The potential of amniotic fluid stem cells in prenatal gene and cell therapy

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2013

A thesis submitted for the degree of Doctor of Philosophy

Declaration

I, Sheng-Wen Steven Shaw, 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

Amniotic fluid stem (AFS) cells can be expanded without feeder layers and can differentiate into mesenchymal and haematopoietic lineages. Long term engraftment has been difficult to achieve after prenatal stem cell transplantation mainly because of allogeneic rejection. Autologous cells could be obtained from amniotic fluid (AF) with minimal risk. My thesis aims to define the potential of human, sheep and mouse AFS cells as an autologous stem cell source for prenatal cell/gene therapy.

Using pregnant sheep, I explored using AF mesenchymal stem cells (AFMSCs) and CD34+ cells for autologous in utero therapy. AF was collected under ultrasound-guided amniocentesis in early gestation. Those cells were transduced with enhanced green-fluorescent-protein (GFP) using lentivirus vector. After expansion, transduced AFMSCs were injected into peritoneal cavity of each donor fetal sheep. Widespread transgenic GFP expression was detected in fetal tissue. For looking into haematopoietic potential, I transplanted autologous fresh and frozen CD34+AFS, and bone marrow cells into immunocompromised mice. Sheep CD34+AFS cells formed colonies, and were positive for CD45, but negative for CD14/CD31/CD4/ CD58. Flow cytometric analysis at 3 months showed GFP positive cells in all haematopoietic organs.

To prove congenic transplantation is better than allogeneic, I collected AF from YFP+/C57BL/6 mice at E13. CKit+/Lin- cells were injected into peritoneal cavity of every mouse fetus. Peripheral blood engraftment was significantly higher in mice transplanted with congenic, versus allogeneic cells, as was liver and spleen engraftment.

Finally, looking ahead to clinical translation, human AF cells could be cultured, transduced, sorted and expanded in vitro by using conditional medium in adherent plates. Xenogeneic transplantation of human Ckit+ AFS cells into fetal mice also showed minimal engraftment in peripheral blood.

In conclusion, AF derived stem cells are an important source of autologous cells that could have prenatal therapeutic value in cell or cell-based gene therapy in the future.

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Publications

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Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Anna David. Her mentorship, encouragement, supports and enthusiasm to teach goes beyond what may be expected, and this is genuinely appreciated. She would often tell me during the animal surgeries and fetal scanning that she would make a fetal medicine specialist and scientist out of me, and did succeed at it.

I am also very grateful to my secondary supervisor, Dr. Paolo De Coppi, for his guidance, encouragement and help with experimental design during the course of this Ph.D. He gave me all of his knowledge about stem cell biology from the beginning with plenty of patience. I have learnt most of the stem cell techniques in the lab of Institute of Child Health.

I would like to thank Prof. Donald Peebles, Prof. Agotino Pierro and Prof. Adrian Thrasher for the very helpful discussions.

I am grateful to a number of colleagues in the lab, who were ever willing to help. Of particular mention are Khalil Abi-Nader, Vedanta Mehta, Elisa Filippi, Marco Ghionzoli, GiorgiaTotonelli, Massimo Garriboli, Caterina Pipino, Fanos Georgiades, Mike Blundell, Simon Waddington and Suzy Buckley.

During the course of this Ph.D., I had an opportunity to supervise several wonderful students in the lab and really enjoyed the experience. These included Durrgah Ramachandra, Kuang Han Lee, and Jane Lin from Taiwan. I am thankful to them for their hard work.

I would also like to take this opportunity to thank all the staff at the BSU, Royal Veterinary College for taking excellent care of the experimental animals, particularly Michael Boyd, David Manners, Tom Bellamy, Adnan, Hannah and Pauline Reading.

I am particularly grateful to Chang Gung Memorial Hospital and National Science Council of Taiwan, who funded many of the experiments described in this thesis.

Last but not the least, I owe it to my family for their encouragement, support and belief; particularly my wife Ann, two kids Max and Alex, also my Mum and Dad, for their full understanding of how difficult the PhD work is.

Selective Abbreviations

AC	abdominal circumference
ACM	amniotic culture medium
AF	amniotic fluid, the fluid in the amniotic cavity
AFMSC	amniotic fluid mesenchymal stem cells, the mesenchymal stem cells lineage isolated from the amniotic fluid with mesenchymal stem cell surface markers.
AFP	alpha fetoprotein
AFS	amniotic fluid stem cells, the pluripotent stem cells isolated from amniotic fluid with positive for CD117 or Ckit surface marker.
AFHSC	amniotic fluid haematopoietic stem cells, the haematopoietic stem cells lineage isolated from the amniotic fluid with haematopoietic stem cell surface markers and could be cultured in the liquid condition.
BFU	burst forming unit
BM	bone marrow
BPD	biparietal diameter
BSU	biological service unit
CB	cord blood
CFU	colony forming unit
CFU-G/M/GM	colony forming unit-granulocyte, monocyte
CFU-GEMM	colony forming unit-granulocyte, erythrocyte, monocyte, megakaricyte
CK18	cytokeratin 18
cPPT	central polypurine tract
CVS	chorionic villus sampling
DAPI	4'-6-Diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic Acid
eGFP	enhanced green fluorescent protein
EIAV	equine infectious anemia virus
EPC	endothelial progenitor cells
ESC	embryonic stem cells
eYFP	enhanced yellow fluorescent protein
FACS	Fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FL	Femur length
FMU	fetal medicine unit
GA	gestational age
GFP	green fluorescent protein
GVHD	graft-versus-host disease
Hb	haemoglobin
Hct	haematocrit
het	heterozygous
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSC	haematopoietic stem cells

ICH	Institute for Child Health
IF	immunofluorescence
IL	interleukin
IM	intramuscular
IP	intraperitoneal
IUSCT	in utero stem cell transplantation
IUT	in utero transplantation
IV	intravascular
IVC	individually ventilated cages
IVIS	in vivo imaging system
Klf-4	Kruppel-like factors 4
LIN	Lineage
LTR	long terminal repeat
LUC	luciferase
MCV	mean corpuscular volume
MEM	Minimum Essential Medium
MHC	Major histocompatibility complex
MOI	multiplicity of infection
MOMA	metallophilic macrophages antibody
MSC	mesenchymal stem cells
NSG	NOD-SCID gamma mice
OCT	Optimal Cutting Temperature
Oct-4	octamer-binding transcription factor 4
OI	Osteogenesis Imperfecta
OSL	occipito-snout length
PB	peripheral blood
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PE	Phycoerythrin
RBC	red blood cells
RDW	red cell distribution
RLU	relative light unit
RVC	Royal Veterinary College
SCF	stem cell factor
SCID	Severe Combined Immunodeficiency
SD	standard deviation
SFFV	the spleen focus-forming virus
SMA	spinal muscular atrophy
Sox-2	SRY-box 2
SSEA-4	stage-specific embryonic antigen-4
TBE	tris-borate-EDTA
TBST	mixture of Tris-Buffered Saline and Tween
TDL	The Doctors Laboratory
TPO	thrombopoietin
UCL	University College London
UCLH	University College London Hospital
UCOE	Ubiquitous Chromatin Opening Element
WPRE	Woodchuck post transcriptional regulatory elements
WT	wild type
YFP	yellow fluorescent protein

Chapter 1

Introduction

1 Chapter 1: Introduction

1.1 The burden of congenital disease

Many congenital diseases have a relatively low prevalence, but collectively they represent a large burden of disease (McCandless, 2004). It is estimated that they are responsible for over a third of all paediatric hospital admissions, and for up to 50% of the total cost of paediatric hospital treatment (McCandless, 2004). Due to the improvement in medical care, affected patients can now survive to adulthood requiring continued therapy (Figure 1.1).

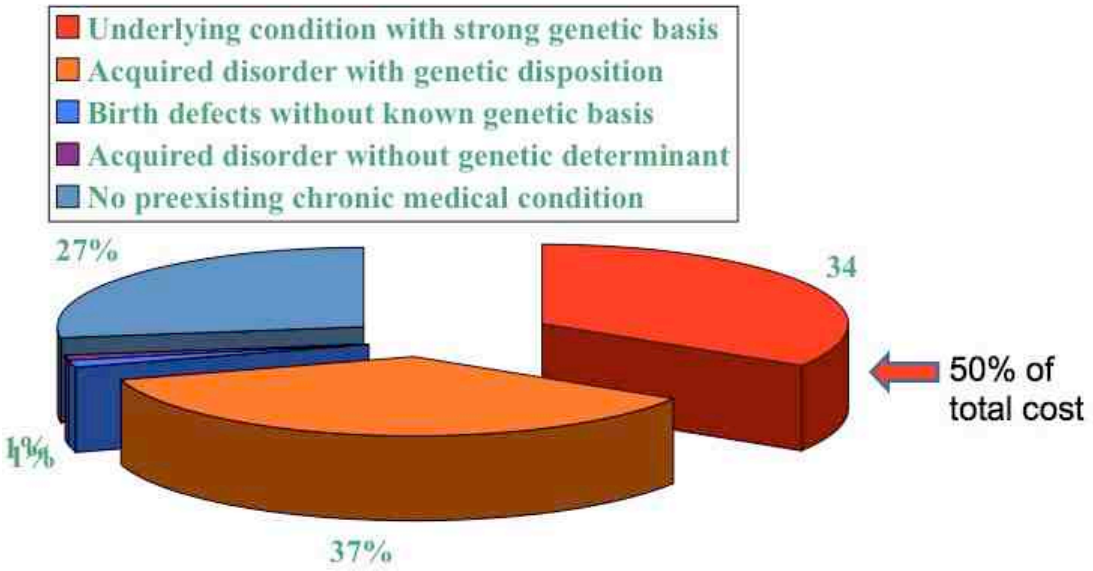


Figure 1.1 Reason for admission to a paediatric hospital.
From 5447 hospital admissions, genetic conditions were responsible for a third of admissions, but for 50% of the total cost of these admissions. (Adapted from the figures of McCandless, 2004)

Currently most pregnant women and their partners facing a prenatal diagnosis of severe life-threatening congenital disease in their fetus can only choose either termination of pregnancy, or to carry on with the pregnancy to deliver an affected baby. With progress in fetal medicine, such couples might be offered prenatal therapy. Possible therapeutic options might include *in utero* stem cell transplantation, gene transfer to modify the disease genetically, or a combined gene therapy stem cell approach. Some fetuses with congenital structural defects could also benefit from a

tissue engineering approach to improve surgical repair of the defect either during prenatal life or postnatally.

It is now possible to derive stem cells with pluripotential ability from the amniotic fluid and other fetal tissues. This opens up the possibility to use these stem cells in an autologous or allogeneic approach for prenatal therapy of congenital diseases.

1.2 Advantages of prenatal over postnatal therapy for severe life-threatening early onset congenital disease

There are a number of reasons why for some life-threatening early onset congenital diseases, performing *in utero* therapy may provide an advantage over neonatal or adult treatment (Waddington et al., 2007).

1. Preventing disease damage before birth: The advantage of prenatal therapy is that pathological damage to the fetus during prenatal development might be prevented (David and Peebles, 2008). Many severe life-threatening congenital diseases have their onset while the baby is *in utero.* Organ damage is often irreversible, to that postnatal treatment cannot improve the quality of life. Any pathological effect may make the newborn dependent on medicines for the rest of their lives and have severe handicap. For example, a fetus carried with alpha thalassaemia major will die *in utero* due to severe fetal edema that occurs by the second trimester.
2. Target stem cell and progenitors: Stem cells and progenitors rapidly expand *in utero* making them accessible to gene transfer that could provide a large pool of corrected cells. Compared to adult bone marrow stem cells or peripheral blood, fetal stem cells have a much better ability to differentiate and expand (Harrison et al., 1997).
3. Dose scaling: The small size of the fetus is an advantage in prenatal therapy. In adult gene therapy or stem cell transplantation, the dose of vector or cells is far higher than would be required during fetal life (Burt, 1999). According to animal studies, the dosage usually is calculated on a per kg body mass basis. Production of usable vector or cells is time consuming and expensive, thus the requirement of a lower dose or cell number is advantageous. In one study the amount of cell number for *in utero* transplantation could be reduced in mice, sheep or human experiments (Troeger et al., 2007, Perillo et al., 2008, Surbek et al., 2002).

4. Immunological immaturity: The immunological naivety in the early gestation fetus has given rise to the concept of fetal tolerance, the inability to raise an immunological response against foreign antigens (Gaunt and Ramin, 2001). Introduction of a foreign protein or antigen and maintenance of its expression during gestation leads to the development of immune tolerance to the foreign protein. This has been demonstrated to occur in fetal gene therapy experiments in the mouse. Waddington et al. demonstrated a permanent correction of hemophilia B mice after prenatal gene therapy. The plasma factor IX antigen remained at around 9, 13 and 16% of normal in hemophilia B mice but fell to undetectable levels when animals were injected postnatally (Waddington et al., 2004). Rejection of donor cells plays a critical role in transplantation, and has a major effect on success rate (Flake and Zanjani, 1999).
5. Finally for structural congenital abnormalities, repair *in utero* may provide a better correction of the defect because there is less scarring, and the pathological process may be interrupted. There is evidence of benefit for example, for *in utero* treatment of spina bifida (Meuli et al., 1996).

Prenatal therapy for congenital disease can be applied in a variety of ways, using gene therapy, stem cell therapy or a combination of the two techniques.

1.3 Gene therapy

Gene therapy is a technique that uses genetic material to correct the genetic defect in congenital disease (Howe and Thrasher, 2003). Scientists may use one of several approaches for correcting the genetic defect. Firstly, a normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene. This approach is most common. Secondary, an abnormal gene could be swapped for a normal gene through homologous recombination. Thirdly, the abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function. Finally, the regulation (the degree to which a gene is turned on or off) of a particular gene could be altered.

1.3.1 Vectors for gene therapy

Vectors are used to deliver the therapeutic gene to the target cell population. The ideal vector for fetal somatic gene therapy is one that can produce long-term regulated

expression of the transferred gene using a single and efficient gene delivery method. The idea vector should also be safe to the mother and fetus, allowing incorporation into clinical practice. Other preferential characteristics include a vector with high transduction efficiency, a specific tropism to the target organ, a carrying capacity large enough to incorporate the therapeutic gene and regulatory elements, a low immunogenicity and a low teratogenic and mutagenic potential (Campos and Barry, 2007, Sinn et al., 2005).

Vector used in gene therapy have been classically subdivided into replication-deficient viral vectors and nonviral vectors. Table 1.1 summarizes the main characteristics of the different vector systems using for prenatal applications.
There are five commonly used viral vectors for gene therapy.

1. Retroviruses: A class of viruses that can create double-stranded DNA copies of their RNA genomes. These copies of its genome can be integrated into the chromosomes of host cells and so run the risk of insertional mutagenesis. The vector only infects the host cell when the cell is dividing (Howe and Chandrashekran, 2012).
2. Lentiviruses: A class of viruses that can create double-stranded DNA copies of their RNA genomes (also a member of the retrovirus family). These copies of its genome can be integrated into the chromosomes of host cells and so run the risk of insertional mutagenesis. The vector infects the host cell whether or not the cell is dividing. Human immunodeficiency virus (HIV) is a lentivirus.
3. Adenoviruses: A class of viruses with double-stranded DNA genomes that cause respiratory, intestinal, and eye infections in humans. The virus that causes the common cold is an adenovirus.
4. Adeno-associated viruses: A class of small, single-stranded DNA viruses that can insert their genetic material at a specific site on chromosome 19 (Rutledge and Russell, 1997).
5. Herpes simplex viruses: A class of double-stranded DNA viruses that infect a particular cell type, neurons. Herpes simplex virus type 1 is a common human pathogen that causes cold sores.

Non-viral vectors can also be used but they tend to result in less efficient gene transfer.

Table 1.1 Characteristics of the different vector systems. (Table and data were modified from David et al., 2008) +: weak efficiency; ++: moderate efficiency; +++: good efficiency.

Vector	DNA (kb)	Efficiency	Advantages	Disadvantages
Nonviral DNA complexes	No limit	+	Low toxicity Low immunogenicity	Low transduction efficiency
Adenovirus	7.5	+++	Can grow to high titer Highly efficient gene transfer	Short-term expression and immunogenic, associated with fetal abnormalities
Adeno-associated virus	4	++	Low immunogenicity Long-term expression	Liver toxicity, miscarriage risk with some subtypes
Retrovirus	10	+	Long-term gene transfer	Insertional mutagenesis, infect dividing cells only
Lentivirus	10	++	Long-term gene transfer Infects dividing and nondividing cells	Insertional mutagenesis, potential for germ line transmission
Herpes simples	30	++	Retra-axonal transduction, Infects nondividing cells	Latent infection

1.3.2 Lentivirus vectors

Lentivirus is an integrating viral vector that provides long-term transgenic protein expression because it is not diluted by cell division.

Lentivirus dose, however, have the potential to cause mutagenesis, and based on the murine leukaemia virus have been implicated as a cause of T-cell leukaemia when used in human trials of neonatal stem cell gene therapy for SCID (Hacein-Bey-Abina et al., 2003). Lentiviruses have been associated with insertional mutagenesis when applied fetally. Mice that were prenatally treated with the equine infectious anemia virus (EIAV) lentivirus vector developed a high incidence of postnatal liver tumors (Themis et al., 2005). Insertion sites, in these instances, were preferentially associated with genes involved in development and cell growth, suggesting that the fetus and

neonate may be particularly sensitive. Integration-deficient lentiviral vectors have thus been developed to avoid this problem. These vectors can sustain expression in post-mitotic tissues, such as the brain, while virtually eliminating the risk of insertional mutagenesis (Philpott and Thrasher, 2007).

1.3.3 In utero gene therapy

There are a number of *in utero* gene therapy pre-clinical studies using animal models that have shown the efficacy of this approach but there has not been a move into clinical trial yet. Figure 1.2 showed the candidate diseases that have been treated prenatally in animal models including mucopolysaccharidosis VII, Leber's congenital amaurosis type 2 (mutation of RPE65 gene), Glycogen storage disease type II, Hemophilia B, and deletion of UDP glucuronosyltransferase 1 family polypeptide A cluster (Rucker et al., 2004, Dejneka et al., 2004, Karolewski and Wolfe, 2006, Seppen et al., 2003, Waddington et al., 2004). Waddington's work in the haemophilia mouse model suggested that *in utero* gene therapy could generate therapeutic levels of human factor IX without development of immune reactions when compared to adult gene therapy in this model. The human factor IX level was maintained for up to 14 months follow-up (Waddington et al., 2004).

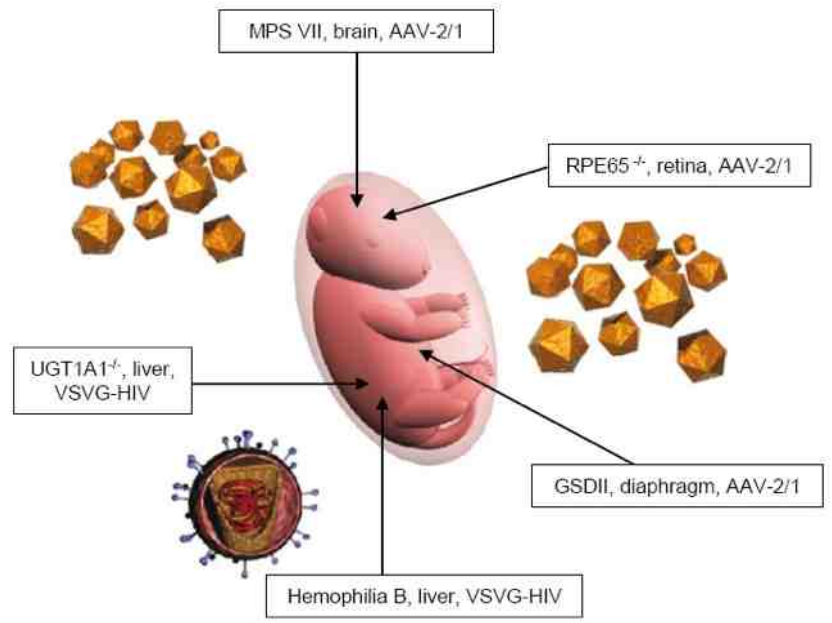


Figure 1.2 The summary of 5 disease models of prenatal gene therapy in mice fetuses.

In each box, the disease, followed by the injection sites and vector types are indicated. (Figure taken from (Waddington et al., 2007))

1.3.4 Translating fetal gene therapy into the clinic

There are a number of issues facing fetal gene therapy that renders it complex to translate into clinical practice. Important safety concerns exist (Billings, 1999, King et al., 1999). One major concern is the risk of transduction of gonadal cells which might result in genetic germ line transduction. There is also a potential risk for the mother because transduction of her somatic or germ cells through transplacental migration of the vector-gene construct could theoretically occur. An additional important safety aspect is the possibility of insertional mutagenesis in fetal cells resulting in a functional gene defect leading to a genetic disease or to the formation of a malignant tumor (Howe et al., 2008). Other problems include the risk of developmental aberrations should be noted and monitored for potential adverse effects of prenatal gene therapy (Coutelle et al., 2012). Some of these risks could be avoided by delivering stem cells rather than vectors to the fetus. The likelihood of insertional mutagenesis can be reduced by the use of self inactivating lentivirus vectors or by the use of gene corrected stem cells in which insertion sites can be examined (Schambach et al., 2007, Modlich et al., 2009).

1.4 Stem cells

Stem cells are essential for the development of any living organism (Reya et al., 2001). Stem cells have the ability to develop and grow during early life and into adulthood, the ability to differentiate into different lineages such as osteogenic, myogenic, chondrogenic and adipocyte, and to self-renew by cell division. These are the key characteristics that make stem cells different from other cell types (Wagers and Weissman, 2004, Reya et al., 2001).

Stem cells can be classified according to their potency: pluripotent, multipotent and totipotent (Wagers and Weissman, 2004, Serafini and Verfaillie, 2006). Totipotency is the ability of all living cells potentially to regenerate whole new individuals as cloning. Pluripotency is the potential of a cell to develop into more than one type of mature cell, depending on environment. A pluripotent cell can create all cell types

except for extra embryonic tissue. Multipotency is the ability a cell to develop into closely related family of cells. For example a blood stem cell can develop into types of blood cells like erythrocytes, leucocytes but cannot develop into any other kind of cells. Stem cells can be derived from many different accessible sources such as bone marrow (BM), peripheral blood (PB), cord blood (CB), chorionic villi and amniotic fluid (AF) (Ljungman et al., 2006, Schmitz and Barrett, 2002). There are also stem cells that reside within tissues and organs. Embryonic stem cells (ESC), mesenchymal stem cells (MSC), endothelial progenitor cells (EPC), haematopoietic stem cells (HSC) and amniotic fluid-derived stem (AFS) cells are some of the types of stem cells that have been studied (Le Blanc et al., 2005, Fauza, 2004, Balsam et al., 2004).

Stem cells are likely to be important in the repair of tissues and organs, and for diseases such as myocardial infarction, diabetes and ischaemic disease where aging is occurring (Balsam et al., 2004, Stamm et al., 2003, Hussain and Theise, 2004). They may also provide a renewable source of cells for tissue engineering (Perry et al., 2003).

1.4.1 Stem cells from the fetal liver

Fetal liver is an abundant source of both haematopoietic and mesenchymal stem cells (Fukumitsu et al., 2009, Le Blanc et al., 2005). One problem for their clinical application is their collection, since they can be easily obtained only from women undergoing either surgical termination of pregnancy procedures, or less commonly from women having ultrasound guided fetal liver sampling for prenatal diagnosis of liver disorders (Kleijer, 2001) which carries a high risk of miscarriage. Sampling of fetal liver cells followed by autologous transplantation was shown to carry a high risk of miscarriage in the fetal sheep (73%) (Schoeberlein et al., 2004). This is probably due to the damage to the liver that occurs when a relatively large needle is inserted within it, and which sucks up some of the cells. It is likely that the fetal loss rate would be similarly high in clinical use. Therefore, fetal liver cells are not likely to be useful for therapy using autologous transplantation but may be available if considered ethically acceptable, from termination of pregnancy collection, for allogeneic transplantation.

1.4.2 Stem cells from the placenta

Placental tissue can be collected prenatally via chorionic villus sampling (CVS) for prenatal diagnosis (Rhoads et al., 1989), or from the placenta at birth (Castrechini et al., 2010). The overall miscarriage rate after CVS is approximately one in 100 (Tabor and Alfirevic, 2010) and is related to the skill and experience of the operator. This invasive procedure needs to be carefully performed to sample the fetal side of the placenta to prevent significant maternal contamination. Also, it was not suggested to perform the CVS before 11 weeks of gestational age as fetal limbs deformity combined with high miscarriage rate was noted. Villi are cleared of any adherent maternal blood afterwards under the microscope before cell culture for karyotyping studies.

The placenta contains a number of stem cell types such as MSC (Portmann-Lanz et al., 2006, Brooke et al., 2009) and HSCs (Lee et al., 2010). MSC showed the typical phenotype with positive for CD166, CD105, CD90, CD73, CD44, CD29, CD13, MHC I, and negative for CD14, CD34, CD45, MHC II. HSC derived from placenta showed the evidence of growing all lineages of haematopoietic colonies.

The advantage for CVS is the timing; it could be performed as early as 11th weeks of gestation in humans. This would allow sufficient time to differentiate, expand and transduce these cells, and inject them back into the donor fetus in an autologous approach before immune competence develops from around 16 weeks of gestation. This may also be an advantage where evidence suggests that it is better to treat congenital fetal disease as early as possible to prevent the irreversible organ damage *in utero*.

