow and fetal liver. Their cells were cultured in a modified embryonic stem cell medium and analysed before and after expansion. Table 4-1 shows the initial experiments done with the corresponding number of cells and source.

| Dam Number | Number of fetuses injected | Number of cells injected, type of cells | Fresh /Cultured | Number of live pups |
|---|---|---|---|---|
| B382001 | 8 | PBS | Control | 4 |
| B382002 | 9 | PBS | Control | 0 |
| B382003 | 9 | 10000, mAF HSC YFP | cultured | 2 |
| B382004 | 9 | 10000, mBM HSC YFP | cultured | 1 |
| B382005 | 10 | 10000, mBM HSC YFP | cultured | 4 |
| B382007 | 8 | 10000, mBM HSC YFP | Fresh | 0 |
| B382008 | 8 | 10000, mBM HSC YFP | Fresh | 1 |
| B382011 | 10 | 10000, mBM HSC YFP | fresh | 0 |
| B382012 | 7 | 10000, mAF HSC YFP | fresh | 1 |
| B382015 | 9 | 10000, mAF HSC YFP | fresh | 0 |
| B382017 | 8 | 50000, AF HSC YFP | Fresh | 3 |

**Table 4-1 IUT attempts using mHSCs**

To avoid maternal rejection and attack the newborn pups were cross-fostered to time mated Cd1 dams which had delivered one day earlier. The pups were mixed in the CD1's litter and identified later from their colour (CD1 mice are white while Humanised thalassaemia mice are grey/black).

Experiments using fresh and cultured cells from a beta-actin-cre-YFP mouse into the CA heterozygote B382 time-mated dam with a CA B382 heterozygote male.

Initial experiments were performed as per table 4-1, and the pups were analysed 16 weeks postnatally. The three pups which had PBS injected were all wild type as per genotype. The animals which had either mAF HSC or mBM HSCs showed minimal engraftment in the various organs, and the heterozygous animals were still symptomatic of thalassaemia, i.e. splenomegaly, extramedullary haematopoiesis see Table 4-2.

| Dam Animal number | Weight in grams | Hb | Spleen | Spleen weight | Genotype | Transplanted cells | Engraftment in the spleen |
|---|---|---|---|---|---|---|---|
| B382001A | 31.84 | 12.5 | normal | 0.11 | WT | PBS | 0.71 |
| B382001B | 29.78 | 9.8 | normal | 0.13 | WT | PBS | 0.81 |
| B382001C | 33.55 | 11.2 | normal | 0.15 | WT | PBS | 0.4 |
| B382003A | 30.46 | 12.5 | normal | 0.14 | WT | mAF cult | 1.1 |
| B382003B | 26.28 | 9.1 | enlarged | 0.48 | HET | mAF cult | 3.15 |
| B382005A | 30.7 | 12 | normal | 0.13 | WT | mBM cult | 0.69 |
| B382005B | 33.06 | 9 | enlarged | 0.56 | HET | mBM cult | 1.37 |
| B382005C | 32.89 | 8 | normal | 0.16 | HET | mBM cult | 0.83 |
| B382008A | 24.6 | 13 | Normal | 0.09 | WT | mBM cult | 0.5 |
| B382012A | 24.5 | 8 | Enlarged | 0.3 | HET | mAF cult | 0.3 |
| B382017A | 28.5 | 8.2 | Enlarged | 0.19 | HET | mAF fresh | 2.17 |
| B382017B | 26.59 | 12.3 | Normal | 0.11 | WT | mAF fresh | 2.09 |

**Table 4-2 Results of mHSC IUT**

## 4.3 Histological Analysis and EMH assessment

The animal tissues were sent to the Cambridge Veterinary School laboratory and had histological examination (H&E) done. A veterinary tissue expert (Dr Joy Archer, University of Cambridge) examined the slides and commented on the presence of Lymphoid hyperplasia, spleen myeloid hyperplasia, bone marrow erythroid hyperplasia and GIT lymphoid hyperplasia as per Table 4-3.

| Offspring Animal Number | Genotype | EMH Spleen | EMH Liver | Spleen Lymphoid Hyperplasia | Bone Marrow Myeloid Hyperplasia | Bone Marrow Erythroid Hyperplasia | GIT Lymphoid hyperplasia |
|---|---|---|---|---|---|---|---|
| B382001A | WT | * | | * | * | | |
| B382001B | WT | * | | | * | | * |
| B382001C | WT | * | | | | * | |
| B382003A | WT | * | | | | | * |
| B382003B | HET | * | * | * | | * | |
| B382005A | WT | | | * | | | |
| B382005B | HET | * | * | | | * | |
| B382005C | HET | * | * | | | | |
| B382008A | WT | | | * | | | |
| B382012A | HET | | * | * | | * | |
| B382017A | HET | | | | | | |
| B382017B | WT | | | | | | |

**Table 4-3 Histological examinations of post-mortem tissues**

HPLC Analysis of each animal's blood sample to test for globins was performed as described in the methods chapter. The analysis was performed with the help of Dr Simon Eaton, ICH, Stem Cells and Regenerative Section. No visible correction was identified in the HPLC analysis.

## 4.4 Discussion

Unfortunately, the engraftment was minimal in these animals with no phenotypic correction in any of the treated animals. In all the heterozygote animals except one, there was splenomegaly and EMH. Evidence of severe EMH was also observed in the liver of the heterozygote animals. Three of the heterozygotes and one of the wild type animals showed bone marrow erythroid hyperplasia, which also corresponded to the low haemoglobin levels. The low engraftment in this animal model might be due to the requirement of enough "space". Other groups have shown that conditioning of the fetus, using an anti-c-kit antibody could create space and improve engraftment (Derderian et al., 2014). This can be done in case we don't reach therapeutic engraftment. Another way to perform "fetal myeloablation" is by preconditioning *in utero* the fetuses with an *in utero* injection of a CD45.2 SAP, haematopoietic cell-specific immunotoxin consisting of saporin (SAP) conjugated to a CD45.2 targeting antibody. This will

be injected together with the HSC transplant(Palchaudhuri et al., 2016). The CD45.2 SAP will target only the host HSCs and HSC cells expressing CD45.1 can be used as donor cells. This has been successfully applied to treat a sickle cell mouse model of disease postnatally(Palchaudhuri et al., 2016).

The maternal immune response might also play a role, but since the new-borns were cross-fostered, I expected this to be minimal (Merianos et al., 2009; Nijagal et al., 2011). After studying the B382 CA mouse model, I realised that the YFP donor animal model was not an actual congenic animal to B382. The B382 is chimeric of C57BL6 and 0129 and it is also humanised (Huo et al., 2017). Transplanting mouse cells from, eventually, a non-congenic donor would lead to rejection, which was the case in these experiments. This can be taken in to account when re-designing the experiments. A right congenic donor can be used (the humanised B383) or enhancement of the allogenic cells to achieve tolerance and engraftment; either using T regulatory cells co-transplantation or engineering (Passerini and Bacchetta, 2017; Pilat et al., 2010), or nanoparticle assisted delivery of the cells into the haematopoietic compartment (Dobson, 2006; Huo et al., 2014; Yang et al., 2011).

This could be part of a future experimental design. This is also described in detail in the future work section. Ideally, the ultimate goal is to rescue the homozygote animal and minimise the need for regular blood transfusions. It is essential, before moving the treatment in larger animal models and non-human primates, to demonstrate that this *in-utero* stem cell therapy approach works in a small animal model. The cells transplanted need to be functional, repopulate the haematopoietic system and produce therapeutic levels of haemoglobin for the survival of the homozygous animal.

# Chapter 5 *In utero* Therapy for Thalassaemia and MRI Follow Up

## 5.1 Introduction

β-thalassaemia is caused by mutations in the β-globin (*HBB*) locus giving β-globin chain reduction. It is a common severe disease with 56,000 β-thalassaemia major (TM) births per year worldwide and an estimated 5-7% carrier frequency in the general population, with increased frequency in risk ethnic groups such as Mediterranean, Middle Eastern, and South Asian(Modell et al., 2008). TM requires regular blood transfusion and if left untreated is a life-threatening condition for which the only curative therapy - allogenic haematopoietic stem cell (HSC) transplantation - is limited to a small percentage of patients with available HSC donors. It can be diagnosed *in utero*, and many countries have efficient screening and prenatal diagnosis programs(Modell Bernadette and Darlison Matthew, 2008) that may lead to a termination of pregnancy (TOP) if an affected fetus is found. Prenatal diagnosis (NIPD) could detect an affected fetus as early as ten weeks of gestation(Rund and Rachmilewitz, 2005a).

Fetal therapy of an affected fetus would avoid termination of pregnancy, in this case, may prevent early death or life-threatening complications for patients with no compatible HSC donor. This would have an impact in countries where α-and β-thalassaemia are most prevalent, even those where termination of pregnancy may not be available, and blood transfusions are prohibitively expensive(Weatherall, 2005).

Possible curative options that show promise are (i) the autologous correction of β-thalassaemia patient HSCs through gene augmentation studies mediated by β-globin expressing lentiviral vectors and (ii) reactivation of the endogenous *HBG* through lentiviral-mediated gene silencing of *HBG* silencer genes (e.g. *BCL11A*)(Wilber et al., 2011).

Various groups have been trying to construct an ideal gene delivery vehicle, which will be able to correct the disease either *in utero* or postnatally(Breda et al., 2012; Finotti et al., 2015; Malik

et al., 2005; Pawliuk et al., 2001; Ye et al., 2009). I chose the GLOBE(Miccio et al., 2008; Roselli et al., 2010) lentiviral vector for an *in utero* therapy approach in a novel humanised model of β-thalassaemia(Huo et al., 2010, 2017; Huo, McConnell and Ryan, 2009; McConnell et al., 2011). The GLOBE LV, which is now in phase I/II clinical trial (NCT02453477) was previously shown to correct β-thalassaemia in murine (Miccio et al., 2008) and human (Roselli et al., 2010) haematopoietic progenitor cells. Recently A.A. Thompson and colleagues showed correction of transfusion-depended β-thalassaemia and reduction or elimination of transfusion requirements using *ex vivo* gene therapy with LentiGlobin BB305 vector (Thompson et al., 2018).

In this study, I used the humanised mouse model of β-thalassaemia which had been generated by replacing the mouse adult α- and β-globin genes with adult human α-globin genes and a human fetal to adult haemoglobin-switching cassette(Huo et al., 2017; Huo, McConnell and Ryan, 2009; Huo, McConnell, Liu, et al., 2009). Two mouse models were used, one with functional HBB (B383: hBThal-Control, α2α1/α2/α1, $\gamma\beta^{A}/\gamma\beta^{A}$) and one with non-functional HBB (CA Mice B382: hBThal, α2α1/α2/α1, $\gamma^{HPFH}\beta/\gamma\beta^{A}$). The B382 with human fetal to adult haemoglobin-switching cassette (α2α1/α2/α1, $\gamma^{HPFH}\beta/\gamma\beta^{A}$, non-functional *HBB*) retained the homozygous human alpha globin and heterozygous human fetal to adult Hb-switching cassette. The homozygous animals from the latter mouse strain die of anaemia within two weeks if left untreated. This provides a similar temporal onset of the disease as in humans (early years) if left untreated. However, animals can be rescued from lethal anaemia by weekly blood transfusions (Huo, McConnell and Ryan, 2009), or postnatal bone marrow transplantation (Huo et al., 2017). Heterozygous animals show features of thalassaemia intermedia such as splenomegaly, extramedullary haematopoiesis, anaemia, and anisocytosis.

## 5.2 Aims of the study

Postnatal genetic rescue of β-thalassaemia requires early intervention with high titres of therapeutic virus(Cavazzana-Calvo et al., 2010; Thompson et al., 2018), high transduction efficiency in HSCs and long term HSC engraftment before the onset of irreversible pathological damage. Correcting the disease before birth may be beneficial in preventing and ameliorating this early-onset disease.

I tested the efficacy of the SIN-LV GLOBE vector expressing a β-LCR HS2/HS3-mini-β-globin gene cassette in rescuing a thalassaemia intermedia CA mouse model pre-natally. The GLOBE vectors (Miccio et al., 2008) as described in the literature were used.

Unlike other thalassaemia mouse models, the mice Cooley's anaemia (CA mouse) mirrors the human disease with two Hemoglobin switches during development and survive to birth. This preclinical humanised mouse model is thus amenable to a prenatal gene or stem cell therapy. At E13.5, the vector carrying the gene was injected directly into the intrahepatic region to target the fetal liver harbouring the HSCs. Twelve and thirty-two weeks after birth, the animals were analysed.

**I investigated the following questions:**

1. **Is the phenotype of the mouse model after transfer to UCL the same as that described by Huo et al.?**
2. **Is *in utero* gene transfer of β-globin to the CA fetus effective in rescuing the thalassaemia phenotype, and if so, how is this achieved?**
3. **Is the Fetal γ-globin switched off after birth?**
4. **Is there a balance between mouse and human β-globin chains?**
5. **Can the therapeutic effect of IUGT, be monitored using MRI techniques?**

## 5.3 Characterization of the phenotype of the humanised mouse model of thalassaemia

I planned to use a novel Humanised heterozygous mouse model of thalassaemia to investigate the effect of *in utero* gene therapy. This model was developed at the University of Alabama at Birmingham, USA (Huo et al., 2010, 2017; Huo, McConnell, Liu, et al., 2009).

Initially, I was planning to use the *th3* mouse model of β-thalassaemia (Yang et al., 1995). In this mouse model, homozygous C57BL/6/Hbb$^{th3}$ mice have a deletion of both fetal and adult β-globin genes, causing β$^{o}$-thalassaemia in homozygotes, that die either *in utero* or perinatally within 12 hours of birth, a similar condition to $\alpha^0$ thalassaemias in humans. The short gestation length of a mouse and the severe phenotype meant that I considered that it was not possible to assess whether my stem cell, gene therapy approach was likely to rescue thalassaemia in this model.

I discussed this with my supervisors, and I established a collaboration with the University of Alabama at Birmingham (UAB). I was awarded a Bogue travel fellowship, which allowed me to visit UAB for six weeks and familiarised myself with the humanised mouse model of thalassaemia. I set up a Material Transfer Agreement which allowed the transportation of those animals from Alabama, USA, to London for my experiments. The animals arrived in November 2012 due to the excessive paperwork. The animals had to be re-derived, and the colony was established in March 2013. The first intrauterine injections into these animals were performed in June 2013.

When the first animals arrived, I performed some experiments using control animals to confirm that the re-derived animal phenotype was the same as that described by the group in UAB.

### 5.3.1 Comparison of Animal weight between Humanised heterozygous mouse model of thalassaemia (B382) and humanised non-thalassaemia control (B383)

First, I studied the growth and development of the transgenic thalassaemia and normal control mice. To do this, I chose aged matched animals, control (B383) and Heterozygote Thalassaemia (B382) at four months of age that had been born at UCL. The following

parameters were measured and compared with data from UAB: weight, full blood count, protein electrophoresis, spleen weight and level of extramedullary haematopoiesis.

Humanised heterozygote animals were compared with humanised control animals. There was no weight difference between the two groups at four months of age (27.04±1.60 vs 28.09±2.12, n=8, p=0.48) as seen in Figure 5-1.

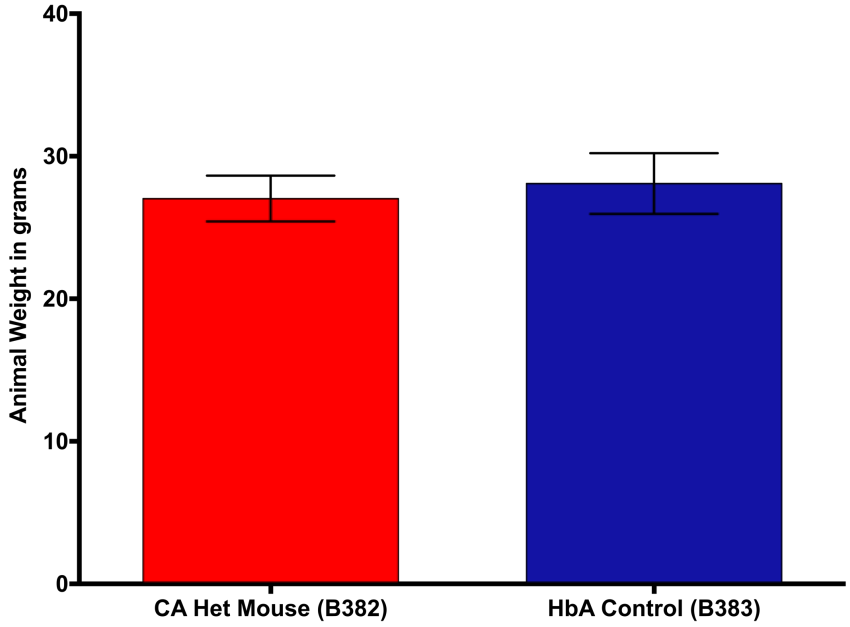
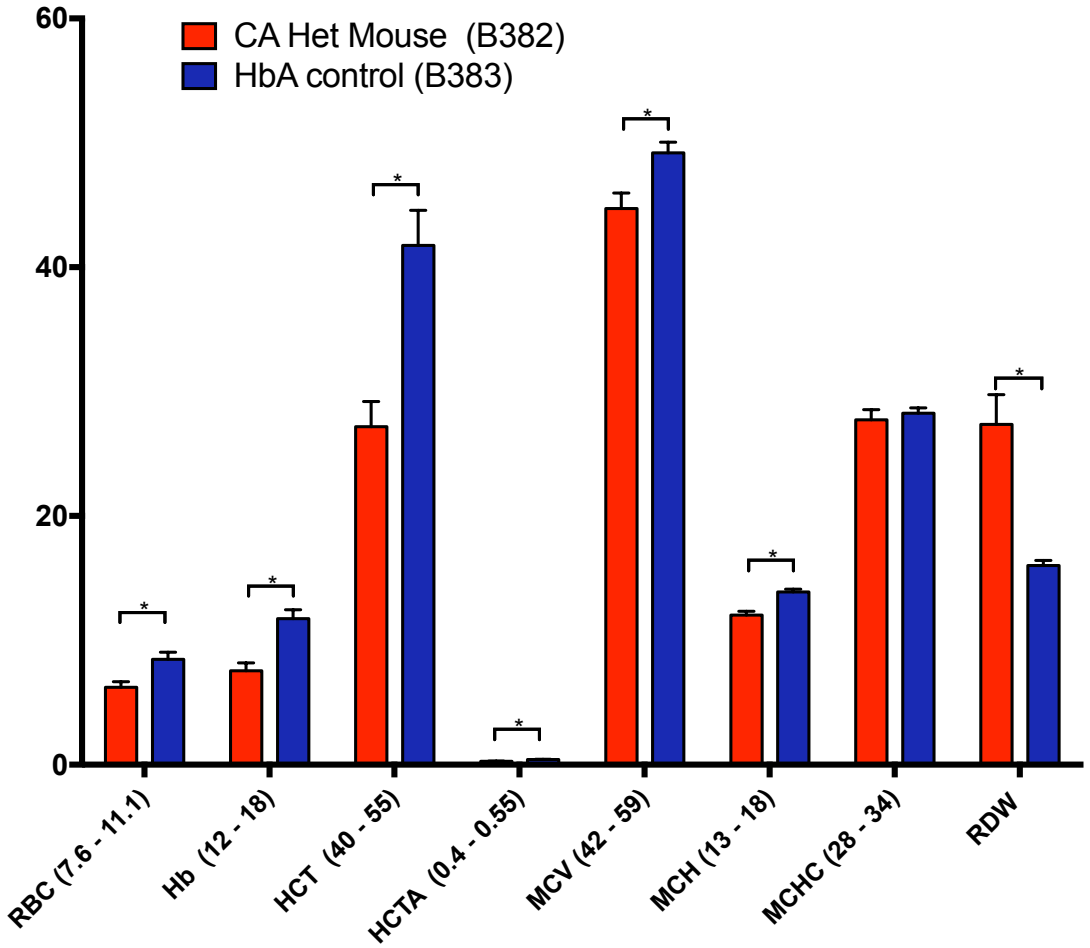



**Figure 5-1 Weight comparison between heterozygote humanised thalassaemia (B382, (27±0.04, n=8)) mouse model and humanised control (B383, 28±2.12, n=8) at four months of age (p=0.48)**

In summary, I found that the Humanised heterozygous mouse model of thalassaemia (B382) and humanised non-thalassaemia control (B383) did not show any difference in weight at four months of age. Haematological analysis of blood indicates differences in full blood count indices between humanised heterozygous mouse modes of thalassaemia (CA Het Mouse, B382) and humanised non-thalassemia control (HbA Control, B383). There were differences in the haematological indices of the humanised heterozygous mouse (B382) model compared with the humanised non-thalassaemia control (B383).

The Red Blood Cell fraction of the blood in these humanised heterozygote animals, showed differences such as lower haemoglobin level (7.55±0.64 vs 11.75±0.70, n=8, p=0.01), see figure Figure 5-2.

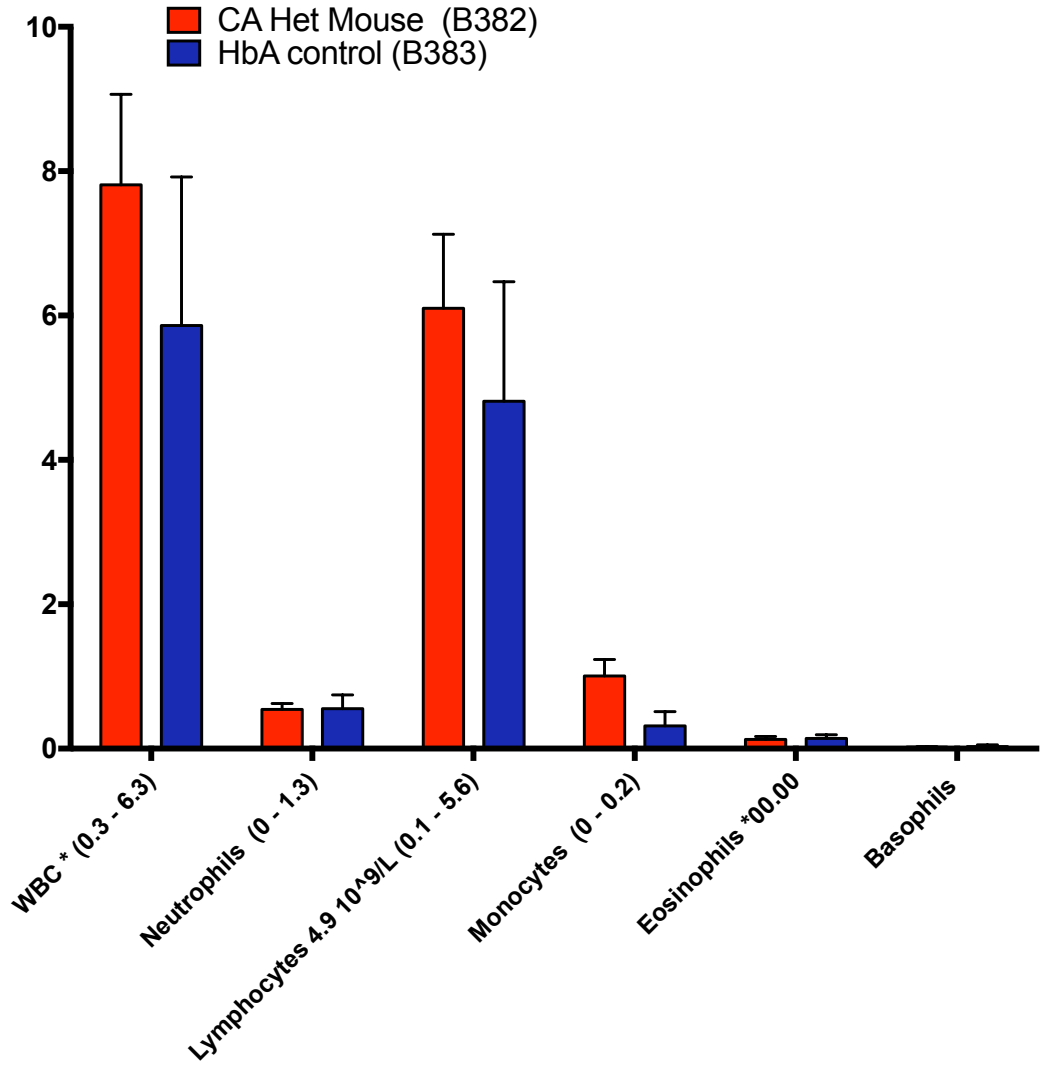



| | CA Mouse (B382) | | | HbA control (B383) | | |
|---|---|---|---|---|---|---|
| | Mean | SEM | N | Mean | SEM | N |
| RBC (7.6 - 11.1) | 6.23 | 0.45 | 8 | 8.47 | 0.57 | 8 |
| Hb (12 - 18) | 7.55 | 0.64 | 8 | 11.75 | 0.70 | 8 |
| HCT (40 - 55) | 27.18 | 2.02 | 8 | 41.76 | 2.80 | 8 |
| HCTA (0.4 - 0.55) | 0.27 | 0.02 | 8 | 0.41 | 0.02 | 8 |
| MCV (42 - 59) | 44.71 | 1.25 | 8 | 49.20 | 0.85 | 8 |
| MCH (13 - 18) | 12.03 | 0.30 | 8 | 13.90 | 0.20 | 8 |
| MCHC (28 - 34) | 27.72 | 0.83 | 8 | 28.26 | 0.43 | 8 |
| RDW | 27.36 | 2.39 | 8 | 16.01 | 0.42 | 8 |

**Figure 5-2 Red cell fraction from B382 vs B383 humanised thalassaemia mouse models. A total of 8 animals from each group were used. There were differences in almost all parameters with P<0.05 except MCHC which had a p=0.57. The significant differences were marked with an * on the figure above. The numbers next to the haematological parameters abbreviations indicate the normal range of each marker.**

I also examined indices of the white cell fraction as shown below in Figure 5-3. No differences were found in the two groups which confirms the mouse model only has a defect in the red cell fraction of blood. It also confirms that engraftment after transplantation will not be because of

immune suppression of the animals but instead because of tolerance, since no evidence of immunocompromise was seen.




| | CA Mouse (B382) | | | HbA control (B383) | | |
|---|---|---|---|---|---|---|
| | Mean | SEM | N | Mean | SEM | N |
| WBC * (0.3 - 6.3) | 7.81 | 1.25 | 8 | 5.86 | 2.06 | 8 |
| Neutrophils (0 - 1.3) | 0.54 | 0.08 | 8 | 0.56 | 0.19 | 8 |
| Lymphocytes 4.9 10^9/L (0.1 - 5.6) | 6.10 | 1.03 | 8 | 4.81 | 1.66 | 8 |
| Monocytes (0 - 0.2) | 1.01 | 0.23 | 8 | 0.32 | 0.19 | 8 |
| Eosinophils *00.00 | 0.13 | 0.04 | 8 | 0.14 | 0.05 | 8 |
| Basophils | 0.02 | 0.01 | 8 | 0.03 | 0.02 | 8 |

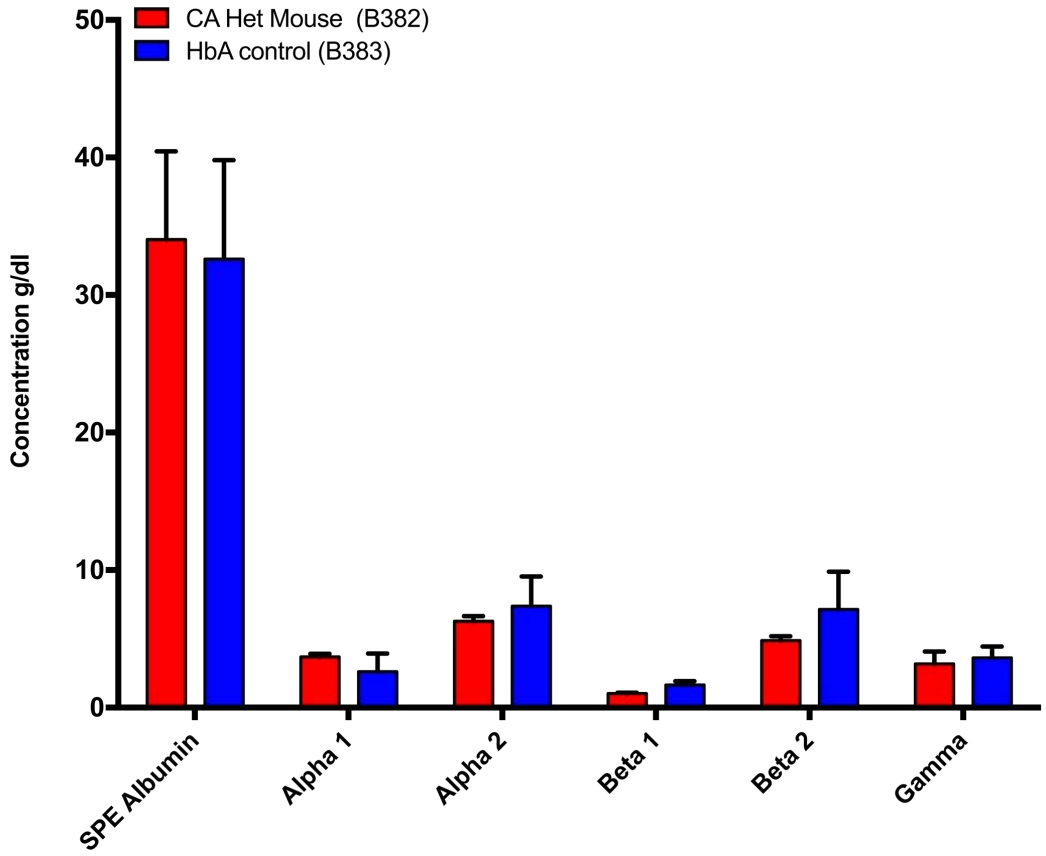
**Figure 5-3 White cell fraction of blood of the humanised thalassaemia model and control. None of the white cell parameters had any difference between the two groups, n=8, p>0.05. The numbers next to the haematological parameters indicate the normal range of each marker.**

In conclusion, despite the differences in the red blood cell component, no differences were observed in the white cell component of the blood from humanised heterozygous thalassaemia. This was expected since thalassaemia affects mainly only the red cell

component of blood (Deborah Rund, M.D., and Eliezer Rachmilewitz, 2011). This was encouraging since it would exclude any immune-associated reason for graft acceptance in these humanised mouse models.