1.4.3 Cord blood stem cells

HSCs can be easily derived from cord blood, and have been used clinically for many years. They have several advantages compared to adult bone marrow including less rejection, less invasion, and less contamination with virus (Koestenbauer et al., 2009). This study also showed the expansion protocol of cord blood HSC (CD34+) that has been standardised by many study groups. While it is common practice to derive HSC from cord blood at birth, they may be derived during pregnancy using ultrasound cordocentesis that can be performed with a low rate of miscarriage (approximately 1%) from 20 weeks of gestation (Antsaklis et al., 1998). The most common reason for

performing cordocentesis in current clinical practice is for treatment of fetal anemia using fetal blood transfusion. The miscarriage rate depends on the indication for fetal blood sampling and is higher in anaemic fetuses (Antsaklis et al., 1998), but overall it is only slightly higher than following CVS or amniocentesis (Orlandi et al., 1990). Their mesenchymal counterpart can be isolated in a limited number of cord blood samples presenting positive CD44, CD73, CD90, and CD105 MSC phenotype. (Weiss and Troyer, 2006).

1.4.4 Stem cells derived from amniotic fluid

A variety of stem cells can be derived from the amniotic fluid, including MSCs and cells with haematopoietic potential and pluripotent cells.

Amniotic fluid (AF) is the clear, watery liquid that surrounds the growing fetus within the amniotic cavity and which has a number of roles. The fluid allows the fetus to grow and move inside the uterus, it protects the fetus from outside injury by cushioning sudden blows or movements, it acts as a vehicle for the exchange of body chemicals with mother and is vital for the development of some organs such as the lungs, in which inhalation of the AF via fetal breathing movements allows normal lung development. The AF contains cells of fetal origin such as the amnion, skin, and respiratory system (Prusa and Hengstschlager, 2002, Tsai et al., 2006b). AF is commonly used in prenatal diagnosis of chromosomal or genetic defects in the fetus (1976, Gosden, 1983, Delo et al., 2006). Amniocentesis is a well-established, and least minimally invasive procedure in clinical practice (Figure 1.3). The miscarriage rate has been reported as low as 1 in 769 when performed by a trained fetal medicine specialist (Odibo et al., 2008). The timing of amniocentesis is from 15 weeks of gestation in human since earlier sampling is associated with abnormalities of limb development.

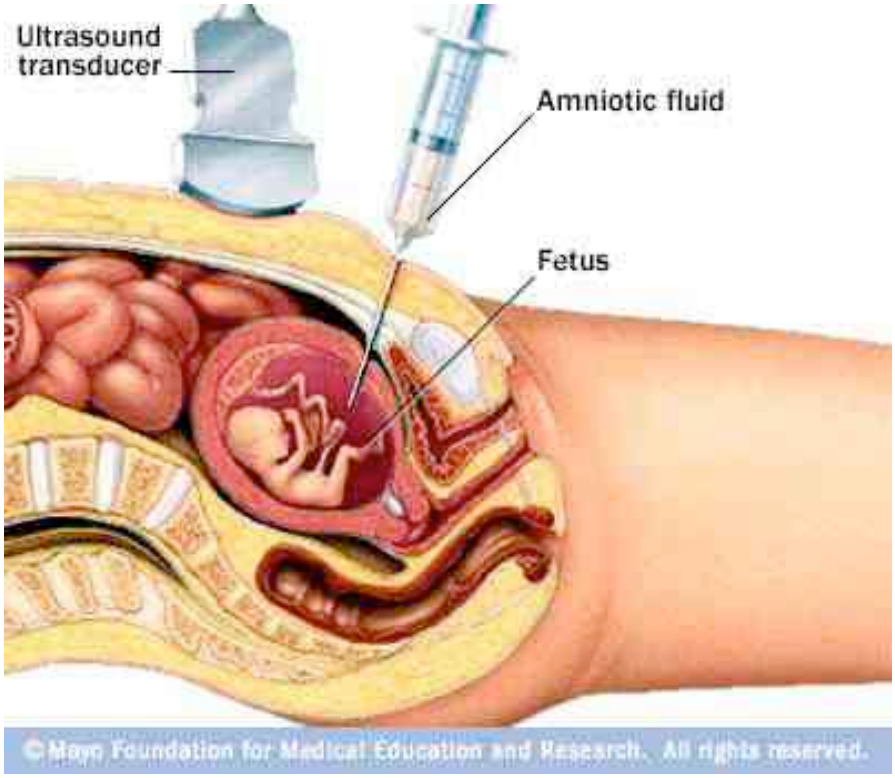


Figure 1.3 Ultrasound Guided amniocentesis.
(Picture was modified from Mayo Foundation for Medical Education and Research)

The AF contains stem cells which can differentiate into the three embryonic layers, including adipogenic, osteogeneic, myogenic, neural, epithelial, and hepatocyte lineages. The cells have both MSC and HSC differentiation potential (Ditadi et al., 2009). Studies on transplanted AF cells do not report any teratoma formation. In the last few years there is strong evidence that AF cannot only be used as a diagnostic tool but also as a potential source for therapeutic applications in a multitude of disorders (Delo et al., 2006).

1.4.5 CKit+ amniotic fluid stem (AFS) cells

The first evidence that the amniotic fluid could contain pluripotent stem cells was provided when Prusa et al. described the presence of a distinct sub-population of proliferating amniotic fluid cells (0.1–0.5%) expressing the pluripotency marker Oct4 at both transcriptional and protein levels (Prusa et al., 2003). Oct-4 (octamer binding transcription factor 4) is a nuclear transcription factor that plays a critical role in maintaining embryonic stem cells (ESC) differentiation potential and capacity for self-renewal (Scholer et al., 1989, Nichols et al., 1998, Niwa et al., 2000). Other than its expression by ESC, Oct4 is specifically expressed by germ cells, where its

inactivation results in apoptosis, and by embryonal carcinoma cells and tumours of germ cell origin, where it acts as an oncogenic fate determinant (Donovan, 2001, Pesce and Scholer, 2001, Gidekel et al., 2003, Looijenga et al., 2003). While its role in stem cells of fetal origin has not been completely addressed, it has been recently demonstrated that Oct4 is neither expressed nor required by somatic stem cells or progenitors (Berg and Goodell, 2007, Lengner et al., 2007, Liedtke et al., 2007).

After the study presented by Prusa et al., different groups confirmed the expression of Oct4 and of its transcriptional targets (e.g. Rex-1) in the amniotic fluid (Bossolasco et al., 2006, Stefanidis et al., 2008). Remarkably, Karlmark et al. transfected human amniotic fluid cells with the green fluorescent protein gene under either the Oct4 or the Rex-1 promoter and established that some amniotic fluid cells were able to activate these promoters (Karlmark et al., 2005). Several authors subsequently reported the possibility of harvesting amniotic fluid cells displaying features of pluripotent stem cells (Tsai et al., 2006b, Kim et al., 2007). Thereafter, the presence of a cell population able to generate clonal cell lines capable of differentiating into lineages representative of all three embryonic germ layers was definitively demonstrated (De Coppi et al., 2007a). These cells, named amniotic fluid stem cells (AFS cells), are characterized by the expression of the surface antigen c-kit (CD117), the type III tyrosine kinase receptor of the stem cell factor (Zsebo et al., 1990).

Pluripotent cells displaying CKit+ have so far been isolated from human and rodent AF (De Coppi et al., 2007a). CKit (CD117) is a surface antigen that is the receptor for stem cell factor (Delo et al., 2006) (Figure 1.4). CKit is a Type III tyrosine kinase receptor for the ligand of cytokine stem cell factor and these receptors locate on the cell membrane of germ cells, haematopoietic stem cells, neuroectodermal cells (Ashman, 1999), cardiac cells (Beltrami et al., 2003) and retinal stem cells (Koso et al., 2007). Approximately 0.8% to 1.4% of the cells from human AF possess the CKit+ surface antigen and they can be sorted using immunoselection by magnetic microspheres. CKit+ stem cells from AF were named “AFS” cells (De Coppi et al., 2007a). The expansion of human AFS cells has been well established with a high population doubling rate and their phenotype has been well characterized (De Coppi et al., 2007a, Delo et al., 2006, Tsai et al., 2006b, Pozzobon et al., 2009, Cananzi et al., 2009).

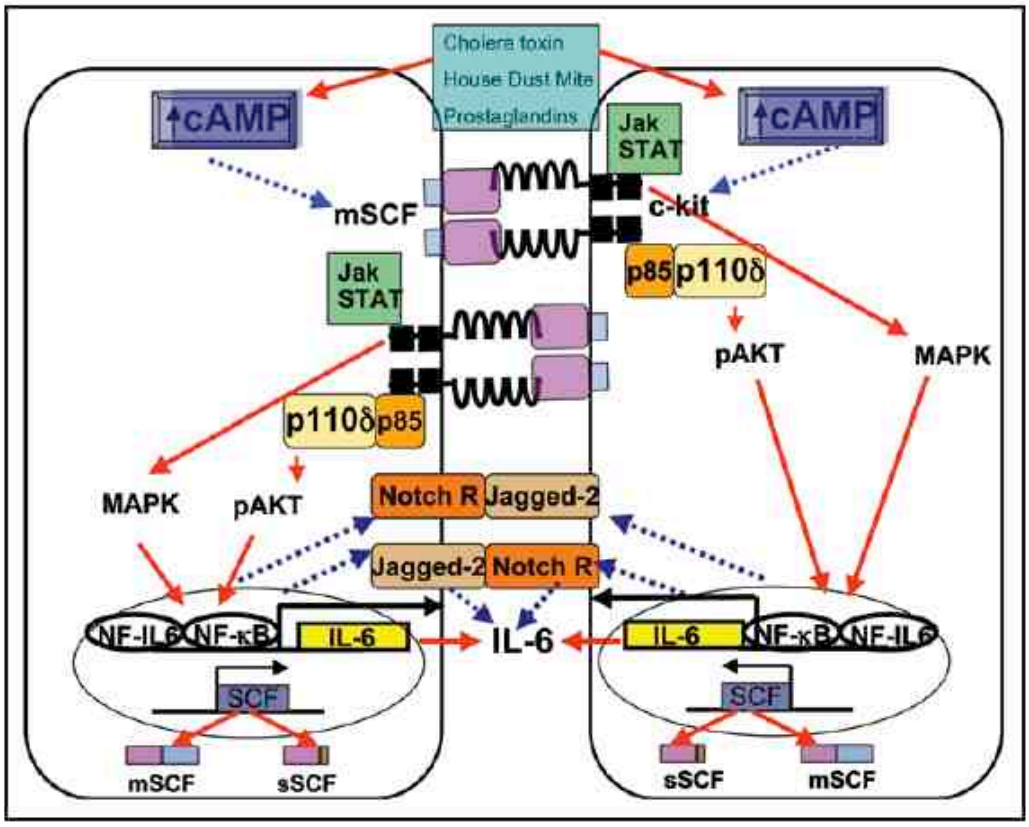


Figure 1.4 The illustration showed the functional role of Ckit as a type III tyrosine kinase receptor. Dual upregulation of Ckit and membrane-bound SCF (mSCF) on dendritic cells by allergens and allergy-inducing adjuvants promotes IL-6 and Jagged-2 expression. Dendritic cells stimulated with Cholera toxin, house dust mite or prostaglandins upregulate c-kit and mSCF expression that is mediated by cAMP. Ckit activation by mSCF via cell-cell interactions, triggers sustained downstream activation of the PI3 kinase/AKT pathway and possibly MAPK and JAK-STAT pathways resulting in increased production of IL-6, whose transcription is known to be dependent on NFκB and NF-IL6, and may also involve Notch/Notch ligand. Activation of c-kit also upregulates expression of Jagged-2, and Notch receptors (NotchR). The red arrows show known mechanisms of activation while the broken blue arrows indicate activation pathways for which the mechanisms have yet to be determined. (The figure was modified from (Ray et al., 2008))

These cells could maintain the potential up to 250 passages without telomerase length change. But they did not show the teratoma formation while in vivo injection performed which is the main concern and ethical issue in embryonic stem cells (De Coppi et al., 2007a). Moreover it has been shown that amniotic fluid derived stem cells can be easily transduced without losing their potential in human, mice and sheep (De Coppi et al., 2007b, Grisafi et al., 2008). Rodent and murine AFS have similar characteristics to human AFS cells. Rodent AFS cells demonstrate similar growth properties and differentiation potential (in vitro) while murine AFS cells express markers of embryonic and adult stem cell types (De Coppi et al., 2007a). Finally,

freshly isolated AFS cells can generate various haematopoietic lineages both in vitro and in vivo (Tiblad and Westgren, 2008). AFS cells have an estimated doubling time of 36 hours and no feeder layers are required for the culture of these cells. The high capacity of self-renewal is impressive where AFS cells may proliferate over 300 times (exceeding Hayflick's limit) (Hayflick and Moorhead, 1961) and retain the same karyotypes without developing chromosomal anueploidies (Delo et al., 2006).

As mentioned previously, the surface antigen CKit (CD117) is known to be the receptor of stem cell factor and to play an essential role in gametogenesis, melanogenesis and haematopoiesis (Fleischman, 1993). Human AFS cells express surface markers of mesenchymal and/or neural stem cells origin, stage-specific embryonic antigen (SSEA)-4, a marker that is usually present in ES cells and Oct-4, a transcription factor (De Coppi et al., 2007a). A number of MSC markers are positive on AFS cells that include CD29, CD44, CD73, CD90, CD105 and vimentin. HSC markers like CD34, CD45 and CD133 are negative on AFS cells (Perin et al., 2008, Walther et al., 2009). Since MSCs are less immunologically competent than their haematopoietic counterparts (O'Donoghue and Fisk, 2004), human AFS cells might be a good candidate for transplantation since they may result in less transplantation-related rejection. Human AFS cells can either expand or be induced to differentiate into various cells of 3 embryonic layers that include adipocytes, osteocytes, endothelial cells, hepatocytes, myocytes, and neuronal cells (De Coppi et al., 2007a, Delo et al., 2006, Perin et al., 2008). Importantly, our study group recently have demonstrated that, when injected into irradiated Rag -/- immunodeficient mice, murine AFS cells are able to reconstitute the entire bone marrow and haematopoietic lineages (Ditadi et al., 2009). This was the first paper showing the HSC like characterization could be found in amniotic fluid cell samples. Table 1.2 showed the summary of characterization of AFS cells, and compared to other stem cells types. The AFS cells have ES cell like pluripotent markers including SSEA-3, SSEA-4, and Tra-1-60, but AFMSC or HSC don't have.

Table 1.2 Characteristics of different kinds of stem cells.
AFS: amniotic fluid stem cells; AFMSC: amniotic fluid mesenchymal stem cells; HSC: haematopoietic stem cells; ESC: embryonic stem cells.

	AFS	AFMSC	HSC	ESC
SSEA-3	+	-	-	+
SSEA-4	+	-	-	+
Tra-1-60	+	-	-	+
Tra-1-81	-	-	-	+
CD73	+	+	-	
CD90	+	+	+	
CD117	+	-	+	
CD105	+	+	-	
CD14	-	-	+	
CD34	-	-	+	
CD45	-	-	+	
MHC I	+	+	-	
MHC II	-	-	+	

1.4.6 Amniotic fluid mesenchymal stem cells (AFMSC)

AFMSC can be easily obtained in humans from second-trimester and third trimester amniotic fluid where their percentage is estimated to be 0.9-1.5% (Roubelakis et al., 2007); and in rodents from the amniotic fluid collected during the second and third week of pregnancy (De Coppi et al., 2007a).

Various protocols have been proposed for their isolation; all are based on the expansion of unselected populations of amniotic fluid cells in serum-rich conditions without feeder layers, allowing cell selection by culture conditions. The success rate of the isolation of AFMSC is reported by different authors to be 100% (Tsai et al., 2004, Nadri and Soleimani, 2007). AFMSC grow in basic medium containing fetal bovine serum (20%). Importantly, it has been shown that human AFMSC can also be cultured without animal serum without losing their properties (Kunisaki et al., 2006); this finding is a fundamental prerequisite for the beginning of clinical trials in humans where culture in serum free medium is usually required.

Figure 1.5 shows the characteristic uniform spindle-shaped fibroblast-like morphology of AFMSC that is similar to that of other MSC populations. AFMSC expand rapidly in culture (Tsai et al., 2004). Human cells derived from a single 2 ml amniotic fluid sample can increase up to 180×10^6 cells within 4 weeks (three passages) and, as demonstrated by growth kinetics assays, possess a greater proliferative potential (average doubling time 25–38 h) in comparison to that of bone marrow-derived MSC (average doubling time 30–90 h) (In 't Anker et al., 2003, Roubelakis et al., 2007). Moreover, the clonogenic potential of AFMSC has been shown to exceed that of MSC isolated from the bone marrow (86 ± 4.3 versus 70 ± 5.1 colonies) (Nadri and Soleimani, 2007). Despite their high proliferation rate, AFMSC retain a normal karyotype and do not display tumourigenic potential even after extensive expansion in culture for over 3 months (Roubelakis et al., 2007). These cells are positive for CD73, CD90, and CD105 that are standard mesenchymal stem cell markers.

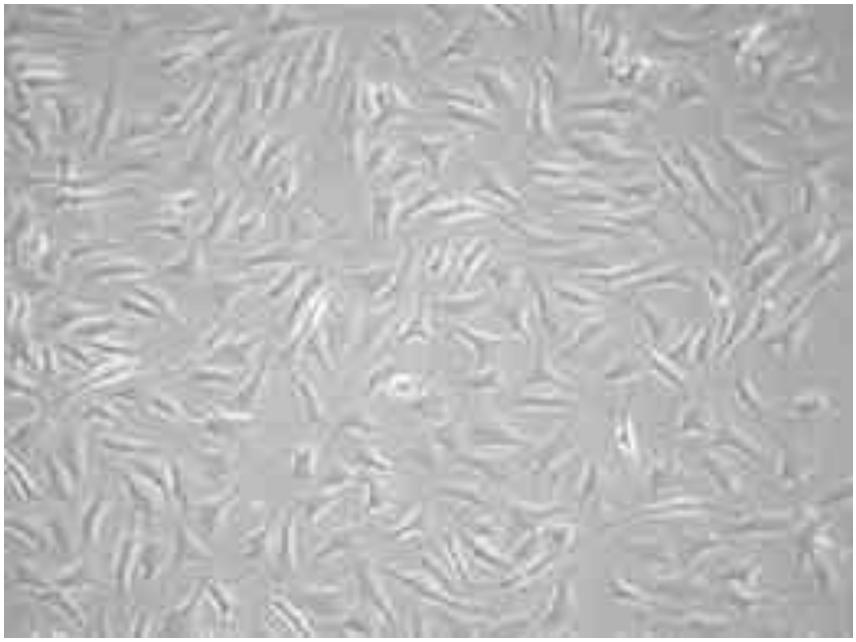


Figure 1.5 The morphology of amniotic fluid mesenchymal stem cells. The cells display a uniform spindle-shape fibroblast-like images. 40X

1.5 Autologous, allogeneic and xenogeneic stem cell transplantation

There are two major types of stem cell transplants: autologous and allogeneic. Each of these stem cell procedures has its own distinct process. When patients or animals receive cells that have been donated by someone else, these transplants are called

allogeneic transplantation, and the cells used are called allogeneic cells. On the other hands, when the cells used for transplantation came from your own or same genetic background animals, we called this autologous or congenic transplantation. Xenogeneic transplantation meant the cells injection from two different species. Moreover, autologous stem cell transplants and allogeneic stem cell transplants are each associated with distinct benefits and risks that should be taken into account when deciding which of these stem cell treatments a patient should undergo. One advantage of this stem cell treatment procedure is that in an autologous stem cell transplant, the graft would attack the host, an occurrence known as Graft-versus-Host Disease (GVHD). In addition, autologous transplants have the added advantage of avoiding the sometimes difficult process of finding a donor for stem cell treatment (Pozzobon et al., 2009, Peranteau et al., 2007).

1.6 In utero stem cell transplantation

1.6.1 Preclinical experiments on in utero stem cell transplantation

Fetal mice injected with congenic HSCs maintained stable, long-term, multilineage chimerism compared with less than 20% of allogeneic recipients (Peranteau et al., 2007). In addition, in mice, early allogeneic fetal stem cell chimerism may be enhanced by pretreatment of the allogeneic stem cells with cytokines, although long term engraftment was unchanged. It is possible that this is due to a host innate response to class I alloantigens or a lack of strain specific stromal support for the allogeneic transplanted cells (Sugiura et al., 1991, Hashimoto et al., 1997). In addition maternal T cells have been shown in mice to cross the placenta and destroy engrafted allogenic cells (Nijagal et al., 2011b).

Schoeberlein at el. demonstrated the first sheep in utero transplantation model comparing autologous and allogeneic stem cell transplantation (Schoeberlein et al., 2004). Under ultrasound guidance they collected a fetal liver sample and cultured the cells in MSC condition. They injected intraperitoneally at 48 to 64 days of gestational age back to the donor fetus in the autologous group, and fetal liver from another animal in the allogeneic group. The autologous group had a much higher loss rate (73% versus 29%).

1.6.2 IUSCT in humans

In utero stem cell transplantation (IUSCT) aims to treat congenital disorders in the fetus using cells capable of self-renewal that can enhance or substitute the affected tissues/organs of the fetus. Unlike *in utero* gene therapy, there is no viral/vector issue in stem cell transplantation. Around 50 human cases of in utero stem cell therapy have been reported using fetal liver or parental CD34+ bone marrow cells (Tiblad and Westgren, 2008, Shaw et al., 2011b). Table 1.3 showed the 4 categories of diseases that have been addressed including hemoglobinopathies, immunodeficiencies, enzyme storage disease, and osteogenesis imperfect (OI). Engraftment has been reported in 10 patients with several having a benign clinical course (Tiblad and Westgren, 2008). Attempts to treat diseases such as sickle cell disease (Westgren et al., 1996) or metabolic storage disorders for example, have been unsuccessful, even where a suitably matched donor has been available.

Table 1.3 Summary of in utero stem cell transplantation cases in humans. (Summary from Tiblad and Westgren, 2008; Shaw et al., 2011b)

	Number of cases	Number of cases demonstrating engraftment	Cell type of transplantation
Hemoglobinopathies	22	0	Fetal liver or parental CD34+ BM
Immunodeficiencies	12	8	Fetal liver or parental CD34+ BM
Enzyme storage disease	7	0	Fetal liver or parental CD34+ BM
Osteogenesis Imperfecta	5	2	Fetal liver MSCs

Successful engraftment has only been demonstrated in cases of immunodeficiencies or OI who received *in utero* stem cell transplantation.

In OI, allogeneic mesenchymal stem cells sourced from fetal liver were injected into affected fetuses by intrahepatic route of injection. Successful engraftment was observed in 2 out of 5 cases (Tiblad and Westgren, 2008). In this case, a female fetus affected by type III of OI received an intraperitoneal injection of human fetal liver mesenchymal stem cells at 26 weeks of gestational age. The donor cells showed persistent bone marrow engraftment around 7% up to three years-old (Le Blanc et al., 2005).

As mentioned previously, all the cells were donated from allogeneic sources rather than being of autologous origin and it is this perhaps that has resulted in the low

engraftment success in congenital diseases such as sickle cell or metabolic storage disorers where the immune system is relatively intact. The stem cells derived from autologous or syngeneic (identical twin) origin could be a possible solution for *in utero* stem cell transplantation.

1.7 Development of the human fetal immune system

The presence of a functionally developed immune system as early as the second trimester of pregnancy calls for alternative strategies (Westgren et al., 1996). The immunological naivety of the early gestation fetus has given rise to the concept of fetal tolerance, the inability to raise an immunological response against foreign antigens (Gaunt and Ramin, 2001). It is believed that the fetal immune system may play a larger role in preventing allogeneic fetal stem cell engraftment than was once thought.

1.8 Clinical applications of post-natal haematopoietic stem cell transplantation and gene transfer

Haematopoietic stem cell transplantation involves the use of HSC derived from bone marrow, cord blood or peripheral blood and transplanted into the host with the intention of reconstituting part or all of the bone marrow (Ljungman et al., 2006). Transplants may be xenogeneic from one species to another although this is rarely used in clinical practice. Most transplants are allogeneic, where a human leukocyte antigen (HLA) matched donor graft is harvested and infused into the recipient.

Autologous stem cell transplantation involves the harvesting and isolation of a graft derived from the recipient and infused back into the recipient, usually after a treatment (Ljungman et al., 2006). Therefore, autologous cells can be gene corrected *ex vivo* before transplantation, as has been used successfully in the recent trials of retroviral gene therapy of bone marrow in SCID that is either X-linked (Hacein-Bey-Abina et al., 2002) or due to adenosine deaminase deficiency (Aiuti et al., 2009). The advantages of autologous transplants include a low incidence of graft-versus-host disease (GVHD) or graft failure, low risk of acquiring infections and the difficulty of finding a suitable donor.

Haematopoietic stem cells are attractive targets for cell-based gene therapy, because they have the potential to produce progeny cells containing a therapeutic gene lifelong. Current clinical protocols of post-natal gene therapy in paediatric patients with genetic diseases are based on ex-vivo retrovial transduction of lymphocyte (Bordignon et al., 1995) or haematopoietic stem cells from cord blood or bone marrow, followed by autologous transplantation of the engineered cells back in to the patients (Kohn et al., 1995). Initial trials showed the feasibility and safety of gene therapy using cord blood cells in patients with ADA-deficiency, although only very limited clinical efficiency has been achieved as reported in a follow-up study (Kohn et al., 1998).

The main obstacles concern transduction efficiency, random integration of vector-gene-construct into host genome, duration of expression of the therapeutic gene (gene silencing), host immune response against vector, gene or gene product, and reproducible production of safe replication-free high-titre vectors (Verma and Somia, 1997). Gene expression can be severely impaired by spontaneous cessation of regulatory sequence activity that control gene expression, by inactivation of promoters in the transduced host cell, by specific host defence mechanisms or by elimination of the transduced cells by the host immune system recognizing the foreign gene product (Bestor, 2000).

1.9 Animal models

The use of animal models is essential in the field of clinical research particularly when translating therapies into the clinic. Animal models can provide knowledge on the safety and efficacy of drugs, cells and other treatments. A variety of animal models such as sheep, macaques and rodents such as mice and rats have been used to study stem cell transplantation. Isolated cells from various sources can be injected into animal models to identify their target and function in the body (Prusa and Hengstschlager, 2002). The behaviour, participation and development of transplanted cells can be closely monitored. The usefulness of each model has limitations as no animal model can truly recapitulate the human body (Donahue et al., 2005) and there are advantages and disadvantages to any animal model used to study IUSCT. Table

1.4 showed the characteristics of experimental animals commonly used in prenatal therapy preclinical assessment.