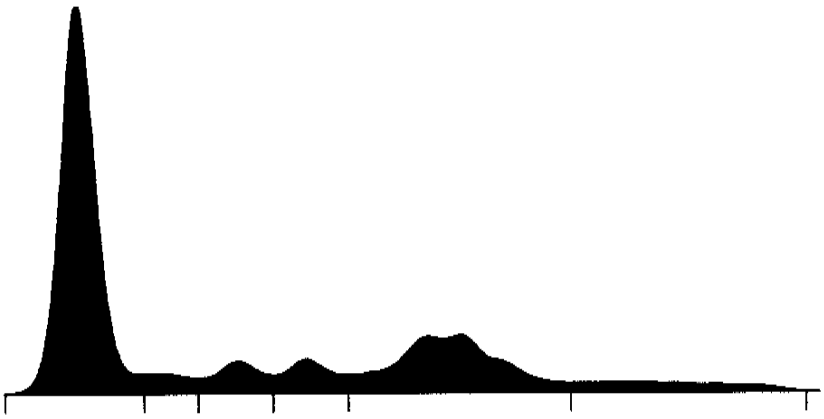
### 5.3.2 Serum Protein Electrophoresis (SPE) of the Humanised heterozygous mouse model of thalassaemia (B382) and the humanised non-thalassaemia control (B383) confirms lower levels of Beta 1 globin chain

In addition to the Full blood count, I also performed serum protein electrophoresis (SPE) for the two groups. SPE separates proteins based on their physical properties. The subsets of these proteins are used in interpreting the results. The technique is used to identify patients with multiple myeloma and other disorders of serum protein(O'Connell et al., 2005). Predictable changes can be seen in response to acute inflammation, malignancy, trauma, chemical injury, liver or renal failure and also hereditary protein disorders. (O'Connell et al., 2005; Vavricka et al., 2009). SPE was performed in the blood from humanised heterozygote and control animals. No differences were observed, which confirms that the two animal models are not affected by any other pathology, and their SPE is similar Figure 5-4 & Figure 5-5.




| | CA Mouse (B382) | | | HbA control (B383) | | |
|---|---|---|---|---|---|---|
| | Mean | SEM | N | Mean | SEM | N |
| SPE Albumin | 34.04 | 2.27 | 8 | 32.61 | 2.28 | 10 |
| Alpha 1 | 3.70 | 0.08 | 8 | 2.61 | 0.47 | 8 |
| Alpha 2 | 6.29 | 0.13 | 8 | 7.39 | 0.76 | 8 |
| Beta 1 | 1.04 | 0.02 | 8 | 1.65 | 0.09 | 8 |
| Beta 2 | 4.88 | 0.11 | 8 | 7.15 | 0.96 | 8 |
| Gamma | 3.18 | 0.32 | 8 | 3.62 | 0.29 | 8 |

**Figure 5-4 Serum Protein Electrophoresis in the two control animal models. The serum was tested for Albumin, Alpha 1, Alpha 2, Beta 1, Beta 2 and Gamma serum proteins. No significant difference was observed in the two animal models (n=8, p>0.05)**



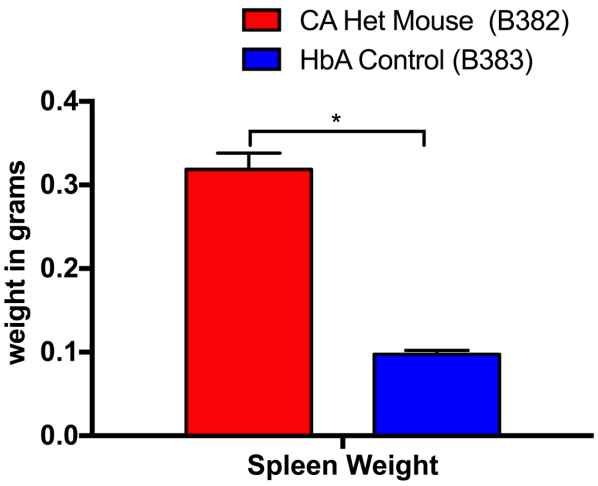
***Serum protein electrophoresis***

| Fractions | % | | Conc. |
|---|---|---|---|
| Albumin | 54.1 | > | 26.0 |
| Alpha 1 | 3.3 | > | 1.6 |
| Alpha 2 | 5.9 | > | 2.8 |
| Beta 1 | 6.3 | > | 3.0 |
| Beta 2 | 24.3 | > | 11.7 |
| Gamma | 6.1 | > | 2.9 |

**Figure 5-5 SPE representative graph from one animal showing analysis for an animal. Each peak represents a different fraction of the serum proteins**

### 5.3.3 Comparison of Spleen Weight between Humanised heterozygous mouse modes of thalassaemia (B382) and humanised non-thalassaemia control (B383)

A significant difference in the spleen weight was observed in the animals suffered from thalassaemia. This agrees with differences seen in other models of thalassaemia, such as the *th3* (Yang et al., 1995). The spleen weight measured at post mortem using a sensitive scale. The measurements from a total of 8 spleens in each group were used. The mean spleen weight in the B382 mice was significantly higher than the B383 controls, as seen in Figure 5-6.




| | CA Het Mouse (B382) | | | HbA control (B383) | | |
|---|---|---|---|---|---|---|
| | Mean | SEM | N | Mean | SEM | N |
| Spleen Weight | 0.32 | 0.02 | 8 | 0.10 | 4.53e-003 | 8 |

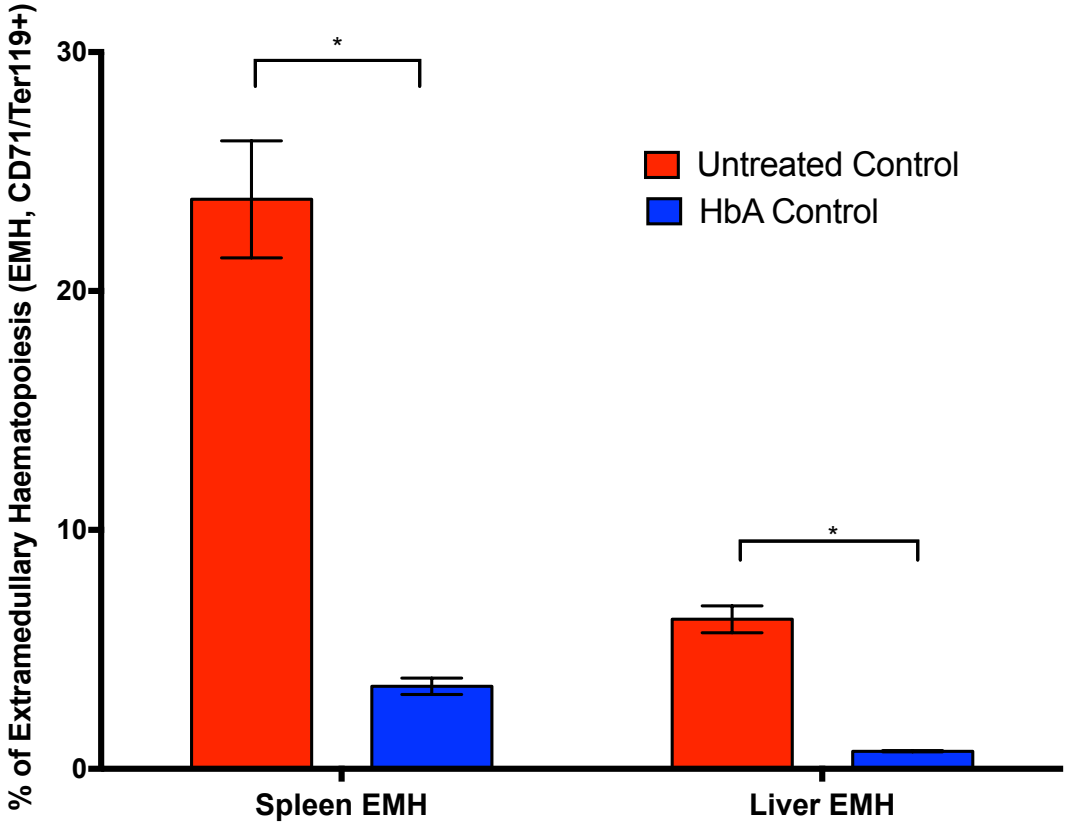
**Figure 5-6 Comparison between spleen weight of humanised heterozygote and control animals (n=8). A difference was seen between them, with the heterozygote having three times heavier spleen than the control, (p<0.0001).**

### 5.3.4 Comparison of extramedullary haematopoiesis between Humanised heterozygous mouse model of thalassaemia (B382) and humanised non-thalassaemia control (B383)

In thalassaemia, ineffective erythropoiesis in combination with peripheral haemolysis leads to anaemia, hypoxia and reactive increase of erythropoietin (EPO) production with subsequent hyperplasia of the bone marrow and extramedullary haematopoiesis (EMH) with hepatosplenomegaly (Makis et al., 2016). This can also be seen in this humanised mouse model. I studied the level of extramedullary haematopoiesis in this mouse model, and I compared it to humanised control. A total of eight, age-matched (4 months) animals, were compared.

The level of EMH was measured as a percentage of double positive cells expressing CD71 and Ter119 markers. This marker identifies the blood-forming cells, or erythroid precursors(Negre et al., 2011). Thus, the percentage of blood-forming cells outside the bone marrow was increased in the humanised heterozygote animal model compared to humanised

control, both in the spleen (*p<0.0001, n=8) and liver (*p<0.0093, n=8) as shown in Figure 5-7. This is another reason why CA Het mice, B382, suffer from splenomegaly (Rivella, 2009).




| | Untreated Control | | | HbA Control | | |
|---|---|---|---|---|---|---|
| | Mean | SEM | N | Mean | SEM | N |
| Spleen EMH | 23.83 | 2.45 | 8 | 3.45 | 0.34 | 8 |
| Liver EMH | 6.26 | 0.56 | 8 | 0.73 | 0.02 | 8 |

**Figure 5-7 Level of EMH in the spleen and liver of humanised heterozygote animal (untreated control) versus humanised controls. The level of EMH was higher in the untreated control animals (B382) compared to humanised controls (B383) in both the spleen (*p<0.0001, n=8) and liver (*p<0.0093, n=8).**

In conclusion, the mouse model, which was imported from UAB, does have a thalassaemia phenotype, showing evidence of significant EMH, splenomegaly anaemia compared to the humanised healthy control mouse. Thus, if a therapy is successful, a complete correction should make this model identical to the humanised control (HbA, B383).

## 5.4 GLOBE Virus Production, sequencing and testing

I used the GLOBE vector which has been used to treat adult thalassaemia mouse models, stem cells from thalassaemia patients and currently used in a clinical trial for the ex-vivo correction of stem cells from thalassaemia major patients (Miccio et al., 2008; Roselli et al., 2010; Thompson et al., 2018).

I produced the viral vectors using plasmids, which were provided, by Dr Michael Antoniou, at King's College London. The plasmids used were the MA821 (GLOBE) and MA1047 (GFP) as described in the methods section. The identity of the plasmids was confirmed using enzyme digestion. This process takes advantage of naturally occurring enzymes that cleave DNA at specific sequences. This allowed me to target specific recognition sites which I identify using the Vencor NTI software with the help of Dr Michael Blundell (Henikoff, 1984).

I used the enzymes EcoRI for MA821 and Hpal, Nhel for MA1047.

The MA821 plasmid was then analysed via restriction digest using EcoRI enzyme showing the expected fragments of 8677bp and 1094bm as see in Figure 5-8.

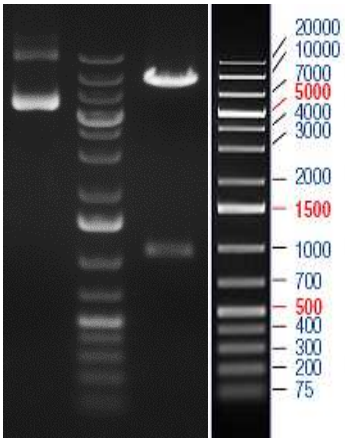



**Figure 5-8 An agarose gel check of DNA: from left to right: undigested DNA, 1kb+ Gene Ruler (Fermentas), digested DNA (fragments of 8677bp and 1094bp for MA821); fragments of Gene Ruler**

The MA1047 plasmid was analysed via restriction digest using Hpal and Nhel enzymes showing the expected fragments of 8677bp and 1094bm as seen in Figure 5-9.

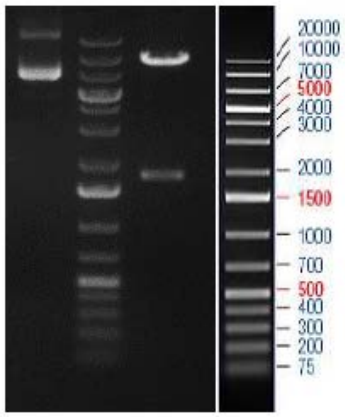



**Figure 5-9 Agarose gel check of DNA: from left to right: undigested DNA, 1kb+ Gene Ruler (Fermentas), digested DNA (fragments of 9331bp and 1589bp for MA1047); fragments of Gene Ruler**

The virus was then produced as described in the methods section.

## 5.5 Correction of a Heterozygous Humanised Thalassaemia Mouse Model after *in utero* gene therapy

### 5.5.1 Study Design

Having shown that the humanised heterozygous and control mouse model of thalassaemia behaved as they had in the source lab in Alabama, USA, I then went on to perform *in utero* injection experiments using the GLOBE vector that I had produced.

To achieve a statistically meaningful result, for each group we aimed to have at least six mice pups in each group, born by more than three different dams, to have more than three independent biological replicates (see methods for sample size calculation). The animal experiments were done according to the project license PPL No: 70/7408 and Animals [Scientific Procedures] Act 1986 and the NC3Rs ARRIVE guidelines 2013(Drummond et al., 2010; Kilkenny et al., 2013). My personal license was PIL 70/23796.

At E13.5 a "GLOBE" vector was injected into the liver of each fetus via a midline laparotomy to expose the uterine horns under sterile conditions. 20µl ($10^7$ VP/ml) of Globe LV viral particles were injected through the myometrium into the hepatic region of each fetus using a 33-gauge needle (Hamilton, Switzerland). The liver was identified as it is the only bright red organ in the

fetal mouse, seen through the transparent uterus. The successful intrahepatic injection was confirmed by seeing a small blop inside the liver. The dams were closely monitored to identify the time of delivery. At delivery, the pups were cross-fostered to time-mated CD1 dams which had already littered a day before. I did this to avoid maternal cannibalism and antibody formation from the mother to the fetus via breast milk(Nijagal et al., 2011). At 12 and 32 weeks of age, recipient blood, liver, spleen and bone marrow were collected for complete blood count, blood film, as well as RNA and DNA isolation. Extra-medullary haematopoiesis was examined in the spleen and liver using flow cytometry (CD71+/Ter119+ cells) and histo-pathological analysis. Gene expression of human and mouse alpha and beta globins, as well as human gamma globin, was assessed by quantitative polymerase chain reaction (qPCR). High-performance liquid chromatography (HPLC) was used to quantify the presence of human beta globin in the peripheral blood of treated animals. Using MRI, we assessed relaxation times T1, T2*. A representative flowchart of the study design can be seen in Figure 5-10. Results are expressed as Mean±SEM, and statistical analysis was performed using ANOVA with post hoc tests. Experimental protocols were approved by the ethical committee on animal experimentation at University College London.

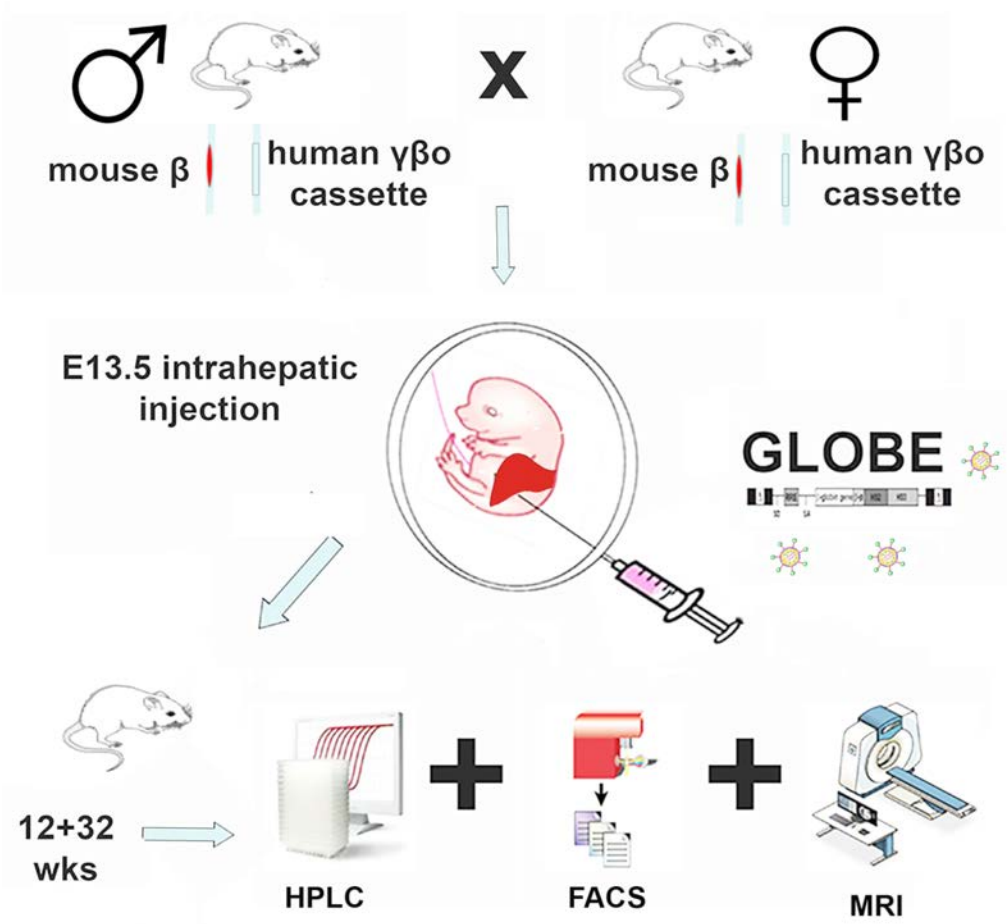



**Figure 5-10 Study design: Heterozygous males were mated with heterozygous females. At E13.5 *in utero* gene therapy was performed by injecting 20µl of the GLOBE vector into the intrahepatic space of each fetus in the litter after exposure of the uterus at laparotomy. The dams were allowed to litter, and the pups were cross-fostered into CD1 time time-mated dams to avoid maternal antibodies towards the virus. Post-mortem and analysis using HPLC, FACS and MRI were performed at 12 weeks in the first study and 32 weeks in the second. Any wild-type animals were excluded from the analysis.**

### 5.5.2 Survival Analysis after IUGT and Maternal Cannibalism

To identify the true fetal and dam survival after, *in utero* gene therapy delivery, I analysed the fetuses at E18 after IUGT in two dams. In a representative dam, where all ten fetuses were injected, at post-mortem, a total of 3 fetuses were identified as alive, where the rest of the injected fetuses suffered from a fetal demise. In another dam where all six fetuses were injected, three were identified as alive at E18. This gives a survival rate after injections of 37%. This was similar to the survival rate of 36% from IUGT to post mortem analysis at either 12 or 32 weeks postnatally, suggesting that the loss was due to the injection itself.

Examination of demised fetuses revealed that those fetuses die immediately after the injection since the fetuses were barely seen at the pilot E18 post-mortem and only the placenta was visible when the dam was sacrificed after cross-fostering.

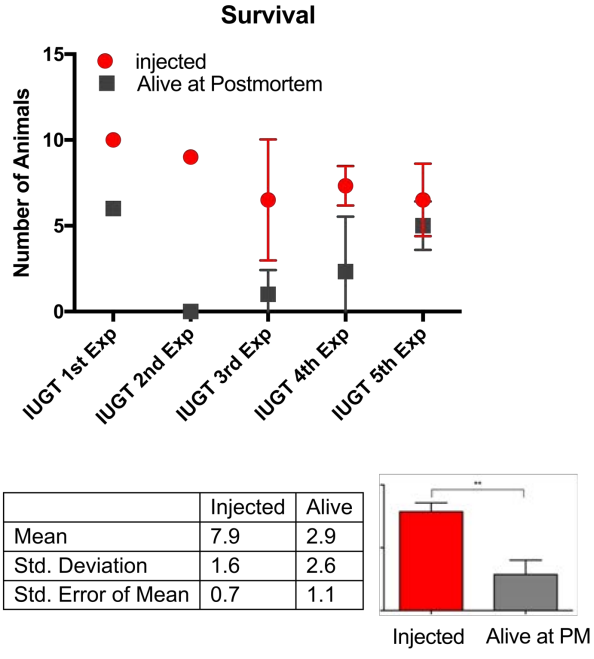
The fetuses which were collected at E18 were also genotyped to identify the homozygotes, heterozygotes and wild type animals as seen in Figure 5-11 and Figure 5-12. The dam mortality was 0%.


**Figure 5-11 Genotype at E18: All fetuses, including those that demised, were genotyped HTZ: Heterozygote, WT: Wild Type, Misc: Miscarriage (Fetal Demise), left and right-hand side 1000b+ Gene Ruler**


**Figure 5-12 Genotype at E18: All fetuses, including those that demised, were genotyped. HTZ: Heterozygote, HMZ: Homozygote, WT: Wild Type, Misc: Miscarriage (Fetal Demise), left and right-hand side 1000b+ Gene Ruler**

### 5.5.3 Survival Post In-Utero Gene Therapy

Dam survival after IUGT was 100% (n=9). All fetuses were injected in all litters n=67 total injection; survival to cross-fostering was n=25. There was no difference in the weight of all groups at 12 Weeks (Untreated thalassaemia Control 28.79±2.08 vs Humanised Control 28.09±2.12 vs IUGT 28.74±1.41, p=0.82, One Way ANOVA, Figure 5-15). Overall, pup survival from cross-fostering at day 2-3 postnatal to post-mortem analysis was 37% for all experiments (Figure 5-13). The animals were cross-fostered to CD1 time mated dams which delivered one day earlier. This was done to avoid maternal cannibalism and also to prevent the passage of maternal antibody against the transgenic protein in the milk. Genotyping at three weeks after birth identified thalassaemia animals, all of which were heterozygous (Figure 5-14). There were no homozygous pups determined by genotyping suggesting that they had demised either *in utero* after the intrahepatic injection or just before cross-fostering as all live cross-fostered pups survived to genotype. The proportion of homozygous pups in uninjected litters at E18 was 25% from n=3 dams. Wild-type animals were excluded from further analysis once identified.




| | Injected | Alive |
|---|---|---|
| Mean | 7.9 | 2.9 |
| Std. Deviation | 1.6 | 2.6 |
| Std. Error of Mean | 0.7 | 1.1 |

**Figure 5-13 Survival rate after IUGT to Post Mortem analysis. All animals were cross-fostered to avoid maternal cannibalism and maternal antibody response against the transgene. The average survival rate was 37%**

### 5.5.4 Genotyping of Animals postnatally

The animals were genotyped after birth and wild type animals were excluded from the analysis. As seen in Figure 5-14, Wild type animals: Globe5F & 1A were excluded from the study.