Table 1.4 The characteristics of experimental animals used in prenatal therapy preclinical studies

Species	Gestational length (Days)	Placenta shape	Maternofetal indigitation	Fetal number
Human (3rd Trimester)	280	discoidal	villous	1-2
Human (1st/2nd trimester)	280	discoidal	villous	1-2
Primates (higher): great apes	240	discoidal	villous	1-2
Primates (lower): Galago	130	diffuse	folded	1-2
Rabbit	31	discoidal	labyrinthine	5-10
Guinea pig	67	discoidal	labyrinthine	2-5
Rat	21	discoidal	labyrinthine	5-10
Mouse	21	discoidal	labyrinthine	5-10
Sheep	145	cotyledonary	villous	1-2

1.9.1 Gestation length

Stem cell culture, transduction and differentiation takes at least 2 weeks *in vitro*. In a mouse or rat model, it would be impossible to use autologous cells due to the short gestational length of only 21 days. Congenic transplantation to an affected mouse is an alternative feasible way to study autologous transplantation using mice of the same genetic background to provide the cells for transplantation into the target affected mice.

Large animals have relative long gestation lengths, for example the sheep has a 145 days pregnancy or the primates has a 240 days (Table 1.4), giving enough time to prepare the autologous stem cells and transplant them. Serial transplantation and/or repeated sampling can be performed later in gestation (Porada et al., 2008a). Considerable insight has been gained on donor cell activity over a period of time in the same model post transplantation.

1.9.2 Maternal fetal cell trafficking

Studies in humans show that fetal cells transferred into maternal blood during pregnancy engraft maternal tissues and persist for decades. Fetal microchimerism was initially implicated in autoimmune disease, such as systemic sclerosis (Nelson, 2002),

but was subsequently also associated with non-autoimmne conditions such as thyroid adenomas (Srivatsa et al., 2001, Johnson et al., 2002). However, fetomaternal trafficking occurs in all pregnancies, and there is increasing evidence that microchimerism occurs in healthy women as well (Khosrotehrani et al., 2003, Lambert and Nelson, 2003). The frequency of fetal microchimerism in healthy women is unknown, with autoimmune studies suggesting wide variation.
Murine models of microchimerism provide some insight (Bianchi and Fisk, 2007). Fetal cells from an earlier pregnancy persisted in lymphoid tissue (Liegeois et al., 1981) and microchimeric fetal cells found in all tissues tested in pregnant mice declined in frequency postpartum, but were still present in retired breeders (Khosrotehrani et al., 2005). Similarly, fetal cells have been demonstrated in murine maternal brains, with a peak frequency of 1.8% at 4 weeks postpartum (Tan et al., 2005). A study finding male presumed-fetal cells in bone marrow and bone from all post-reproductive women who had sons decades earlier (O'Donoghue et al., 2004) suggests that fetal microchimerism in human is as frequent as that found in animal studies, and may even be ubiquitous.

1.9.3 Open or ultrasound guided delivery of stem cells to the fetus

Based on the concept of acquired neonatal tolerance to foreign antigens (Billingham et al., 1953), initial experiments have been performed in a mouse model using intraplacental injection of haematopoietic stem cells and showed the potential of this method to cure genetic anemias prenatally (Fleischman and Mintz, 1979). Delivery of stem cells to fetal mice, rats and other rodents requires open surgery, which carries a significant mortality rate of miscarriages. Later, Flake et al. successfully developed the sheep model with intraperitoneal allogeneic stem cell transplantation of fetal liver cells into a fetal recipient (Flake et al., 1986). Subsequently, several different animal models have been used, including mice (Blazar et al., 1995, Archer et al., 1997) and monkeys (Harrison et al., 1989).
Large animals such as the sheep model provide advantages since they allow surgical manipulation and ultrasound imaging can be used for prenatal assessment and to guide invasive procedures. Unlike the mouse model, performing a laparotomy for fetal sheep IUSCT is not needed. Ultrasound guided invasive procedures could be performed by an experienced obstetrician without any difficulty. The use of sheep as

a predictive model for the study of stem cell transplantation using ultrasound has been successfully established (Porada et al., 2008b). The sheep model has also been used to investigate minimally invasive ultrasound guided prenatal gene transfer (David et al., 2006b, Weisz et al., 2005). Techniques carry a low morbidity and mortality rate when performed under ultrasound guidance.

1.9.4 Route of administration

It is uncertain whether intravascular (IV) rather than intraperitoneal (IP) injection is associated with a higher frequency of donor cells in the target organ haematopoietic microenvironment in the fetal liver and marrow (Westgren et al., 1997). However, even if the intravascular route was more favourable, it may be technically difficult and risky at <14 weeks of gestation in human (Surbek et al., 2000) or early stage in rodent pregnancy, and therefore the IP route for *in utero* stem cell transplantation would be preferred by most investigators if stem cell delivery is to be performed early in pregnancy. Nevertheless, it is unknown to what extent the transplanted cells enter the circulation to reach the target organs. In sheep models, ultrasound guided intramuscular and intrahepatic routes of delivery of a viral vector to fetal sheep were also demonstrated and found to target the systemic circulation (David et al., 2006a, David et al., 2003b, David et al., 2010).

1.9.5 The development of the immune system in animals

The fetal immune response seems unlikely to play a significant role during early engraftment events after *in utero* cell transplantation while comparing allogeneic or autologous transplantation (Schoeberlein et al., 2004). Reports that show that fetal sheep are not capable of reacting to foreign antigens before 60 to 65 days of gestation, while the cell-mediated immune response is elicited at approximately 70 days of gestation, are in line with this observation (Miyasaka and Morris, 1988). Furthermore, Schoeberlein et al. suggested that early engraftment of donor stem cells in haematopoietic niches after *in utero* transplantation does not seem to be MHC-restricted. Other studies also found there was no difference in short-term engraftment between congenic and allogeneic donor stem cells in non-deficient (Shaaban et al., 1999) and deficient mice (Barker et al., 2003). In fetal sheep, it has been shown that co-transplantation of donor stromal cells as well as allogeneic stromal cells enhances

engraftment of donor haematopoietic stem cells (Almeida-Porada et al., 1999). Other mechanism such as the competitive marrow population by host haematopoiesis in later development are more likely to be responsible for the limited engraftment of donor cells in recipients with a quantitatively normal haematopoiesis. Nevertheless, it remains open whether these results can be translated to human fetuses, because reactive lymphocytes can be found already at 10 weeks in human fetuses.

1.10 The concept and clinical applications of amniotic fluid stem cells in prenatal and postnatal autologous transplantation

Stem cells derived from amniotic fluid could be collected, cultured, differentiated into cells with MSC or HSC potential and then used for stem cell therapy applications. The major advantage of amniotic fluid stem cells is the timing, which means that potential cells could be obtained as early as the second trimester of gestation providing a chance /time window for autologous transplantation back into the same fetus. In addition, the amniotic fluid stem cells could be stored for further postnatal use with autologous stem cell origin. Figure 1.6 shows this concept of prenatal and postnatal autologous transplantation using human amniotic fluid stem cells.

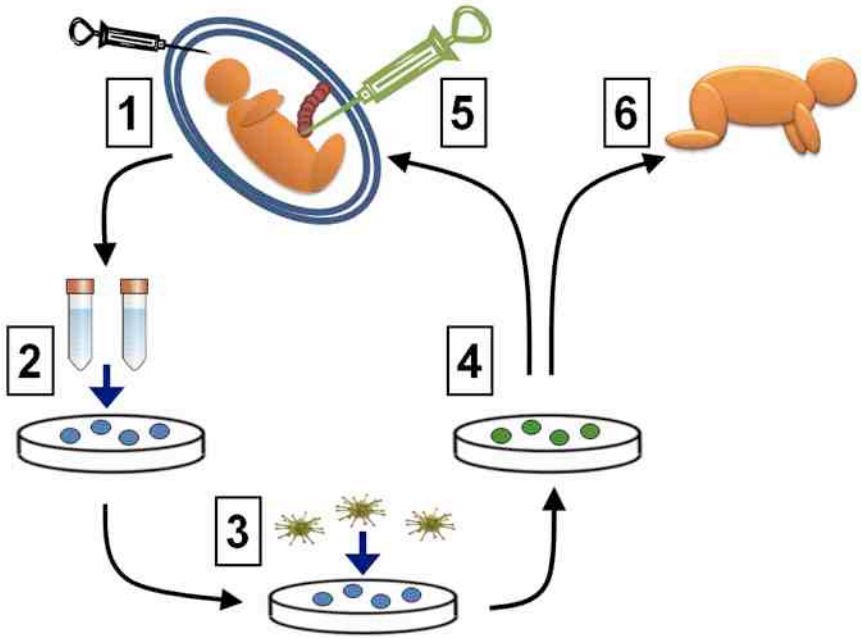


Figure 1.6 The concept of *in utero* and postnatal autologous transplantation using amniotic fluid stem cells.
The amniotic fluid could be collected under ultrasound guided amniocentesis (1), and cultured *in vitro* (2). These cells could be transduced with a vector encoding a therapeutic gene (3) then the transduced cells are expanded *in vitro* (4). Expanded/transduced cells could be transplanted back into the same fetus after a few weeks as a fetal autolgous transplantation (5), alternatively, stored stem cells or engineered tissues could be provided for an affected newborn postnatally (6).
(Figure was taken from Shaw et al, 2011b)

1.11 Clinical applications of amniotic fluid stem cells: Postnatal tissue repair

In previous section (Chapter 1.6), *in utero* stem cells transplantation was discussed by using amniotic fluid stem cells. Here, amniotic fluid derived stem cells have been used in variety of tissue repair studies. Amniotic fluid stem cells have been cultured and isolated from human, mice, rat, rabbit, and sheep. Those cells showed the potential to repair the chest wall injury, cardiac injury, neurologic disorder/injury, lung injury, bladder injury, hematopoietic disorder, tracheal repair and diaphragmatic hernia repair (Table 1.5) (De Coppi et al., 2007b, Ditadi et al., 2009, Carraro et al., 2008, Cheng et al., 2010, Fuchs et al., 2004, Ghionzoli et al., 2010, Iop et al., 2008, Klein et al., 2010, Kunisaki et al., 2006, Pan et al., 2009, Perin et al., 2010, Rehni et al., 2007, Schmidt et al., 2008, Yang et al., 2010, Yeh et al., 2010, Zani et al., 2009, Shaw et al., 2011a). Most of them are demonstrated the ability of tissue repair in adult or neonate stage. There are only few studies using amniotic fluid stem cells to do transplantation or tissue repairing prenatally (Shaw et al., 2011a, Kunisaki et al., 2006). AFS cells could be a great and potential source to treat congenital diseases in utero in the near future.

For autologous repair, tendon was engineered using sheep mesenchymal amniocytes labelled with green fluorescent protein (GFP), that was expanded and seeded into a collagen hydrogel. This was then used to repair a diaphragm defect postnatally after creation in the donor sheep fetus. Cells that were labelled with GFP were tracked in the sheep and visualised at sacrifice. Repair of the diaphragmatic hernia with the autologous AFMSC engineered tendon had a lower recurrence than repair using the collagen hydrogel alone, Diaphragmatic hernia recurrence was significantly higher in animals with acellular grafts then in animals with cellular ones supporting the autologous approach (Fuchs et al., 2004).

Table 1.5 Prenatal and postnatal application of amniotic fluid stem cells.
(Modified from Shaw et al., 2011b)

Target disease or model	Animal	Donor cells	Transplantation	Route	Reference
Prenatal approach					
In utero autologous transplantation	Sheep	Sheep AFMSC	Autologous	intraperitoneal	Shaw et al., 2011a
Fetal tracheal reconstruction	Sheep	Sheep AFMSC	Autologous	Cartilaginous graft	Kunisaki et al., 2006
Postnatal approach					
Cryoinjured bladder	Rat	Rat AFS	Allogeneic	Intravascular injection	De Coppi et al., 2007b
Hematopoietic system repair	Immuno-suppressed mice	Human and mice AFS	Xenogeneic or Allogeneic	Intravascular injection	Ditadi et al., 2009
Postnatal chest wall repair	Rabbit	Rabbit AFMSC	Autologous	osseous graft with scaffold	Klein et al., 2010
Sciatic nerve crush injury	Rats	Human AFMSC	Xenogeneic	Direct injury site injection	Cheng et al., 2009
Postnatal diaphragm reconstruction	Sheep	Sheep AFMSC	Autologous	Autologous tendon	Fuchs et al., 2004
Cell migration in abdomen	Rat	Rat AFS	Allogeneic	Intraperitoneal injection	Ghionzoli et al., 2010
Heart cryoinjury	Rat	Rat AFMSC	Allogeneic	Intracardiac injection	Lop et al., 2008
Sciatic nerve crush injury	Rats	Human AFMSC	Xenogeneic	Direct injury site injection	Pan et al., 2009

Actue tubular necrosis	Immune-deficient mouse	Human AFMSC	Xenogeneic	Intravascular injection	Perin et al., 2010
Cerebral ischaemia-reperfusion injury	Mice	Human AFS	Xenogeneic	Intracerebroventricular injection	Rehni et al., 2007
Heart valve tissue engineering	-	Human AFS		In vitro study	Schmidt et al., 2008
Parkinson's disease	Rat	Human AFS	Xenogeneic	Intracerebroventricular injection	Yang et al., 2010
Postnatal cardiac repair for myocardiac infarction	Immune-suppressed Rat	Human AFMSC	Xenogeneic	Intracardiac injection	Yeh et al., 2010
Hyperoxia lung injury	Mice	Human AFS	Xenogeneic	Intravascular injection	Carraro et al., 2008

Several studies also have been performed to find an appropriate cell source for the treatment of a wide range of disorders. For instance, the chondrogenic potential of AFS have been studied and established in order to carry out the regeneration of damaged articular cartilage (Kolambkar et al., 2007). The feasibility of using ovine BM-MSC as a source of heart valve engineering has been demonstrated (Perry et al., 2003). This was supported by the ability of cells to populate in scaffolds and possess similar biomechanical properties. Many fetuses with structural defects undergo amniocentesis for prenatal diagnosis, because of the associated high risk of fetal aneuploidy. This includes such abnormalities as exomphalos, congenital diaphragmiatic hernia and major cardiac defects. Amniotic fluid stem cells could be collected either via amniocentesis earlier in gestation or at Caesarean delivery, if sufficient time was available between their collection and requirement for organ repair. Much will depend on the different characteristics of cells collected at different gestational age time points and further research is needed to explore this.

Therefore, autologous transplantation using amniotic fluid stem cells could be an ideal way and strategy in the near future as figure 1.6. These cells could be injected back into the same fetus within few weeks before the disease progression or into newborn afterwards if longer time needs for tissue engineering. Stem cells derived from amniotic fluid are a relative new source of cells which is still relatively unknown but that could have a therapeutic value in various diseases diagnosed prenatally and treated pre and/or postnatally.

1.12 Study aims

In my thesis, I investigated the feasibility and potential of amniotic fluid for prenatal therapy in order to combine IUSCT and gene therapy into one therapeutic strategy.

1. to determine whether *in utero* transplantation of autologous amniotic fluid stem cells was feasible.
2. to compare engraftment after *in utero* transplantation of amniotic fluid stem cells from congenic and allogeneic sources and to investigate whether this technique can correct a mouse model of haematological disease.
3. to investigate whether human amniotic fluid stem cells can be transduced and used for *in utero* stem cell transplantation.

For Aim 1, I explored amniotic fluid mesenchymal stem cells and used the fetal sheep, which has the advantages of long gestational age, similar fetal size and comparable fetal development and physiology to human pregnancy (David et al., 2006b, David and Peebles, 2008) and in which there is wide experience with fetal interventional procedures (David et al., 2006a, David et al., 2003a). Second, novel sheep CD34 antibody was used for sorting the CD34+ sheep AFS cells that would be autologous transplanted back into the sheep fetus as the same procedure.

For Aim 2, I used a small animal model the mouse, to investigate the prenatal cell or cell-based gene therapy using marking vectors. Congenic versus allogeneic transplantation were also compared in mice model using mice AFS cells. I further investigated a disease model of thalassemia for prenatal therapy of small animals.

For Aim 3, I collected and cultured human amniotic fluid samples in vitro to determine their pluripotency and characterization. Ex vivo expandable cells could be transduced with viral vectors and used for xenotransplantation.

Chapter 2

Material and Methods

2 Chapter 2: Material and Methods

2.1 Sheep experiments

2.1.1 Generation of time mated sheep

Sheep of Romney breed were used. Each ewe was ear tagged twice with an identification number and the sheep flock was vaccinated against Toxoplasma, Chlamydia and Clostridium annually. All procedures on animals were conducted in accordance with the UK Home Office regulations and the Guidance for the Operation of Animals (Scientific Procedures) Act (1986) under Project Licence number 70/6546 titled 'Fetal gene therapy *in utero'*.

All experiments were conducted on time-mated sheep to ensure the gestational age of fetus was consistent. Tupping of sheep was begun in early autumn during the sheep breeding season and approximately 20 sheep were tupped each year. To enable timed mating of sheep, Chronogest® sponges (Flugestone acetate) containing 30mg of progesterone were placed in the vagina of ewes for 2 weeks to induce ovulation. Forty-eight hours after removal of the progesterone sponges, ewes were placed in a pen with the ram overnight. The abdomen of the ram had been marked beforehand with Ram Raddle®, a coloured powder mixed with liquid paraffin that permanently marks the fleece on the back of the ewe once she has been tupped. Ewes that had been marked the next morning were presumed to have been successfully tupped. Two weeks later ewes were placed back in with the ram overnight in case of unsuccessful tupping the first time. A different coloured Ram Raddle was used to be able to differentiate the two tupping dates. Ewes were scanned by the technical staff at the Royal Veterinary College (RVC) at approximately 50 days of gestation to confirm pregnancy. Non-pregnant ewes were re-sponged and re-tupped a month later. The success rate of sheep pregnancy on tupping was 85%.

Pregnant ewes were sent from the RVC, Hawkshead Campus to the Biological Services Unit (BSU), RVC Camden Campus in a lorry one week before use to allow acclimatization. At the BSU in Camden, they were housed together in pairs in

specially designed sheep pens at ambient temperature. The floor of the pens was covered with straw and the ewes had ad lib access to hay and running water and were given a daily portion of Super Ewe and Lamb compound feed. The feed is a concentrated source of oil, protein and vitamins and the amount given to sheep was increased as gestation progressed or when there was more than one fetus. This ensures adequate nutrition for the ewe and reduces the risk of toxaemia caused by the large late gestation fetus compressing the rumen and reducing the ability of the ewe to digest enough hay for nutrition.

Total of 19 ewes (Romney breed) were used in my projects. All pregnant sheep were scanned the day after arrival at the BSU, Camden to confirm pregnancy, fetal number and gestational age. Ewes were caught, turned up and held in a sitting position for scanning by an experienced animal technician. The fleece was then clipped from the abdomen. Using gel (Ultragel Vet, Transpharma Sas, Italy) applied to the suprapubic area and lower abdomen, an experienced fetal medicine specialist (Anna David or myself) scanned the uterus using an Acuson 128 XP10 ultrasound scanner (Siemens, Bracknell, United Kingdom) with a C3 3.5 MHz curvilinear transducer. Fetal number was checked and the gestational age of the fetuses was confirmed by comparing the biparietal diameter (BPD) and occipito-snout length (OSL) with published data (Barbera et al., 1995b).

2.1.2 Sheep anaesthesia and preparation for surgery

Prior to surgery, the sheep were starved overnight on wood chip bedding with free access to water. During this fasting period, a companion animal was also placed and fasted with the sheep that had to undergo surgery the next morning to prevent distress. The overnight fasting was to prevent bloating that may occur when sheep under anaesthesia are unable to belch (eructate) normally and release methane gas, which is a by-product of the fermentation of their food.

On the morning of surgery, the sheep was run down the corridor to the theatre room where she was ‘turned’ and restrained. The wool was clipped from her neck, the jugular vein was cannulated using a 19 Gauge butterfly winged perfusion set (Terumo Europe NV, Leuven, Belgium) and general anaesthesia was induced with thiopental

sodium 20mg/kg intravenously (IV, Thiovet, Novartis, Animal Health UK Ltd., Hertfordshire, UK). Once asleep, the ewe was lifted onto the operating table and intubated supine with a 9.0 mm cuffed endotracheal tube (Portex, UK) using a laryngoscope (Penlon, UK) to visualize the vocal cords. A cuff was inflated on the endotracheal tube to prevent inhalation of regurgitated ruminal contents and the ewe's head was lowered to allow drainage of saliva and ruminal fluid. Because access to the whole abdomen was needed for procedures, the animals were kept in dorsal recumbency. Anaesthesia was maintained with 2%-2.5% Isoflurane in oxygen (Isoflurane-vet, Merial Animal Health Ltd., Essex, UK) using a Magill circuit (Medishield ventilator, Manley Serovent). The induction and maintenance of general anaesthesia was performed by Mr. Michael Boyd, Theatre Manager, Biological Services Unit, RVC Camden.

Once anaesthetized, the wool was clipped from the ewe's abdomen and a detailed ultrasound examination of the uterus and its contents was performed using a 3.5MHz probe as before. Fetal measurements were collected, namely biparietal diameteter, occipito-snout length, abdominal circumference, femur length and umbilical artery Doppler pulsatility and resistance indices. The abdominal surface was scrubbed with 'Hibiscrub' (Chlorhexidine gluconate 4% w/v, Regent Medical, Manchester, UK) followed by 'Povidone' (1% w/w Iodine solution, Vetasept Animal Care Ltd., York, UK). The abdomen was exposed with sterile drapes.

A pulse oximeter (5250 RGM, Ohmeda) placed on the ewe's ear was used to monitor oxygen saturation and pulse rate during general anaesthesia and this data was documented every 15 minutes together with respiration rate, isoflurane concentration and level of anaesthesia. Maternal blood was taken for pre-operative haematological and biochemical analysis. Blood was collected into BD Vacutainer tubes (BD Vacutainer systems, Plymouth, UK) containing 0.105 M sodium citrate for plasma (9C, blue topped bottle), silica clot activator polymer gel for serum (yellow top bottle) and potassium EDTA (K3EDTA) for whole blood (purple top bottle).

2.1.3 Collection of amniotic fluid at necropsy

Some amniotic fluid samples were collected at necropsy using the uninjected sheep of a colleague's PhD project. These amniotic fluid samples were prepared for xenotransplantation into immunocompromised mice or *in vitro* stem cell characterization. For these samples, at the end of gestation (136 to 144 days) the sheep underwent terminal general anesthesia. The abdomen was cleaned and draped to maintain sterility and the abdomen was opened via a longitudinal incision. The uterine wall was incised to expose the amniotic membrane which was held carefully with sterile tissue clamps without touching the chorionic vessels. A 1 to 2 cm incision on the membrane was made and a sterile 50ml syringe was inserted into the amniotic cavity to collect all of the amniotic fluid. The collected amniotic fluid was placed on site at room temperature for further experiments. Fetal biometry and weight were noted.

2.1.4 Amniocentesis in sheep

For the amniocentesis collection procedure, sheep were prepared for starvation overnight before surgery. General anesthesia was induced with Ketamine (4mg/kg, Merial, UK) intravenously and after intubation, the ewes were then maintained on Isoflurane-Vet 3% (Merial, UK) in oxygen. Fetal measurements were confirmed by ultrasound as described (David et al., 2003b). The abdomen was cleaned and draped to maintain sterility. The first procedure, amniocentesis (Figure 2.1), was performed in early pregnancy (57 - 61 days of gestation, term = 145 days) under ultrasound guidance using a 22 Gauge, 15cm echo-tip needle (Cook Medical, USA) and 10 to 20 ml of amniotic fluid was withdrawn from each amniotic sac.

The ewe was recovered and two days later fetal wellbeing was confirmed using ultrasound examination. After amniocentesis collection, amniotic fluid mesenchymal stem cells (AFMSCs) were selected and cultured in adherence in defined conditions (see later). AFMSCs were transfected with lentivirus GFP (see later) and in a subset of animals were re-injected into the peritoneal cavity of the fetal donor (see later).

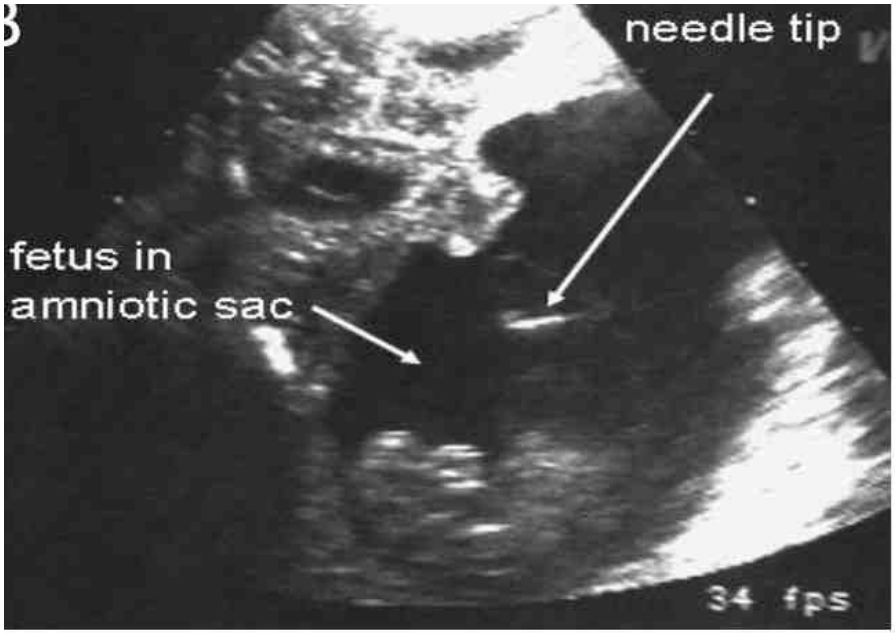


Figure 2.1 Sonogram of ultrasound-guided amniocentesis in sheep.