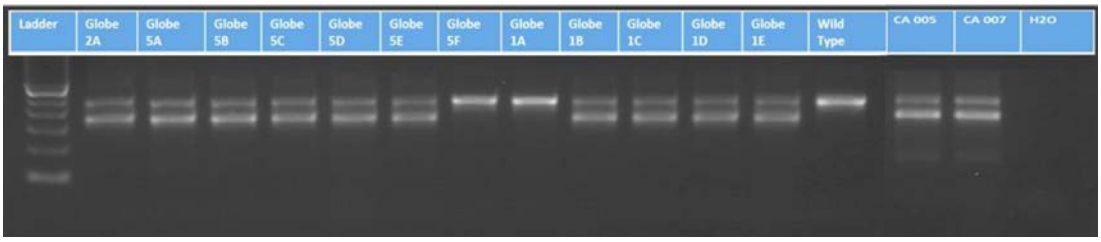



**Figure 5-14 Agarose Gel with PCR product from animal genotype. The double line indicated a heterozygote animal; single upper line indicated a wild type animal and single lower line a homozygote animal**

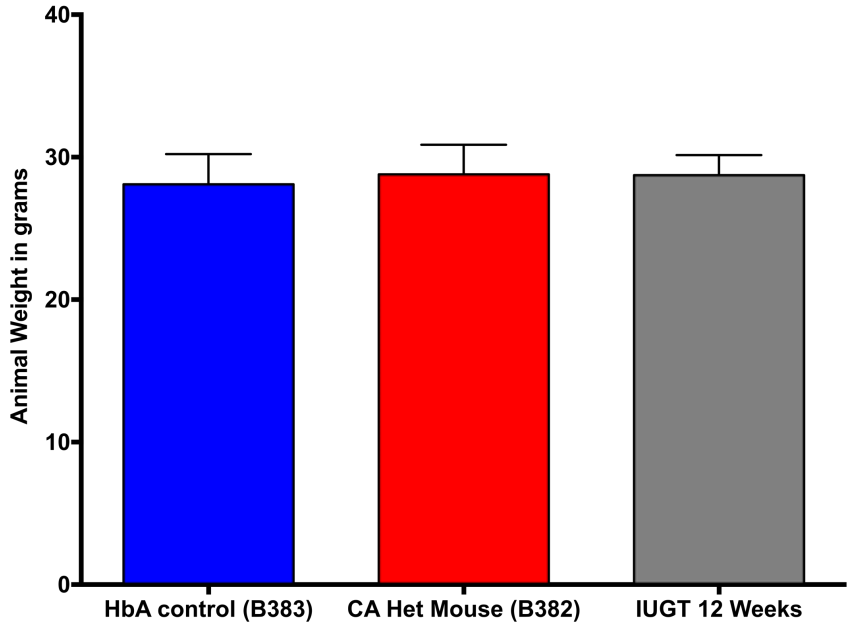



**Figure 5-15 Comparison of weight between the HbA control, B382 and IUGT at twelve weeks. There was no difference between the groups (p=0.96-0.99, Tukey's multiple comparison test)**

### 5.5.5 Successful correction of Haemoglobin after IUGT 12 Weeks

At 12 weeks of age, the peripheral blood haemoglobin concentration of treated heterozygous pups was not different to that of humanised non-thalassaemia control animals (11± 0.21 vs 11± 0.22, p>0.99**,** One Way ANOVA)**,** and was higher than the haemoglobin concentration of untreated heterozygous control pups (8.4±0.17, p=0.0001). This confirms the successful correction of haemoglobin, which reached healthy levels, similar to HbA control.

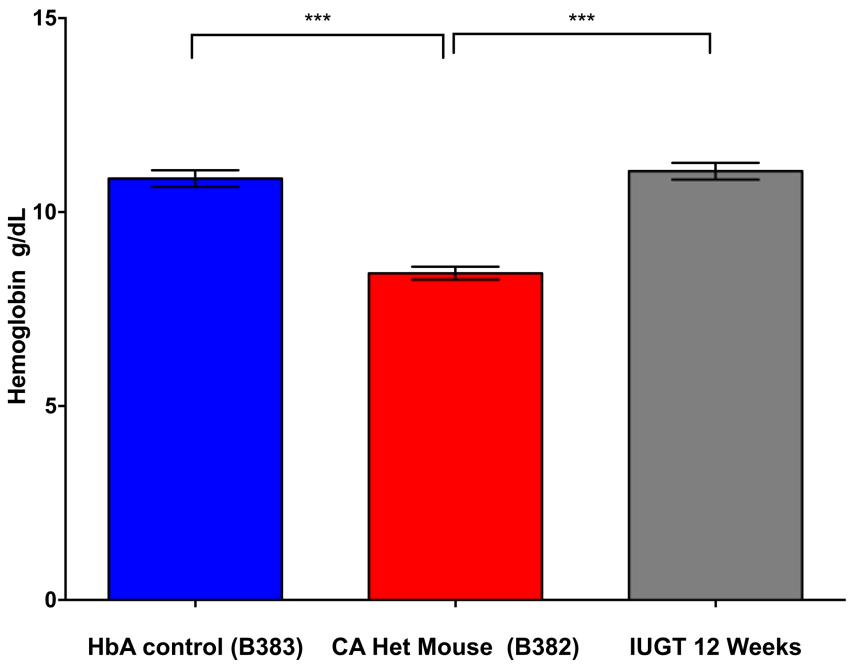



**Figure 5-16 Haemoglobin measurement in animal groups twelve weeks post IUGT. An increase was observed in the measurement of Haemoglobin in Humanised Non-Thalassaemia Control, B383 11± 0.21 Versus Uninjected Thalassaemia Control, B382, 8.4±0.17 Versus IUGT 12 Weeks 11±0.22, n=8, *p<0.0001. No difference was observed in the HbA controls vs IUGT 12 weeks**

### 5.5.6 Analysis of Human Beta Globin Expression in IUGT 12 Weeks Animals by Real Time PCR (these experiments were done together with Dr Sindhu Subramaniam, MSc Student, ICH)

Real-Time PCR of human beta-globin relative gene expression showing upregulation of the beta-globin gene in the IUGT treated animals compared to untreated thalassaemia controls. Wild-type C57BL6 and human blood were used as positive and negative controls.

#### 5.5.6.1 Beta Globin Expression Cumulative

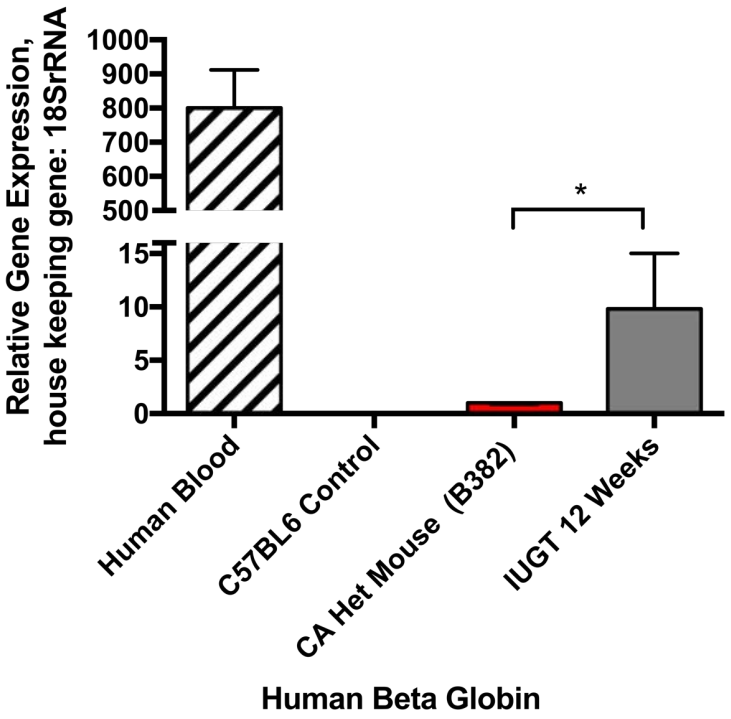



**Figure 5-17 The relative expression of the human beta-globin gene in the peripheral blood confirmed that the normalised haemoglobin concentration was due to the increased expression of the transgenic protein (n=10, p=0.039, One Way ANOVA, with Kruskal-Wallis post hoc test)**

#### 5.5.6.2 Human Beta Globin Expression Individual Animals

Figure 5-18 shows the increase in beta-globin gene expression in individual animals (IUGT 12 Weeks) compared to controls. There was an increase in beta-globin expression in all animals, even though the expression varied across the animals.

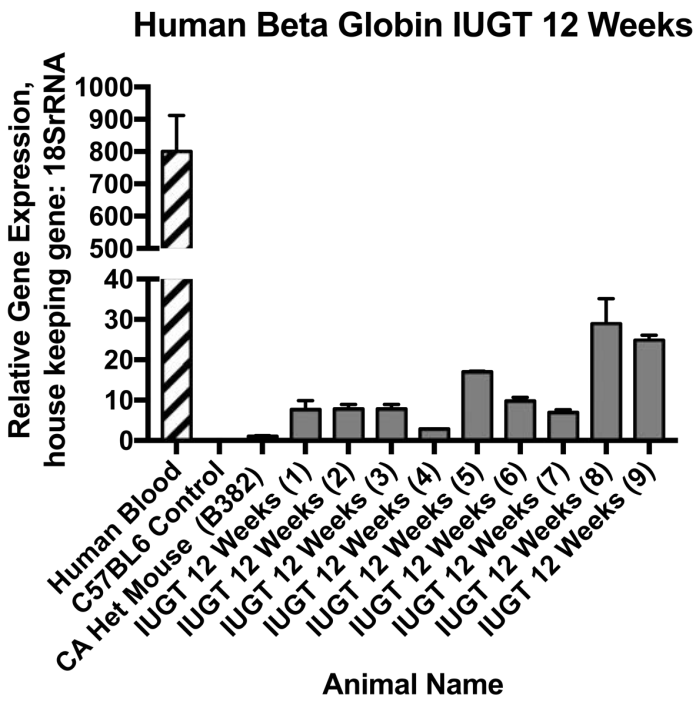



**Figure 5-18 Beta Globin Gene Expression, individual animals, human blood was used as positive control and C57BL6 as a negative control**

### 5.5.6.3 Expression of Gamma Globin Gene

At 12 weeks of age, the relative gene expression of human gamma-globin was lower in GLOBE, IUGT, treated compared to untreated heterozygous thalassaemia pups (0.11±0.024 versus 1.0±0, n=10, p=0.0002, one-way ANOVA, Kruskal-Wallis post hoc test) as seen in Figure 5-19. This confirmed that in treated heterozygous thalassaemia pups, the human gamma to beta-globin switching cassette successfully switched human gamma-globin to beta and subsequently fetal to adult haemoglobin. i.e. there was no hereditary persistence of fetal haemoglobin which could have been responsible for the higher haemoglobin.

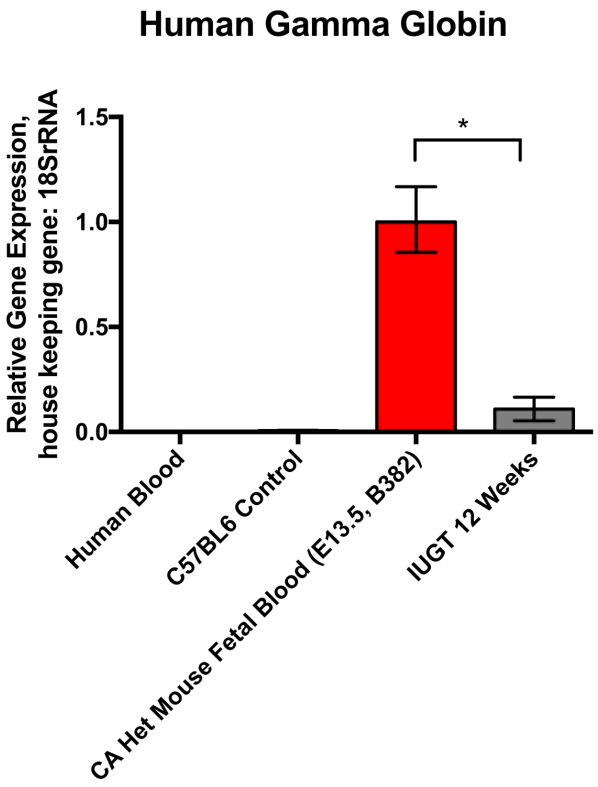



**Figure 5-19 Real-Time PCR of human gamma-globin relative gene expression showing downregulation of the gamma-globin gene in the IUGT treated animals compared to untreated fetal thalassaemia controls at E13.5, 0.11±0.08 versus 1.0±0, n=10, p=<0.0001. Wild-type C57BL6 and human blood were used as negative primer controls.**

#### 5.5.6.4 Gene Expression from All Globin Genes

To have a stable production of haemoglobin, the equivalent mouse globin was also increased in the IUGT 12 Weeks treated animals. As seen in Figure 5-20, most of the animals were compound heterozygotes. Human gamma globin was also present which might have contributed in the normalization of haemoglobin but not as high as present in the fetus. This was also seen in the human gene therapy trial by Cavazzana et al. (Cavazzana-Calvo et al., 2010).

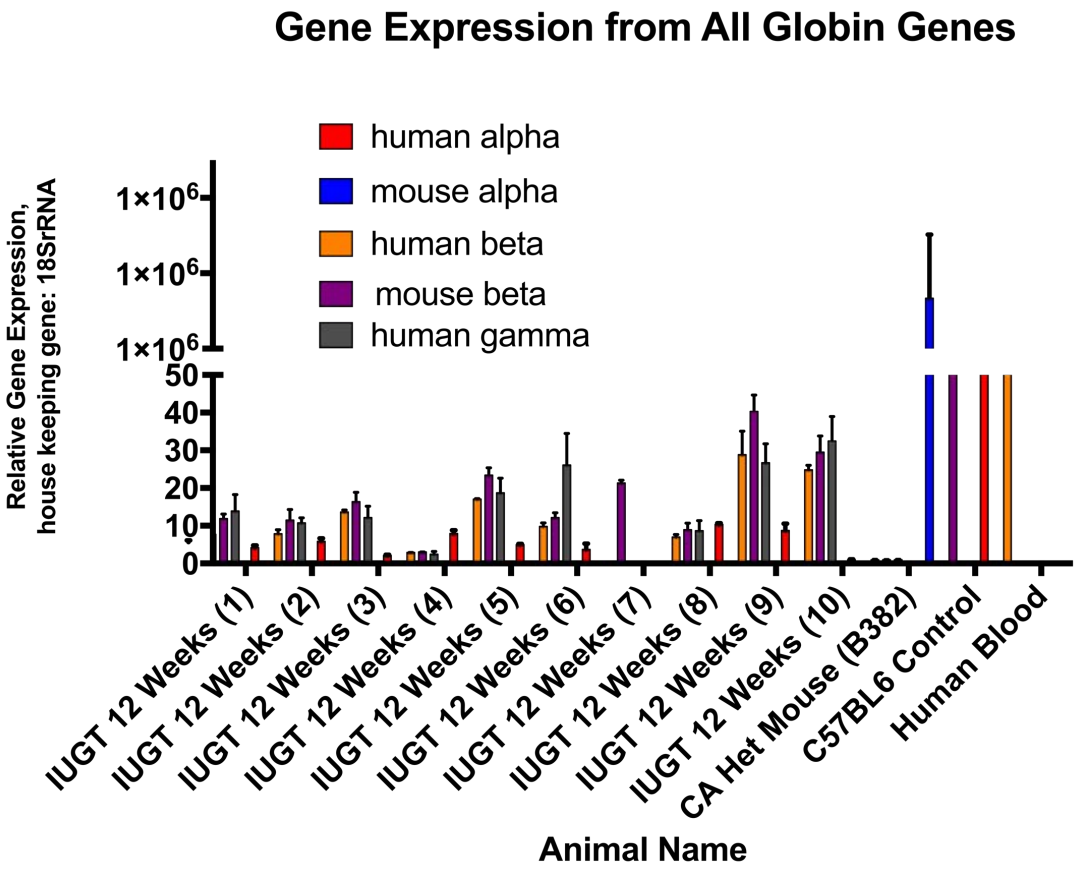



**Figure 5-20 Gene Expression of all globins for each animal**

### 5.5.7 Globin Analysis Using High-Performance Liquid Chromatography

The production of transgenic beta-haemoglobin protein was also verified at a protein level using high-performance liquid chromatography where the presence of a human beta chain peak was seen in the treated heterozygous thalassaemia pups 12 weeks after injection animals; this can be seen in the representative Figure 5-21.

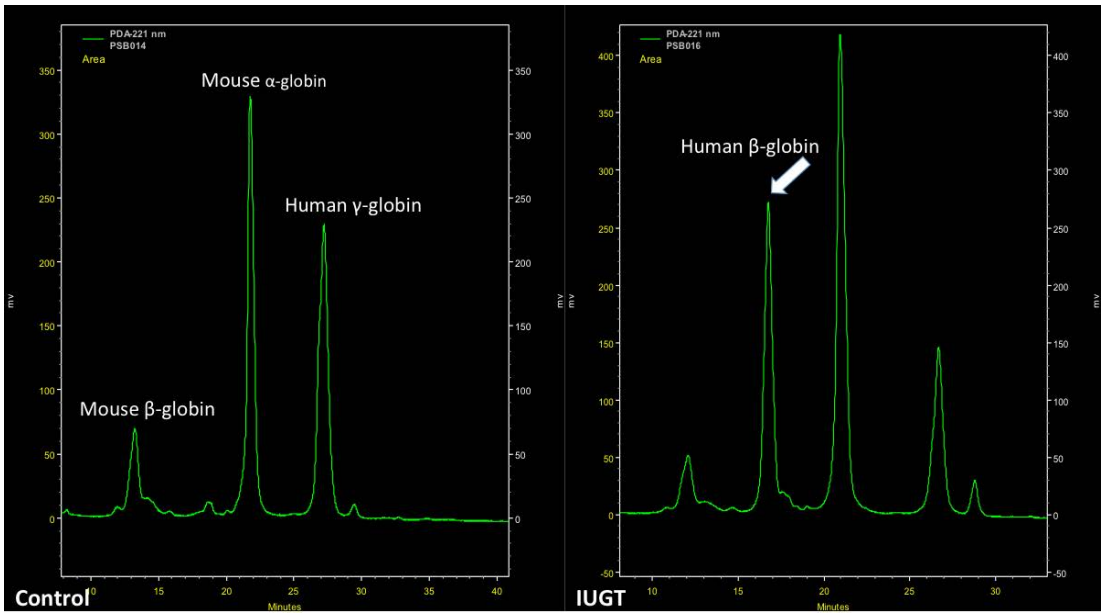



**Figure 5-21 Representative figure of High-Performance Liquid Chromatography showing the presence of a human beta-globin chain pick in the IUGT group**

Data from HPLC were quantified and show an increase in the expression of human beta-globin chain at a protein level. The alpha globin in the treated animals was also higher than untreated, and the level of gamma-globin was not affected by IUGT.

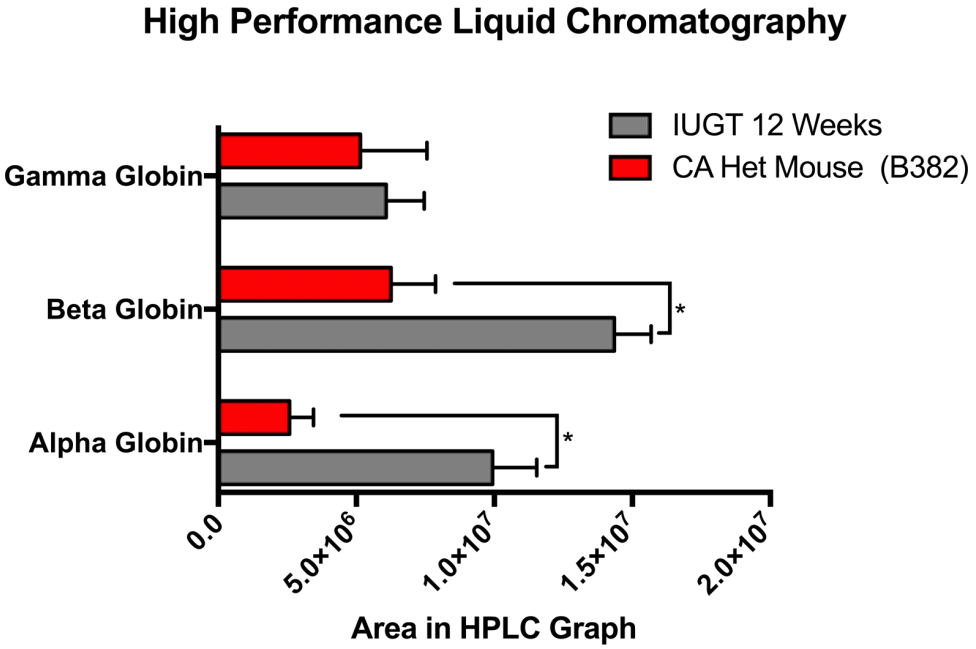



**Figure 5-22 Quantification of HPLC data showing an increase of both beta and alpha human globin (*p=0.0044) with gamma globin remaining stable after IUGT**

### 5.5.8 Confirmation of human beta-globin presence on paraffin-embedded tissue samples using a specific anti-human-beta globin antibody

Tissues from individual animals were stained, and histology slides were prepared. I used a specific human anti-beta globin antibody, as described in the methods section, to trace human beta globin. Immunofluorescence Images from Bone Marrow, Spleen, Liver showing the presence of human beta globin in the samples treated with IUGT and the absence of human beta globin in the untreated samples. Humanised Non-Thalassaemia Animals which produce human beta-globin were used as positive controls. Representative pictures can be seen in Figure 5-23, Figure 5-24 & Figure 5-25.

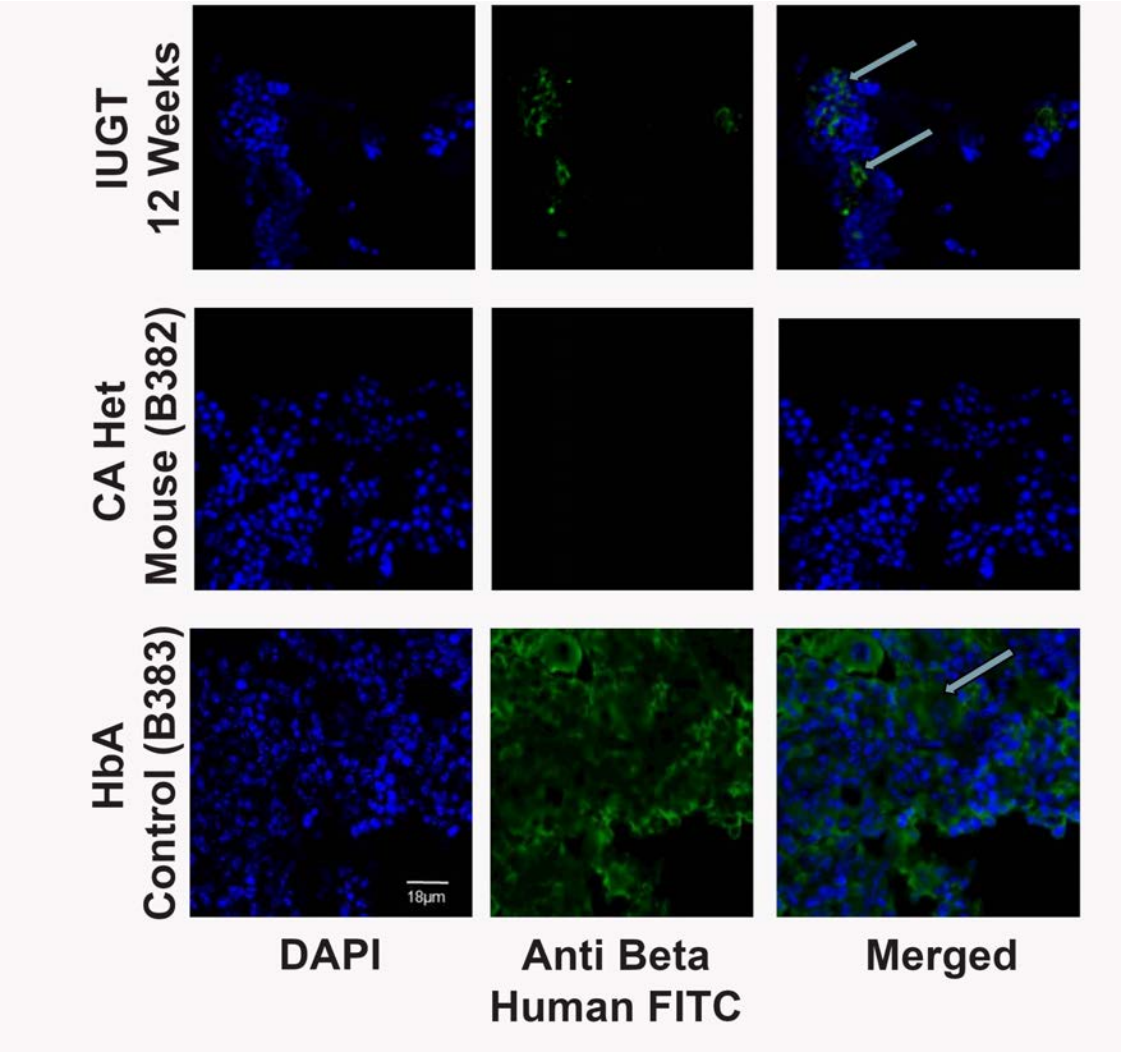



**Figure 5-23 Immunofluorescence imaging of paraffin-embedded slides from bone marrow, stained with a specific human anti-beta antibody. Arrows indicate green fluorescent protein, which is the human anti-beta globin. Blue colour indicates DAPI**