2.1.5 Ultrasound-guided in utero reinjection of transduced amniotic fluid stem cells

2.1.5.1 Sheep amniotic fluid mesenchymal stem cells project

In a subset of animals, approximately two weeks later (71-83 days of gestation) sheep were re-anaesthetized and prepared as above for a second procedure. Transduced GFP+AFMSCs (average $7.5X10^6$ cells in 1-2 ml PBS) were injected under ultrasound guidance into the peritoneal cavity of the fetus from which they were derived (Figure 2.2) using a 20 gauge 15cm echo-tip disposable needle (Cook Medical, USA). To ensure correct needle placement within the peritoneal cavity, the needle was inserted through the anterior abdominal wall of the fetal sheep superior and lateral to the fetal bladder to avoid the umbilical arteries, and microbubbles were observed moving within the peritoneal cavity as the vector was instilled.

2.1.5.2 Sheep amniotic fluid CD34+ cells project

In a second group of animals the 2 procedures (amniocentesis and transplantation) were schedule only 48 hours aside to evaluate haematopoietic engraftment. In those animals, amniocentesis (first procedure) was performed between 60 and 64 days of gestational age but transplantation of freshly isolated CD34+ amniotic fluid stem cells (20000) back in to the fetal donor was performed 2 days later. Fetal wellbeing was confirmed after the procedure using ultrasound examination. Animals were recovered in theatre and extubated before being placed back in their pen.

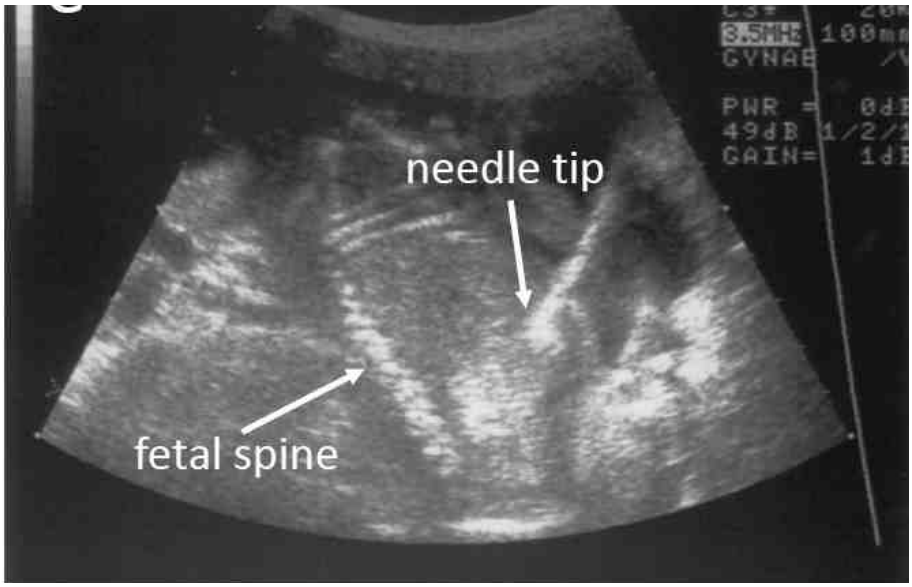


Figure 2.2 Sonogram showed ultrasound guided intraperitoneal injection.

2.1.6 Follow up after amniocentesis or ultrasound guided reinjection of transduced amniotic fluid stem cells

Following recovery the ewes were scanned regularly to check for fetal wellbeing and fetal growth every 20 days for long-term follow up project by ultrasound examination. Occipital snout length (OSL), biparietal diameter (BPD), Femur length (FL), and abdominal circumference (AC) were checked by ultrasound direct measurement.
For analysis of MSC engraftment, animals underwent scheduled necropsy three weeks after *in utero* cell injection. For analysis of HSC engraftment we monitored the ewes and lambs up to 6 months after delivery. All the delivery data were recorded immediately including birth weight, initial assessment form, time to standing, girth at umbilicus, biparietal diameter, standing height to shoulder (using a ruler), gender, heart rate, respiratory rate and rectal temperature **(Appendix I).** Lambs were fed naturally with maternal breast milk. All the growth data were also recorded monthly including weight, shoulder height, and umbilical girth by ruler. Lamb peripheral blood was taken from jugular veins using No. 18 needles with 20ml syringe every 2 weeks after birth. The blood samples were collected in EDTA tubes for flow cytometry analysis.

2.1.7 Postmortem examination of sheep and lambs

The post-mortem examination was performed approximately five days before the expected date of delivery (range 3 to 7 days, delivery usually at 145 days of gestation) or three weeks after intraperitoneal AFMSC transplantation (89-103 days of gestation). The ewe was anaesthetized as before, and fetal measurements were recorded by ultrasound. Maternal blood was sampled for biochemistry and haematological analysis. The ewe then received an overdose of phenobarbitone
The abdomen was opened, the uterus exposed and amniotic fluid collected into a syringe and snap frozen. The umbilical cord was tied and cut and the fetal sheep was removed from the uterine cavity. Fetal blood was sampled from the umbilical veins for engraftment analysis, biochemistry and haematological analysis. Maternal and fetal organs were sampled widely according to a standard procedure, organs were weighed and measurement of fetal parameters was undertaken **(Appendix II).**
Samples from each tissue were snap-frozen in liquid nitrogen for future nucleic acid/protein extraction. One sample from each tissue was placed in a histology cassette and dropped into 4% paraformaldehyde (pH 7.4) overnight before transfer into 70% ethanol.

For the long term follow up project, in which maintenance of postnatal engraftment, the ewe and lamb underwent scheduled necropsy at 3 and 6 months after birth respectively. Surgical and anesthetic procedures were the same as previously described, with the same protocol of collecting all organ samples. For the lamb necropsy, general anesthesia was set up for the final procedure of this experiment to collect bone marrow and all internal organs under sterile conditions. The sternum was cut using automatic or manual saws, and then bone marrow was collected directly by a surgical spoon.

All the post-mortem procedures were followed as written protocol **(Appendix III)**. The comprehensive postmortem included all the fetal and maternal organ sampling, and fetal biometry measurement. There was widespread sampling of maternal and fetal tissues, that were fixed in 4% paraformaldehyde (pH7.4) overnight and processed into wax for immunohistochemistry analysis. In parallel, tissues were also snap-frozen for protein extraction and PCR analysis. Cord blood, spleen, liver and

bone marrow of fetal sheep and maternal peripheral blood, bone marrow were collected and placed in PBS for FACS flow cytometry.

2.2 Sheep stem cell culture

2.2.1 Ex vivo expansion of sheep amniotic fluid mesenchymal stem cells

Amniotic fluid samples collected from fetal sheep were centrifuged (300g) for 5 minutes. Pellets were resuspended in the amniotic culture medium (ACM) which consisted of αMEM (63%, Life Technology, Gaithersburg, MD), Chang Medium (20%, Chang B plus Chang C; Irvine Scientific, Santa Ana, CA), fetal bovine serum (FBS, 15%, Invitrogen, UK) and streptomycin, penicillin and L-glutamine (1% each, Invitrogen, UK). Cell seeding was performed on single 35 mm Falcon petri dishes (Becton Dickinson, Los Angeles, Ca) that incubated at 37°C with 95% air and 5% carbon dioxide. After 3 days, non-adherent cells and debris were discharged while changing new medium and the adherent cells were cultured until pre-confluence. Adherent cells were detached from the plastic substrate using 0.05% trypsin and 0.02% sodium-EDTA (Life Tech). Cells were passaged into 100mm petri dishes manufacturer and further split one to four once the dish reached 70% confluence. Cells were counted by using a haemocytometer (Marienfeld, Germany) before viral transduction (Figure 2.3). The formula used for the doubling time (hours) calculation was Doubling time = h*log(2)/log(c2/c1) c1: initial seeding cell number, c2: cell number after growth, h: hours of culture.

Figure 2.3 Haemocytometer.

2.2.2 Differentiation and characterization of sheep amniotic fluid mesenchymal stem cells

In order to characterize the isolated and cultured AFMSC, the following surface markers were used. Mouse anti-sheep CD31, CD44, CD45 and CD58 antibodies conjugated with fluorescein isothiocyanate (FITC) (AbD Serotec, UK) were used for characterization of the sheep AFMSC. 10µl antibodies were stained with $5X10^5$cells in 100µl ice cold PBS. The incubation time was 15 minutes. The stained cells were analyzed with flow cytometry. To induce adipogenic differentiation, cells were seeded at a density of 3,000 cells/cm2 and were cultured in DMEM low-glucose medium with 10% FBS, antibiotics (Pen/Strep, Gibco/BRL), and adipogenic supplements (1 mM dexamethasone, 1 mM 3-isobutyl-1-methylxanthine, 10 mg/ml insulin, 60 mM indomethacin (Sigma-Aldrich, UK). The cells were stained with Oil red O (Sigma-Aldrich, UK).

For osteogenic differentiation, cells were seeded at a density of 3,000 cells/cm2 and were cultured in DMEM low-glucose medium with 10% FBS (FBS, Gibco/BRL), Pen/Strep and osteogenic supplements (100 nM dexamethasone, 10 mM betaglycerophosphate (Sigma-Aldrich, UK), 0.05 mM ascorbic acid-2-phosphate (Wako Chemicals, UK). Alkaline phosphatase was used for osteogenic cell staining.

2.2.3 Isolation and culturing of sheep CD34+ amniotic fluid cells and adult bone marrow cells

Fresh sheep amniotic fluid was collected during the first procedure-amniocentesis between 60 to 64 days. The primary CD34 monoclonal IgG1 antibody, not available in the market and just recently described, was kindly provided by a collaborator, Dr Christopher Porada, Wake Forest Institute of Regenrative Medicine, North Carolina, USA (Porada et al., 2008a). Adult sheep bone marrow cells, used as control, were collected from the sternum of uninjected adult ewes as previously described at post-mortem examination. Then the bone marrow was washed by passing through the cell strainer with PBS. Either sheep amniotic fluid cells or bone marrow cells were prepared as single cell suspension in PBS and counted the cell number before sorting. The sorting procedure was performed using MACS system (Miltenyi Biotec, Germany) followed by the protocol published by the. Briefly, primary CD34 antibody

was incubated with sheep amniotic fluid total cells or BM cells for 15 minutes on ice at 1 in 100 concentration. After washing out the primary antibody, the secondary antibody (Rat anti-Mouse IgG1 MicroBeads, cat #130-047-101) was incubated with the previous cell suspension for 15 minutes on ice at the same concentration. Before starting the sorting, the MS column was rinsed by PBS. The CD34 positive fraction conjugated with microbeads were collected after three times PBS washing out the negative fraction through MS cell column. The sorted CD34 positive cells were characterized with the sheep specific surface antibodies including FITC or PE conjugated CD14, CD31, CD44, CD58, CD34, and CD45 (all from AbD Serotec, UK). The setting of the flow cytometry to analyses amniotic fluid or bone marrow CD34 positive cells was the same as the previous section 2.2.2.

The fresh, defrosted amniotic fluid cells or bone marrow CD34+ cells were collected and cultured for 48 hours with viral transduction using the lentivirus. For the fresh frozen amniotic fluid cells, the cells was pelleted and stored in the liquid nitrogen 3 to 6 month containing 90% FBS and 10% DMSO. These cells were defrosted immediately after returning to room temperature. The cell suspension was centrifuged 1500rpm for 5 minutes, then the supernatant was discarded. The defrosted amniotic fluid cells were suspended in the cultured medium and for further viral transduction. The culture medium contained basal IMEM, 10% FBS with StemSpan CC100 with a combination of cytokines including Flt-3, Stem cell factor (SCF), IL3, and IL6 (Stemcell Technologies, UK). 100000 CD34+ cells sorted from fresh or frozen amniotic fluid, or bone marrow were seeded in the 24-well low attachment plates with culturing medium for 48 hours. The incubation condition was 37°C containing 5% CO^2. The 10ul lentivirus was added while seeding the cells into each well of 24-well plate for transduction (MOI=50). After viral transduction, the cell suspension was washed and re-suspended in PBS for in utero injection or mice injection.

2.3 Mouse experiments

2.3.1 Generation of pregnant mice and surgical procedures

All the mice works were recorded in Chapter 5 and the animal procedures were carried out either in Imperial College South Kensington Campus or University College London Institute of Child Health under UK Home Office Project License number 70/6629 (Mechanisms of mammalian birth defects) or 70/6014 (Gene delivery to small animals).

Before the mice could be made available for experiment, the indicated colony was expanded. I set up the breeding cage, which contained one female and one male from the same genetic background for long-term breeding. For the breeding cages, it was not important to know which day the mice were mated and pregnant until the female animal gave birth. The newborns were kept in the parental cage till 21 days after delivery. The ear marking for labeling the newborns was performed before moving them into the new individual cages. For each background colony I set up some breeding cages to maintain the different genetic mice for all the experiments.

For mouse experiments it was necessary to know the exact mating day for in utero injection procedure or amniotic fluid collection. The timing of amniotic fluid collection and in utero transplantation was between E12 and E14. Therefore I put two female mice in the male cage that only contain one male and then I checked for a vaginal plug of female mice on the following morning which would indicate that mating had occurred. If a vaginal plug was found, I separated the female mouse into another cage and marked this day as E0. If no vaginal plug was found, I put the female mouse back into the original female cage and re-mated again two weeks later. Pregnant mice were always observed and examined by palpation to confirm the pregnancy status before the surgery.

For the in utero cell transplantation (using mice or human cells), the pregnant mouse on E14 was sedated initially by vaporised Isoflurane-Vet 5% in oxygen (VetTech Solutions Ltd, UK) and maintained by vaporised Isoflurane-Vet 3% in oxygen. The abdominal wall of mice was sterilized by Povidone-Iodine surgical scrub (Vetasept, UK). Via longitudinal laparotomy, the abdominal cavity was exposed and the number

of fetal mice within the bicornuate uterus was counted. 10 to 20 ul amniotic fluid stem cells or gene-transduced cells in PBS were injected through the uterus surrounding with amniotic fluid into the belly and beneath the liver (intraperitoneal, IP) of each pup or via yolk sac vessels (intravascular, IV) via 33 gauge needles (Hamilton, UK) (Figure 2.4).

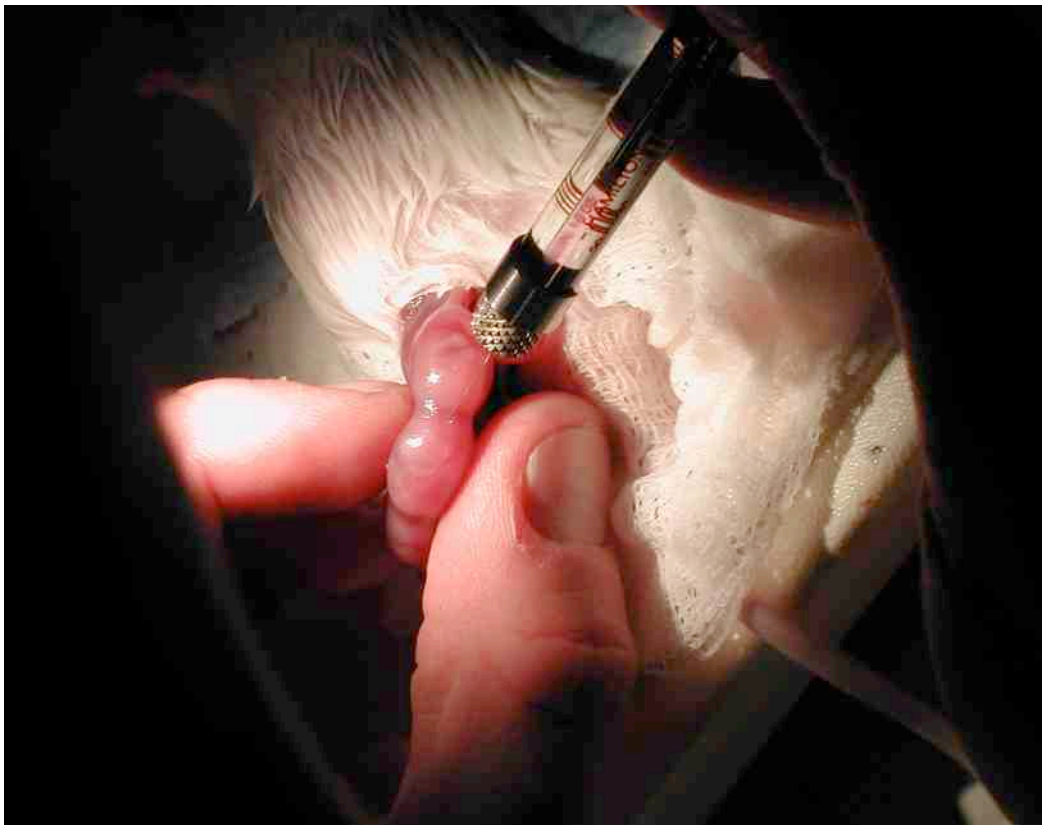


Figure 2.4 Intraperitoneal injection using Hamilton needle.
(The photo was provided by Dr. Waddington)

After injection of the cells, Isoflurane was stopped and transplanted mice were placed in the heating chamber for 30 minutes till full recovery from the surgery. Every transplanted mouse was fed with normal food in the individual cage and regularly checked up by animal house staff or me. After injection of the cells, the transplanted mouse were recovered by stopping the Isoflurane then placing them in the heating chamber for 30 minutes till full recovery from the surgery. Every transplanted mouse was fed with normal food in the individual cage and regularly checked up by animal house staff or me daily.

The delivery rate and newborns survival were recorded immediately after birth around E20 to E21. The newborns would be weaned and move to new cages 21 days after birth. The post-mortem procedure and blood or organ sampling of all the mice work were described in the following sections.

2.3.2 Experimental mice used

In this section, I will list all the experimental mice that I used in the thesis.

beta-actin/YFP mice

The original R26-stop-EYFP mutant mice have a loxP-flanked STOP sequence followed by the Enhanced Yellow Fluorescent Protein gene (EYFP) inserted into the Gt(ROSA)26Sor locus. When bred to mice expressing Cre recombinase, the STOP sequence is deleted and EYFP expression is observed in the cre-expressing tissues of the double mutant offspring. This strain was held in the UCL institute of Child Health that were purchased from The Jackson Laboratory USA. This mouse was mated with beta-actin Cre recombinase strain in house that expresses Cre recombinase in all cells of the embryo by the blastocyst stage of development to produce the beta-actin/YFP mice. I used beta-actin/YFP mice for most of the amniotic fluid collection experiments and congenic or allogeneic prenatal transplantation.

MF1 mice

These outbred mice were held in Imperial College London and used as the wild type control. These albino mice, which were originally developed at Olac prior to 1970, came from a cross between the Laboratory Animal Centre A-strain mice and from Scientific Products Farm (acquired by Charles River Laboratories, UK). The stock was re-derived by Charles River Laboratories in 2004 and subsequently transferred to Charles River Laboratories UK in April 2005. I used this colony mainly on the experiments of prenatal cell-based gene delivery model and tracking the luciferase activity after transplantation as described in Chapter 5.2.

CD1 mice

This CD mouse is also an outbred colony that I used for most of the experiments in UCL Institute of Child Health. CD1 could be purchased directly from Charles River

UK and set for time-mated experiments. This wild type female mouse is easy to get pregnant and has low surgery-related death rate. The original group of Swiss mice that served as progenitors of this stock consisted of two male and seven female albino mice derived from a non-inbred stock in the laboratory of Centre Anticancereux Romand, Lausanne, Switzerland. These animals were imported into the United States in 1926, United Kingdom in 1959. The strain code is 022 in Charles River Laboratory, UK.

B6 mice

This is the most common wild type with black skin color colony could be available either UCL or Imperial College. Unlike CD1 or MF1 mice, B6 mice are inbred which could be applied for the congenic transplantation experiments if the donors/recipients also have the same genetic background and black color. B6 are developed in 1921, from a mating of Miss Abby Lathrop's stock that also gave rise to strains C57BR and C57L. Strains 6 and 10 separated about 1937 and shipped to Charles River Laboratory UK in 1974. The strain code is 027.

Th3 thalassemia mice

The thalassemia mice I used in the project were held in Imperial College London, South Kensington Campus. Dr. Simon Waddington initially managed and set up these mice for human globin gene therapy experiments and I started to expand this colony for prenatal cell therapy since early 2009. The colony could also be purchased at Jackson Laboratory US, B6.129P2-Hbb-b1^{tm1Unc} Hbb-b2^{tm1Unc}/J (stock number: 002683). Th3 thalassemia mice firstly developed in 1995 by Yang et al. Homozygous mice die prenatally or perinatally. Heterozygous mice show characteristics typical of severe thalassemia (anemia) and are fertile. (Yang et al., 1995)

NOD-SCID gamma mice (NSG)

Mutant NOD-SCID gamma (NSG) mice were used for transplantation experiments that combine the features of the NOD/ShiLtJ background, the severe combined immune deficiency mutation (SCID) and IL2 receptor gamma chain deficiency. As a result, the NSG mice lack mature T cells, B cells, or functional NK cells, and are deficient in cytokine signaling, leading to better engraftment of human hematopoietic

stem cells and peripheral-blood mononuclear cells than any other published mouse strain. The youngest age of NSG mice we used was 12 weeks.

2.3.3 Xenotransplantation to adult NOD-SCID gamma mice using sheep cells

The youngest age of NSG mice I used was 12 weeks. All the animal procedure was licensed by UK Home Office Project Licence number 70/6629. Before xenotransplantation, sublethal dose irradiation (300 Rads for 67 seconds) to the NSG mice was performed in Institute of Child Health (ICH) of UCL. 300000 sheep CD34+ fresh or frozen AF cells, or adult BM cells in 0.2ml PBS were injected into the tail vein of primary or secondary NSG mice 1 hour after irradiation under isolation hood. The mice were restrained to limit the movement of mice and to expose the tail. The injection needle here I used was 1ml insulin syringe with needle. At 12 weeks of age, all recipient mice were sedated by vaporised Isoflurane-Vet 5% in oxygen (VetTech Solutions Ltd, UK) and the blood samples from central circulation were collected via intracardiac puncture by a 22 gauge needle. The mice were sacrificed by manual dislocation of cervical cord and were dissected through midline incision on chest and belly. Tissues from liver, heart, striated muscle on thigh, lung, kidney, spleen, adrenal gland and gonad were harvested. Femur bones were collected for retrieval of bone marrow.

2.3.4 Follow up after in utero transplantation and neonatal care

After injection of the cells, the transplanted mouse were recovered by stopping the Isoflurane then placing them in the heating chamber for 30 minutes till full recovery from the surgery. Every transplanted mouse was fed with normal food in the individual cage and regularly checked up by animal house staff or me daily.
The livebirth rate and newborn survival were recorded immediately after birth around E20 to E21. Mothers were checked daily and the neonatal mice were counted. Touching the newborn mice was avoided as far as possible to reduce the risk of maternal rejection. The newborns had usually weaned themselves and were moved to new cages 21 days after birth.

2.3.5 Blood sampling of alive mice

After being sedated by vaporised Isoflurane-Vet 5% in oxygen (VetTech Solutions Ltd, UK), the peripheral blood was sampled from an incision on the tail of recipient mouse. The incision was made using a scalpel (No. 11 blade), and the blood was squeezed out and collected by pipette into a heparin-rinsed microcentrifuge tube (50 to 100ul of blood). 1ml RBC lysis solution (5 PRIME Inc, USA) was added into each blood sample and the mixture was kept under room temperature for 5 minutes. The sample was centrifuged under 1500 rpm for 5 minutes then the supernatant was removed. A further 1ml RBC lysis solution was added and the same procedures were repeated till the pellet was clean. The pellet was resuspended in 300µl PBS for flow cytometry analysis.

2.3.6 Postmortem examination of mice

At different stage of age, all recipient mice were sedated by vaporised Isoflurane-Vet 5% in oxygen (VetTech Solutions Ltd, UK) and the blood samples from central circulation were collected via intracardiac puncture by a 22 gauge needle. The mice were sacrificed by manual dislocation of cervical cord and were dissected through midline incision on chest and belly. Tissues from liver, heart, striated muscle on thigh, lung, kidney, spleen, adrenal gland and gonad were harvested. Femur bones were collected for retrieval of bone marrow. All the post mortem procedure was written in the **Appendix IV.** The comprehensive postmortem included all the mice organ sampling. There was widespread sampling of mice tissues, that were fixed in 4% paraformaldehyde (pH7.4) overnight and processed into wax for immunohistochemistry analysis. Fresh tissues of NSG mice from liver and spleen were ground by a plunger of the syringe in a sterile dish, rinsed by PBS then filtered through a cell strainer of 40µm nylon mesh (BD, UK). Bone marrow of femur bones were chopped at bilateral ends and flushed out by PBS via 22 gauge needle with syringe then strained as well. 2ml RBC lysis buffer was added into each sample and the mixture was kept under room temperature for 10 minutes then centrifuged at 1500 rpm for 5 minutes. Supernatant was removed and the pellet was resuspended in 300µl PBS. 1ml PBS was added again to each tube and centrifuged at 1500 rpm for 5 minutes. Supernatant was discarded and the pellet was dispersed in 300µl PBS under 4°C for flow cytometry.

2.4 Mouse stem cell culture

2.4.1 Collection of mouse amniotic fluid and isolation of Ckit+/Lin- AFS cells

All the mice amniotic fluid cells for in utero injection were sorted for Ckit+/Lin- population before injection. Amniotic fluid was collected at E12 to E14 gestational age of YFP or MF1 mice. The pregnant dams were killed by cervical dislocation. Midline laparotomy was performed to expose the uterus. The two horns of uterus were dissected out carefully by surgical scissors and rinsed with PBS to remove the maternal blood. From each gestation sac containing one fetus, the amniotic membranes were peeled off to release the amniotic fluid (approximately 20ul) that was collected by gravity in a sterile petri dish (Figure 2.5). Red blood cell lysis buffer (2ml) was added to the fluid to lyse erythrocytes.