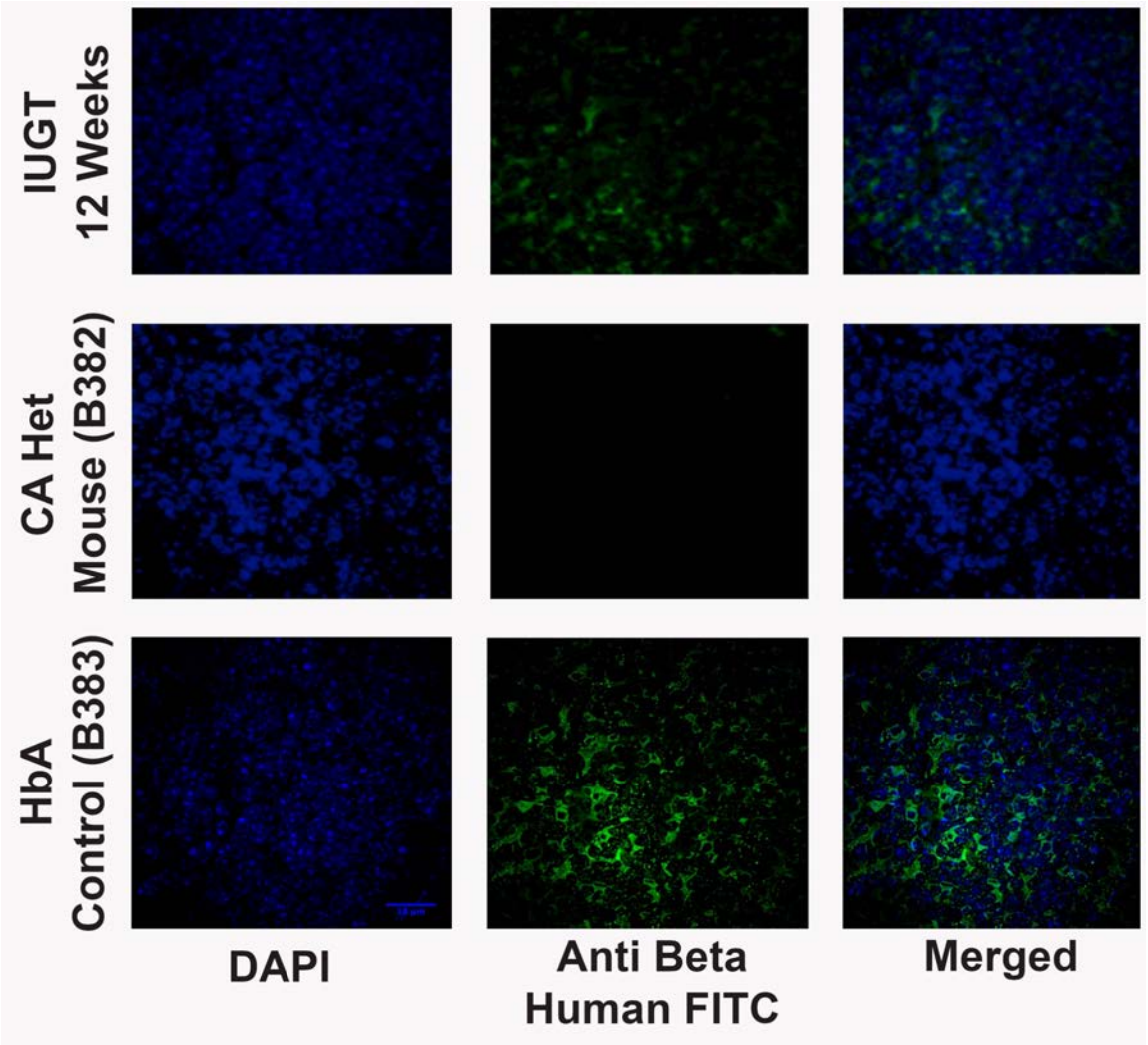



**Figure 5-24 Immunofluorescence imaging of paraffin-embedded slides from spleen, stained with a specific human anti-beta antibody. Green fluorescent protein indicates the human anti-beta globin. Blue colour indicates DAPI**

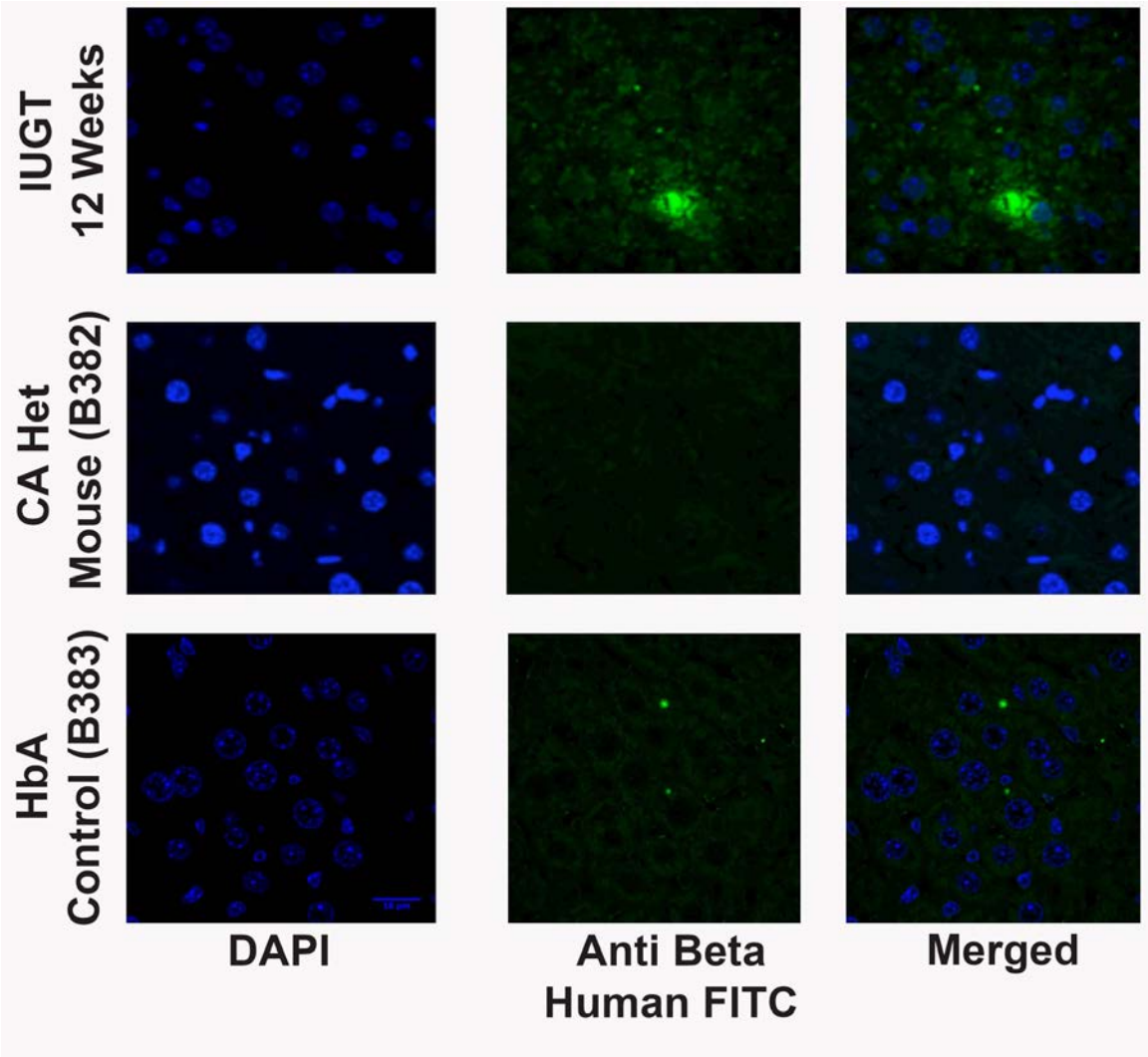



**Figure 5-25 Immunofluorescence imaging of paraffin-embedded slides from Liver, stained with a specific human anti-beta antibody. Green fluorescence indicates human anti-beta globin. Blue colour indicates DAPI**

In conclusion, I showed that IUGT successfully increased the Hb in the heterozygote mouse model. The effect is due to the transgene which, is integrated into the haematopoietic system, and this was confirmed by qPCR, HPLC and immunofluorescence.

### 5.5.9 Successful Reduction of Spleen Weight after IUGT 12 Weeks

Heterozygous humanised thalassaemia animals show evidence of splenomegaly, extramedullary and abnormal full blood count indices. In IUGT 12 Weeks animals the spleen weight was less (0.12±0.0065) than non-treated animals (0.31±0.017, n=8, p<0.0001, One Way ANOVA), and similar to Non-thalassaemia control animal (Imren et al., 2002) as seen in Figure 5-26. This demonstrates absent or minimal EMH, which is expected in a healthy animal.

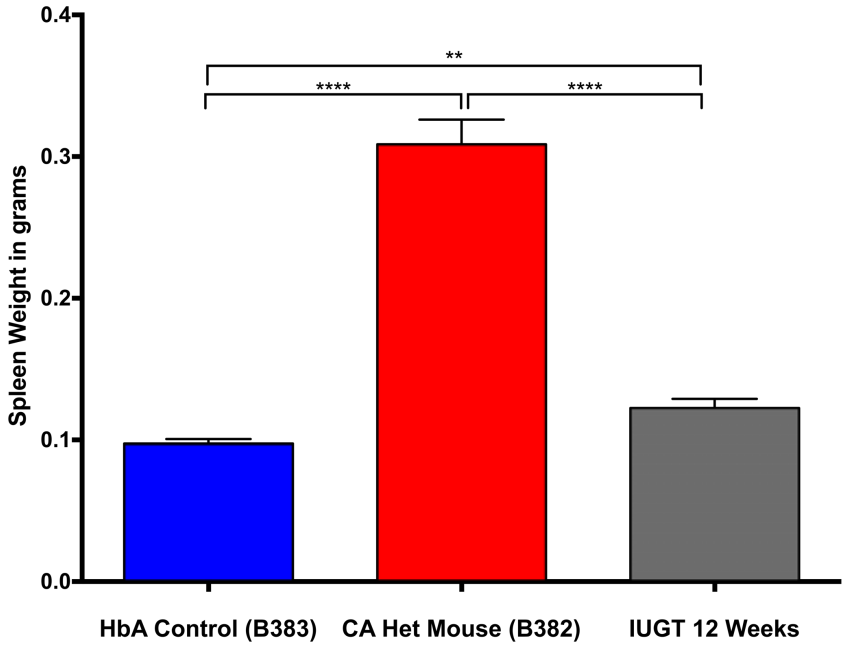



**Figure 5-26 Spleen weight in IUGT 12 Weeks animals was lower 0.12±0.0065 than untreated thalassaemia controls 0.31±0.017, n=8, p<0.0001 but not different to Humanised Non-Thalassaemia Control 0.098±0.0031, p=0.0016.**

### 5.5.10 Successful Correction of Red Blood Cell Count and Haematocrit IUGT 12 Weeks

IUGT mice haematological indices were also corrected at 12 Weeks, in which, Red Blood Cell Count (Figure 5-28) and Haematocrit (Figure 5-27) were at the levels of the Humanised Non-Thalassaemia Control animals.

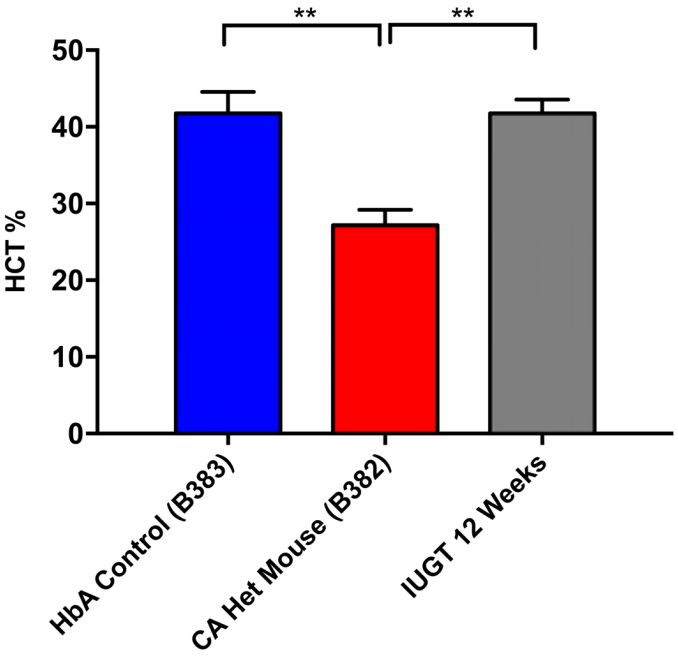



**Figure 5-27 The Haematocrit in IUGT 12 Week animals was higher 41.79±1.76%, n=8 than untreated thalassaemia controls 27.19±2.02, n=8, p=0.0025 and similar to humanised non-thalassaemia controls 41.76±2.80, n=8, p>0.99, One Way ANOVA, Bonferroni's Multiple comparisons test**

#### 5.5.10.1 Red Blood Cell Count

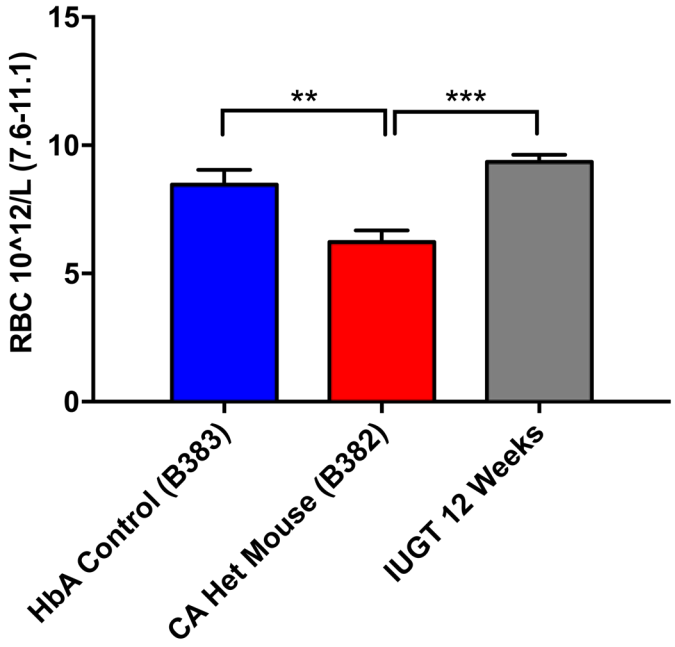



**Figure 5-28 Red Blood Cell Count in IUGT 12 Week animals was higher 9.36±0.27 10^12/L, n=8 than untreated thalassaemia controls 6.23±0.45 10^12/L, n=8, p=0.0015 and similar to humanised non-thalassaemia controls 8.47±0.57 10^12/L, n=8, p=0.089, One Way ANOVA, Bonferroni's Multiple comparisons test**

### 5.5.11 Reduction in the Extramedullary Haematopoiesis in the Spleen and Liver in IUGT 12 Weeks animals

The level of extramedullary haematopoiesis (ineffective erythropoiesis), indicated by the ratio of erythroid progenitor cells (CD71+Ter119+, see Figure 5-29) outside the bone marrow was also lower in IUGT 12 Weeks animals (p=0.00075, One Way ANOVA) compared to untreated thalassaemia controls and not different from Humanised Non-Thalassaemia Controls (p=0.1, One Way ANOVA)(Rivella, 2009) as seen in Figure 5-30. The reduction of extramedullary haematopoiesis was also confirmed by H&E staining of spleen and liver slides as can be seen in Figure 5-31. These results are comparable to previous studies done on adult thalassaemia mice (Malik et al., 2005; Puthenveetil et al., 2004; Roselli et al., 2010).

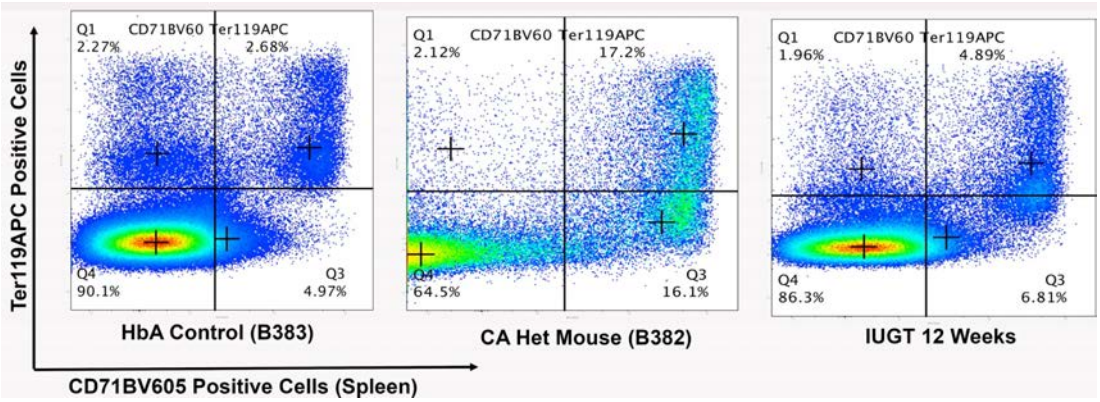



**Figure 5-29 Gating strategy used to detect extramedullary haematopoiesis in the liver and spleen. Cells double positive for CD71 and Ter119 were identified using the corresponding antibodies and flow cytometry. The three groups were compared using ANOVA, HbA control (negative control) CA Het Mouse (positive control) IUGT at twelve weeks.**

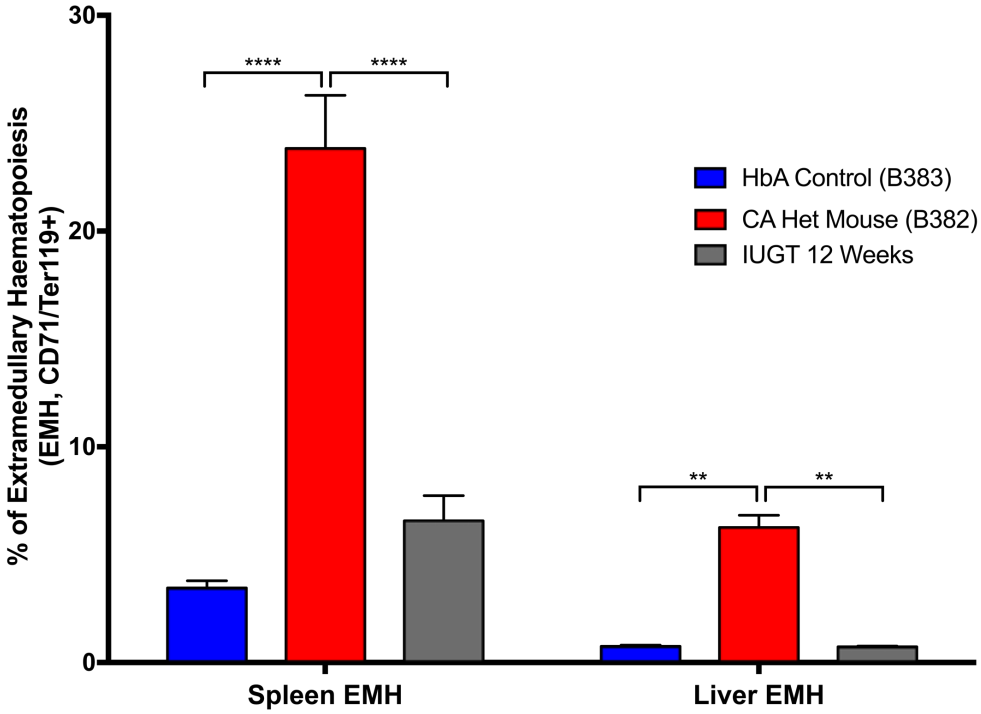



**Figure 5-30 Extramedullary Haematopoiesis in the Spleen was lower in IUGT 12 Week animals 6.57±1.78, n=4, versus uninjected thalassaemia controls 23.10±4.57, n=3, p=0.00075 and similar to humanised non-thalassaemia controls 3.69±0.41, n=6, p=0.64. Similarly, in the Liver, the level of EMH was lower in the IUGT 12 Week animals 0.74±0.042, versus uninjected thalassaemia controls 5.99±0.87, n=5, p=0.10 and comparable to humanised non-thalassaemia controls 0.75±0.091, n=4, p>0.99, One Way ANOVA, Bonferroni's Multiple comparisons test**

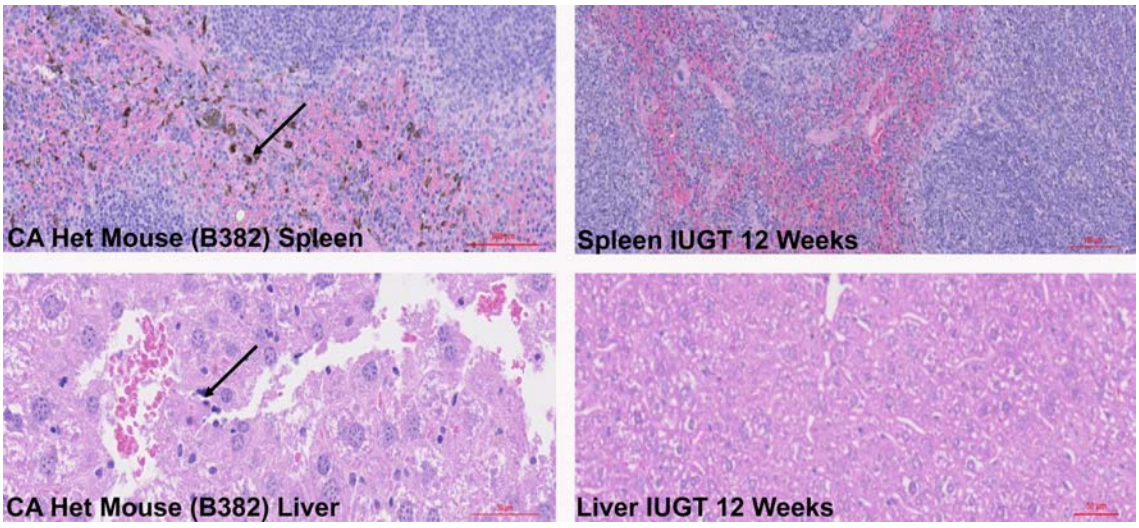



**Figure 5-31 Hematoxylin & Eosin light microscope images showing areas of extramedullary haematopoiesis (arrows) in the Spleen (Scale Bar 100µm) and Liver (Scale Bar 50µm).**

In conclusion, in this section, I showed that there was a phenotypic correction of the heterozygote mouse model of thalassaemia. The various haematological indices reached a healthy level.

### 5.5.12 Use of MRI techniques to monitor progress in animals treated with IUGT 32 Weeks

I then planned to examine the second cohort of animals. The in-utero gene transfer was performed as before, but the animals were sacrificed at 32 weeks of age having had MRI assessment two weeks before post-mortem to evaluate iron deposition, cardiac function and organ volume. The animals were also genotyped, and wild-type animals were excluded from the analysis. There was a difference in animal weight in the IUGT 32 Weeks group which was higher (38.03±2.93, n=6, p=0.01, One Way ANOVA, Bonferroni's Multiple comparisons test) from the age-matched control groups, uninjected Thalassaemia (28.97±1.4, n=9) and humanised Non-Thalassaemia (28.46±1.53, n=8) (Figure 5-32).

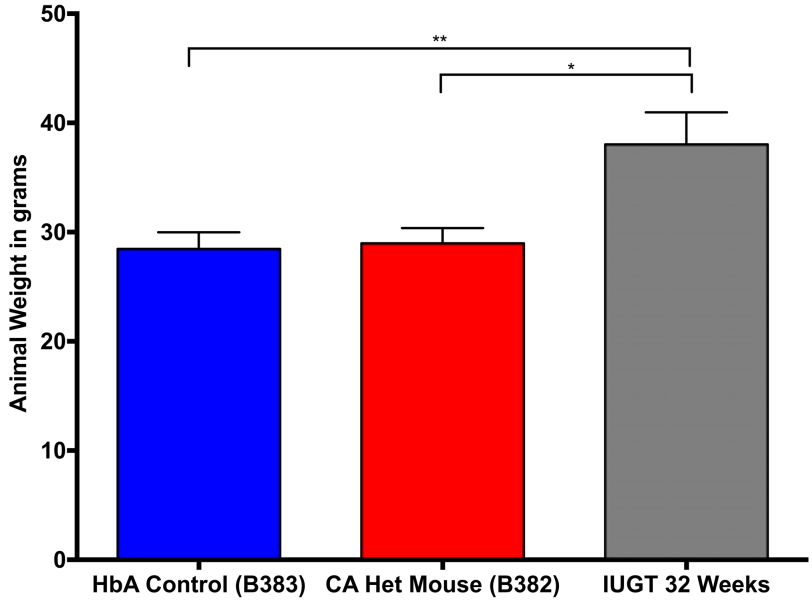



**Figure 5-32 Animal weight was similar in CA Het animals and humanised Non-Thalassaemia (HbA Control) control animals, but higher in IUGT 32-Week-old animals 38.03±2.93**

Spleen weight in long term animals (IUGT 32 Weeks) was lower that Ca Het mouse (untreated control) but also higher that HbA Control (Humanised Non-Thalassaemia), see Figure 5-33. This was not the case in the 12-week old animals, where a complete correction was observed. This might be a sign that the IUGT effect might be fading away, or the IUGT techniques in this cohort did not achieve complete correction.

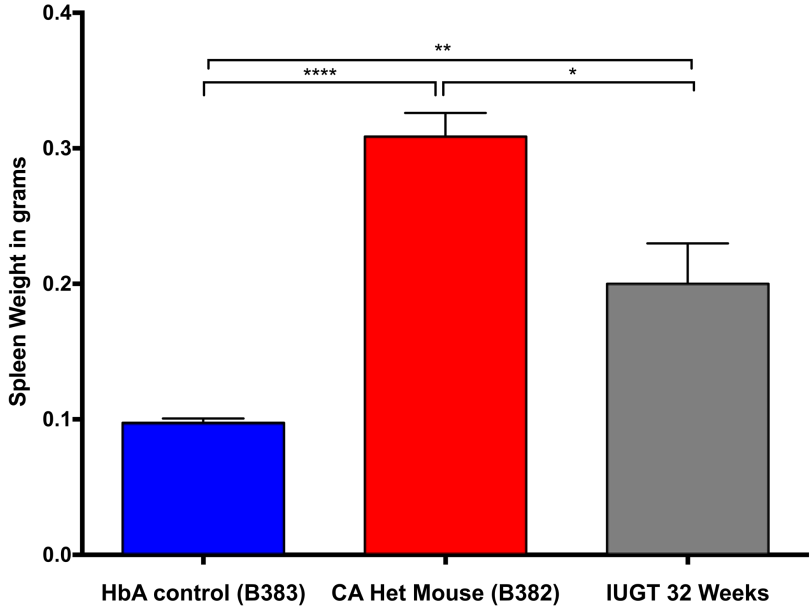



**Figure 5-33 Spleen weight in the IUGT 32 Week old animals was lower 0.2±0.03, n=8 than un-injected thalassaemia controls 0.309±0.017, n=8 p=0.006 and similar but also different to humanised non-thalassaemia control 0.1±0.0031, n=8, p=0.01, One Way ANOVA, Bonferroni's Multiple comparisons test**

The haemoglobin levels in the IUGT 32 Week old group were also higher than the uninjected thalassaemia control animals but not different from the humanised non-thalassaemia control, as seen in Figure 5-34.

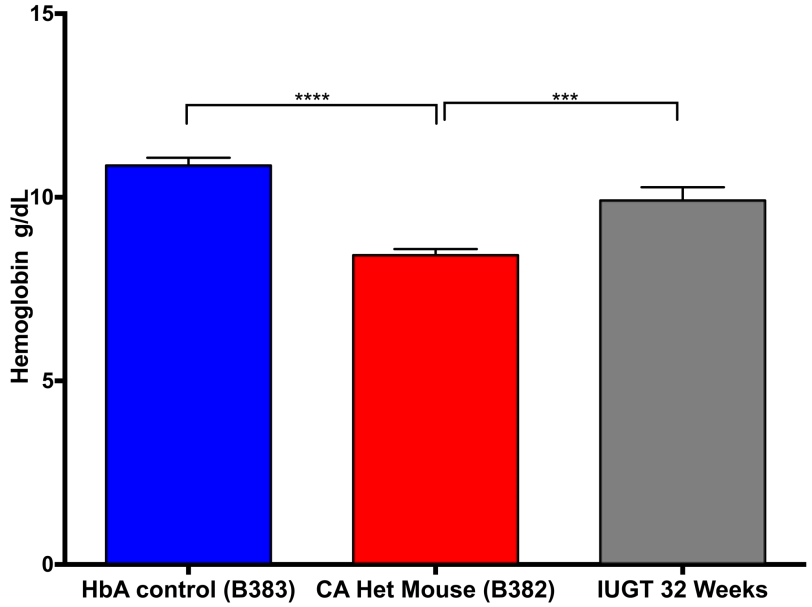



**Figure 5-34 Haemoglobin levels in the IUGT 32 Weeks animals were higher 9.91±0.36, n=8, than un-injected thalassaemia controls 8.42±0.167, n=8 p=0.0053 and similar to humanised non-thalassaemia control 10.87±0.21, n=8, p=0.1, One Way ANOVA, Bonferroni's Multiple comparisons test.**

### 5.5.13 Spleen Volume using MRI techniques can detect normalisation of Spleen size after IUGT 32 Weeks

Spleen Volume to Animal Mass Ration ($mm^3/g$) as measured by MRI was lower in IUGT 32 Weeks animals 5.19±1.20, n=7 than Uninjected Thalassaemia controls 10.97±1.78, n=7, **p=0.015 and similar to Humanised non-thalassaemia control 3.54±0.29, p=0.24. This was a confirmation that spleen volume was reduced, without the need to sacrifice any of the animals (Figure 5-35).

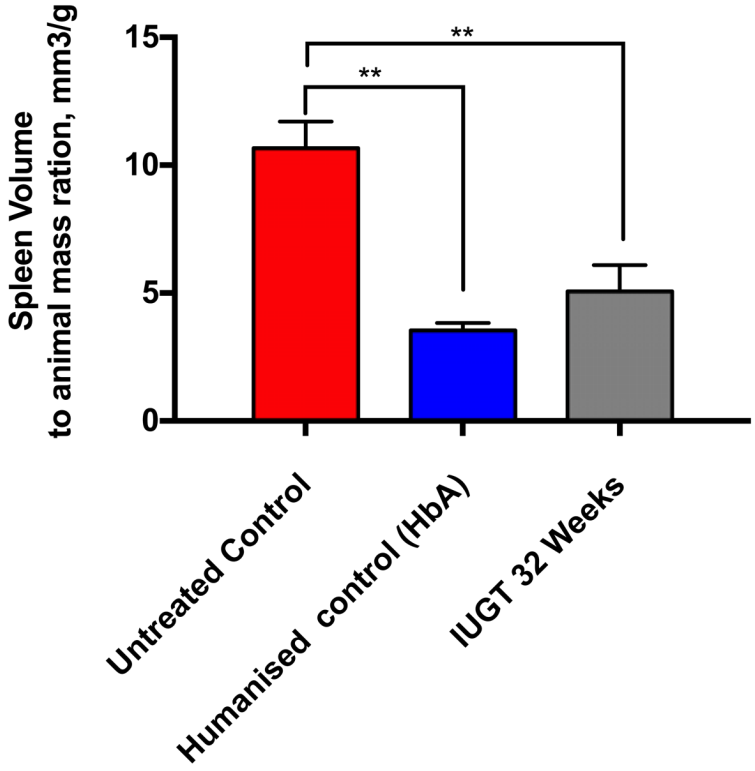



**Figure 5-35 MRI assessment of Spleen Volume to animal mass ratio. IUGT reduced the spleen volume to normal level (ANOVA with Holm-Sidak's multiple comparisons test was used to compare each group, IUGT 32 wks vs Untreated control **p=0.015, HbA vs untreated control **p=0.0021, IUGT 32 wks vs HbA p=0.24**

### 5.5.14 Assessing Heart function using MRI to detect amelioration of disease

The heart stroke volume remained the same after IUGT (0.029±0.0022ml, n=6 versus Uninjected Thalassaemia control 0.03±0.0047ml, n=6, p=0.987 versus Humanised Non-thalassaemia controls 0.032±0.0021ml, n=6, p=0.902, One Way ANOVA, Bonferroni's Multiple comparisons test, Figure 5-36). This confirms that IUGT does not cause any structural defect, which was also confirmed on histopathological examination.

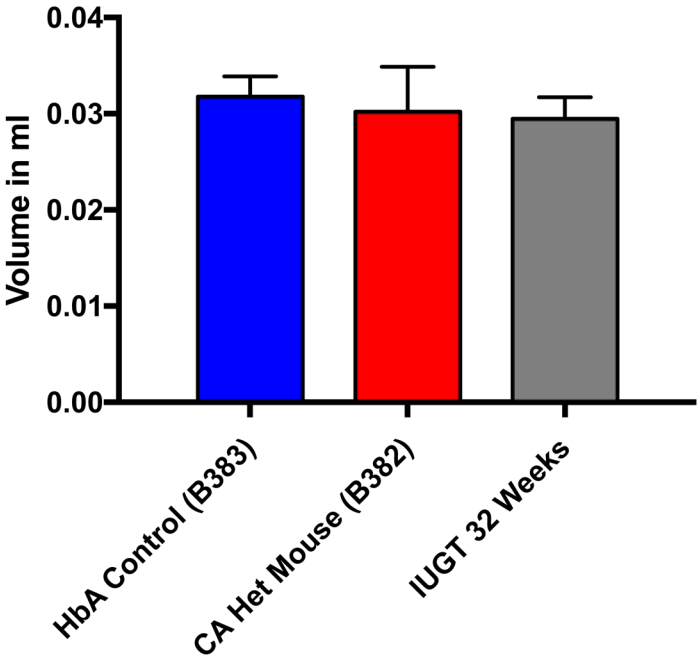



**Figure 5-36 Stroke Volume (in ml) as measured by MRI was not different in any of the groups at 32 weeks of age. IUGT 32 Weeks 0.029±0.0022, n=6 versus Uninjected Thalassaemia control 0.03±0.0047, n=6, p=0.987 versus Humanised Non-thalassaemia control 0.032±0.0021, n=6, p=0.902, One Way ANOVA, Bonferroni's Multiple comparisons test**

Encouraging results have arisen from the finding that the left ventricular ejection fraction (%) was increased in the IUGT 32 Week old group (70.85±1.58, n=6) compared to uninjected thalassaemia (50.40±2.83, n=6, p=0.0024) but was not different from the Humanised non-thalassaemia control (68.00±0.41, n=6, p=0.34, One Way ANOVA, Bonferroni's Multiple comparisons test, Figure 5-37). I hypothesise that the iron accumulation in control non-injected group caused a reduction in the ejection fraction, which did not occur in the IUGT treated animals that had improved cardiac function (Davis et al., 2004; Maggio et al., 2012; Westwood et al., 2007).

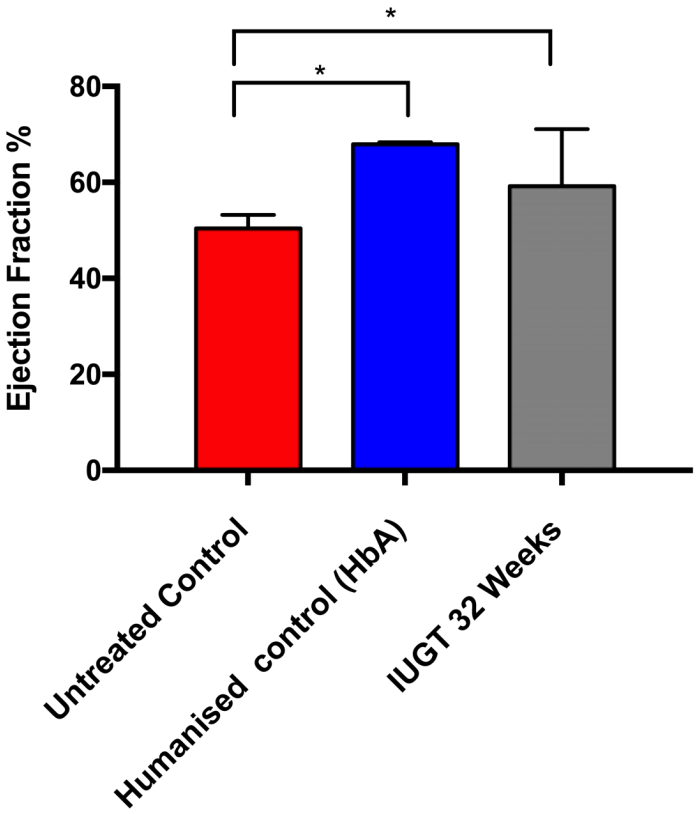



**Figure 5-37 Left Ventricular Ejection Fraction as measured by MRI (%). This was higher in the IUGT 32 Weeks group compared to uninjected thalassaemia control but not different from the Humanised non-thalassaemia control**

The IUGT 32 Weeks animals as well the control animals, were scanned using MRI T1, T2 and T2* relaxation times. The iron accumulation was identified using the method described in the methods section. This technique was correlated with organ dry weight iron, which was done in collaboration with another group at UCL with whom I helped optimise the method(Jackson et al., 2017).

The MRI scanning was done by Dr Laurence Jackson, who provided me with the data collected during the MRI (see representative MRI image Figure 5-38). I analysed the various relaxation times (during MRI), and significant differences were found between the groups.

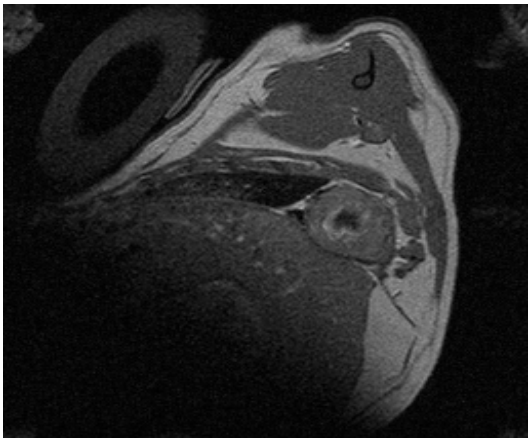


**Figure 5-38 Representative MRI Image used to calculate Left ventricular ejection fraction The left ventricular blood pool was segmented at systole and diastole using Segment v1.8 R0462(Stuckey et al., 2012) and the corresponding volumes used to calculate left ventricular ejection fraction**

T1 relaxation times were shortened in IUGT 32 Weeks in the Heart 1054±118, n=6 compared to Uninjected Thalassaemia Control 1421±91, n=6, p=0.03 but comparable to Humanised Non-Thalassaemia Control 1069 ±44, n=6, p=99, ANOVA with Turkey's multiple comparison test. This highlights the excessive iron accumulation in the B382 heterozygote animals and normalisation of the IUGT animals.

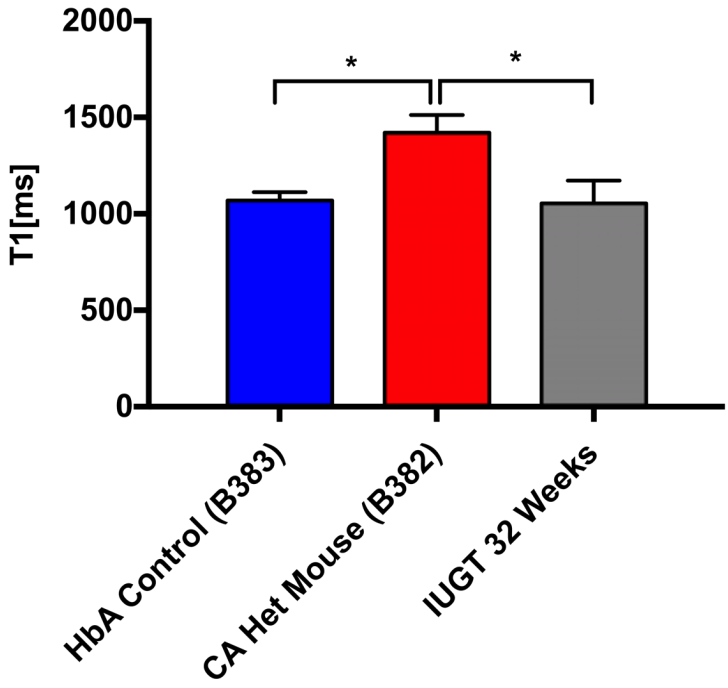



**Figure 5-39 T1 Relaxation time in the heart, showing an increase in the T1 relaxation time of the B382**

T1 relaxation time was shortened in IUGT 32 Weeks in the Liver 858.3±49, n=6 compared to Uninjected Thalassaemia Control 1190±139, n=6, p=0.04 but comparable to Humanised Non-Thalassaemia Control 853±33, n=6, p=0.99. The T1 relaxation time between the untreated control and the humanised non-thalassaemia control was also higher (p=0.03). This can be seen in Figure 5-40. This was not the case for T2 relaxation times, which were shortened in the B382 group (untreated control) instead Figure 5-43.

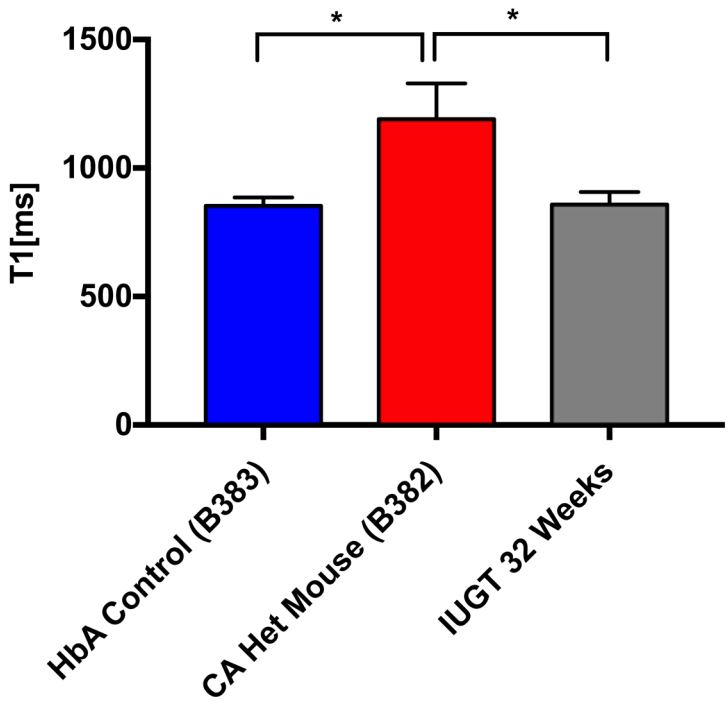



**Figure 5-40 T1 Relaxation times after MRI scanning in the Liver of the animal groups. Healthy Non-thalassaemia control had similar T1 relaxation times with IUGT at 32 weeks of age while the Untreated thalassaemia control had higher relaxation times from both the IUGT 32 weeks and the healthy control groups.**

T2 relaxation time was longer in IUGT 32 Weeks in the Heart 24±5.5, n=6 compared to Uninjected Thalassaemia Control 13±0.83, n=6, p=0.26 and shorter, compared to Humanised Non-Thalassaemia Control 28±2.8, n=6, p=0.49. The T2 relaxation time of the untreated thalassaemia control was lower (p=0.012) than the humanised non-thalassaemia control. This can be seen in Figure 5-41.

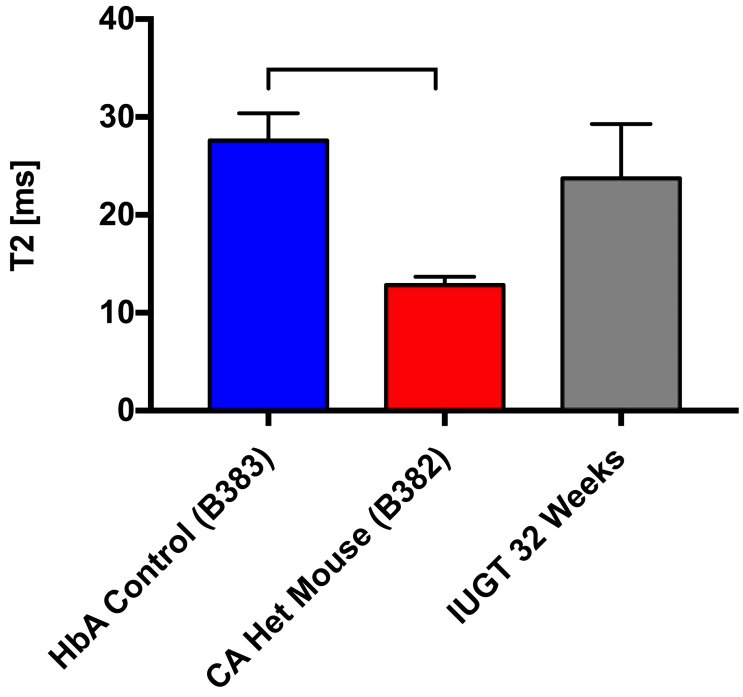



**Figure 5-41 T2 Relaxation times after MRI scanning in the Heart of the animal groups. Healthy Non-thalassaemia control (B383) had higher T2 relaxation times (28±2.8ms) compared to untreated thalassaemia control (B382) (13±0.83ms) (n=6, p=0.03). The T2 relaxation time of the 32 Weeks IUGT group did not have any difference to the HbA control group (24±5.5ms, n=6, p=0.65)**

The T2 and T2* relaxation times in the spleen of the IUGT group were shortened compared to HbA control group, as seen in Figure 5-42 and Figure 5-46. No difference was seen between the IUGT group and the B382 control, which was unexpected. This might be because of the increased breaking down of erythrocytes and subsequently iron accumulation in the spleens of the thalassaemia animals (IUGT and B382). The excess iron accumulation in the thalassaemia animals could be due to dietary iron, and excess accumulation could be specific to the animal model.

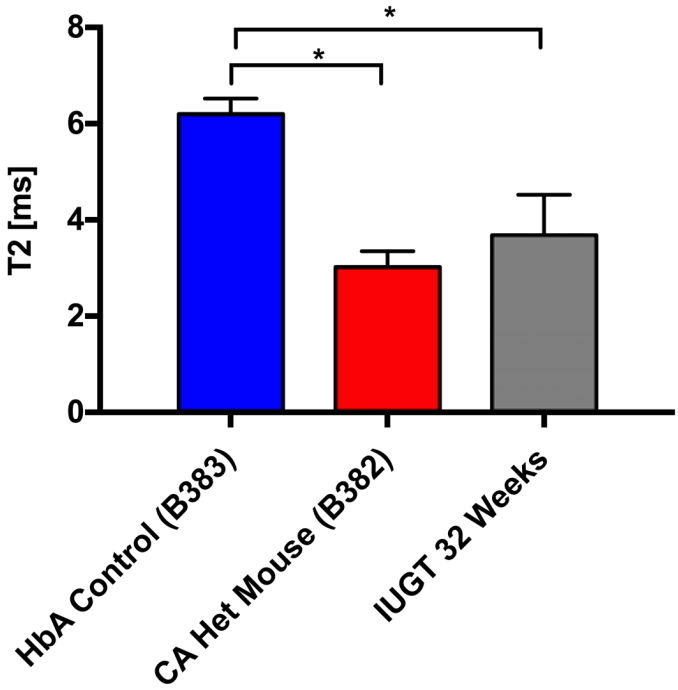



**Figure 5-42 T2 relaxation times after MRI scanning in the Spleen. Healthy Non-Thalassaemia control (B383) had higher T2 relaxation times (6.2±0.32ms) compared to untreated thalassaemia control (B382) (3±0.33ms) (n=6, p=0.03). The T2 relaxation time of the 32 Weeks IUGT group (3.7±0.84ms, n=6) was shortened compared to the HbA control group (*p<0.005)**

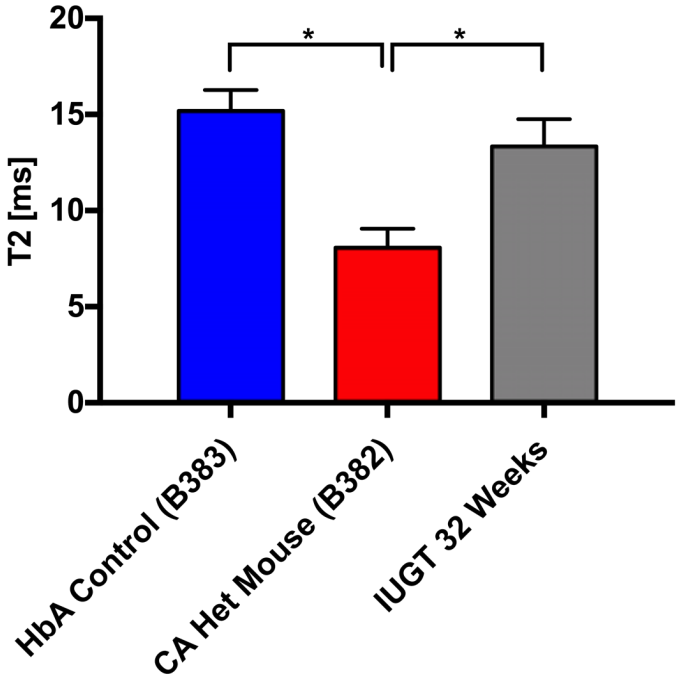



**Figure 5-43 T2 relaxation times after MRI scanning in the Liver. The T2 relaxation time of the 32 Weeks IUGT group (13±1.4ms, n=6) was shorter than the untreated control group (8.1±0.99ms, n=6). (*p<0.05) Healthy Non-Thalassaemia control (B383) had longer T2 relaxation times (15±1.1ms) compared to untreated thalassaemia control (B382)**

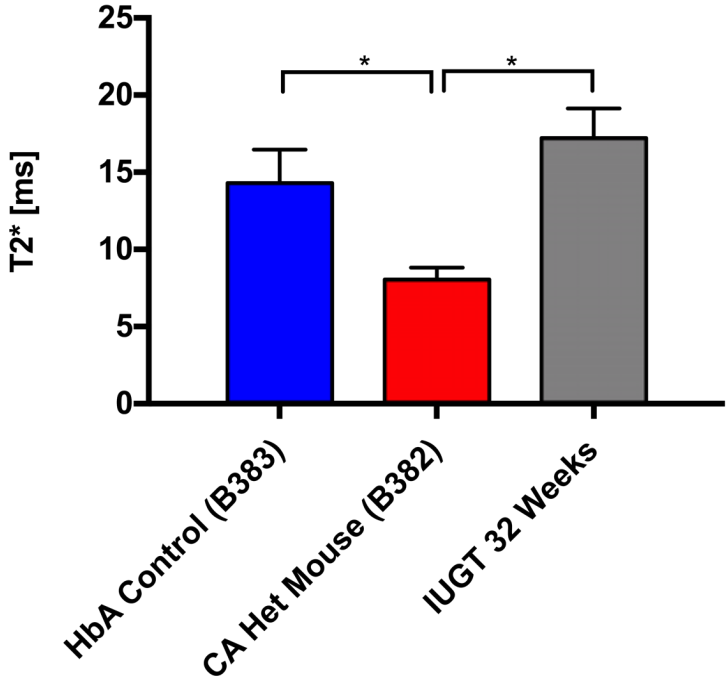



**Figure 5-44 T2* relaxation time in the heart. The T2* relaxation time of the 32 Weeks IUGT group (17±1.9ms, n=6) was prolonged compared to the untreated control group (8±0.77ms, n=6) (*p<0.05). Healthy Non-Thalassaemia control (B383) also had higher T2 relaxation times (14±2.2ms) compared to untreated thalassaemia control (B382)**

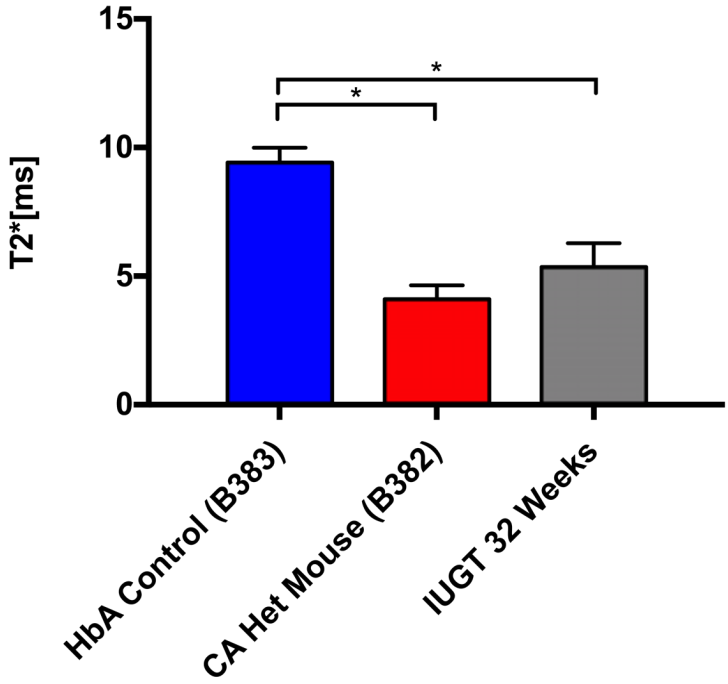



**Figure 5-45 T2* Relaxation time in the Liver. The T2* relaxation time of the 32 Weeks IUGT group (5.4±0.92ms, n=6) had no difference to the untreated control group (4.1±0.55ms, n=6). Healthy Non-Thalassaemia control (B383) had higher T2 relaxation times (9.4±0.57ms) compared to untreated thalassaemia control (B382) (*p<0.05).**

T2* in the spleen of IUGT 32 Weeks were shorter (0.81±0.27, n=6 compared to Humanised Non-Thalassaemia Control 1.72±0.09, n=4, p=0.049) but not different from the uninjected Thalassaemia Control group, 0.60±0.21, n=6, p=0.76

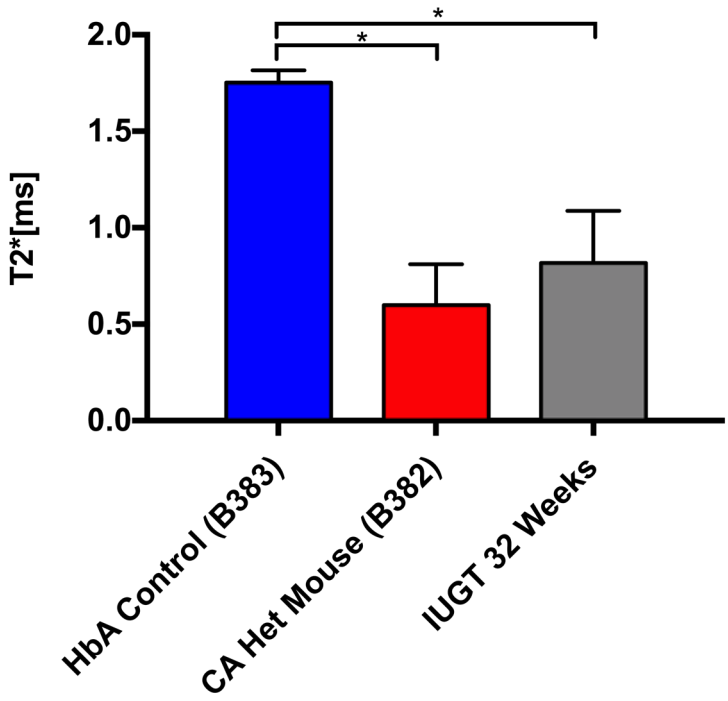



**Figure 5-46 T2* Relaxation time in the spleen. The T2* relaxation time of the 32 Weeks IUGT group (0.82±0.27ms, n=6) had no difference to the untreated control group (0.6±0.21ms, n=6, p=0.16). Healthy Non-Thalassaemia control (B383) had significantly higher T2 relaxation times (1.75±0.063ms) compared to untreated thalassaemia control (B382) (p=0.0011) and IUGT 32 weeks(p=0.0027)**

Even though the T2* in IUGT 32 Weeks was similar to uninjected Thalassaemia Control group, representative Pearl stains from an animal with successful correction of the defect (HB:12.5g/dl) showed less iron pigmentation compared to an untreated thalassaemia control (Hb:9.0g/dl) as seen in Figure 5-47.