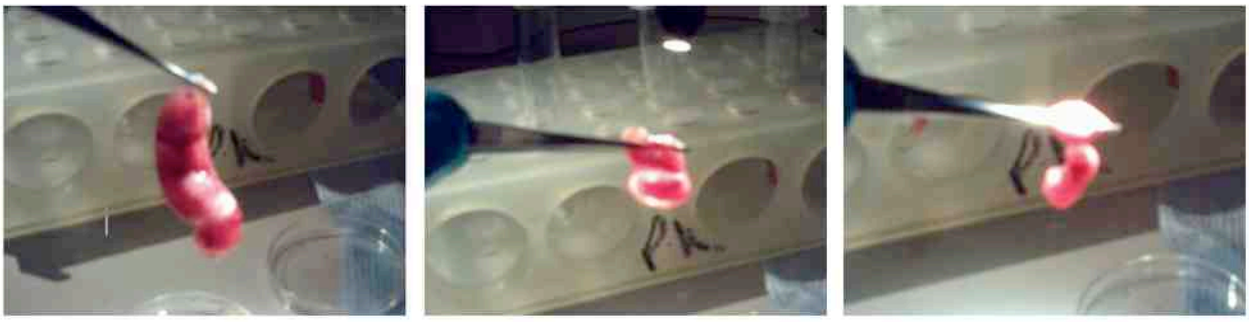


Figure 2.5 The method of collecting mice amniotic fluid.
Left panel, one horn of the uterus; middle panel, forceps lifting up the placenta to expose the sac; right panel, fetus exposure after peeling off the amniotic membrane

The cells were firstly negative selected with mice Lineage cell depletion kit (130-090-858, Miltenyi, UK). After adding Biotin-Antibody cocktail and Anti-Biotin MicroBeads, the negative fraction of cells was collected. Then these Lin- cells were positively selected with mice CD117 MicroBead (Miltenyi, UK) sorting system to have positive population following the procedure suggested by the manufacture. Fresh cell fraction was prepared in PBS on ice for prenatal IP injection on the same day. 10000 to 50000 cells were resuspended in 10 to 20ul PBS to be used for injection of a single pup.

2.4.2 Culturing and viral transduction of the Ckit+/Lin- AFS cells

Following the previous section, the Ckit+/Lin- sorted mice AFS cells were collected in PBS first. For most of the experiments, the sorted cells were freshly injected into the peritoneal cavity of fetal mice immediately without any culture. For viral transduction experiment, lentivirus vector with UCO-E promoter driving luciferase (LUC) were used for 24 hours amniotic fluid cells transduction (MOI=50). 50000 sorted and transduced AFS cells in 20µl PBS were prepared and injected intravenously into each MF1 fetal mouse at E14. To preserve the stem cell activity, cells were cultured with StemSpan (StemCell Technologies, UK) culture medium in 24-well ultra-low attachment plate with essential cytokines including IL3, IL6, TPO, FL, and SCF (all from StemCell Technologies, UK). 10µl vector was added into each single well of cells. The plate was placed in the incubator for 24 hours.

2.5 Human amniotic fluid collection

2.5.1 Collection of human amniotic fluid by amniocentesis

Human amniotic fluid samples were collected from the patients having amniocentesis or amniodrainage in Fetal Medicine Unit of University College London Hospital (UCLH). All enrolled cases were consented by Sheng-Wen Steven Shaw or Dr. Anna David. Ethical approval (08/0304). Human amniocentesis was performed with sterile condition and under ultrasound guidance. The 22G echotip needle (Cock, USA) was inserted through maternal abdominal wall into the amniotic cavity. A total of 15 to 20 ml of amniotic fluid was withdrawn using 20ml syringe. The collected amniotic fluid was placed at room temperature.

Most of the cases were primary cultured or freshly isolated at ICH lab, while some of the amniotic fluid samples were freshly frozen in the liquid nitrogen with 90% FBS plus 10% DMSO for further use. Some extra fluid (1 to 5ml) could be obtained at the same day of the procedure in those cases above 15 weeks of gestation or when amniodrainage was performed.

The samples (10 to 15ml) were sent to TDL for clinical karyotyping study. If surplus AF was available ("fresh") it was used immediately for research purposes. The majority of the AF cells were set up in culture and underwent trypsinisation to harvest the cells for karyotyping by G banding ("harvested cells"). A proportion of the fluid was set up in culture without harvesting for G banding to provide a backup cell source ("cultured cells"). Both types of cells were made available for research use after clinical care was complete. Therefore, human amniotic fluid cells used into our study would have been handled in three different ways: fresh, cultured and harvested cells.

2.5.2 Collection of human amniotic fluid by amniodrainage

Under the same setting and condition, the pregnant women who had polyhydramnios due to twin-to-twin transfusion syndrome, gastrointestinal atresia, dysfunctional genitourinary system or other congenital abnormalities referred to UCLH Fetal Medicine Unit would undergo amniodrainage to reduce intrauterine pressure and

prevent preterm labor. Women were consented prior to the procedure. The amniodrainage was performed under ultrasound guidance. Local anaesthetic (lignocaine 1%, 10ml)_was injected into the skin and subcutaneous tissues and an Echotip needle (Cook UK Ltd, 18-20Gauge) was inserted into the amniotic sac. The needle was connected to a negative pressure vacuum bottles (Armstrong Medical Ltd., UK) (Figure 2.6) by sterile tubing to speed up the collection process. The fluid collected in the bottles were placed at room temperature and delivered back to ICH for cell experiments.

Figure 2.6 The vacuum bottles for amniodrainage.

2.6 Human stem cell culture

2.6.1 Clinical culture of amniotic fluid cells

Cultured amniotic fluid cells were also available from the laboratory (TDL, London) where clinical analysis of the cells was made two to three weeks after amniocentesis. The TDL laboratory set up two parallel cell cultures from the amniotic fluid. They harvested one of the cell plates (100mm Cell Culture Dish, Cat. 353003, BD, UK) once cells reached full confluence by trypsinisation then examined the cells for karyotyping "harvested cells". Another plate was only cultured as a backup by changing Chang medium every 3 to 4 days "cultured cells". These two kinds of cell culture were set up from every patient and after clinical use had been completed, cells were collected for further cell culture and analysis. All procedures had been approved by the Joint UCL/UCLH Committees of Ethics on Human Research.

Figure 2.7 showed the flowchart of the processing the second trimester amniotic fluid after collecting from the hospital. Extra fresh AF 1 to 5 ml were collected and selected for Ckit+ population by using MACS column (Normally 15 to 20 ml of amniotic fluid will be collected during amniocentesis, so we took the fluid over 15 ml that was for karyotyping). Both Ckit+ and Ckit- cells were seeded in the adherent plate with conditioning medium. The morphology of Ckit+ cells was smaller with heart shape compared with spindle shape Ckit- cells (Figure 2.8). I only used Ckit+ cells for further experiments including doubling time calculation, cell size measurement, viral transduction, and HSC expansion. The cultured and harvested cells from TDL 3 weeks later after karyotyping were selected with Ckit antibody as well (Figure 6-2).

For the third trimester amniotic fluid from amniodrainage, I selected and cultured the Ckit+ cells first as previous method, and then stored the rest of the cells. After 3 months storage in the liquid nitrogen, I defrosted the Ckit+ cells and recovered them with conditioning medium for study.

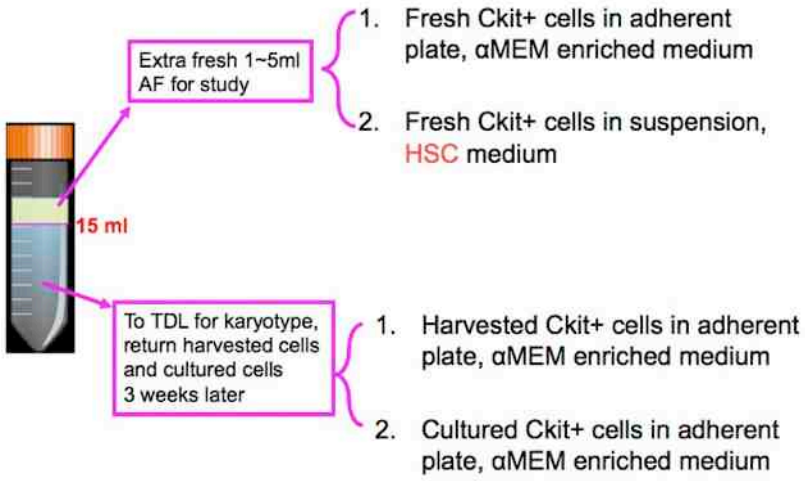


Figure 2.7 The flowchart of second trimester amniotic fluid collection and culturing.

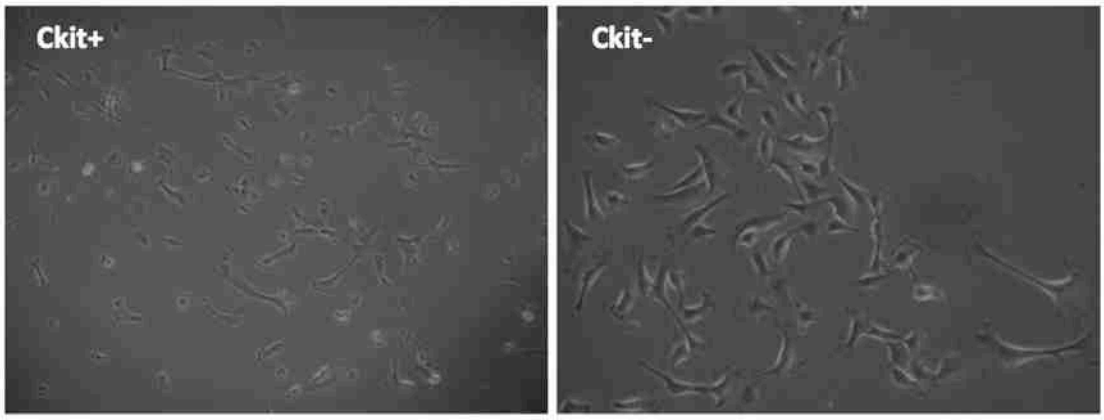


Figure 2.8 The morphology of Ckit+ and Ckit- cells cultured in the adherent plates.
After magnetic sorting, both Ckit+ and Ckit- cells were seeded in the adherent plates with conditioning medium. The morphology of Ckit+ cells is round shape, and smaller in size. The Ckit- cells look more like fibroblast cells with spindle shape.

The cell cultures were incubated for 5 days after the initial set up at TDL. After 5 days the culture fluid was changed with fresh media, then the media change was performed twice a week until ready to harvest. Before first fluid change the cultures were examined for signs of contamination, e.g. dramatic change of colour from pink / orange to yellow, cloudiness of medium, visual evidence of fungal growth.

The amniotic fluid cells were checked using the inverted microscope. The actively dividing cells were rounded and look slightly lifted from the bottom of the culture. A amount of 2.5ml PBS was added to wash each culture. Then PBS was discarded from first culture tube and added 1.0ml warmed trypsin. The first culture tube was knocked against the palm of hand to dislodge cells and poured the contents into the centrifuge

tube that labeled with the corresponding number after 25 minutes incubation. We then transferred the contents of the remaining trypsinised culture tubes to their corresponding centrifuge tubes. The culture tubes was refilled with 2.5ml post harvest medium. The fix solution was added to the remainder of the tubes in the same order and at the same speed that they were trypsinised. Then we spin the tubes in bench-top centrifuge at 1500rpm for 10minutes.

2.6.2 Culture of human amniotic fluid stem cells in adherence

The cells were centrifuged to obtain pellets that were suspended in amniotic culture medium and 10 μ l of the suspension was checked by 0.4% trypnan blue exclusion assay on Neubauer haemocytometer. The cells in the suspension were implanted in Falcon Integrid Petri dishes (Becton Dickinson, USA). The amniotic culture medium is composed of Chang medium B and Chang medium C (Irvine Scientific, USA), MEM Alpha solution (63%, Life Technology, UK), fetal bovine serum (15%, Invitrogen, UK), antibiotics (penicillin and streptomycin) and L-glutamine (1%, Invitrogen, UK). The dishes were incubated in an incubator that was constantly under 37ºC, 95% air and 5% carbon dioxide.

Every 3 days non-adherent cells and debris were removed and the remnant medium was replaced by new medium. Once 60~80% confluence was achieved the adherent cells were detached from the plate by 0.05% trypsin and 0.02% sodium-EDTA (Life Technologies, UK) and were passaged into a bigger dish or dispensed evenly into 2 dishes. When 60~80% confluence was achieved again one of the dishes was used to get the pellet for CKit sorting. The selected CKit(+) cells and CKit(-) cells were seeded on new dishes separately. In accordance with the same principles before selection, all the unsorted, C-kit(+) and C-kit(-) dishes were managed and passaged thereafter.

Because the availability of human AF either from diagnostic amniocentesis or therapeutic amniodrainage was not predictable, it was challenging to obtain both fresh human AF and E14 pregnant mice exactly on the same day and freezing was necessary. In the group of fresh human AFS cells, the pellets were suspended in fetal bovine serum (15%, Invitrogen, UK) and 10 μ l of the suspension was checked by

0.4% trypnan blue exclusion assay on Neubauer haemocytometer. The suspension was transferred to cryotubes. Dimethyl sulphoxide (DMSO) (Sigma, UK) was added and mixed in each cryotube with 1:9 ratio to fetal bovine serum in volume. The cryotubes were stored in a Nalgene Cryo 1ºC freezing container (Thermal Scientific, USA) and were frozen in –80°C freezer. Once IUT was ready when the plugged mice reached E14, the cryotubes were thawed in 37°C water bath and the cells were resuspended in amniotic culture medium. After centrifuging at 1500rpm for 5 minutes, supernatant was discarded and pellet was collected for C-Kit sorting.

2.6.3 Culture of human haematopoietic stem cells from amniotic fluid

For culturing the human amniotic fluid stem cells under haematopoietic condition, I tried to culture the cells in suspension (liquid culture) with HSC conditioning medium, IMDM with 10% FBS (Invitrogen, UK) containing all essential cytokines including IL3 (10ng/ml), IL6 (10ng/ml), TPO (10U/ml), and FL (50ng/ml) (all cytokine were carrier-free and from R&D Systems, UK) in 24-well ultra low attachment plates (BD, UK), following the standard culturing methods for cord blood CD34+ cells (Piacibello et al., 1997). The cells grew in the liquid suspension and culturing medium was changed every three days. The cell number was counted every time when changing the medium.

2.6.4 Human amniotic fluid cell CD117 sorting

Magnetic-activated cell sorting (MACS) (Miltenyi Biotec, USA) was conducted according to the procedures suggested by the manufacturer. The cell pellet was resuspended in sorting buffer (0.5% bovine serum albumin and 2mM EDTA in PBS) from which 10 μ l was withdrawn to count the live cells in order not to exceed the maximal capacity of 2 x 10^8 for each magnetic-sorting column in following steps. FCR blocking reagent and the antibody (CD117, Microbeads, human) were added to the suspension and then the sample was incubated on ice for 15 minutes. After resuspension, centrifugation and resuspension again, the sample was applied onto a magnetic-sorting column within which the magnetically labelled C-Kit(+) cells would be retained, whereas the unlabelled C-Kit(-) cells would pass through the column. For retrieval of C-kit(+) cells, 1ml sorting buffer was pipetted onto the magnetic-sorting

column and the C-Kit(+) cells were flushed out with the buffer by firmly applying the plunger which fitted the column. The sorted Ckit+ cells were cultured, expanded, frozen down or transplanted into target animals directly.

2.7 Lentivirus vector gene transfer

2.7.1 Lentivirus vector preparation and transduction of sheep amniotic fluid mesenchymal stem cells

A lentivirus vector encoding the HIV-1 central polypurine tract element, the Spleen Focus Forming Virus LTR promoter, and the marker gene eGFP was used (Figure 2.9). VSV-G envelope was used as the envelope on the lentivirus. Vector stocks were generated as previously described (Qasim et al., 2007, Demaison et al., 2002), and were concentrated by ultracentrifugation at 100,000 xg for 2 h at 4°C using a Beckmann ultracentrifuge. The virus particles were resuspended in X-Vivo 10 and stored at -80°C. The number of infectious particles was estimated by flow cytometry of 293T cells, 72 h after exposure to serial dilutions of virus stock.

Sheep amniotic fluid mesenchymal stem Cells cultured in ACM (1 x 10^6) and were seeded into 24-well non-tissue culture dishes. After 24 hours they were transduced with the lentivirus vector at a multiplicity of infection of 10 virus particles per cell. Cells were incubated with the vector for 48 hours at 37°C in 95% air and 5% carbon dioxide. Transduced cells were treated with Trypsin and re-plated into 100mm dishes for further growth in ACM.

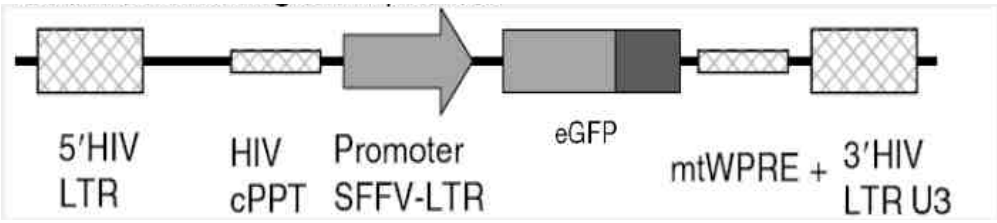


Figure 2.9 The structure of lentivirus vector used for transduction.
LTR, long terminal repeat; SFFV, the spleen focus-forming virus; WPRE, Woodchuck post transcriptional regulatory elements; cPPT, central polypurine tract.

2.7.2 Viral transduction of sheep amniotic fluid and bone marrow CD34+ cells

The fresh, defrosted amniotic fluid cells or bone marrow CD34+ cells were collected and cultured for 48 hours with viral transduction using the same vector. For the fresh frozen amniotic fluid cells, the cells was pelleted and stored in the liquid nitrogen 3 to 6 month containing 90% FBS and 10% DMSO. These cells were defrosted immediately after returning to room temperature. The cell suspension was centrifuged 1500rpm for 5 minutes, and the supernatant discarded. The defrosted amniotic fluid cells were suspended in the cultured medium and for further viral transduction.

The culture medium contained basal IMEM, 10% FBS with StemSpan CC100 with a combination of cytokines including Flt-3, Stem cell factor (SCF), IL3, and IL6 (Stemcell Technologies, UK). 100000 CD34+ cells sorted from fresh or frozen amniotic fluid, or bone marrow were seeded in the 24-well low attachment plates with culturing medium for 48 hours. The incubation condition was 37°C containing 5% CO^2. The 10ul lentivirus was added while seeding the cells into each well of 24-well plate for transduction (MOI=50). After viral transduction, the cell suspension was washed and re-suspended in PBS for in utero injection or mice injection.

2.8 Tissue analysis to assess engraftment, and stem cell spread

2.8.1 Flowcytometry analysis

Single cell suspensions of fetal spleen, liver and bone marrow were prepared immediately after animal sacrifice by straining fetal tissue through 40μm cell strainer (Figure 2.10). After rinsing with PBS, red-blood cell lysis buffer was added for 5 minutes at 37°C. Mononuclear cells were isolated from umbilical cord and maternal blood samples by density gradient centrifugation using Ficoll-Paque solution (Stem Cell Technology, Canada) (Figure 2.11). Single cell suspensions from control untransfected ewes and their fetuses were used as negative controls. The cells were acquired by Becton Dickinson FACSCalibur and LSR II machines (Becton Dickinson, San Jose, CA, Figure 2.12) and analyzed using FlowJo version 5.7.1 software (Tree Star). Ten thousand events were collected per sample and the data were stored as list mode files.

For the GFP detection and engraftment analysis, the method of flowcytometry analysis and single cell suspension preparation were described in previous section. We detected GFP positive signal by using FITC gating from all the organs of NSG mice, sheep and lambs. Un-injected animals of the same age were used as negative control for the GFP(-) gating. The organs from YFP+ mice were used as positive control. To calculate transduction efficiency study, I used the same FITC channel to gate the GFP positive population. Un-transduced amniotic fluid stem cells from the wild type animal were used as a negative control, while the amniotic fluid stem cells of YFP transgenic mice were used as a positive control.

Figure 2.10 Cell strainer.

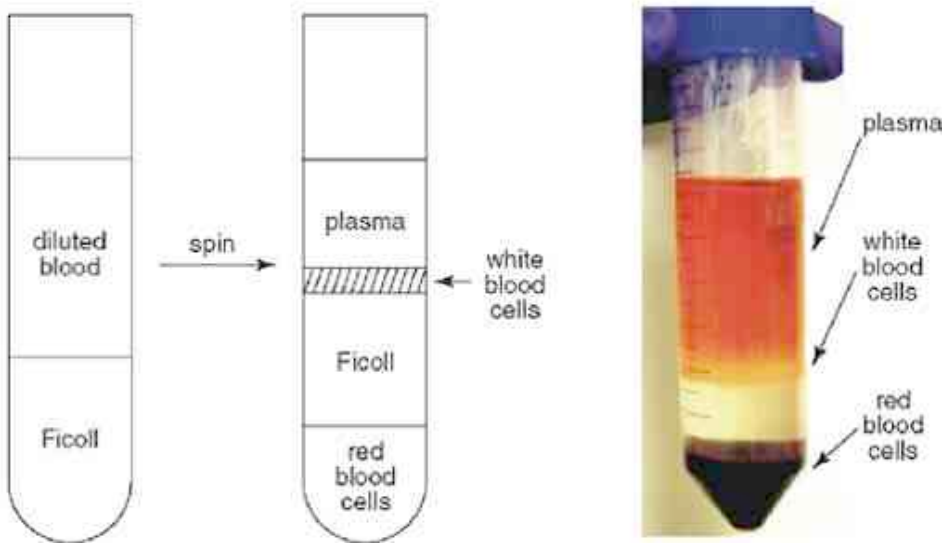


Figure 2.11 Ficoll-Pague solution. The blood showed in the different gradient level.

Figure 2.12 Machine for flowcytometry analysis.

2.8.2 PCR analysis

DNA was extracted from fetal and maternal tissues using a mini kit (Invitrogen, USA) and the quality was confirmed using a nanodrop machine (NanoDrop Technologies, USA). Sample DNA (4μg) was used for the first PCR reaction (30 cycles of 45 seconds at 65°C). The initial denaturation condition was 94°C for 30 seconds with Taq DNA polymerase (Invitrogen, UK), and the primer extension was 72°C for 45 seconds. The following primers were used for first round amplification of eGFP (Forward: 5'-TGAACCGCATCGAGCTGAAGGG-3'; Reverse: 5'-TCCAGCAGGACCATGTGATCGC-3'). A second nested PCR round was performed (25 cycles of 45 seconds at 60°C) using the following second round primers: (Forward: 5'-GGCACAAGCTGGAGTACAACT-3'; Reverse: 5'-CCATGTGATCGCGCTTCT-3'). PCR products were analyzed on 1.2% agarose gel stained with ethidium bromide. DNA from GFP transduced amniotic fluid cells was used as a positive control.

As previously described, all the DNA from the tissues of sheep, lambs or NSG mice were extracted by using a mini kit (Invitrogen, USA) and the quality were confirmed using a nanodrop machine (NanoDrop Technologies, USA). Sample DNA (4μg) was used for the PCR reaction (30 cycles of 45 seconds at 65°C). The initial denaturation condition was 94°C for 20 seconds with Taq DNA polymerase (Invitrogen, UK), and the primer extension was 72°C for 45 seconds. The following primers were used for the amplification of eGFP (Forward: 5'-TGAACCGCATCGAGCTGAAGGG-3'; Reverse: 5'-TCCAGCAGGACCATGTGATCGC-3'). PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide. DNA from GFP transduced amniotic fluid cells were used as a positive control.

For the xenotransplatation mice project, a published method was performed for the PCR of human β2 microglobulin (Chou et al., 2006a). The positive control used was genomic DNA extracted from human amniotic fluid stem cells and negative control was genomic DNA extracted from liver of a wild type mouse without IUT. According to published human DNA sequences (Gussow et al., 1987), the forward primer that is specific for human β2 microglobulin is 5'-GTGTCTGGGTTTCATCAATC-3' and the reverse one is 5'-GGCAGGCATACTCATCTTTT-3'. 0.5μl Taq DNA

polymerase (Invitrogen, UK) was added into each sample. Thermal cycler (Eppendorf, UK) was set to denature double-stranded DNA at 95°C for 1 minute and then to run 35 thermal cycles that are composed of 95°C for 30 seconds, 57.1°C for 1 minute and 72°C for 30 seconds. After thermal cycles the samples will experience 70°C for 5 minutes as a final extension then be kept in 4°C (Chou et al., 2006a). Electrophoresis of PCR products were performed on 1.5% Agarose gel (Agarose 1.5g and ethidium bromide 6μl in 0.5% TBE solution 100ml) under the electric current of 140 volts for 30 minutes.

2.8.3 Western blot analysis

Cell pellets and homogenized tissues were resuspended in 200 μL PBS and 200 μL 2× protein sample buffer (0.5 M Tris-HCl [pH 6.8], 5% glycerol, 2% sodium dodecyl sulfate and 100 mM dithiothreitol). Samples were boiled for 5 minutes and centrifuged at 16,000*g* for 15 minutes to remove insoluble material. Samples were loaded onto a precast 7.5% Ready gel with a 4.5% stacker (BioRad Laboratories, Hercules, CA, USA) and run for 1 hour. After electrophoresis, proteins were transferred to nitrocellulose membranes in a Bio-Rad Electroblotter Apparatus. Membranes were blocked in TBST (TBS, 0.05% Tween 20, Sigma, UK) containing 5% milk for 1 hour at room temperature and then reacted overnight at 4°C with rabbit anti-GFP (Invitrogen, USA) diluted 1:2000 in TBST-milk. After three washes in TBST, the membranes were treated with affinity-purified horseradish peroxidase–conjugated goat anti-rabbit IgG (Promega, Madison, WI, USA) diluted 1:4000. Membranes were washed three times in TBST, and then developed with a 3,3′ diaminobenzidine–based horseradish peroxidase detection kit (Vector Laboratories, USA). β-actin was used as internal control. The protein extracted from GFP transduced sheep amniotic fluid cells were used as positive control.

2.8.4 Immunofluorescence

Fresh fetal sheep tissues embedded in OCT, were snap frozen in methyl-butane and liquid nitrogen and were cut into 10-15 μm sections using a cryostat (OTF, Bright, UK). Blocking solution was prepared with 1% BSA, 0.15% Glycine and 0.1% Triton in PBS and preserved at 4^{o}C. To co-stain hepatocytes expressing GFP, mouse anti-CK18 (Abcam, UK) and rabbit anti-GFP polyclonal (Invitrogen, USA) primary

antibodies were applied at 1:150 concentration in blocking solution overnight at 4^{o}C. Goat anti-rabbit (Alexa Fluor 488, Invitrogen, USA) and goat anti-mouse (Alexa Fluor 568) secondary antibodies were incubated for 2 hours at room temperature with blocking solution at 1:150 concentrations. To co-stain myocytes expressing GFP, rabbit anti-laminin (Abcam, UK) and mouse anti-GFP primary antibodes were applied. Secondary goat anti-rabbit (Alexa Fluor 594) and goat anti-mouse (Alexa Fluor 488) antibodies were used. The conditions for primary and secondary antibody staining were the same as for hepatocyte analysis. The stained tissues were covered by VECTASHIELD HardSet Mounting Medium with DAPI for nuclear staining (Vector Laboratories, USA). The slides were observed under inverted immunofluorescence microscopy (Leica, Germany). In order to quantify the number of AFMSCs engrafted in the fetal liver, we counted GFP and CK18 positive/negative stained cells in four high power fields per fetal liver. Values (mean ± SD) were expressed as the percentage of single or double positive stained cells per total nuclei in each high power field.

For the slides from liver of human AF cell transplanted mice, the primary staining solution is made of mouse anti-human mitochondria antibody (Millipore, USA), rabbit anti-mouse albumin (AbD serotec) and blocking solution (1:1:100 in volume). The secondary staining solution (prepared in dark) is composed of goat anti-mouse IgG with green fluorescence (Alexa Fluor®488, Invitrogen), goat anti-rabbit antibody with red fluorescence (Alexa Fluor®594, Invitrogen) and blocking solution (1:100 in volume). For all other slides rather than liver, the primary staining solution is made of mouse anti-human mitochondria antibody and blocking solution (1:100 in volume). The secondary staining solution (prepared in dark) is composed of goat anti-mouse IgG (green fluorescence) and blocking solution (1:100 in volume). Each slide was stained by 200µl primary staining solution at room temperature for 2 hours and washed by PBS twice. Then each washed slide was stained by 200µl secondary staining solution in dark for 2 hours and washed by PBS twice.
For nuclear staining, each slide was mounted with 3 droplets of diamidino phenylindole (DAPI)-Vectashield® medium (Vector Laboratories, CA) then covered by cover slip. An inverted immunofluorescence microscopy (Zeiss AX10) was applied to observe the stained tissues. Photos under green, red and blue fluorescence

were taken by a digital camera (A650 IS, Cannon). Raw images were collected and analysed by Photoshop CS2 (Adobe). All the antibodies were shown in Table 2.1.

Table 2.1 The antibodies list for immunofluorescence.

Type of staining	Animal	Primary Antibody	Secondary Antibody
GFP	Sheep	rabbit anti-GFP	goat anti-rabbit
CK18	Sheep	mouse anti-sheep CK18	goat anti-mouse
Laminin	Sheep	rabbit anti-laminin	goat anti-rabbit
GFP	Mouse	mouse anti-GFP	goat anti-mouse
Human-mitochondria	Human	mouse anti-human mitochondria	goat anti-mouse

2.8.5 Colony forming unit (CFU) assay

MethoCult® GF H4434 kit and M3434 kit (Stem Cell Technologies) were used to perform CFU assays for sheep amniotic fluid or bone marrow CD34+ cells and NSG mice bone marrow cells. $CD34^+$ cells which were isolated by MACS were added into methylcellulose cocktail containing cytokines and growth factors at a concentration of 1×10^4 cells per plate. The mixture was vortexed and plated into sterile 35mm plates with the use of a blunt needle and syringe. The plates were incubated at 37°C with 5% CO_2 in humidified atmosphere for 14 days. After 14 days, colonies were scored with the help of gridded scoring dishes using an inverted microscope. The 'Atlas of Human Hematopoietic Colonies' by C. Eaves and K. Lambie was used as a guide to identify and record CFU colonies such as colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), colony forming unit-granulocyte, monocyte (CFU-G/M/GM), burst-forming unit/CFU-erythroid (BFU/CFU-E).

2.8.6 The analysis of tissues of transplanted animals by confocal microscopy

The tissues from sheep or transplanted lambs or NSG mice were prepared as the previous section 2.1.9. The liver sample slides were co-stained with mouse monoclonal anti-GFP antibody (A11120, Invitrogen, UK) and rabbit polyclonal anti-

alpha fetoprotein (AFP) antibody (ab74663, abcam, UK). The concentration of these two antibodies was 1:100 with buffer solution. The sample slides were incubated for 2 hours at room temperature. The secondary antibodies, Alexa Fluor 488 goat anti-rabbit IgG and Alexa Flour 594 donkey anti-mouse IgG (A11008 and A21203, Invitrogen, UK) were used in 1:100 concentration for another two hours incubation at room temperature. The sample slides were observed by using the confocal microscopy (Leica TCS SP5 II, Germany), and the images were analyzed with the software ImageJ (NIH, US). The green positive signal indicated the GFP positive cells, and the red signal indicated the hepatocytes staining with AFP.

2.8.7 Analysis for in vivo and ex vivo luciferase activity

For the experiment of Chapter 5.2, mice AFS cells were transduced with UCOE-LUC so that the luciferase activity would need to be analyzed. I used in vivo imaging system (IVIS) to calculate the activity of luciferase after lentivirus transduction. Intraperitoneal injection of 10ul of luciferin was followed by in vivo bioimaging with a Xenogen IVIS-50 Manufacturer cooled charge-coupled-device camera. Luciferase expression was quantitated as photon flux from the upper abdomen.

For the ex vivo luciferase experiment, mice blood cells and liver samples were collected first and prepared for analysis. Cells were harvested by centrifugation and washed once with 1 mL of ice cold lysis buffer (1× PBS pH 7.4, 1 mM PMSF) and then resuspended in 0.3 mL of the same buffer. Typically the concentrations of crude lysates were between 0.1 and 1.0 mg/mL as determined by the Bradford method (BioRad, UK). Luciferase activities were determined using 5 μL of lysate/sample using the Dual-Luciferase Assay System (Promega, UK) and a TD 20/20 luminometer (Turner Designs, UK). The luciferase activity ratio was calculated for each recoding signal. All assays were performed in triplicate at least three times.

Chapter 3

Autologous transplantation of amniotic fluid derived mesenchymal stem cells into fetal sheep

3 *Chapter 3: Autologous transplantation of amniotic fluid derived mesenchymal stem cells into fetal sheep*

Introduction

In utero stem cell transplantation (IUSCT) aims to treat congenital disorders in the fetus using cells capable of self-renewal that can enhance or substitute the affected tissues/organs of the fetus. Therefore, transplantation of genetically corrected autologous amniotic fluid stem cells into a fetus could represent an alternative therapeutic strategy for the treatment of severe congenital diseases.

In this chapter, I investigated the feasibility and potential for combining IUSCT and gene therapy into one therapeutic strategy using amniotic fluid derived mesenchymal stem cells (AFMSCs). With this aim I used the fetal sheep, which has the advantages of long gestational age, similar fetal size and comparable fetal development and physiology to human pregnancy (David et al., 2006b) and in which there is wide experience with fetal interventional procedures (David et al., 2003a, David et al., 2006a). Then I examined whether MSCs derived from amniotic fluid collected from fetal sheep could achieve autologous transplantation *in utero*. The experimental design is shown below (Figure 3.1)

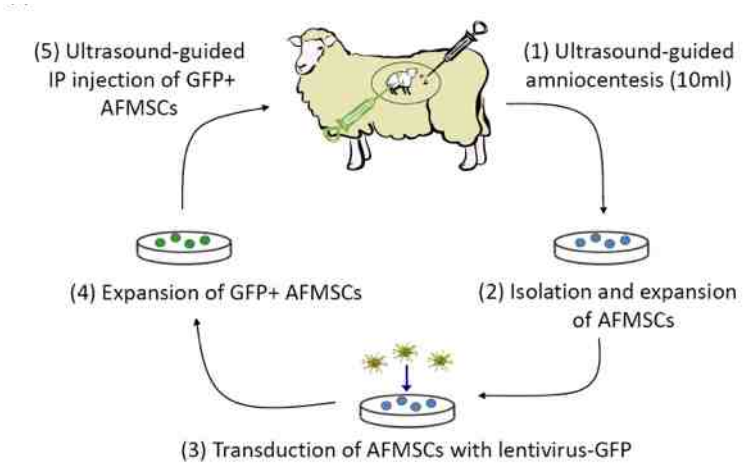


Figure 3.1 Experimental design.
After amniocentesis collection, amniotic fluid mesenchymal stem cells (AFMSCs) were cultured in adherence in defined conditions. Cells were transfected with lentivirus GFP and re-injected into the peritoneal cavity of the fetal donor.

3.1 Ultrasound guided amniocentesis can be used to sample amniotic fluid in first trimester fetal sheep with a low rate of miscarriage

For experiments on autologous AFMSCs, AF was collected using amniocentesis under general anaesthesia from time mated pregnant sheep. Amniotic fluid was successfully sampled in all fetuses attempted (n = 9) at a mean gestational age of 58.3±1.9days (term = 145 days). The procedure was performed in a similar way to amniocentesis procedures performed in clinical fetal medicine units in hospital. During amniocentesis an echo tip 22 Gauge needle was inserted under ultrasound guidance and 10 ml of clear amniotic fluid was collected into a sterile tube for further experiments. All amniocenteses took less than 3 minutes to complete (154±25seconds). A total of 7 sheep were autologous transplanted with amniotic fluid mesenchymal stem cells prenatally via intraperitoneal (IP) route. Table 3.1 showed the summary of the experiments.

One ewe subsequently miscarried twin fetal sheep a few days after the amniocentesis procedure, but before autologous transplantation could be performed. The procedure was performed without incident such as haemorrhage or obvious trauma. Postmortem examination of this ewe was normal, and the tissues sent for microbiology showed no evidence of infection. All remaining fetuses survived to the scheduled postmortem examination at the end of the experiment giving a miscarriage rate of 78% (2 out of 9 procedures) for this small group of experiments.

Here, I found first trimester ultrasound-guided amniocentesis could be easily performed in the fetal sheep with a low miscarriage rate.

Table 3.1 Experimental details of IP *in utero* autologous transplantation of sheep amniotic fluid mesenchymal stem cells.
After sampling of amniotic fluid (amniocentesis), cells were cultured and transduced *in vitro*, before being injected into the peritoneal cavity of the donor fetus (transplantation). Scheduled post mortem examination was performed at least four weeks later to allow time for cell engraftment. SD: standard deviation; nt: not tested.

Sheep	Gestational age (days)			Time (days)		Number of cells injected	Injected cell volume (ml)	AFMSC transduction efficiency (%)
	Amniocentesis	Transplantation	Post mortem	Cell culture and transduction	Engraftment			
1	55	71	89	16	34	1×10^5	1	nt
2	58	76	99	18	41	1.6×10^6	1	64.2
3	59	77	95	18	36	7×10^6	1	53
4	59	77	95	18	36	1.8×10^7	2	38.3
5	57	79	99	22	42	9×10^6	1.5	96.2
6	61	83	103	22	42	1.5×10^7	2	62.3
7	59	72	91	13	32	2×10^6	1.5	65.1
Average ± SD	58.3 ± 1.9	76.4 ± 4.1	95.9 ± 4.9	18.1 ± 3.2	37.6 ± 4.1	7.5×10^6	1.4 ± 0.4	63.2 ± 19.1

3.2 AFMSCs can be isolated from sheep pregnancy

Having collected second trimester sheep AF samples, I set out to characterize their cell growth and differentiation in culture.

AFMSCs could be isolated and expanded in vitro from all the 9 sheep (100%). The initial numbers of viable cells from 10 ml amniotic fluid in all animals were less than 20000 cells, but they were easy to expand in petri dishes without feeder layer and demonstrated a doubling time ranging from 36 to 48 hours (Figure 3.2). The cell number was counted at every passage from 9 sheep. The doubling time could be calculated when the cell number doubled. AFMSC growth velocity and differentiation potential was maintained for up to 10 passages (Figure 3.2).

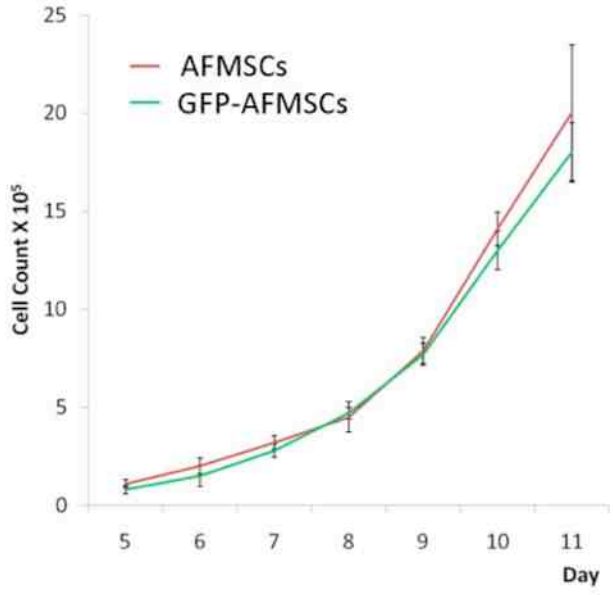


Figure 3.2 The growth rate of sheep amniotic fluid mesenchymal stem cells.
The growth curve demonstrates that both the doubling time of AFMSCs and GFP-AFMSCs were around 36 to 48 hours. (Error bar: standard deviation).

3.3 Sheep AFMSCs could be transduced efficiently with lentivirus vector

Gene transfer to AF derived MSCs was performed in culture using a SFFV lentivirus encoding eGFP. The viral titer was 1 x 10^9 per ml and 10µl of virus was used for 1 x 10^5 cells transduction to reach an MOI=100. The cells showed good tolerance without morphology change after 48 hours incubation. After reaching 70% confluence in 24-well plate, the transduced cells were split into 150mm petri dishes for expansion

Transduction efficiency was assessed 3 days after lentiviral transduction using cytofluorimetric analysis. As can be seen, efficiency of gene transfer was on average 63.2% (range 38.3 to 96.2%, Figure 3.3, panel A and B). GFP expression was also detected when AFMSCs were observed under fluorescence microscopy (Figure 3.3, panel C). The AFMSCs could be easily transduced with a high transduction rate. Cell kinetics of GFP transduced AFMSCs (green line) was similar to non-transduced AFMSCs (red line) (Figure 3.2).

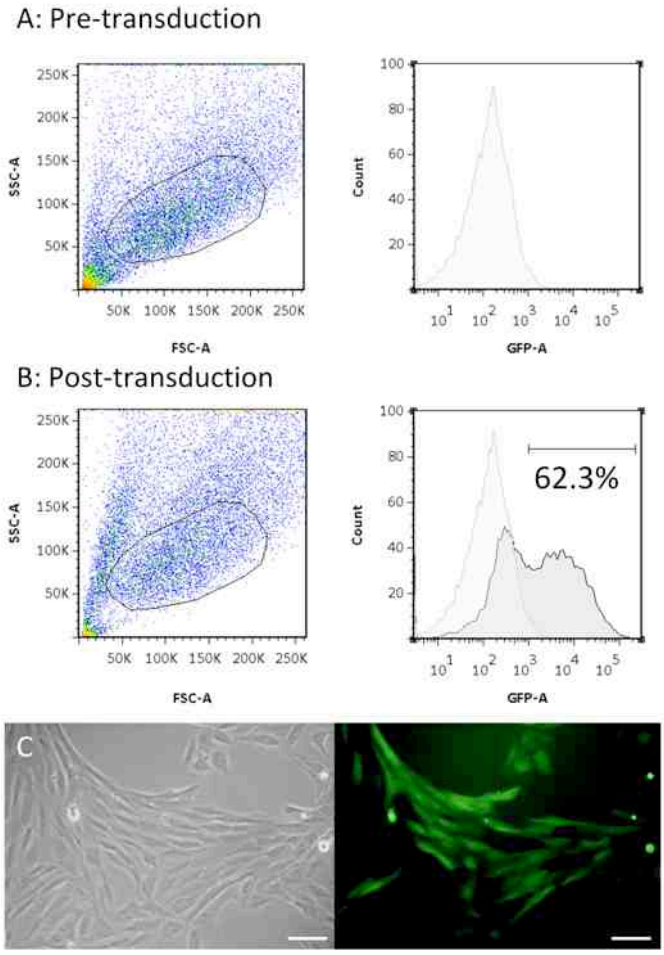


Figure 3.3 Transduction efficiency of AFMSCs.
After transduction with lentivirus vector, AFMSCs were analyzed at the cytofluorimeter for GFP expression to determine the efficiency of gene transfer. The post-transduction cells in the GFP channel of one example can be seen with 62.3% cells expressing GFP (B), the negative control is indicated (A). Cells were gated on the basis of the region shown on the forward (FSC-A) and side (SSC-A) scatter dot plot (left); the percentage of donor cells calculated by selection of the high fluorescence population on the cell count versus fluorescence intensity (GFP-A) histogram (right). AFMSC were observed in bright field (C, left panel) and under a fluorescent microscope where the positive GFP expression could be detected within the cytoplasm of each AFMSCs (C, right panel). Scale bars: 20µm.

3.4 Sheep AFMSCs and lentivirus GFP transduced AFMSCs can be expanded and characterised

In order to achieve a sufficient number of cells for transplantation, cells were cultured for up to 20 passages. The number of injected cells was correlated with the time available for their culture from amniocentesis to transplantation. Sub-confluent cells showed no evidence of spontaneous differentiation. There was no significant difference in cell size and DNA content from cell cycle analysis between pre- and post-transduction AFMSCs (Figure 3.4). AFMSC and GFP-AFMSCs samples had a similar and higher ratio in G0/G1 phase, with low percentage of S phase and G2/M phase (Figure 3.4, panel B).

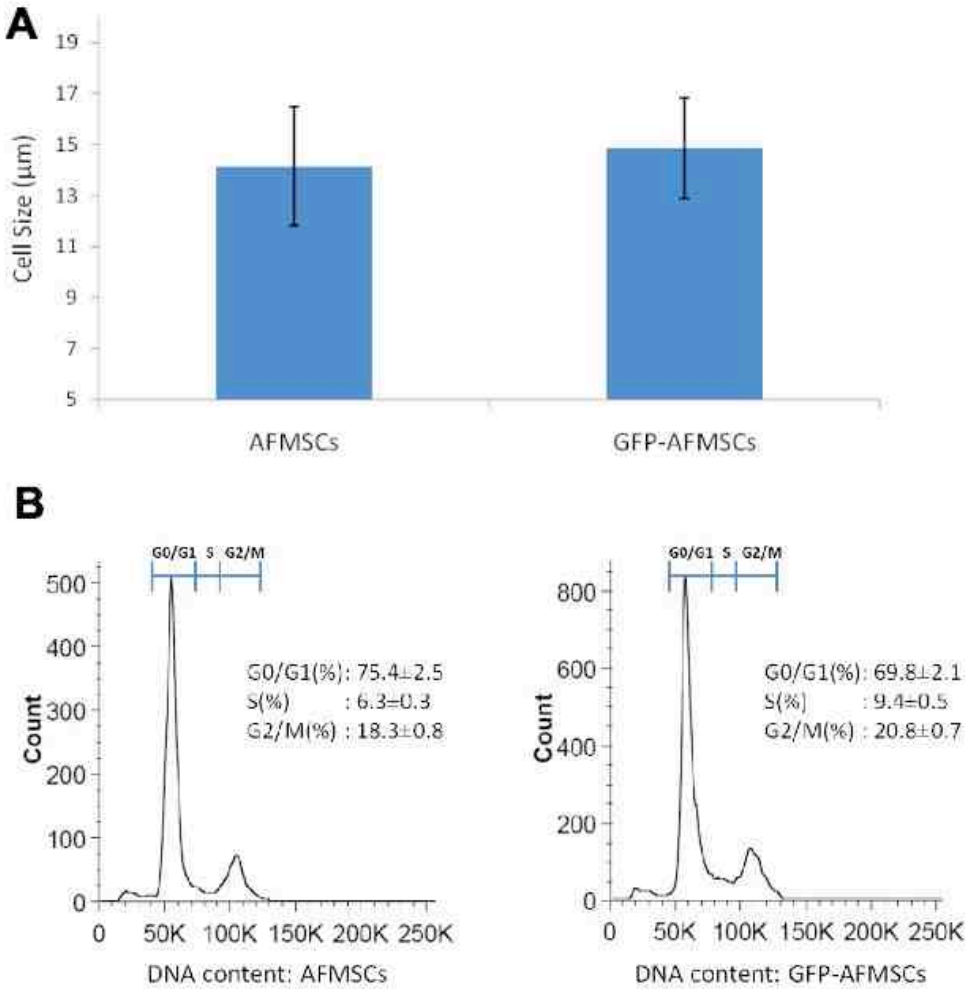


Figure 3.4 Cell size and cell cycle of sheep amniotic fluid mesenchymal stem cells.
In panel (A) the cell size of AFMSCs and GFP-AFMSC is not significantly different. .In Panel (B) the DNA content of cells cycle by using flow cytometry analysis shows no significant difference between AFMSCs and GFP-AFMSCs.

The surface markers of mesenchymal stem cells, anti-sheep CD44, CD58 and CD166, were strongly positive in cultured sheep AF cells. The monocyte and lymphocyte markers, anti-sheep CD14, CD31 and CD45 showed negative in sheep cells. (Figure 3.5). The results were the same in the pre- or post-transduced AFMSCs.

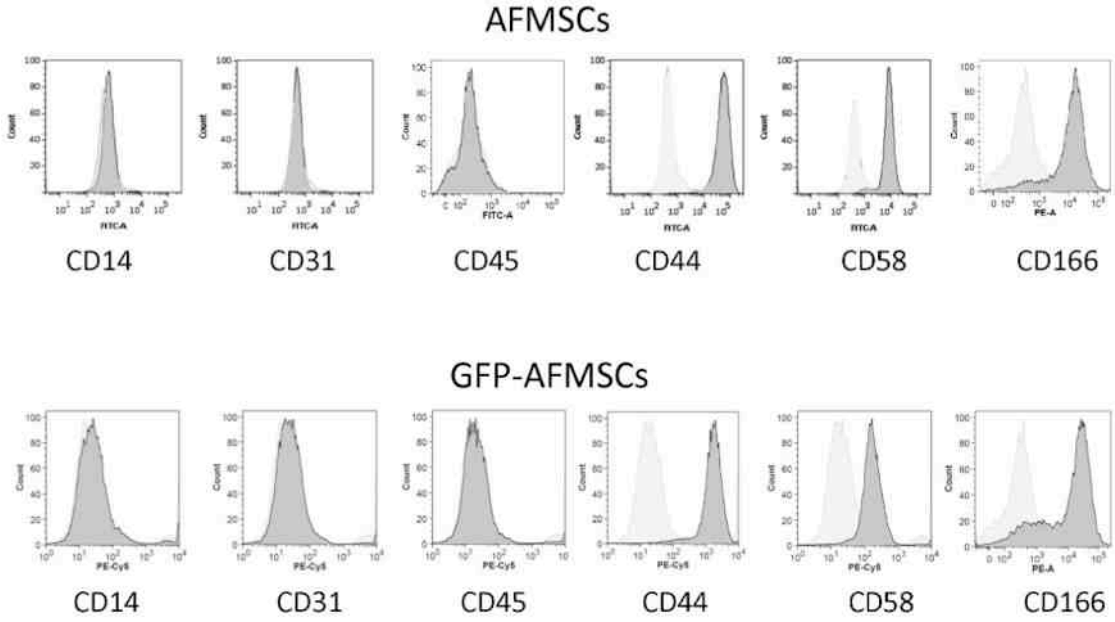


Figure 3.5 Characterization of sheep amniotic fluid mesenchymal stem cells.
The figures of flow cytometry studies demonstrated the sheep AF cells stained strongly positive for MSC surface markers CD44, CD58, and CD166 confirming their mesenchymal origin. Anti-sheep CD14, CD31 and CD45 were negative compared to background staining. (Light gray, negative control; Dark gray, anti-sheep antibodies).

Both of the two groups of cells that could be expanded *in vitro* showed the ability to differentiate into adipogenic and osteogenic lineages following the relevant differentiation protocols (Figure 3.6). After adipogenesis differentiation, cells showed oil drop morphology and staining with oil-red-O. After osteogenesis differentiation, the cells could be stained with alkaline phosphatase.