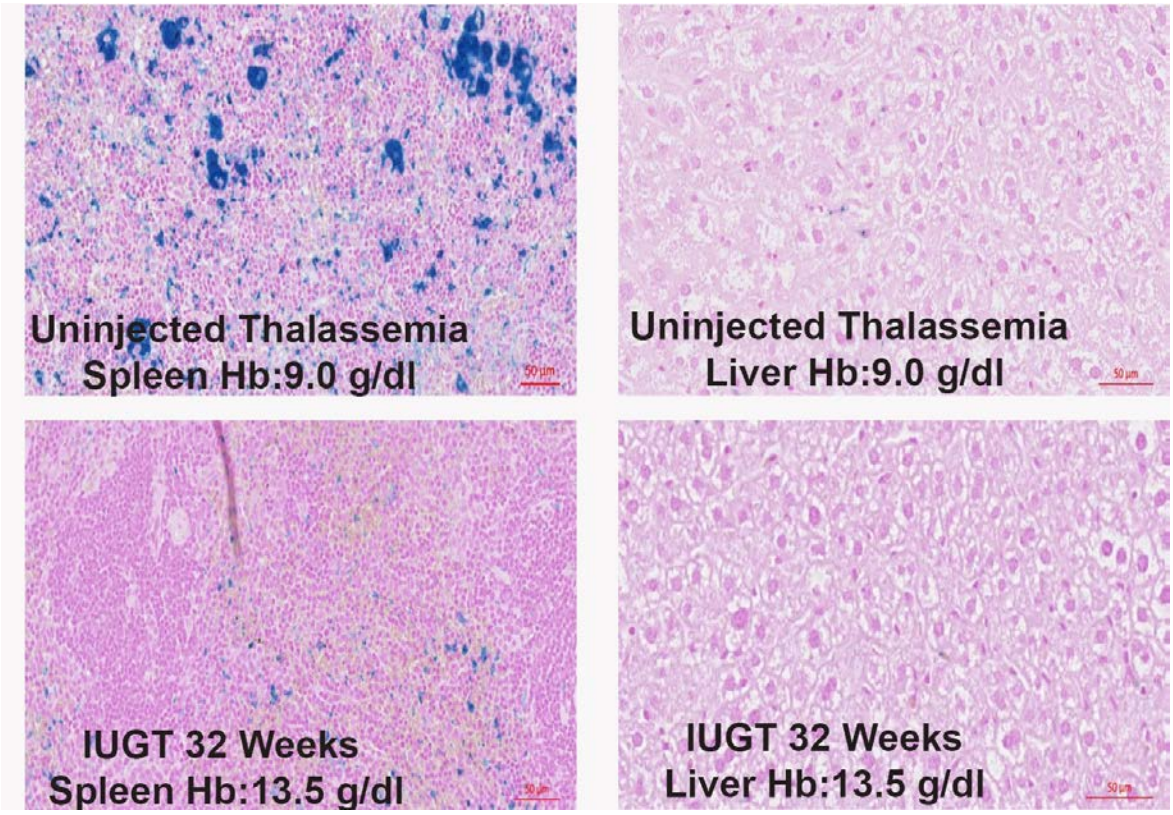



**Figure 5-47 Representative Pearl Stained slides showing increased iron accumulation in the spleen and liver of the uninjected animals compared to IUGT 32 Weeks, (Scale Bar 50µm)**

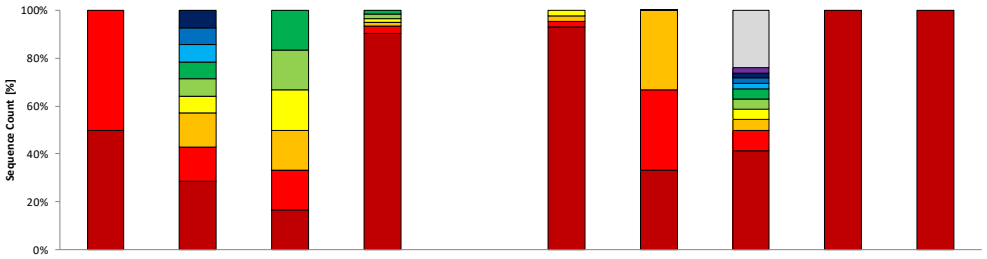
Non Invasive disease progress monitoring is possible with MRI, which corresponds to dry weight measurements (Jackson et al., 2017). For my experiment T2* was more accurate that T1 or T2 in the heart even through T2 had the same trend as T2*. This was also seen when the control experiments were performed with Dr Laurence Jackson (Jackson et al., 2017). The T1 data were paradoxical since the treated and non-thalassaemia control animals had shortened T1 relaxation times instead of longer, which was the case in T2 and T2*. The question is if T1 is able to quantify small iron concentrations as accurate as T2/T2*, or if an error occurred during the acquisition of the data during the MRI scanning in T1, which might be the case since recent data suggest that T1 shows excellent agreement with T2 for determining, at least cardiac iron concentration(Krittayaphong et al., 2017). The presence of iron in the tissue affects the MRI signal by shortening the relaxation times. The discrepancy in th T1 relaxation times was unexpected and this was also reported in the literature where T1 relaxation times were increased in patients with steatohepatitis only but decreased in all

patients with hepatic iron deposition only. While T2 and T2* were consistent T1 relaxation times were increased in the untreated animals which raises the question of a more complex liver, underlying disease, such as concomintant steatosis, in the untreated animals, which was reported in human studies(Henninger et al., 2012).

### 5.5.15 Vector Integration Studies in IUGT 12 Weeks Recipients

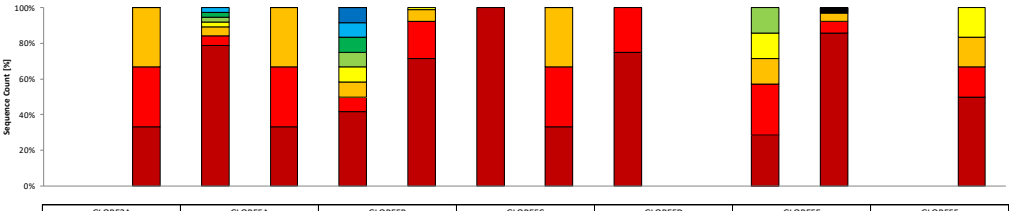
Vector integration studies were done in the animals which were sacrificed at 12 weeks. The integration site analysis was done on bone marrow and liver.

Vector Integration Studies in IUGT 12 Weeks animals showed no homogeneous integration sites. The mean vector copy number (VCN) in the IUGT 12 Weeks group, in the liver, was 0.093±0.14, n=10 and the corresponding integration sites were 60.56±30.73. The VCN in the bone marrow was 0.0059±0.0014, n=10 and the corresponding integration sites were 9.25±4.27.



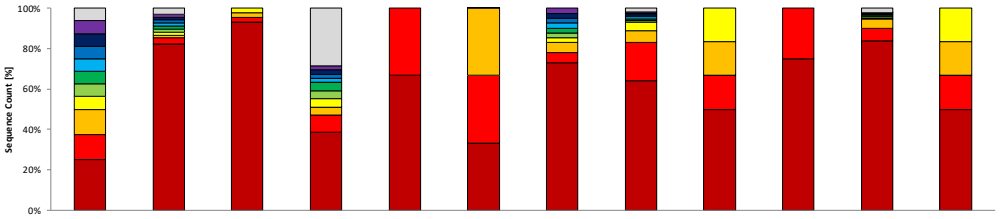

| nr(LAM-PCR) | GL01A | | GL01B | | GL01C | | GL01D | | GL01E | |
|---|---|---|---|---|---|---|---|---|---|---|
| Enzyme | BM | Liver | BM | Liver | BM | Liver | BM | Liver | BM | Liver |
| DNA Input | 200ng | 200ng | 175ng | 200ng | 200ng | 125ng | 100ng | 175ng | 100ng | 75ng |
| **Rank** | | | | | | | | | | |
| Top1 | Tcte1 | Nanos2 | Tasp1 | Scarb2 | | Psma3 | Thsd7b | Batf2 | Oxgr1 | Tpx2 |
| Top2 | Kazn | Cylc2 | Bmp7 | Whsc2 | | Kcnj4 | Lsamp | Cadps | | |
| Top3 | | Ddx6 | D730039F16Rik | Lrrc7 | | Ndfip1 | Cbln2 | Sp110 | | |
| Top4 | | Pde10a | Grid2 | Cdk8 | | Sema3e | | Arhgap9 | | |
| Top5 | | Ddi2 | Nqo1 | Deaf1 | | | | Zfp352 | | |
| Top6 | | 1700009N14Rik | Itm2a | Epm2aip1 | | | | Plcxd2 | | |
| Top7 | | Grm3 | | | | | | Deptor | | |
| Top8 | | AI593442 | | | | | | Alcam | | |
| Top9 | | Magix | | | | | | Dok6 | | |
| Top10 | | | | | | | | Cytip | | |
| #All Other mapp. IS | 0 | 0 | 0 | 0 | | 0 | 0 | 11 | 0 | 0 |
| Seq Count 10 Strongest | 2 | 14 | 6 | 62 | | 44 | 3 | 35 | 1 | 2 |
| Seq Count all other mapp. IS | 0 | 0 | 0 | 0 | | 0 | 0 | 11 | 0 | 0 |
| Total Seq Counts Used | 2 | 14 | 6 | 62 | | 44 | 3 | 46 | 1 | 2 |

**Figure 5-48 Estimation of clonal Size on the 12 week recipients (a)**




| nr(LAM-PCR) | GLOBE2A | | GLOBE5A | | GLOBE5B | | GLOBE5C | | GLOBE5D | | GLOBE5E | | GLOBE5F | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Enzyme | BM | Liver | BM | Liver | BM | Liver | BM | Liver | BM | Liver | BM | Liver | BM | Liver |
| DNA Input | 200ng | 175ng | 250ng | 300ng | 250ng | 22ng | 250ng | 45ng | 75ng | 20ng | 75ng | 50ng | 125ng | 30ng |
| Rank | | | | | | | | | | | | | | |
| Top1 | | B3gnt2 | Grin2a | Akr1cl | Ctnnd2 | Peg12 | Ccnh | Grm1 | Socs6 | | Lsm12 | Lars2 | | Brsk2 |
| Top2 | | Dab1 | Slitrk6 | Setdb2 | Gabrg2 | Pcdh20 | | Lrrc4c | Slc39a11 | | Dpysl3 | Tjap1 | | Ube2e2 |
| Top3 | | 4930544G11Rik | Med13l | Zdhhc14 | 2010015L04Rik | Il3ra | | Mmp16 | | | Gsdmc | Rabgap1l | | Cdh12 |
| Top4 | | | Entpd6 | | Lrrc66 | Retsat | | | | | Actl9 | Glra2 | | Rgma |
| Top5 | | | Itga6 | | Tfec | | | | | | Nhs | Bcas3 | | |
| Top6 | | | Nkd1 | | Gabrg3 | | | | | | | Abca13 | | |
| Top7 | | | D330045A20Rik | | BC030500 | | | | | | | Flrt2 | | |
| Top8 | | | | | Gadl1 | | | | | | | Lrrc3b | | |
| Top9 | | | | | | | | | | | | Exosc10 | | |
| Top10 | | | | | | | | | | | | Cntnap4 | | |
| #All Other mapp. IS | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 1 | | 0 |
| Seq Count 10 Strongest | | 3 | 38 | 3 | 12 | 105 | 3 | 3 | 4 | | 7 | 288 | | 6 |
| Seq Count all other mapp. IS | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | 0 | 1 | | 0 |
| Total Seq Counts Used | | 3 | 38 | 3 | 12 | 105 | 3 | 3 | 4 | | 7 | 289 | | 6 |

**Figure 5-49 Estimation of clonal Size on the 12-week recipients (b)**




| Rank | GL01A | GL01B | GL01C | GL01D | GL01E | GLOBE2A | GLOBE5A | GLOBE5B | GLOBE5C | GLOBE5D | GLOBE5E | GLOBE5F |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Top1 | Nanos2 | Scarb2 | Psma3 | Batf2 | Tpx2 | B3gnt2 | Grin2a | Peg12 | Ccnh | Socs6 | Lars2 | Brsk2 |
| Top2 | Cylc2 | Whsc2 | Kcnj4 | Cadps | Oxgr1 | Dab1 | Slitrk6 | Pcdh20 | Grm1 | Slc39a11 | Tjap1 | Ube2e2 |
| Top3 | Ddx6 | Tasp1 | Ndfip1 | Sp110 | | 4930544G11Rik | Med13l | Il3ra | Lrrc4c | | Rabgap1l | Cdh12 |
| Top4 | Tcte1 | Bmp7 | Sema3e | Arhgap9 | | | Akr1cl | Ctnnd2 | Mmp16 | | Lsm12 | Rgma |
| Top5 | Pde10a | D730039F16Rik | | Zfp352 | | | Setdb2 | Gabrg2 | | | Dpysl3 | |
| Top6 | Ddi2 | Lrrc7 | | Plcxd2 | | | Zdhhc14 | 2010015L04Rik | | | Glra2 | |
| Top7 | Kazn | Cdk8 | | Deptor | | | Entpd6 | Lrrc66 | | | Flrt2 | |
| Top8 | 1700009N14Rik | Grid2 | | Lsamp | | | Itga6 | Tfec | | | Lrrc3b | |
| Top9 | Grm3 | Deaf1 | | Alcam | | | Nkd1 | Retsat | | | Gsdmc | |
| Top10 | AI593442 | Nqo1 | | Cbln2 | | | D330045A20Rik | Gabrg3 | | | Actl9 | |
| #All Other mapp. IS | 1 | 2 | 0 | 14 | 0 | 0 | 0 | 2 | 0 | 0 | 6 | 0 |
| Seq Count 10 Strongest | 15 | 66 | 44 | 35 | 3 | 3 | 41 | 115 | 6 | 4 | 290 | 6 |
| Seq Count all other mapp. IS | 1 | 2 | 0 | 14 | 0 | 0 | 0 | 2 | 0 | 0 | 6 | 0 |
| Total Seq Counts Used | 16 | 68 | 44 | 49 | 3 | 3 | 41 | 117 | 6 | 4 | 296 | 6 |

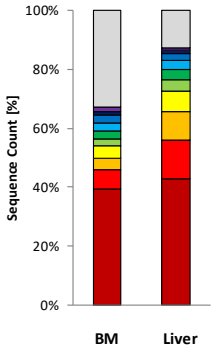
**Figure 5-50 Estimation of clonal Size by subject on the 12-week recipients**

No homogeneous integration sites were found during the vector integration site analysis. Cumulatively the number of integration sites in the liver were 504 and in the bone marrow 51. The strongest integration sites were (www.genecards.com):

- **Grin2a0:** provides instructions for making a protein GLuN2A which is found in nerve cells
- **Ctnnd2:** The *CTNND2* gene provides instructions for making a protein called delta-catenin. This protein is active in the nervous system, where it likely helps cells stick together (cell adhesion) and plays a role in cell movement
- **Socs6:** (Suppressor of Cytokine Signalling 6) is a Protein Coding gene. Among its related pathways are Jak-Stat and Kit receptor signalling pathway.

- **Ccnh:** A protein coding gene. The protein encoded by this gene belongs to the highly conserved cyclin family, whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle.
- **Lsm12:** Aprotein coding gene.
- **Dpysl3:** DPYSL3 (Dihydropyrimidinase Like 3) is a Protein Coding gene associated with hydrolase activity and phosphoprotein binding.
- **Slitrk6:** This gene encodes a member of the SLITRK protein family. Members of this family are integral membrane proteins that are characterized by two N-terminal leucine-rich repeat (LRR) domains and a C-terminal region that shares homology with trk neurotrophin receptors. This protein functions as a regulator of neurite outgrowth required for normal hearing and vision. Mutations in this gene are a cause of myopia and deafness.
- **Med12l:** The protein encoded by this gene is part of the Mediator complex, which is involved in transcriptional coactivation of nearly all RNA polymerase II-dependent genes. The Mediator complex links gene-specific transcriptional activators with the basal transcription machinery.
- **Gsdmc:** A protein coding gene. The N-terminal moiety promotes pyroptosis. May be acting by homooligomerizing within the membrane and forming pores.
- **Lsamp:** This gene encodes a member of the immunoglobulin LAMP, OBCAM and neurotrimin (IgLON) family of proteins. The encoded preproprotein is proteolytically processed to generate a neuronal surface glycoprotein. This protein may act as a selective homophilic adhesion molecule during axon guidance and neuronal growth in the developing limbic system. The encoded protein may also function as a tumor suppressor and may play a role in neuropsychiatric disorders.
- **Lars2:** This gene encodes a class 1 aminoacyl-tRNA synthetase, mitochondrial leucyl-tRNA synthetase. Each of the twenty aminoacyl-tRNA synthetases catalyzes the aminoacylation of a specific tRNA or tRNA isoaccepting family with the cognate amino acid.

- **Peg12:** The protein encoded by this gene belongs to the GSK-3-binding protein family. The protein inhibits GSK-3-mediated phosphorylation of beta-catenin and positively regulates the Wnt signaling pathway. It may function in tumor progression and in lymphomagenesis.
- **Scarb2:** The protein encoded by this gene is a type III glycoprotein that is located primarily in limiting membranes of lysosomes and endosomes.
- **Psma3:** This is a protein coding gene related to cell cycle.
- **Pcdh20:** This gene belongs to the protocadherin gene family, a subfamily of the cadherin superfamily. This gene encodes a protein which contains 6 extracellular cadherin domains, a transmembrane domain and a cytoplasmic tail differing from those of the classical cadherins. Although its specific function is undetermined, the cadherin-related neuronal receptor is thought to play a role in the establishment and function of specific cell-cell connections in the brain
- **Tjap1:** This gene encodes a tight junction-associated protein
- **Batf2:** This is a protein-coding gene associated with DNA binding transcription factor activity and sequence-specific DNA binding.
- **Rabgap:** This is a protein-coding gene.
- **Il3ra:** This is a protein-coding gene associated with cytokine receptor activity and interleukin-3 receptor activity.
- **Nanos2:** Plays a key role in the sexual differentiation of germ cells by promoting male fate but suppressing female fate. Represses the female fate pathways by suppressing meiosis, which in turn results in the promotion of the male fate — required for the maintenance of the spermatogonial stem cell population.




| Rank | | BM | Liver |
|---|---|---|---|
| Top1 | | Grin2a | Lars2 |
| Top2 | | Ctnnd2 | Peg12 |
| Top3 | | Socs6 | Scarb2 |
| Top4 | | Ccnh | Psma3 |
| Top5 | | Lsm12 | Pcdh20 |
| Top6 | | Dpysl3 | Tjap1 |
| Top7 | | Slitrk6 | Batf2 |
| Top8 | | Med13l | Rabgap1l |
| Top9 | | Gsdmc | Il3ra |
| Top10 | | Lsamp | Nanos2 |
| #All Other mapp. IS | | 25 | 59 |
| Seq Count 10 Strongest | | 51 | 504 |
| Seq Count all other mapp. IS | | 25 | 73 |
| Total Seq Counts Used | | 76 | 577 |

**Figure 5-51 Estimation of clonal size by tissue**

In conclusion, the SI study highlighted the importance of investigating the specific vector in each biological application (IUGT) before human use. Even though oncogenesis through viral genome integration is intrinsically less likely in terminally differentiated cells, this is not the case for rapidly dividing cells of the haematopoietic lineage (Alton et al., 2017).

Themis and colleagues found a high incidence of hepatocellular carcinoma in mice receiving intravenous vitelline vein injection of the Equine Immune Anaemia Virus (EIAV) SMART 2hFIX, SMART 2Z, and SMART 3NZ lentiviral vectors *in* utero, but no oncogenesis was observed in HIV-1 based vector injections using the same route (Themis et al., 2005). Thus the particular vector type may be of importance in the development of oncogenesis. Further studies suggest that the fetal mouse may be a sensitive genotoxicity model that exposes particular lentiviral-associated mutagenesis resulting in liver oncogenesis(Nowrouzi et al., 2013). I observed integration to Peg12 gene, which is associated with tumorigenesis, but no hepatocellular carcinomas were observed at any time point. I did observe that IUGT animals were significantly heavier than non-thalassemic controls at 32 weeks. A possible explanation is an integration to the gene SOCS-6, which was observed during the site integration studies. Socs-6 is involved in IGF-1 signalling and integration to its site might have caused

overexpression of the gene resulting in a significant weight difference(Kabir et al., 2014; Krebs et al., 2002). Also, SI was observed in genes which are related to the development and function of the nervous system and also spermatogenesis. A future repeat study should include an assessment of the neural development of the offspring and also their reproductive potential, especially in males.

Vectors need to be assessed for safety and show that they have low oncogenic potential, before gene therapy application (Montini et al., 2006). This is greatly important since, in *in-vivo* transduction, the integration profile differs from ex-vivo gene delivery, especially because of the different genome organisation. The safety of such a system needs to be addressed(Bartholomae et al., 2011).

### 5.5.16 Vector Copy number in comparison with Vector Integration in IUGT 12 Weeks

Vector integration was compared with Vector copy number in individual animals in both liver and bone marrow, as demonstrated in Figure 5-52 and Figure 5-53. The vector copy number was relatively low compared to other similar published studies (Levasseur et al., 2003; Pawliuk et al., 2001), where the Vector copy number was 1.9. This might be a reason why the rescue of the homozygote animals was not possible.

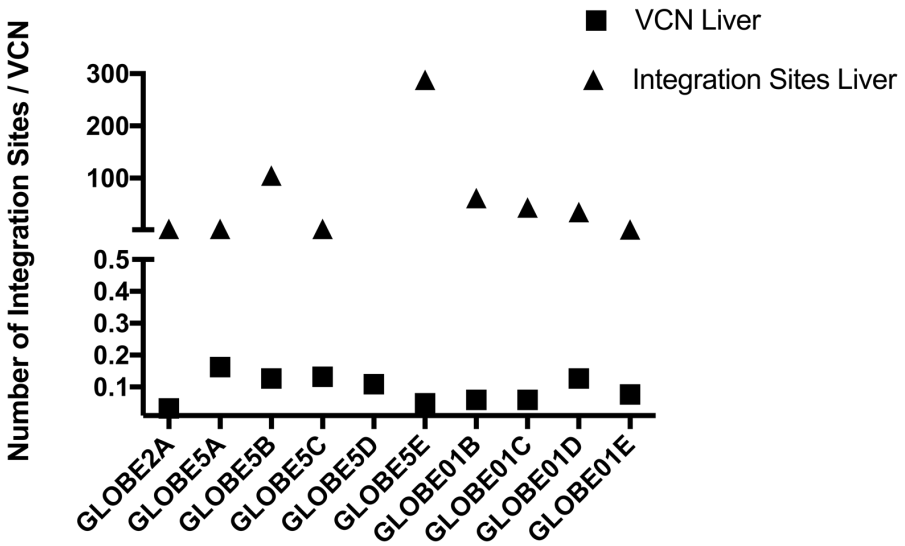



**Figure 5-52 Number of SI in the Liver showing an average VCN in the liver of 0.093±0.013. The SI was 60.56±30.73.**

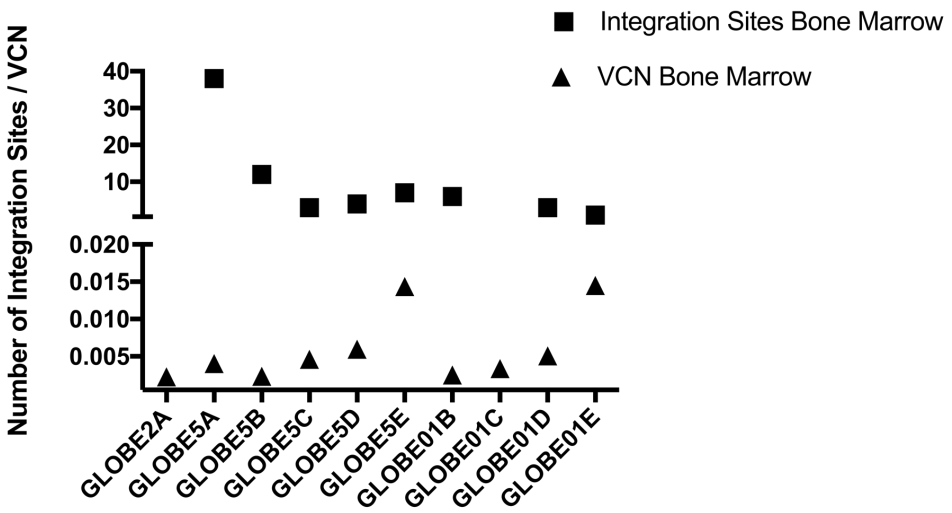



**Figure 5-53 Number of SI in the Bone Marrow showing an average SI of 9.25±4.27 with a VCN of 0.006±0.0015**

### 5.5.17 Vector Copy number in homozygote CA animal treated with IUGT (experiment done with Dr Christina Flouri)

A male homozygote animal identified (Figure 5-54) at P14 when it showed signs of illness. The weight of the animal was 3.4 grams, while the heterozygote littermates' weight was 6.9 grams. The haemoglobin was measured by HemoCue, and it was low at 6g/dL.