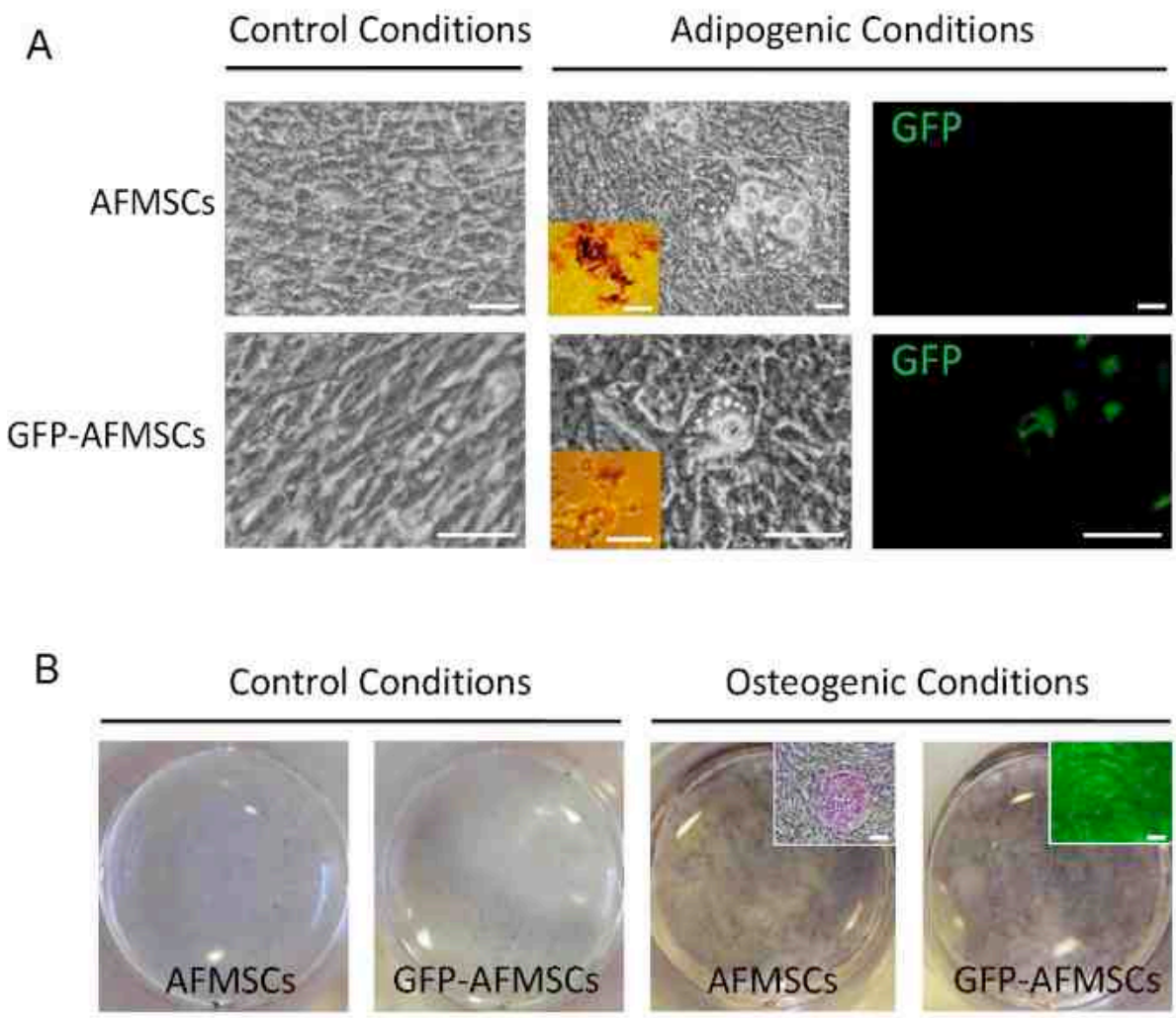


Figure 3.6 Differentiation potential of sheep AFMSCs after culture of AF in mesenchymal conditions.
Adipogenesis differentiation showing lipid drops with positive staining of Oil red O (Panel A and insert) present in AFMSCs and GFP-AFMSCs. Both groups of cells could undergo osteogenic differentiation (Panel B), expressing alkaline phosphatase and forming lamellar-like structures (insert). AFMSCs and GFP-AFMSCs cultured in control conditions did not show adipogenic (Panel A) and osteogenic differentiation (Panel B). Scale bars: 20µm.

Here, I showed that sheep AF cells grown in mesenchymal conditions have the markers and differentiation characteristics of MSCs.

3.5 Transduced AFMSCs could be successfully injected in a sheep prenatal autologous transplantation model

Transduced cells were injected back into the donor fetus (autologous transplantation) via intraperitoneal (n = 7) and intramuscular (n = 1) routes. The initial plan was to compare results from intramuscular with intraperitoneal injection. It was felt that the intramuscular route of injection could provide a route that incorporated an element of cell damage to enhance engraftment, where the intraperitoneal route of injection would not. Intrahepatic injection was also considered but it had been associated with a high rate of miscarriage in previous experiments by the research group using injection of viral vectors (David et al., 2003a), and this was a concern.

The AF cells were expanded in ACM culture medium for approximately 2 weeks time after gene transfer before injection back into the donor fetus. The time points varied slightly since the surgery needed to be fit into the theatre availability. The cells were suspended in 1 to 2 ml PBS.

Intramuscular delivery was found to be more technically difficult. The procedure of transplantation the cells took us over 20 minutes to complete. The angle of needle entrance into legs of sheep fetus was relative sharp. Not like the intraperitoneal injection, the microbubble could not be seen clearly in the muscle area after transplantation under ultrasound guided. The volume of cells we injected intramuscularly was the same as IP route. There was only one case of sheep received IM transplantation so I did not put the IM data in Table 3.1 to compare the IP group.

The intraperitoneal route was by comparison, more straightforward and allowed a larger volume of cell suspension to be delivered. Because of the difficulties with the intramuscular route of injection, it was decided that intraperitoneal injection would be performed for the remaining experiments. Intraperitoneal injection (n =7) was successfully performed at all attempts. Compared to the first amniocentesis procedure, the injection difficulty was related to the position of the fetus. Since the best needle approach is via the anterior abdominal wall of the fetus, in those fetuses that were lying anterior side down (n=4), the uterus was gently manipulated to improve peritoneal access, and this increased the injection time. Mean injection time was 320 ± 55 seconds.

All animals were checked by ultrasound examination the day after injection to check for fetal wellbeing and maternal condition. There was no fetal or maternal mortality until planned post mortem examination in all 7 fetal sheep. The survival rate of the full procedure (amniocentesis and intraperitoneal injection) was thus 78%. Experimental details are listed in Table 3.1. There was no maternal morbidity including fever, abdominal distension, haemorrhage or ruptured amniotic membranes. There was no evidence of fetal haemorrhage or trauma relative to the injection procedure and no fetal abnormality detected at scheduled post mortem examination.

In summary, all the fetuses could be injected using autologous AFMSCs smoothly

3.6 GFP gene could be detected in sheep fetal organs after IP injection of AFMSCs

In order to detect the GFP gene in the injected fetuses, DNA extraction and PCR were performed on tissues taken at post-mortem examination. All the fetal and maternal organs sampled were minced for extraction of DNA and subjected to two rounds of PCR. GFP DNA was detected in many fetal organs using nested PCR, suggesting systemic spread and survival of GFP positive cells after fetal injection (Table 3.2 and Figure 3.7). The amniotic membrane and placentomes were most frequently positive (85.7%), followed by the fetal liver, which was positive in 5 fetuses (71.4%). GFP DNA was also detected in fetal muscle, umbilical cord, heart, adrenal gland, gonad, bone marrow and peritoneum.

Table 3.2 PCR analysis of GFP DNA in 7 sheep fetuses.

Fetal tissue	1	2	3	4	5	6	7	No. of fetuses with +ve PCR in organ (%)
Liver	+	-	+	+	+	-	+	5 (71%)
Muscle (thigh)	+	-	-	-	+	-	-	2 (29%)
AM	+	+	+	+	+	+	-	6 (86%)
Placentome	-	+	+	+	+	+	+	6 (86%)
Umbilical cord	+	-	-	+	-	-	-	2 (29%)
Heart	+	-	+	-	+	-	-	3 (43%)
Adrenal gland	+	-	-	-	+	+	-	3 (43%)
Gonad	-	-	+	+	-	-	-	2 (29%)
Bone marrow	+	-	-	-	-	-	+	2 (29%)
AF	-	+	+	+	-	-	+	4 (57%)
Peritoneum	-	+	-	-	+	-	+	3 (43%)
Brain	-	-	-	-	-	-	-	0
Spleen	-	-	-	-	-	-	-	0
Intestine	-	-	-	-	-	-	-	0
Thyroid	-	-	-	-	-	-	-	0
Thymus	-	-	-	-	-	-	-	0
Lung	-	-	-	-	-	-	-	0
Kidney	-	-	-	-	-	-	-	0
No. of +ve organs per animal (total = 18)	7 (39%)	4 (22%)	6 (33%)	6 (33%)	7 (39%)	3 (17%)	5 (28%)	

AM: amniotic membrane; AF: amniotic fluid

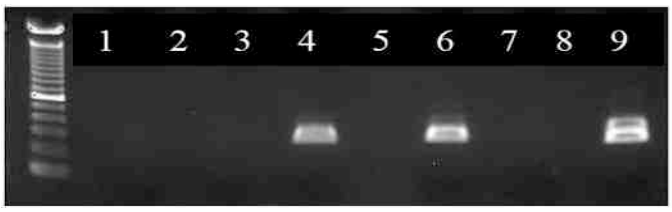


Figure 3.7 PCR gel after IP injection of GFP transduced AFMSCs in one fetus.
1. Heart, 2. Gonad, 3. Spleen, 4. Placentome, 5. Brain, 6. Liver, 7. Thyroid, 8. Negative control: uninjected sheep fetus, 9. Positive control: transduced sheep AFMSCs. Ladder: 100bp.

The cell number injected in each animal varied because of differences in the time gap between cell collection and reinjection and differences in cell growth kinetics. The length of cell culturing and final cell number for injection showed moderate correlation (r=0.59, Figure 3.8A). There was no significant correlation between the number of injected cells and the presence of GFP positive cells in the different organs as analysed by PCR (r=-0.18, Figure 3.8B). Maternal liver, bone marrow, kidney, lung, heart and adrenal gland were examined using GFP nested PCR but were negative, indicating no evidence of maternal engraftment after fetal transplantation.

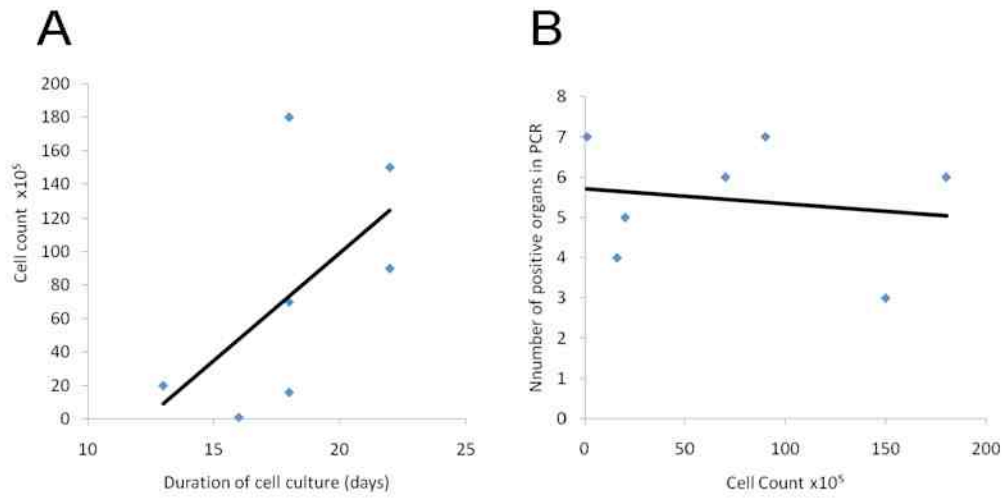


Figure 3.8 The correlation between the number of injected cells and presence of GFP positive cells in fetal organs, and evidence of maternal spread of GFP-AFMSCs. There was a moderate correlation (r=0.59) between the final cell number and days of cell expansion in vitro (Panel A). The correlation between the number of injected cells and the number of detectable GFP positive organs did not showed significance in PCR (r=-0.18, Panel B).

In summary, the GFP gene could be detected in many organs of injected fetuses but not in maternal organs.

3.7 GFP protein expression is detected in sheep fetal organs after in utero IP injection of GFP transduced AFMSCs

GFP gene detected by PCR could only demonstrate the transduced cells in the recipient but not show any function or protein level expression of those cells. We performed flow cytometry, and western blotting to detect evidence of GFP protein expression from donor cells.

Cytofluorimetric analysis for GFP expression showed low level engraftment of transduced AFMSC in fetal bone marrow (0.85±0.13%) and liver (1.24±0.08%). Figure 3.9 is an example of one of the animals with positive engraftment in bone marrow and liver. There were no GFP positive cells in fetal umbilical cord blood and spleen, or maternal blood and bone marrow.

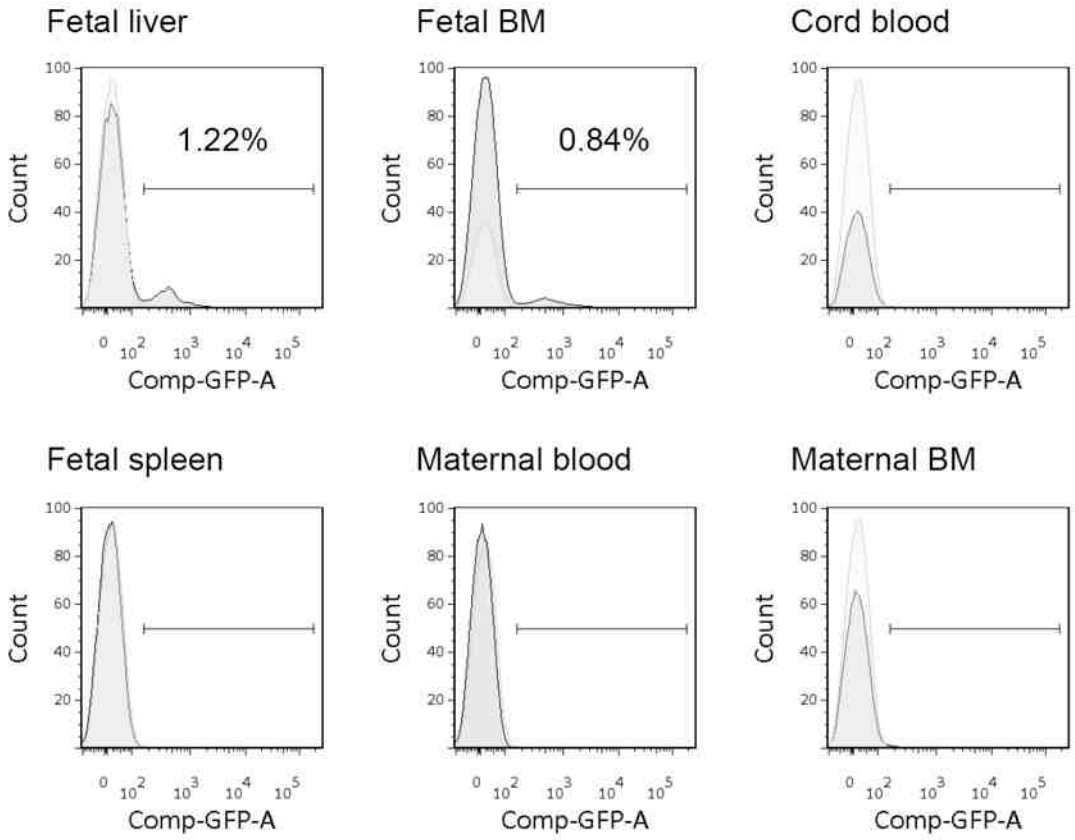


Figure 3.9 Flow cytometric analysis for GFP expression in fetal blood, tissues and maternal samples taken at post mortem examination.
Histograms demonstrate positive GFP detection in the fetal liver (1.22%), and fetal bone marrow (0.84). Cells from a control gestational age matched fetal sheep that was not transduced were used as negative control (light gray). Fetal cord blood, fetal spleen, maternal blood and maternal bone marrow are negative for GFP protein. BM: bone marrow.

GFP protein expression was also assessed by western blot in those fetal tissues in which GFP DNA was detected (Table 3.3, Figure 3.10). There was insufficient protein available in samples of fetal gonad and peritoneum for western blot analysis. The most frequent tissues to have positive GFP protein expression were the placenta (6/7, 86%), the fetal liver and the amniotic membranes (5/7 fetuses, 71%, Table 3.3). Of all tissue samples that were PCR positive for GFP, western blot confirmed GFP protein in 77.8 % (21/27).

The presence of GFP protein detected 3 weeks after injection of transduced AFMSCs was strong evidence of cell engraftment in these tissues after transplantation.

Table 3.3 GFP expression in tissues from 7 fetuses analysed using western blot.

Fetal tissues	1	2	3	4	5	6	7	No. of fetuses with detection in organ (%)
Liver	+	-	+	+	+	-	+	5 (71%)
Muscle (thigh)	+	-	-	-	+	-	+	3 (43%)
AM	+	+	+	-	+	+	-	5 (71%)
Placentome	+	+	+	-	+	+	+	6 (86%)
Umbilical cord	+	-	+	+	-	-	+	4 (57%)
Heart	-	-	+	-	+	-	-	2 (26%)
Adrenal	-	-	-	-	-	+	-	1 (14%)

AM: amniotic membrane

Figure 3.10 Western blot for GFP protein after IP injection of GFP transduced AFMSCs into fetal sheep.
P: positive control. IP: Intraperitoenal.

There was no significant correlation between the number of injected cells and the presence of GFP positive cells in the different organs as analysed Western blot (r=-0.4, Figure 3.11).

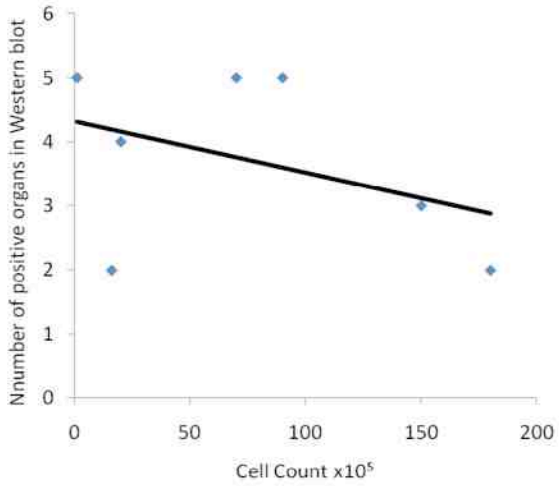


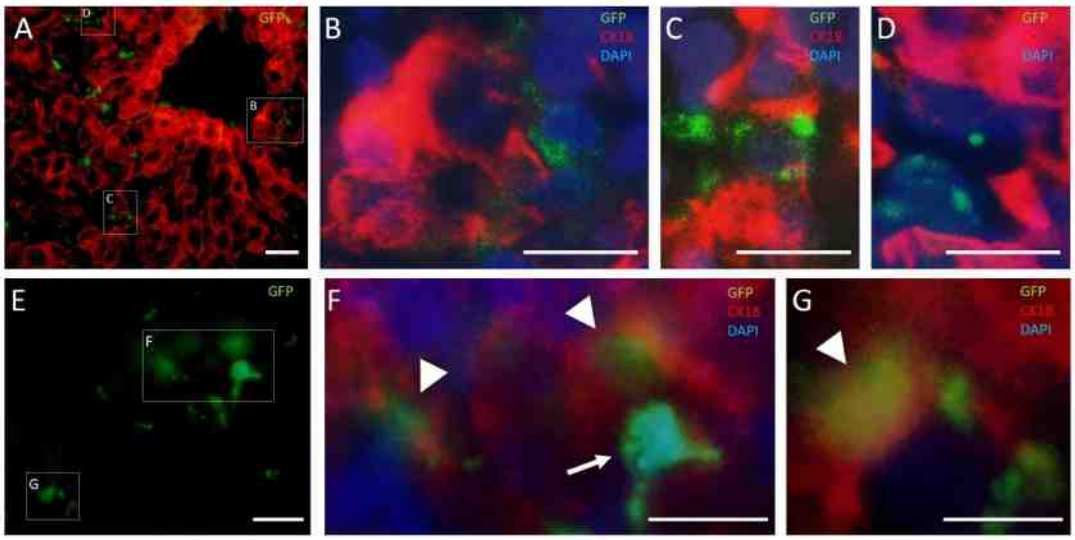
Figure 3.11 The correlation between the number of injected cells and presence of GFP positive cells in fetal organs.
The correlation between the number of injected cells and the number of detectable GFP positive organs did not showed significance in Western blot (r=-0.4).

3.8 Engraftment of GFP cells in the fetal liver and other organs after IP injection of AFMSCs

To analyse for engraftment, the fetal and maternal organs were all prepared as fresh frozen section during post-mortem. The sections were stained with tissue specific antibodies to investigate for engraftment. To confirm the presence of engrafted GFP+ AFMSCs in fetal tissue, I used immunofluorescence to analyse selective organs including fetal liver, skeletal and cardiac muscle and placenta. GFP+ cells could be easily observed under fluorescence microscopy.

For hepatocyte differentiation, I analysed expression of GFP, cytokeratin 18 (CK18), and AFP in the liver of the four fetuses that had GFP positive staining. Two liver lobes were examined in each fetus. In some sections, GFP positive cells did not co-stain with CK18 (Figure 3.12, panel A to D). In other sections a few GFP positive cells were seen co-stained with CK18 positive cells in both liver lobes, suggestive of hepatocyte differentiation (Figure 3.12, panel E-G). The percentage of GFP positive cells per high powered field was 2.97±1.15% for GFP+/CK18- cells and 1.08±0.33% for GFP+/CK18+ cells in the fetal liver (Figure 3.12, panel H).

AFMSCs and GFP-AFMSCs did not show CK18 expression from our study, while the non-injected fetal sheep as a positive control showed strong expression of CK18 in the liver (Figure 3.13A). AFP, another liver specific marker, also demonstrated the presence of GFP-AFMSCs in the liver after autologous transplantation (Figure 3.13B).



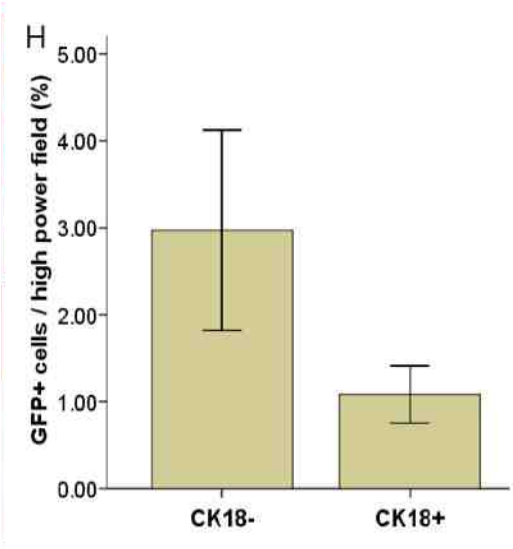


Figure 3.12 Immunofluorescence for GFP expression and markers of MSC differentiation. GFP+ AFMSC migration and differentiation was studied using GFP immunofluorescence and co-staining with tissue markers in fetal organs such as the liver (CK18 A-G), and skeletal muscle and heart (laminin; H-I). In fetal liver, GFP+ cells (green) mainly localized in the stroma (A-D) and did not co-stain with CK18, a marker of hepatocytes stained red. In another section, however, a few rare cells co-stained for GFP and CK-18 (E, F, G, indicated by the arrow head). Some cells that only stained for GFP, remained in the stroma (F, indicated by the arrow with tail). Histogram showed percentages of positive GFP cells with/without CK18 staining detected per high power field in the liver section (H). Error bar: standard deviation.

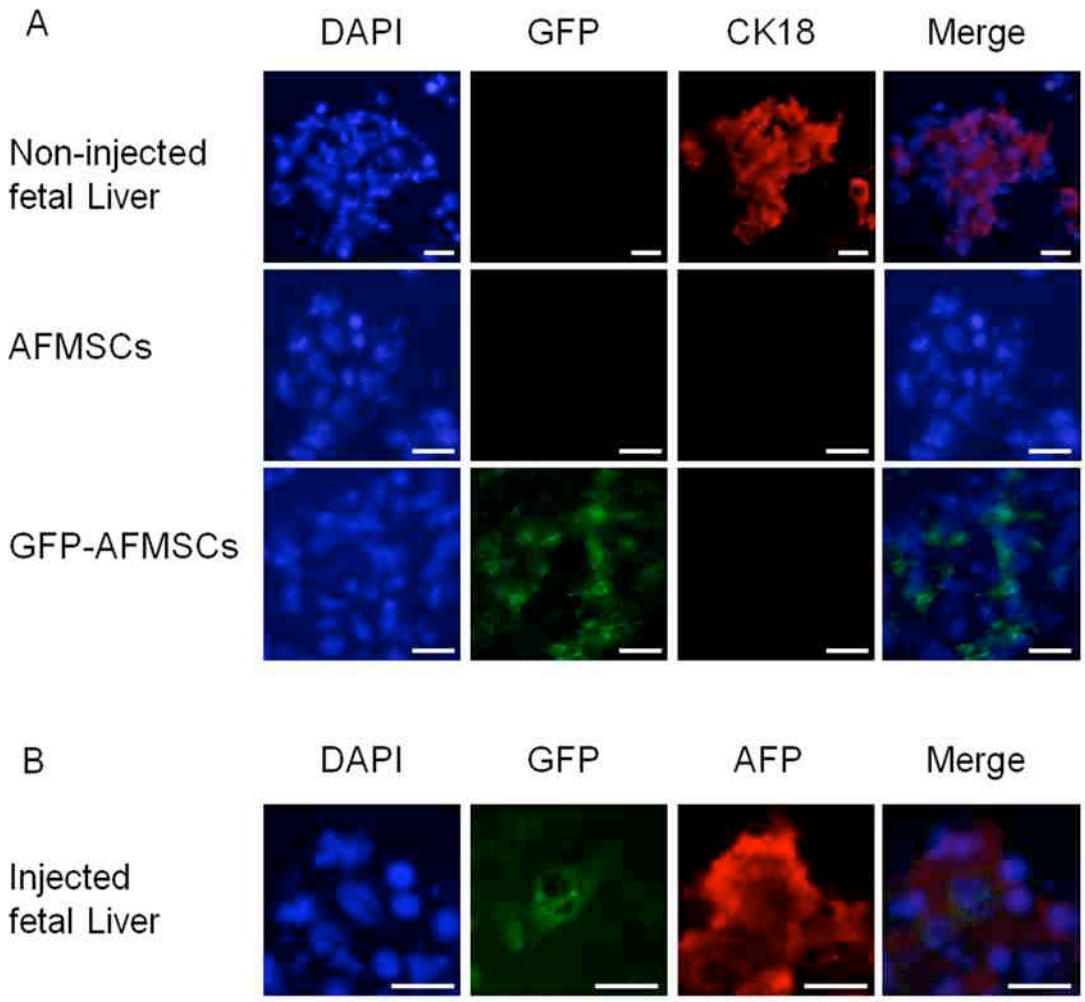


Figure 3.13 Immunofluorescence for CK18 and AFP expression in fetal liver and amniotic fluid stem cells.
The upper panel shows positive CK18 expression in cells cultured from a control fetal sheep liver but no expression in amniotic fluid mesenchymal stem cells (AFMSCs) or transduced cells (GFP-AFMSCs) before injection. An uninjected sheep fetus of comparable gestational age was used as the control. The lower panel shows co-expression of GFP with expression of Alpha fetoprotein (AFP) another liver-specific marker in the fetal liver after transplantation of transduced AFMSCs. Scale bars: 20µm.