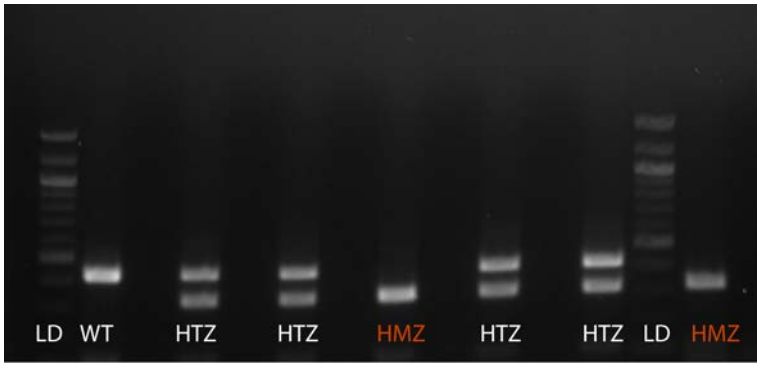



**Figure 5-54 Genotyping gel showing the presence of a homozygote animal culled at 14 days of gestation. WT: Wild Type, HTZ: Heterozygote, HMZ: Homozygote, LD: Ladder**

The VCN per cell was calculated as seen in Table 5-1. The VCN in the liver (0.0075) was lower than the VCN of the animals sacrificed at 12 weeks (0.093±0.013). The VCN in the bone marrow was also lower (0.0026) than the 12-week animals (0.006±0.0015). This low VCN might contribute to the inability of the IUGT to rescue the homozygote animal.

| P14 Homozygote | Peripheral Blood | Bone Marrow | Liver | Spleen | Heart |
|---|---|---|---|---|---|
| **Average VCN per Cell** | 0.048 | 0.0026 | 0.0075 | 0.0012 | 0.0041 |

**Table 5-1 Average VCN per cell in the organs of a homozygote humanised mouse model culled at P14**

In conclusion, the VCN, as well as the SI, were low. This could be due to the IUGT technique itself, i.e. an IV route into the vitelline vein might give a higher VCN (Boelig et al., 2016). A higher viral titre would also be beneficial to improve the transduction efficiency of the HSCs. An ex-vivo approach could be ideal since the gene delivery will be targeted toward haematopoietic cells only and unnecessary SI could be prevented, similarly to the adult gene therapy clinical trial (Thompson et al., 2018). This might be challenging because of the timeframe and selection of the appropriate cells for correction, i.e. amniotic fluid stem cells, fetal liver stem cells, bone marrow, autologous or allogenic (Loukogeorgakis and Flake, 2014; Ramachandra et al., 2014).

## 5.6 Discussion

In this study, I show successful phenotypic correction of thalassaemia intermedia in a humanised mouse model using an *in-utero* gene therapy approach. The normalisation of haemoglobin and spleen size and a reduction in the level of extramedullary haematopoiesis were the main findings of this preclinical study. I proved that there was a successful fetal to adult haemoglobin switch in the treated mice, as this was necessary to demonstrate that the improved phenotype was due to the IUGT. *In vivo* mouse MRI was employed to monitor the disease(Jackson et al., 2017), and showed improved cardiac function in the IUGT treated pups, even at 32 weeks after birth. I hypothesise that this was mediated through reduced iron accumulation in the heart.

The target cell niche in this study was the expanding population of fetal liver haematopoietic stem cells. There are intrinsic differences between fetal and adult HSCs properties especially fetal liver HSCs which can give rise to thymocytes, especially Tregs which are responsible for promoting tolerance to self and potentially foreign antigen(Mold et al., 2010). The fetal to

adult switch in these HSC properties occurs between week one and two after birth in mice. During fetal life, the HSC pools in the fetal liver are actively cycling and rapidly expanding, whereas, once the HSCs reach the fetal bone marrow, they become quiescent. This property of fetal HSC pools in the liver and their potential of thymic repopulation may be an added advantage for intrahepatic IUGT (Burt et al., 2010) which is easily able to direct gene transfer to the fetal liver through direct injection. Fetal liver HSCs are also capable of repopulating five times more effectively long term, than short term(Harrison et al., 1997). Any manipulation of liver HSCs should have a long term effect, ideally permanent, especially if these cells migrate to the thymus(Mold et al., 2010). Intrahepatic fetal gene transfer to haematopoietic progenitors has also been demonstrated in primates, where the maximum effect was achieved using lentiviral vectors(Tarantal, O'Rourke, et al., 2001).

In this study, I achieved correction of haemoglobin with a low VCN of 0.093 in the liver. This is similar to the findings of Miccio et al. (Miccio et al., 2008) where correction of haemoglobin was achieved with an HSC VCN of <1 (0.2-0.9)(Miccio et al., 2008) in adult mice using *ex vivo* transduction of CD34+ HSCs before reinfusion in an autologous setting. The liver VCN in this study is 5-10 times lower than the HSC VCN that Miccio et al. observed(Miccio et al., 2008). This may be due to the use of direct *in utero* gene therapy, taking into advantage the high proliferation of the fetal HSCs which resulted in a large number of transduced daughter cells that were able to correct the phenotypic Hb defect. It was not possible to do individual sorting of HSCs from each transduced mouse due to the minimal amount of tissue collected and the analysis initially planned such as EMH, histology and HPLC. Thus, I was unable to calculate the VCN in the HSC population and instead present data on the VCN for the whole heterogeneous population of hepatocytes and whole bone marrow. Previous studies have found low genotoxicity of lentiviral vector integration in mice that are prone to developing tumours (Montini et al., 2006). Nevertheless, it is an important safety consideration to keep the VCN as low as possible while maintaining the efficacy of the gene transfer.

The humanised mouse model of beta thalassaemia (Huo et al., 2010, 2017; Huo, McConnell, Liu, et al., 2009; McConnell et al., 2011) that I used is unique in that the fetal mice produce human gamma-globin (human fetal haemoglobin) *in utero* (Huo et al., 2010), and have a

human gamma to beta switching cassette which results in the death of homozygous animals within two weeks of birth, as the production of defective beta globin takes over. Homozygous animals can be rescued postnatally using regular blood transfusions(Huo, McConnell and Ryan, 2009). This model allows human beta-globin producing vectors to be tested *in utero* (Bank et al., 2005; Frittoli et al., 2011; Miccio et al., 2008; Roselli et al., 2010), and since the *in utero* phenotype is less severe than of other thalassaemia mouse models, e.g. *th3*, it allows the evaluation of IUGT to rescue the mouse model *in utero* and post-natally(Rivella et al., 2003; Vogiatzi et al., 2010).

In the first cohort (12 Weeks), where the animals were analysed at 12 weeks after birth, I observed normalisation of the phenotype with functional correction of anaemia, excessive extramedullary haematopoiesis and the other abnormal haematological indices. The presence of normal blood film in the treated animals compared to untreated was also a confirmatory result. To confirm the presence of normal Hb I used real-time PCR and high-performance liquid chromatography to demonstrate beta-globin gene expression with downregulation of the gamma-globin gene as well as the extra peak from the beta globin chain in HPLC. These results excluded the hereditary persistence of fetal haemoglobin, which could have contributed to the phenotypic correction of the overall haemoglobin.

In the long term 32-week postnatal group, the phenotypic correction appeared to be less effective as in the group analysed at 12 weeks after birth, suggesting that the effect may reduce over time. This could be due to transcriptional silencing of the transgene (Bestor, 2000) or even due to epigenetic changes leading to deactivation of the beta-globin gene as the pups grew (Costa et al., 2015; Ginder, 2014). This was evident in the lower Hb concentration at the later time point (9.91±0.36g/dl at 32 weeks' vs 11± 0.22g/dl at 12 weeks), although the haemoglobin concentration at 32 weeks post-delivery was still significantly higher than that of age-matched controls. Histological analysis of the animals by Dr Joy Archer at the University of Cambridge showed the correlation of spleen hyperplasia with iron deposits in the spleen, moderate anisocytosis and anaemia.

In the long term group, in collaboration with the group of Dr Laurence Jackson, I used MRI as a novel method of monitoring *in utero* gene therapy functional success (left ventricular

ejection fraction, spleen volume, iron overload)(Anderson et al., 2001; Delo et al., 2008; Porter and Shah, 2010; Stuckey et al., 2012). I found that MRI can be used to monitor the disease progress, using T2 or T2* without sacrificing the animal.

I did not observe any rescued homozygous pups in the genotyped litters; indeed, given the dam numbers and from previous studies in this specific humanised mouse model, I would have expected to have at least 5-6 (25% of the total) homozygous pups if they were corrected. The only homozygote pup had to be culled at 14 days of age, due to severe illness, and haemoglobin measurement showed severe anaemia. All live cross-fostered pups survived to genotype. It is possible either that the injected homozygous pups demised *in utero* after the intrahepatic surgery due to a higher susceptibility to miscarriage which, was also seen in the control experiments I performed initially, or in the first hours after birth before they were cross-fostered. This might be because homozygote animals, if not rescued, will become unwell, after the *in-utero* injection and die. If born, the mother will recognise interference, and subsequently, the new-born pups get rejected or attacked by their mother. This was reported in other studies where fetal intervention occurred(Hutton et al., 2009).

Future studies using a higher titre of the vector will be conducted to determine whether this improves survival in the homozygous pups.

In this study, I show that targeting the specific stem cell niche(Coutelle et al., 2005; Waddington et al., 2007) early enough in gestation with gene therapy could be critical especially if most of the haematopoietic stem cells are within the compartment, in this case, the liver. Alternative routes of injection such as intraperitoneal or intravascular, may not deliver a sufficiently high number of vector particles to the liver compartment and its HSCs to achieve phenotypic cure. An IV route might be ideal since it can improve the survival rate and delivery of the transgene (Boelig et al., 2016). Importantly for clinical translation, the tissues were studied by an expert (Dr Joy Archer), and no animals were found to have malignancy, which is in keeping with other studies using this stable GLOBE vector (Miccio et al., 2008; Thompson et al., 2018). It is reassuring that the GLOBE vector is currently used in a Phase II clinical trial, using autologous stem cell/ gene therapy approach in adult patients (Thompson et al., 2018). The number of vector integration sites corresponded to the vector

copy number, even though this was low. This could be a sign that if higher VCN is achieved, the level of haemoglobin could also rise. The integration site analysis revealed integration associated with the neural system, stem cells of spermatogenesis and also to an oncogene. This must be carefully studied since the potential of tumour formation is high(Themis et al., 2005). This also supports the use of an *ex-vivo* gene therapy approach using amniotic fluid stem cells or other highly efficient HSC progenitors (Ramachandra et al., 2014).

I chose to deliver the IUGT at embryonic day (E13.5) just before the time of HSC fetal bone marrow colonization in mice, which occurs around E16-E17(Coşxkun et al., 2014), and which corresponds to an equivalent of 10 to 12 weeks of gestation in a human fetus(Otis and Brent, 1954). Fetal gene transfer to the haematopoietic compartment (Klein et al., 2003) may offer a third option to couples with a prenatal diagnosis of a fetal congenital blood disorder, especially in the beta haemoglobinopathies (Payen and Leboulch, 2012). For human therapy, non-invasive prenatal diagnostic techniques are being developed using the maternal blood to diagnose thalassaemia from 8-10 weeks of gestation (Zafari et al., 2016). This would allow enough time to deliver gene therapy to the human fetus using ultrasound-guided intrahepatic injection. Since no *ex-vivo* expansion and correction of the appropriate haematopoietic progenitor is required, this could even be applied in low-income countries where the palliative cost of homozygous patients is unsustainable for the local health systems(Ho et al., 2006; Karnon et al., 1999; Riewpaiboon et al., 2010).

# Chapter 6 Discussion

## 6.1 Summary of main findings

During my PhD, I performed a series of experiments in normal mice investigating whether there was a difference in engraftment between IUT of congenic and allogenic derived mAF HSCs. I demonstrated the haematopoietic properties of mAF and the production of haemoglobin when cultured in erythroid differentiation conditions. Freshly isolated mAF HSCs express pluripotency and haematopoietic markers. I showed that there is an engraftment advantage if congenic mAF HSCs are used and that most of the cells populate the haematopoietic system. Donor cell engraftment and characterisation was assessed by flow cytometry.

Chimerism in the blood and bone marrow was observed in 100% of pups transplanted with congenic cells and in 29% of pups with allogenic cells. At 4 and 16 weeks of age, engraftment was higher in congenic vs allogenic transplanted animals.

I optimised mAF HSC culture and developed a method to expand cells and maintain their haematopoietic potential *in vitro* for up to 7 days. I studied the phenotype of these cells using flow cytometry.

I then hypothesised that IUGT to the fetal HSC compartment with the corrected β globin gene might cure thalassaemia disease before birth**.** Compared to noninjected heterozygous pups (control), IUGT increased haemoglobin levels. Moreover, treated animals had reduced spleen weight, as well as reduced extramedullary haematopoiesis in the liver and spleen. qPCR analysis demonstrated increased gene expression of human β globin and reduced expression of human γ globin of IUGT offspring. HPLC analysis confirmed these findings at the protein level. IUGT in this study resulted in phenotypic normalisation in a heterozygous humanised mouse model of thalassaemia. Increased levels of β globin and associated downregulation of γ globin confirmed successful prenatal correction of the genetic defect.

## 6.2 Expansion of HSCs and IUT using mAF HSCs

### 6.2.1 The purity of mAF HSCs

Initially, I isolated mAF from pregnant dams at E13.5. Isolation of human and mouse AF HSCs was previously described by De Coppi et al. (De Coppi, Bartsch, et al., 2007). While their haematopoietic properties cannot be maintained if cultured in Chang C medium as described by previous authors (De Coppi, Bartsch, et al., 2007; Delo et al., 2006; Grisafi et al., 2008), fresh hAF & mAF HSCs do have haematopoietic potential (Ditadi et al., 2009; Shangaris et al., 2018; Shaw et al., 2015). Isolation of mAF was challenging. This was done carefully to ensure that the fluid collected was coming from the fetus itself and that maternal contamination was excluded. This could have been better designed by using GFP or YFP males and C57BL6 (non-GFP/YFP) females. This would have given me a pure cell population originating from the fetal mAF if selected for YFP/GFP, similar to the study by A. Ditadi et al. (Ditadi et al., 2009). Unfortunately, the transplantation model I used, did not allow this breeding since I used the CD45.1/45.2 (Ha et al., 2010), as congenic and $H2K^{d/b}$ HSC (allogenic) transplantation models instead. This could have been done by using the CD45.1/45.2 models and breeding C57BL6 female mice to CD45.1 male and selecting for CD45.1 during the isolation of mAF HSC (Merianos and Tiblad, 2009). This was only considered after the end of the research time and when the first paper went out for peer review; this could be considered a weakness of the study.

The purity of mAF HSC was determined using flow cytometry after MACS selection and negative depletion. Only 1-2% of the AF cells were positive for the CD117 marker when studied using flow cytometry. This was in line with findings from other groups (Ditadi et al., 2009; Shaw et al., 2015). When the purity of the MACS selected cells was tested, the result was not as encouraging, with only around 82% positive for CD117. This was addressed by using FACS whenever possible instead of MACS. FACS allows sorting of the cell population of interest with minimal contamination from debris or other non-$CD117^{+}$ cells. This was also shown in the paper by Yan et al. where the two techniques were compared (Yan et al., 2009). The group showed that when purifying CD4+CD25+ Tregs, the sorting time using MACS was twice that

of FACS with lower purity. The MACS method can also lead to a decrease in cell viability, while FACS gives a higher number of the pure and live cell population (Yan et al., 2009).

### 6.2.2 Comparison of amniotic fluid stem cells to bone marrow and fetal liver haematopoietic stem cells

The initial characterisation of the fresh cells showed expression of haematopoietic markers such as CD117, Sca-1, and CD45. While differences were seen in the expression of CD117, no difference was observed in the CD45 marker. This was also highlighted when the cells were characterised using $RT^2$ PCR Array, which showed variable gene expression for each freshly isolated group(Loukogeorgakis et al., 2019). This could be due to the origin/niche of each HSCs group (Tavian and Péault, 2005a).

Furthermore, to explore if these cells were functional, I stimulated them after isolation *in vitro*, using an erythroid differentiation medium and a semisolid medium. Production of haemoglobin and formation of haematopoietic colonies emphasised the functionality of these cells and their haematopoietic properties (Dolznig et al., 2005; Wang et al., 2012).

### 6.2.3 Expansion media and techniques

The difficulty in culturing amniotic fluid stem cells (human) is that once the cells are cultured fresh, in Chang C medium, the mesenchymal portion of the stem cells attach to the petri dish, while the haematopoietic component is floating in the medium. When the medium is changed, the haematopoietic component is discarded together with debris and dead cells.

I tried to culture the mAF cells in a similar environment to the hAF unsuccessfully, due to the above reason. Since my main interest was the haematopoietic component, I tried to culture them using published haematopoietic cytokines, in a feeder-free medium, again unsuccessfully (Pineault and Abu-Khader, 2015).

Since AF HSCs originate in a fetal environment, the culture techniques used for culturing embryonic stem cells were selected to test the ability to maintain their haematopoietic potential. This was also shown by another group where mBM HSCs were cultured successfully on MEFs similarly to embryonic stem cells (Yuan et al., 2004).

Creating an embryonic environment is essential for the expansion and growth of primitive cells such as ESCs or IPSs. Amniocytes have also been shown to be able to create this environment and even serve as feeders for the growth of IPSs and ESCs(Anchan et al., 2011; Lai et al., 2009). When culturing cord blood progenitors, their repopulating potential is impaired especially when the culture is performed in suspension. When the HSCs are co-cultured with MSCs, their short -term engraftment potential is improved (Holmes et al., 2012). This was also seen in the studies of other groups which showed that stromal cells in combination with growth factors are needed for the culture of HSCs (Ji et al., 2008; Walenda et al., 2011). Another group demonstrated the need for removing the produced cytokines during an HSC culture using an automated closed system process by employing a controlled fed-batch media dilution approach(Csaszar et al., 2012). By using this system, they achieved an impressive 11 fold increase in HSCs in just 12 days with multilineage repopulating activity.

### 6.2.4 Expansion in a modified ESC medium and challenges for clinical translation

For clinical translation in an autologous *in utero* therapy concept, the fetal cells will need to be collected at around twelve or fourteen weeks (before the migration of primitive HSCs to the liver finalises), corrected and expanded in seven days before transplanting them back to the same fetus. This requires a highly efficient culture mechanism. I showed that culture of mAF HSCs in the modified ESC medium could give a 6-7 expansion fold. The question is if this is feasible clinically and if a higher expansion fold is needed. The other obstacle is that the cells need to be separated from the MEFs using FACS, which again introduces manipulation and mortality.

A relatively new method has been introduced in cell culture, using microfluidics, which may improve cell culture yield. The microfluidic cell culture can handle small volumes for more dynamic control, a continuous exchange of media, accuracy and high-throughput (Mehling and Tay, 2014; Meyvantsson et al., 2008), which might be used for the culture and expansion of mAF HSCs.

### 6.2.5 Expansion using modified MEFs with ANG

In an attempt to improve the expansion fold of the mAF HSCs I collaborated with Dr Steve Howe who provided the Angiopoietin-Like Protein 3 mouse vector. It has been previously shown that Angiopoietin improves the expansion potential of cells in liquid cultures(Drake et al., 2011; Farahbakhshian et al., 2014; Zhang et al., 2006; Zheng et al., 2011). I then transduced the MEFs and produced a MEF line which produced angptl3. The expansion fold was higher when compared to expanding on normal MEFs. These results were similar to a study by Farahbakhashian et al. who observed an increased expansion of murine HSC populations from 6 or 10 fold to 17 or 32 fold following the addition of angptl3 in their cultures and was cell media dependent (Farahbakhshian et al., 2014).

### 6.2.6 *In vitro* functional assays

Following the *in vitro* expansion of the cells, their differentiation properties were demonstrated by culturing them in a semisolid medium (M3434) for two weeks. Formation of haematopoietic colonies confirmed their stemness and plasticity(Loukogeorgakis et al., 2019; Shangaris et al., 2018). Ideally, this should have been done *in vivo* as well, by transplanting the cells into sub-lethally irradiated NSG mice. Repopulation of the animals' haematopoietic system and even survival would have confirmed the stemness and plasticity of the expanded cells *in vivo* similar to the study by Ditadi and colleagues(Ditadi et al., 2009).

### 6.2.7 Neonatal and Fetal Mortality after IUT

The mice survival rate in IUT was around 48%. This was after cross-fostering the pups to non-injected time mated dams to correct for maternal cannibalism and maternal antibody response (Merianos et al., 2009). Maternal cannibalism was also observed in the study by Guillot et al. (Guillot et al., 2008) where 26 out of the 32 osteogenesis imperfecta mouse model females that underwent IUT suckled their offspring.

The survival rate could have been improved if a different route of administration was used. In this study, I used the IP route injecting 20µl of cell suspension in each fetus. If a future study is performed, the IV route might improve the survival rate as well as the engraftment rate. This

was demonstrated in the comparison study performed by M.M. Boelig et al. (Boelig et al., 2016) where the IP, IV and the IH (intrahepatic) routes were compared. In this study, the survival rate was 39.2% for IV, 37.2% for IP, 32.2% for IH; P>0.4 (Boelig et al., 2016). The survival rate when injecting 20µl IP was similar to this study. The engraftment advantage was demonstrated in my collaborative study with Dr Stavros Loukogeorgakis. In particular, 100% of animals receiving intravenous IUT of congenic mAF HSC showed stable hematopoietic macrochimerism for up to 6 months, with engraftment in blood and BM exceeding 20% (Loukogeorgakis et al., 2019).

### 6.2.8 *In utero* transplantation of amniotic fluid stem cells, congenic cells are superior to allogenic

The congenic and allogenic transplantation study was adapted from Merianos et al. experiments (Merianos and Tiblad, 2009) where they demonstrated the presence of a maternal immune response, that limits the engraftment of IUT cells. Merianos et al. demonstrated that maternal immune response towards the transplanted cells could be partly addressed by cross-fostering new-born pups to non-injected dams. This was done in this study. I similarly noticed an increased advantage of the congenic cells with all the congenic recipients being chimeric. The initial analysis was performed at four weeks, and further analysis was done at sixteen weeks. Engraftment of the cells was observed in the significant haematopoietic organs. The delivery of the cells at a specific time was decided based on the physiology of the fetal haematopoietic system. As seen in Figure 6-1 adapted from Irina B. Mazo et al. (Mazo et al., 2011), the organs; bone marrow, thymus, spleen and liver have a significant haematopoietic role during fetal life. Primitive HSCs initially originate in the yolk sac at (E7.5), and then they migrate to the aorta gonad mesonephros (E9) region (AGM). Soon after that, at E10, they colonise the fetal liver (E16), which becomes the major haematopoietic organ in the fetus. From the fetal liver, haematopoietic stem cells migrate to the thymus, spleen, and bone marrow. The BM colonization begins at E16., which makes the bone marrow the main haematopoietic organ during adult life. Even though the bone marrow is the only

haematopoietic organ in adult life, there are haematopoietic stem cells and progenitor trafficking which is possible via the bloodstream or lymphatic vessels (Mazo et al., 2011).

I decided to transplant the cells early enough to correct the primitive HSCs before their migration from mFL to mBM. The high proliferation of the cells would have pushed the transplanted HSCs to migrate to the mBM together with the host's HSCs and function symbiotically in the haematopoietic system.

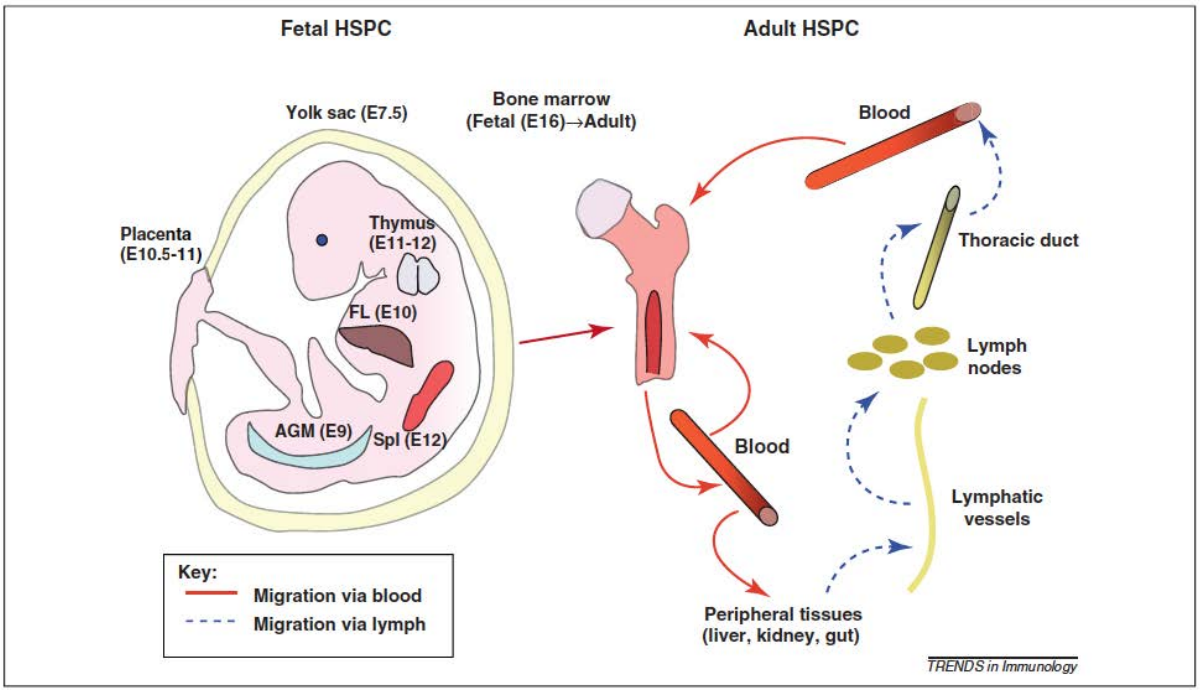



**Figure 6-1 Migration of Fetal progenitor HSCs during fetal life Adapted from Irina B. Mazo et al. (Mazo et al., 2011)**

In any adult HSC transplantation ideally, you create "space" by myeloablation of the host's cells to achieve therapeutic chimerism (Lodi et al., 2011; Seung et al., 2000). In a recent clinical trial for the treatment of β thalassaemia, using ex-vivo autologous HSC gene correction patients underwent myeloablative conditioning with intravenous busulfan for four consecutive days to create space and remove defective HSCs (Thompson et al., 2018). *In utero* myeloablation is difficult but not impossible. Engraftment in my study might have been improved if I had used an *in utero* myeloablative agent towards the HSCs such as murine c-Kit receptor antibody (ACK2). This was used by S. Derderian et al. to create space within the haematopoietic niche to improve donor engraftment (Derderian et al., 2014). In their study, the authors achieved significantly higher levels of chimerism when they depleted the host using

the ACK2 antibody (IUT with ACK2 vs IUT alone, 24.2±2.2 vs 9.9±1.9 respectively, n=4-14 per group, p=0.002). This would not have improved the engraftment in the allogenic group since this technique only addresses the “space” barrier while the “immune” barrier remains intact (Derderian et al., 2014; Witt et al., 2018). This could be possible in humans if a similar antibody is tested for human use. The advantage of such an antibody is that it only attached the stem cell component leaving behind the terminally differentiated cells, avoiding in such a way anaemia. This can be tested in a non-human primate animal model of transplantation before human clinical translation (Derderian et al., 2014).

### 6.2.9 Assessment of engraftment

I assessed engraftment using two different antibodies, CD45.1 and $H2K^d$. CD45 is a phosphatase protein tyrosine phosphatase, receptor type C that is expressed by all nucleated haematopoietic cells (Hermiston et al., 2003). An identical repopulation ability of $CD45.1^+$ and $CD45.2^+$ cells in both CD45.1 and CD45.2 recipients, up to 15 weeks post bone marrow transplantation, was observed when a high dose of irradiation was used (Waterstrat et al., 2010). To detect donor cells in the allogenic recipient, the $H2K^d$ antibody was used since the cells originated from a BABLc mouse expressing $H2K^d$ instead of $H2K^b$, which was expressed in the recipient cells. This model was used by other groups to demonstrate and investigate allogenic stem cell transplantation (Koenecke et al., 2012). The difference in donor detection techniques should not affect the assessment of engraftment since the engraftment was assessed as the percentage of donor cells in the total $CD45^+$ population of the recipient. i.e. only cells expressing CD45 were considered when assessing the engraftment, positive engraftment was considered if the cells were >1% positive for the donor marker.

### 6.2.10 Characterisation of the engrafted cells in the congenic and allogenic recipients

After assessing the engraftment, I characterised the donor cells in the various organs and compared the cell populations between the congenic and allogenic recipients.

The congenic recipients had increased levels of donor-derived cells both at four and sixteen weeks while the engraftment in the allogenic group was minimal.

Characterization of these cells in the sixteen-week recipients in the bone marrow and blood showed that chimerism expressed due to the transplanted cells could form haematopoietic cells from all three haematopoietic lineages, erythroid, myeloid and lymphoid. This was also confirmed after culturing the BM cells from the recipient mice in semisolid medium. The cells formed haematopoietic colonies from all lineages, and the presence of donor cells in these colonies was confirmed by flow cytometry. This confirmed the stemness of the mAF cells and their plasticity. This could have even been confirmed by performing secondary transplantation experiments where the donor bone marrow stem cells from recipient bone marrow are transplanted intravenously and repopulate the haematopoietic system of the NOD-SCID-gamma (NSG) mice. This was demonstrated for sheep AF in the study of S.Shaw et al. (Shaw et al., 2015). The attempt to perform the study is discussed later on in this section.

In murine recipients, as published by A.Flake's group, all the animals with >1% chimerism accept a postnatal booster transplant from the same donor (Ashizuka and Peranteau, 2006; Vrecenak and Flake, 2013). This was also observed in a canine model of leukocyte adhesion deficiency (Peranteau et al., 2002). Postnatal boost transplantations should reproduce the results of other researchers. Unfortunately, due to insufficient time, and this was not possible during my PhD.

### 6.2.11 Immune Response in the recipients of the congenic and allogenic transplants

The recipient's response to these transplants was also investigated by flow cytometry and qRT-PCR. Flow cytometry data suggest an increase in the total CD4 and CD8 cell population in the allogenic recipients at four weeks. This can be correlated to a reaction of the immune system to these cells, something that contradicts the initial theory of prenatal tolerance (Peranteau et al., 2002). This was also supported by the *in vitro* MLR assay where higher T-cell proliferation can be seen in the allogenic group.

Allogenic animals demonstrate higher resistance to donor-transplanted mAF HSCs, which could be related to an immune response by CD4+/CD8+ T-cells; this is supported by flow cytometry data. It seems that the fetal immune system can inhibit the engraftment of allogenic mAF HSCs following IUT. The data are consistent with previous studies which suggest that

immune response is generated by fetuses causing allogenic graft rejection following IUT (Carrier et al., 2000; Donahue et al., 2001; Peranteau et al., 2009).

Two investigators reported that maternal alloantibodies are transmitted via breast milk into pups and maternal T-cells are transferred into fetuses during gestation resulting probably in an immune barrier to engraftment (Merianos et al., 2009; Nijagal et al., 2011). This was addressed by cross-fostering the animals to non-injected dams to avoid maternal antibody response. The other possibility is that fetal intervention itself enhances maternal T cell recognition of the fetus (Wegorzewska et al., 2014).

Also, Durkin and colleagues showed the role of NK cells following allogenic *in utero* HSC transplantation. They found that T cell reactivity is modulated by host functional NK cells resulting in their contribution in allogenic graft rejection (Durkin et al., 2008). They showed that an initial chimerism threshold level of >1.8% is required for durable engraftment, while depletion of recipient NK cells could stabilise the engraftment if the chimerism is <1.8%(Durkin et al., 2008). The role of NK cells is also reported in a previous study where IUT resulted in successful donor stem cell engraftment, in an NK cell deficient patient(Flake et al., 1996) (Flake, 1996).

Analysis of qRT-PCR results provides evidence of acceptance of the transplanted cells. The foxp3 and TGFβ expression in the congenic recipients was higher compared to the allogenic chimeric and allogenic non-chimeric recipients. The levels of expression of Foxp3, which is expressed by Tregs, is directly associated with their potential to suppress T-cell activation and prevent graft rejection as shown by Chauhan et al. (Chauhan et al., 2009). The same group also demonstrated that increased levels of TGFβ are also related to acceptance or tolerance of the transplant (Chauhan et al., 2009). Other groups also have used the higher expression of Foxp3 and TGFβ to show acceptance or tolerance of the graft (Wang et al., 2013).

However, according to the known immunosuppressive function of Treg cells following stem cells and solid organ transplantation, my results in congenic animals are unexpected. The relative expression of Foxp3 is up-regulated in congenic (autologous) recipients compared to allogenic and control animals.

If the transplantation was truly autologous (congenic), no response should have been seen. However, two possible reasons may support this data. Firstly, there are complications with studying autologous stem cell transplantation in the available animal models (mainly including murine and rats models). So far, it is not possible to design an autologous approach as the gestational age of mice is only 21 days while mAF stem cell culture in ESC medium takes approximately one week. Also, the maximum number of mAF HSC cells collected from each fetal mouse is 10000 and that involved collecting the whole amniotic fluid. The fetal mice will not survive if the whole fluid is removed, and the cell number will be minimal if less is collected. Thus, to overcome this limitation, I used a congenic transplantation (donor and recipient mice have the same genetic background) approach, which mimics autologous IUT. In the CD45.1/CD45.2 congenic IUT model, two different isoforms of the same CD45 extracellular marker have been used to detect donor and recipient cells. The donor cells expressed $CD45.1^{+,}$ while recipient cells expressed $CD45.2^{+}$. A weak immune response has previously been shown to be triggered after bone marrow transplantation using the CD45.1/CD45.2 congenic model(Waterstrat et al., 2010; Xu et al., 2004). Also, van Os et al. also explored that an immune response was induced in CD45.1/CD45.2 congenic model (van Os et al., 2001). Consequently, even this minor CD45.1/CD45.2 disparity between donor and recipient cells could result in an immune response.

TGF-β signalling is critical for the development of natural $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells in the thymus (Liu et al., 2008). It also plays a key role in Treg homeostasis, induction and maintenance of Foxp3 expression (Pyzik and Piccirillo, 2007). The TGF-β mRNA expression follows the expression pattern of Foxp3 in examined organs of congenic, allogenic and control animals. Real time-PCR revealed that TGF-β and Foxp3 gene expression is up-regulated in all examined organs of congenic animals.

I also examined the relative expression of IL10 in the thymus, spleen and bone marrow of congenic and allogenic animals using real-time PCR. The mRNA levels of IL-10 were up-regulated in all examined organs of the congenic group. IL10 is important in Treg suppressor function (Hoffmann et al., 2002), required for the functional activity of Tregs(Hara et al., 2001) and to mediate Treg tolerance to alloantigens *in vivo* (Hara et al., 2001). Taken together, my

results can lead to further studies where Tregs could be used to induce tolerance in an *in utero* HSC transplantation model (Di Ianni et al., 2011; Duggleby et al., 2018; Tang and Vincenti, 2017; Witt et al., 2018). Other groups have performed allogenic bone marrow stem cell co-transplantation of host Tregs which lead to prolonged and high levels of chimerism, tolerance to donor cells, and subsequent tolerance to a delayed solid organ transplantation without immunosuppression (Duran-Struuck et al., 2017; Safinia et al., 2016; Vaikunthanathan et al., 2017).

### 6.2.12 Secondary Transplantation experiments not successful

Secondary transplantation experiments were performed where the mBM HSCs selected for donor markers ($CD45.1^+$ and $H2K^{d+}$) from congenic and allogenic recipients were collected and transplanted in NOD-SCID-Gamma (NSG) mice after irradiation. This was to provide evidence of the stemness and plasticity of mAF HSCs. Unfortunately, at the time the experiments were running the Biological Services Unit suffered from an infection, and all the animals had to put down. The experiment was repeated with the same outcome; all animals developed severe illness and had to be put down. No results were collected from these experiments.

### 6.2.13 Weaknesses and Limitations of the IUT of mAFSC study

Attempts to optimise an immunofluorescence technique to trace the donor cells were unsuccessful, as well as molecular techniques, targeting the CD45.1 and $H2K^d$. Primers from published papers were used, but they were unable to work on any of the control tissues, after extensive testing and troubleshooting (Dressel et al., 2009; Skinner et al., 2012; Tichy et al., 2010). Secondary transplantation was unsuccessful, and there was no time for post-natal boost transplantation.

### 6.2.14 Attempts to correct the mouse model of beta thalassaemia not successfully

Attempts to reproduce these results, in a humanised mouse model of thalassaemia, did not work, as described in Chapter 4. The engraftment was minimal and similar to the engraftment of the allogenic cells in the first experiment. The most probable reason is that the B382 model is not truly congenic to the YFP model, which I used as a donor.

When I discussed the CA (B382) mice with the postdoctoral fellow at UAB (the institution which created the CA mice) I discovered that they were originally generated from V6.5 ES cell line (v6.5, Novus Biologicals, NBP1-41162), which is 129xC57BL6 F1 hybrid, and then the mice were bred with C57/BL6 for several times, and then they were brother X sister bred. Most of their background should be C57BL6, but they also have some 129 background. The mouse MHC (i.e. H-2 locus), 129 is the same as C57BL6, and they all should have H-2b haplotype, but there is some minor histocompatibility antigen difference between them (Huo et al., 2017). An ideal congenic source of cell would be the B383 humanised non-thalassaemia control, animal model. This model is truly congenic and produced human haemoglobin. Unfortunately, if this model is used, I will not be able to trace the cells (do not produce any GFP/YFP CD45.1/2 to use flow cytometry to detect them *in utero*). A new animal model could be bred by using YFP/GFP male mouse together with female B383 humanised non-thalassaemia control. In this way, the mAF would express GFP/YFP, and maternal contamination can also be avoided. The only issue is that the fetuses and the mAF will not have full humanised haemoglobin tetramer (will be humanised chimeric) and this might provoke an immune response.

## 6.3 *In Utero* Gene Therapy using GLOBE LV

### 6.3.1 LV virus and ways to improve correction

Improvements to the gene delivery system can also be considered. GLOBE-LV has a minimal regulatory LCR region containing HS2 and HS3. Other β-globin lentiviral vectors, containing large regulatory fragments encompassing HS2, HS3 and HS4 such as TNS9(Arumugam and Malik, 2010; Rivella et al., 2003; Sadelain et al., 2010) could be used instead to test if the higher β-globin expression is possible when delivering gene therapy to the fetal liver. To

improve efficiency and targeted delivery of LV, the use of nanoparticle molecules could be explored during *in utero* delivery (Sirianni, 2018; Zylberberg et al., 2017). An example was shown by Yang et al. when chitosan nanoparticles were used to successfully deliver EGFP plasmid *in vivo, in utero* (Yang et al., 2011). Another group demonstrated, using ultrasmall gold nanoparticles, as carrier vehicles into the nucleus, that gene expression can be regulated by delivering a triplex-forming oligonucleotide (Huo et al., 2014).

In this study, the homozygous CA mouse could not be rescued probably due to relatively low viral titres (~$10^7$ VP/mL) compared to routinely obtained titre values (>$10^9$ VP/mL). Higher titres using nanoparticle delivery methods might be able to rescue the homozygote animal model (Chen et al., 2016; Yang et al., 2011).

Vector copy number of <1 determined from the liver and bone marrow samples of treated pups was found to be sufficient to phenotypically correct the animal model. Since the heterozygous model was corrected with a low VCN, this is an indication that the vector has an excellent safety profile. This was comparable to studies performed by other groups(Han et al., 2007; Miccio et al., 2008) and by the fact that similar vectors are being used in ongoing human clinical trials (Thompson et al., 2018).

### 6.3.2 Humanised mouse model of β- Thalassaemia

Mice carry 6-12 fetuses per pregnancy, and the total gestation is 20-21 days. This offers injection of all fetuses in one experiment. They can serve as replicates for each experiment. Many groups have used mouse models of β-thalassaemia to demonstrate either an *in utero* therapeutic approach or postnatal gene or cell therapy(Rivella et al., 2003; Vogiatzi et al., 2010). The majority of these mouse models lack real fetal haemoglobin and consequently do not mimic the β-thalassaemia disease in humans. Thus, I imported the humanised mouse model that expresses human γ globin and recapitulates β-thalassaemia in humans(Huo et al., 2010, 2017; Huo, McConnell and Ryan, 2009). Much like β-thalassaemia babies, upon Hb switching from γ to non-functional $\beta^0$globin KI allele after birth, homozygote animals need regular blood transfusions to survive. The physiological and anatomical changes observed in

the heterozygous animals were significant enough to allow monitoring of the given treatment (IUGT) and outcomes, even though no homozygote was rescued.

### 6.3.3 IUGT in the Haematopoietic Stem Cell Niche

The attempt to tranduce the cells *in vivo*, was made based on evidence that the primitive HSCs niche at E13.5 resides in the fetal liver (Bertrand et al., 2005; Choi et al., 1998; McGrath and Palis, 2005; Palis et al., 2001; Palis and Yoder, 2001). The fetal liver is the niche of HSCs capable of definitive erythropoiesis. Of course, the ideal scenario is to target the yolk sac, which is where the first primitive erythroid cells are found as seen in Figure 6-2 (Palis et al., 1999; Palis and Yoder, 2001). That would correct the primitive haematopoietic niche even before any HSC migration to the fetal liver. This is a challenging concept even if this is done in a mouse model since it has to be done at around or before E9.0, well before the hepatic clononisation commences (Mazo et al., 2011). In humans, the embryonic liver contains cells able to establish haematopoiesis 32 days after fertilisation, even though the first hepatic colonisation starts at day 23(Tavian et al., 1999; Tavian and Péault, 2005a, 2005b). That is quite early, and the challenge will be if a blood disorder will even be diagnosed by that timepoint. Studies have shown that a prenatal diagnosis using NIPD could be possible from 10 weeks of gestation (Chan et al., 2010), although cell-free DNA could be detected from the 4$^{th}$ week of gestation (Bustamante-Aragonés et al., 2012; Mortarino et al., 2011).

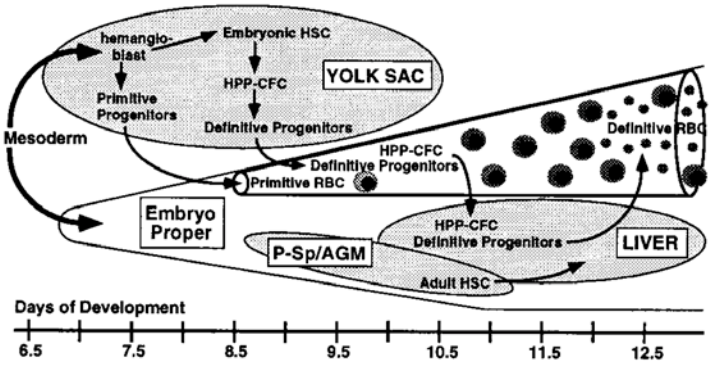



**Figure 6-2 Model of early haematopoietic ontogeny in the mouse embryo as described by Palis and colleagues (Palis et al., 2001) Figure adapted from Palis et al. 2001, P-Sp/AGM, para-aortic splanchnopleura/aorta-gonad-mesonephros region; RBC red blood cells**

Accessing small fetus is also a challenge. The equivalent gestation in humans of the E13.5 murine timepoint is 6.5 weeks (Otis and Brent, 1954) at which stage the human embryo will be approximately 2.4mm(Papaioannou et al., 2010). Groups are currently working on finding ways to access the early fetus in view of delivering fetal therapy(Maneas et al., 2018). Another option is to deliver gene therapy to the yolk sac itself, which has an average diameter of 3.4mm at 42 days (Papaioannou et al., 2010). This was demonstrated by the *in-utero* injection of adenovirus in a canine model of the disease. The virus was injected into the yolk sac and peritoneal cavities of the canine fetuses. This method was efficient and high levels of the functional transgenic protein and proviral DNA was observed and detected up to 10 months postnatally. This illustrated the potential for long-lasting prenatal gene therapy(Meertens et al., 2002).

### 6.3.4 Use of MRI techniques to monitor disease progression in animals treated with IUGT

Non Invasive disease progress monitoring is possible with MRI, which corresponds to dry weight measurements (Jackson et al., 2017). For this experiment T2* was more accurate that T2 in the heart even though T2 had the same trend as T2*. This was also reported by L Jackson et al. (Jackson et al., 2017).

The use of MRI for monitoring patients has been reported previously, and it is currently used clinically(Attili et al., 2010; Papakonstantinou et al., 2009, 2012). To measure the effect of my prenatal therapy method, I collaborated with the imaging group at UCL where a pilot study was initially established to assess the efficiency of mouse MRI to detect iron overload and cardiac function in a mouse model of disease(Jackson et al., 2017). This was a proof of principle study before the treated animals were scanned. The major advantage of this method was the ability to monitor the animals in a non-invasive way (no post mortem required) throughout the study. I was able to assess cardiac function (left ventricular ejection fraction), iron accumulation and organ volume for all treated and control animals. MRI can be used to monitor disease progression in an animal model, post-IUGT or IUT of HSCs. Tissue iron can be detected indirectly by measuring, using MRI, the relaxation times of hydrogen nuclei affected by ferritin and hemosiderin iron. The proton relaxations times are shortened as a result of the iron,

specifically T2. The presence of blood can spoil the myocardium and blur its borders. This can be addressed by using T2* which excludes the signal from blood, using multiecho images in late diastole(He, 2014). The control experiment which was performed by my collaborators, Jackson et al., before scanning the IUGT animals showed that spleen volumetrics, relaxometry and cardiac function could distinguish between the humanised non-thalassemia controls, heterozygous controls and iron-loaded animals. Iron dry weight measurements were used to create a calibration curve to quantify the content of iron in the tissue, and correlate this to T2* relaxation times(Jackson et al., 2017). Currently, this technique is used in clinical practice to monitor thalassaemia patients for cardiac iron accumulation (Cheong et al., 2005). Other groups have also used it to monitor iron accumulation in other tissues such as liver and spleen (Anderson et al., 2001; Cheong et al., 2005; L et al., 2016; Papakonstantinou et al., 2009). The T2 and T2* relaxation times in the spleen of the IUGT group were shortened compared to Humanised Non-Thalassaemia Control. No difference was seen between the IUGT group and the Thalassemia control, which was unexpected. This might be because of the increased breaking down of erythrocytes and subsequently iron accumulation in the spleens of the thalassaemia animals (IUGT and Thalassemia Control). The excess iron accumulation in the thalassaemia animals could be due to dietary iron, and excess accumulation could be specific to the animal model.

### 6.3.5 Site Integration studies, future toxicology studies and weakness of the study

The integration site analysis was performed by Dr Wei Wang at the Department of Translational oncology at the Nationales Centrum für Tumorerkrankungen (NCT) Heidelberg, Germany. The most immediate way to estimate clonal size is by counting the sequence reads belonging to each integration site. For example, it is common to detect oligoclonal populations and dominant clones in the early phases of haematopoietic reconstitution. The reason is the unequal activation of clones sustaining the first haematopoietic wave. Cells with the highest fitness and differentiation potential are selected first. Thus, identifying true insertional mutagenesis events in the first six months after treatment is challenging (Biasco, 2017; Biasco

et al., 2017; Yadak et al., 2018). As expected, there were stronger integration sites in the liver (504) compared to BM (51), which correlates to the VCN.

To determine the percentage of transduced HSCs (instead of the whole mBM) in the bone marrow, repeat experiments were performed. In these experiments, the mBM cells were isolated and then sorted using FACS for lin-/Sca1+/CD117+ (LSK) cells (experiments performed after dedicated research time completed). Unfortunately, the number of mBM LSK cells were inadequate for qPCR processing due to the small amount of BM and further determination of VCN in HSC only population, which is a weakness of the study. Ideally, I wanted to look at the proportion of chimeric HSCs both in the mBM HSC niche and peripheral blood. Having not been able to collect these data, it is not known whether transduced fetal stem cells migrate and colonise the adult bone marrow to pursue haematopoiesis during adulthood, or whether adult stem cells progressively arise from a source of cells that have not been targeted by injection of the fetal liver. I had observed variable levels of correction between two IUGT experiments, with better correction when the analysis was performed at 12 weeks and lower at 32 weeks. Another weakness of the study is that I should have measured individual Hb monthly and followed the mice over several months to determine the stability of the correction. Toxicology studies would need to be performed to confirm the safety of the vector especially since the method of delivery is not the same as the current gene therapy clinical trials (Bartholomae et al., 2011; Ramachandra et al., 2014; Themis et al., 2005; Thompson et al., 2018). The vector (or a modified version of the same) is currently clinically used in human trials for the treatment of β-thalassaemia after *ex-vivo* correction in adults(Thompson et al., 2018).

### 6.3.6 *In Utero* Therapy and Ethics

The clinical application of *in-utero* therapy or IUGT to treat diseases depends on numerous factors. These include the age of onset of the disease, the severity of the disease, the level of irreversible damage and associated lethality, the age of diagnosis and timing of screening as previously discussed. The main issues in prenatal therapy using vectors are the maternal gene transfer, germ-line transfer and ability of the vector to correct the disease

efficiently. LVs in fetal circulation have a high risk of crossing the placental barrier to the maternal blood stream. The likelihood of this event is difficult to deduce as the placental structure differs between species and undergoes significant changes during pregnancy. Maternal and germ line transmission should be assessed. In an experiment where AAV serotype two derived vectors carrying a beta-galactosidase reporter gene or human clotting factor IX cDNA were delivered *in utero*, no detectable vector sequences were found, in either isolated sperm DNA from the treated animals or in their offspring (Jakob et al., 2005). This has to be addressed for each vector on each occasion.

The other question is if it ethical to offer parents the option of prenatal and/or postnatal treatment in severe cases of a blood disorder (for example alpha-thalassaemia), or any other monogenic disorder, when the prognosis for their child is uncertain, the procedure could provide unrealistic hopes and whether the child might survive in poor condition after prenatal treatment when it might otherwise have died. There is a risk of causing harm to the fetus, such as a miscarriage or preterm birth, and harm to the mother, such as an intrauterine infection. Although likely to be uncommon, these risks are not insignificant and must be balanced with the chance of improving the outcome for the child. These issues are general to all fetal interventions that are given during pregnancy to improve that individual's outcome. This includes treatments that are accepted in clinical practice such as fetal blood transfusion for fetal anaemia and fetoscopic laser ablation of placental anastomoses for identical twin placental complications (Chervenak and McCullough, 2018; Pearson and Flake, 2013; Szaflik et al., 2013).

### 6.4 Future Work and plans for further research training

Having shown that an *in utero* approach is possible using congenic cells my next step would be to use the humanized mouse models of thalassaemia to show that this technique works and can be applied in this mouse model. Attempts to correct the mouse model were unsuccessful using "congenic" cells. This was probably because the cells used were not truly congenic. The next step would be to use cells from a true congenic humanised, healthy non-thalassaemia mouse model (383), which has the same background and the thalassemia

mouse model (B382). If this is not successful, the next step is to use cells from the humanised mouse model itself and transplant the cells after *in vitro* correction using the GLOBE vector.

Another option if the above fail is to induce tolerance of the allogenic cells. It is well established that a specialised subset of immunosuppressive cells identified by the lineage-defining transcription factor Foxp3, termed regulatory T cells (Tregs), are strongly associated with tolerance to self, as well as tolerance to non-inherited antigen (Burt, 2013). Tregs are also critical mediators in the induction of transplantation tolerance, where they prevent solid organ transplant rejection when co-transferred with the allograft (Romano et al., 2017; van der Net et al., 2016; Velásquez-Lopera et al., 2008). In a highly relevant pre-clinical macaque model, allogenic bone marrow stem cell co-transplantation with host Tregs led to prolonged and high levels of chimeric engraftment, tolerance to donor cells, and subsequent tolerance to solid organ transplantation without immunosuppression (Duran-Struuck et al., 2017). Using this technique, allogenic HSC co-transplantation (which could be from either cord blood, bone marrow or amniotic fluid) with Tregs *in utero*, can be attempted to assess their engraftment potential. Thus allogenic haematopoietic stem cells transplanted *in utero* can be enhanced and reach therapeutic levels by co-transplantation donor matched (engineered) T-regulatory cells (Duggleby et al., 2018; Vaikunthanathan et al., 2017).

Another option, to avoid a maternal antibody response, is transplantation of maternal bone marrow (BM) HSCs to the fetus. This was attempted at the University of California San Francisco (UCSF) where fetuses with alpha thalassaemia survived to term. UCSF is currently recruiting patients for this clinical trial for *in utero* therapy for alpha thalassaemia (ClinicalTrials.gov - identifier NCT number: NCT02986698) (Kreger et al., 2016). This involves intervention in the mother herself, with mobilisation of stem cells during pregnancy. If the mother is homozygous for sickle cell disease or thalassemia, harvesting stem cells from her is not appropriate since they will need ex-vivo manipulation and correction prior to transplantation. Thus induction of tolerance *in utero* is an ideal method.

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## Appendix A. Sample of Post Mortem Form

**PM Protocol:** **mice: B382…….: (n:…) Control (n:….)**

**Date:**

**Animal No:**

**Weight:**

**Spleen Weight:**

**Haemoglobin (Hemacue):**

**Tissue Sampling**

| number | organ | Code | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Blood EDTA Lab/ HPLC | RNA/ DNA 2X 1.5MLTUBES | | | protein | Histology H&E 4% PFA |
| 1 | Bone Marrow | BM | | | | | | |
| 2 | Spleen | X | | | | | | |
| 3 | Liver | L | | | | | | |
| 4 | GIT | GIT | | | | | | |
| 5 | Blood | Blood | EDTA/HPLC | | | | | |

**Antibodies for flow cytometry:**

**LIVER TO BE STAINED WITH ……**

**For Bone marrow and Blood**

CD45 –

CD3 / B220 / CD11b / Gr1 / Ter119 - APC

Viability Dye: 7AAD PE cy5