For muscle differentiation, we analysed the expression of GFP and muscle-specific laminin. Laminin is a cell membrane marker in muscle fibres and in the brain. GFP positive cells were clearly detected in the skeletal muscle (Figure 3.14, panel A) and heart (Figure 3.14, panel B) of 4 fetuses. No myogenic differentiation could be observed when muscles were co-stained with laminin.

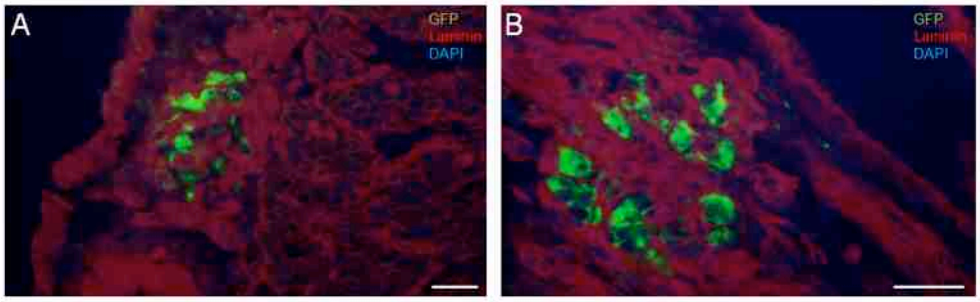


Figure 3.14 Immunofluorescence for GFP expression and markers of muscle differentiation. In sections of fetal skeletal muscle (A) and heart (B), GFP+ cells did not co-stain for laminin (red). Scale bars: 20µm.

Abundant GFP positive cells could be detected in placentome samples (Figure 3.15, A, B, C).

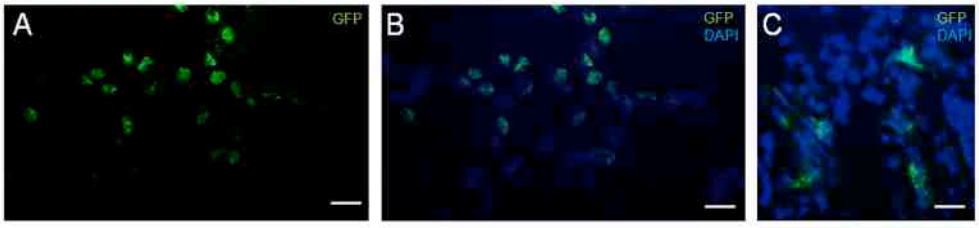


Figure 3.15 Immunofluorescence for GFP expression in placentomes. The sections showed GFP+ cells (A, B, C). Scale bars: 20µm.

3.9 In utero of IM injection of transduced autologous AFMSCs is achievable but results in less system spread than IP injection

We have done the only one prenatal autologous intra-muscular injection in one pup of the first twin pregnancy. Ultrasound guided technique was performed as previous description. The major difference was the echo-tip needle aiming the thigh of the sheep fetus. The procedure needed skilful operator as it cost longer time (15 minutes) than IP injection (Figure 3.16). All the tissues were analyzed as IP series. The PCR study showed the positive GFP DNA could be detected in fetal muscle, liver, membrane, adrenal gland and gonad (Figure 3.17). The western blot confirmed the GFP expression in fetal liver, fetal injected muscle and placenta (Figure 3.18). The local injection of transduced AFMSCs into fetal skeletal muscle showed less widespread engraftment than systemic intraperitoneal injection, even in the same conditions of gestational age of amniocentesis/injection/post mortem, and same injected cell number/volume.

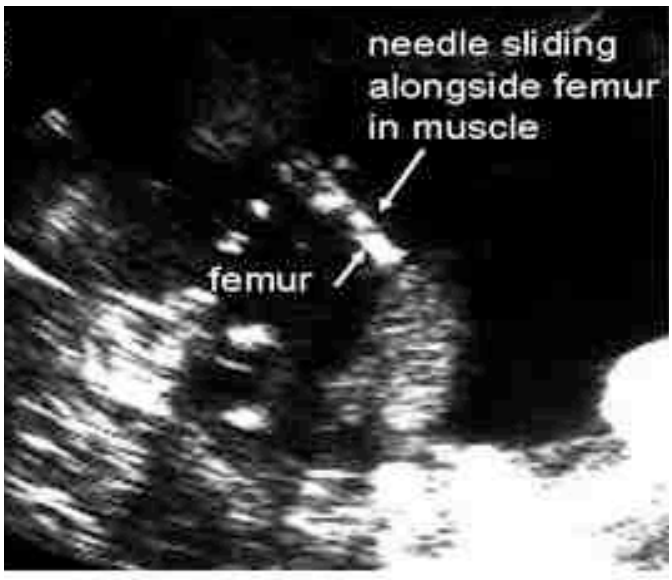


Figure 3.16 Ultrasound guided intramuscular injection.

Figure 3.17 PCR gel showing detection of GFP +ve DNA in fetal tissues after IM injection of autologous transduced AFMSCs.
L: liver, M: muscle, K: kidney, Ad: adrenal gland, G: gonad, N: negative control (water), P: positive control (transduced GFP amniotic fluid cells).

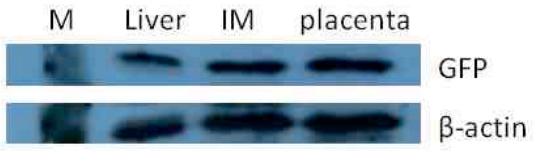


Figure 3.18 Western blot showing detection of GFP protein in fetal tissues after IM injection of autologous transduced AFMSCs.
M: marker, IM: intramuscular injection.

Discussion

This is the first study to describe *in utero* autologous transplantation and migration of AFMSCs. I showed that using this system, it is possible to obtain a widespread migration of transduced autologous cells, with evidence of expression of transgenic protein in major fetal organs, bone marrow and blood. Tissue analysis by PCR, western blot, immunofluorescence and cytofluorimetric assay revealed that AFMSCs injected into the peritoneal cavity preferentially localized in fetal liver, muscle and heart. Moreover, a small number of cells expressing transgenic protein in the liver co-stained with markers of hepatocyte differentiation.

Using ultrasound-guided amniocentesis, a common clinical procedure with a known fetal loss rate of approximately 1% (Tabor et al., 2009), I was able to isolate AFMSCs in 100% of the animals. A miscarriage occurred in one ewe carrying twin pregnancies despite a straightforward ultrasound guided amniocentesis procedure. This compares favourably to the higher rates of fetal loss observed in other studies where different sources of autologous MSCs were used for autologous in utero transplantation. In particular, fetal transplantation of autologous MSC derived from fetal liver in sheep carried an overall mortality rate of 73% (Schoeberlein et al., 2004). For IUSCT in fetal sheep, transuterine ultrasound-guided injection has been shown to achieve a

higher rate of engraftment when compared to an open delivery procedure at hysterotomy (70 versus 20%) (Nagao et al., 2009), in both cases cells were delivered intraperitoneally. Intraperitoneal injection is a useful route of delivery for fetal blood transfusion in clinical practice although it is less commonly performed than cordocentesis (Tongsong et al., 2001). It is usually reserved for fetal blood transfusion in the late first trimester when injection into the umbilical vein may be technically challenging. In my study, I did not observe any miscarriage related to the transplantation procedure. The miscarriage rate was similar to that we have previously reported for both intra-amniotic and intraperitoneal gene transfer to fetal sheep (David et al., 2003a). In comparison, another study of xeno-transplantation of human cord blood and fetal bone marrow MSC into fetal sheep had an overall 37.5% fetal loss rate (Noia et al., 2008).

In my experimental setting, mesenchymal progenitors were derived in 100% of the animals. In keeping with previous studies in sheep (Fuchs et al., 2004), the AFMSCs were maintained in feeder-free cultures, had a doubling time of approximately 36 to 48 hours, and displayed mesenchymal stem cell markers (CD44, CD58, and CD166), while being negative for macrophages, hematopoietic and endothelial markers (CD14, CD31, and CD45). When cultured in conditional medium (Tsai et al., 2004) these cells could differentiate into adipogenic and osteogenic lineages. The potential of differentiation and characterization of all the surface markers did not change even after viral transduction.

Injected cells appeared to migrate mainly to the liver, heart, adrenal gland and umbilical cord of the fetus, and to extra-embryonic tissues such as the amniotic membranes, placentomes and amniotic fluid. Cells delivered to the peritoneal cavity are likely to migrate into the blood stream, from where they can travel to the placenta and membranes via the umbilical vein in the cord. This study was performed in normal fetuses in which there is no engraftment advantage for the injected cells. Different results may be obtained in animal models of disease where damaged organs may provide a niche for injected cells to engraft.

The question of whether autologous IUSCT is superior to allogeneic cells is as yet unresolved. In a previous study in sheep of fetal transplantation of MSC derived from fetal liver, the level of bone marrow engraftment of autologous MSCs achieved was

not significantly different to that observed after allogeneic derived MSCs (0.16% vs 0.56%). However, engraftment in the fetal liver was higher (0.65 versus 0.23%) after autologous transplantation (Schoeberlein et al., 2004). Similarly I observed a good number of injected cells in the fetal liver, confirmed also by the appearance of rare cells co-expressing GFP and CK18 or AFP. Further studies need to be conducted to evaluate the possibility that AFMSCs may engraft in the liver. This could be particularly relevant since hepatocyte differentiation may lead to the treatment of congenital metabolic disorders (Quaglia et al., 2008).

In summary, this chapter demonstrates widespread and systemic migration after fetal injection of transduced autologous AFMSCs in the sheep. I successfully cultured sheep AFMSCs, and achieved a high level of lentivirus vector mediated gene transfer. Longer-term follow-up studies are needed to investigate whether cell engraftment is occurring (Chapter 4). My results however demonstrate that this approach may hold promise for targeted treatment of severe early onset genetic diseases that can be diagnosed in utero.

Chapter 4

Sheep CD34+ amniotic fluid cells show the potential of haematopoiesis in NOD SCID gamma mice and prenatal autologous transplanted lambs

4 Chapter 4: Sheep CD34+ amniotic fluid cells show the potential of haematopoiesis in NOD SCID gamma mice and prenatal autologous transplanted lambs

Introduction

In the previous chapter, I showed that MSCs derived from sheep AF could be isolated, cultured, transduced and transplanted back into the same fetus as a prenatal autologous gene-cell transplant (Shaw et al., 2011a). The transplanted GFP+ cells could be detected in many fetal organs including liver, muscle, adrenal gland, and placenta. Ditadi et al demonstrated the haematopoietic potential of selected AFSCs *in vitro* and *in vivo* (Ditadi et al., 2009). These Lin(-)/Ckit(+) sorted cells derived from human or murine AF could show all the haematopoietic colonies in semi-solid culture. Engraftment of murine and human donor cells in haematopoietic organs was demonstrated after intravascular injection into irradiated immunocompromised recombinant activating gene 1 (RAG1)-/- mice, and furthermore, the engrafted cells could be found after secondary transplantation confirming their haematopoietic potential.

Based on this data, I hypothesised that cells with haematopoietic potential could be demonstrated in sheep AF. At the time of starting this study, and to date, there is no sheep specific Ckit antibody available. At the beginning of my PhD, Porada CD et al. developed a novel sheep CD34 antibody that had been tested in sheep bone marrow and found to be a truly haematopoietic stem cell marker (Porada et al., 2008b). We contacted the author who kindly allowed us to test this antibody in sheep AF cells. To demonstrate their haematopoietic potential, I selected CD34+ cells from sheep AF, and I injected them into NOD SCID gamma (NSG) immunocompromised mice to study their engraftment, and into donor sheep after marking them using a viral vector for prenatal autologous transplantation. NSG mice are severely immunocompromised, featuring absence of mature T or B cells, lack of functional NK cells and deficiency in cytokine signalling (Shultz et al., 2005). The engraftment of xenogeneic hematopoietic stem cells and peripheral-blood mononuclear cells in the NSG mice is greater than that in any other mouse strain (Majeti et al., 2007, Ishikawa et al., 2005). The following chapter describes the results of engraftment analysis to prove the haematopoietic potential of the sheep AF CD34+ cells.

4.1 CD34+ cells can be isolated from sheep amniotic fluid

For these experiments we used fresh and frozen sheep AF, collected from time mated animals in the first trimester at post mortem examination. The average gestational age was 62 days (60-64 days) and the maximum possible volume (300 to 700 ml AF) was collected each time (Figure 4.1, 4.2 and Table 4.1). The methods for fresh AF collection, defrosting of the frozen AF, and bone marrow cell collection are described in Chapter 2. All procedures were carried out carefully using sterile conditions as far as possible to prevent any infection or contamination.

Five to 8 million cells could be collected from fresh unsorted AF or frozen AF (Table 4.1). The AF was frozen down 3 months before this experiment and defrosted for CD34+ sorting and transplantation. After centrifuging the fluid, the cell pellets were pooled and cells were then selected with sheep CD34 antibody. The amount of CD34+ cells could be isolated from each pregnant animal numbered between 200,000 to 500,000. The proportion of CD34+ cells was 5.05% in fresh and 5.38% in the frozen AF group respectively. Bone marrow collected from the sternum of the adult sheep also contained CD34+ cells. The percentage of isolated CD34+ cells from sheep fresh adult bone marrow was 6.48%, which is slightly higher than fresh or frozen AF samples. There was no significant difference between these three groups (ANOVA).

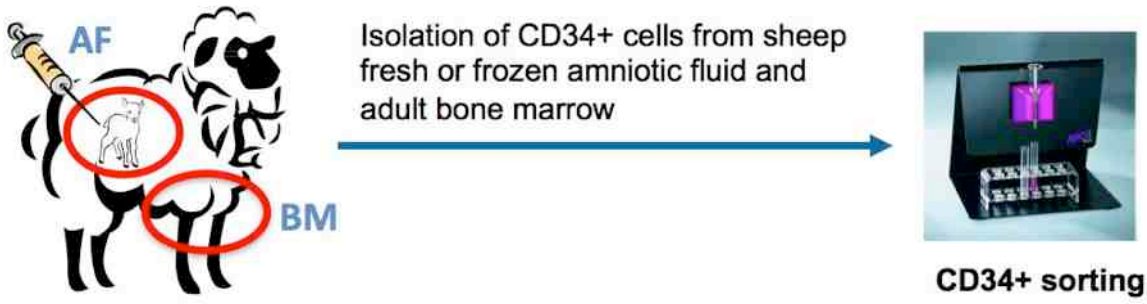


Figure 4.1 Isolation of CD34+ cells from sheep fresh, frozen AF or adult bone marrow.

Table 4.1 Characteristics of first trimester sheep AF and adult bone marrow.
CD34+ cells could be isolated from all three sources. AF: amniotic fluid; BM: bone marrow; SD: Standard deviation.

Cell source	Animal	Gestational age (Day)	AF (ml)	Total Cells (n)	CD34+ cells (n)	%
Fresh	1	61	500	$7X10^6$	$4X10^5$	5.7
	2	63	300	$5X10^6$	$2X10^5$	4
	3	63	350	$5X10^6$	$3.5X10^5$	6.3
	4	61	400	$6X10^6$	$2.5X10^5$	4.2
					Mean	**5.05**
					SD	**1.13**
Frozen	1	64	500	$8X10^6$	$5X10^5$	6.3
	2	64	550	$8X10^6$	$4X10^5$	5
	3	60	600	$5X10^6$	$3X10^5$	6
	4	60	700	$6X10^6$	$2.5X10^5$	4.2
					Mean	**5.38**
					SD	**0.96**
BM	1			$9X10^6$	$6X10^5$	6.7
	2			$9X10^6$	$5.5X10^5$	6.1
	3			$8X10^6$	$6X10^5$	7.5
	4			$8X10^6$	$4.5X10^5$	5.6
					Mean	**6.48**
					SD	**0.82**

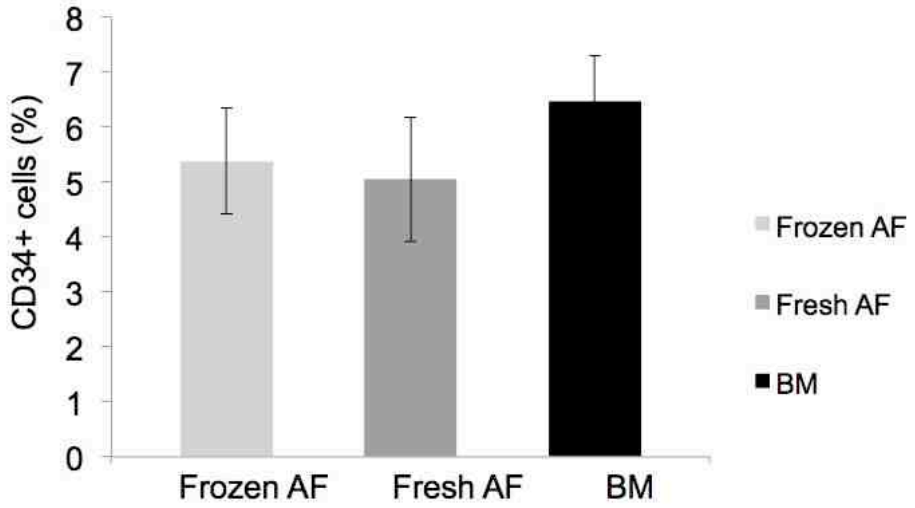


Figure 4.2 Proportion of CD34+ cells isolated from three sheep cell sources.
There was no statistical difference in the proportion of CD34+ cells between the three cell sources by ANOVA. AF: amniotic fluid; BM: bone marrow. Error bar: standard deviation.

In summary, the sheep specific CD34 antibody selected CD34+ cells from fresh and frozen AF and adult bone marrow. In each case the CD34+ fraction was about 5% of the total population.

4.2 Sheep CD34+ AF cells can be cultured under haematopoietic conditions and transduced with lentivirus vector

Having isolated CD34+ cells from sheep AF, I evaluated their transduction efficiency using lentivirus vectors. Following collection of the positive fraction of sheep CD34 cells from magnetic microbead sorting system, cells were transferred into ultra-low attachment 24-well plates at a seeding density of 100000 cells/ml in all three groups and grown with conditional medium containing cytokines as described in Chapter 2. HIV-SFFV vector encoding eGFP was used for transduction (vector dose=10^9/ml, MOI=50, 50µl of vector for 10^6 cells transduction) as described before (Chapter 3). Cells were incubated with the lentivirus vector for 24 hours before the transplantation. The transduction efficiency was tested 72 hours later after incubation by using flow cytometry. The transduction efficiency ranged between 46.9 and 73.2% and was on average 56.63% ± SD 11.0% (n=6) as shown below in Figure 4.3.

In summary, I concluded that sheep CD34+ AF cells could be cultured under haematopoietic condition and efficiently transduced with lentiviral vector.

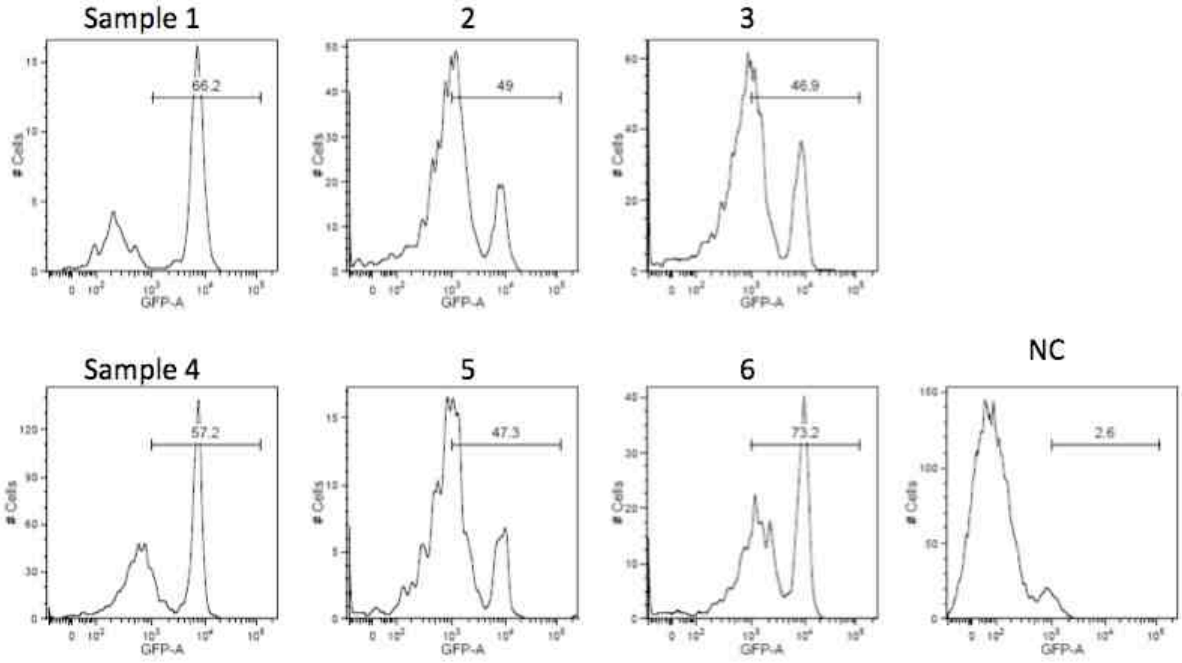


Figure 4.3 Histograms showing flow cytometry results for GFP+ cells after gene transfer to CD34+ sheep AF cells using lentivirus vector containing the eGFP gene after 72 hours infection. The X-axis shows the GFP channel, while the number on the gate represents the percentage of GFP+ positive cells out of total population. Y-axis, cell number count.

4.3 Phenotypic characterization of isolated sheep AF CD34+ cells was comparable to bone marrow CD34+ cells

In order to understand better the hematopoietic potential of the AF CD34+ cells experiments were designed to compare characteristics of CD34+ cells from all three groups (fresh and frozen CD34+ AF and BM).

The fresh or frozen sheep AF CD34+ cells, or sheep adult bone marrow CD34+ cells were cultured in suspension. All the cells from these three groups were characterized after 24 hours culturing. Single cell suspension was prepared and incubated with sheep specific antibodies for 15 minutes on ice followed by flow cytometry study. The CD34+ population of cells were also positive for CD45 (96.5%, 95.1%, and 94.3% in fresh AF, frozen AF, adult BM groups), but were negative for MSC markers including CD44, CD58 (Figure 4.4 and Table 4.2). They were also negative for CD14 (monocyte marker) and CD31 (epithelial cell marker). Amniotic fluid or bone marrow CD34+ cells, which were not incubated with antibodies, were used as negative control.

Table 4.2 Characterisation of sheep CD34+ cells from the first trimester AF and adult bone marrow.
Samples of sheep fresh AF, frozen AF or adult bone marrow (BM) were tested for surface markers and the percentage of cells showing marker positive is provided. The data represented is mean ± standard deviation.

	CD14	CD31	CD34	CD44	CD45	CD58
Fresh AF	1.2±0.9	0.9±0.4	97.9±1.4	0.7±0.2	96.5±2.7	1.1±0.8
Frozen AF	1.0±0.8	1.6±0.3	96.3±2.4	0.5±0.4	95.1±3.0	1.3±0.8
Adult BM	1.1±0.8	0.9±1.0	96.2±2.1	1.1±0.7	94.3±2.7	0.6±0.6

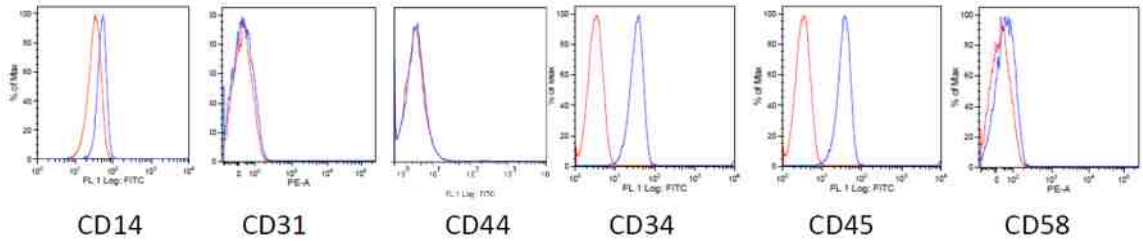


Figure 4.4 Example of a flow cytometry study to characterise sheep CD34+ cells from fresh AF. Cells are positive for CD45, but negative for CD14, CD31, CD44, and CD58. The red line indicates the negative control (AF or BM CD34+ cells without antibodies incubation) and the blue line indicates the cells tested. In negative results, the red and blue lines merged together while they separated as two peaks in positive results.

In summary, the sheep CD34+ cells from fresh or frozen AF, or adult bone marrow were positive for haematopoietic stem cell markers (CD45), but negative for a monocyte marker (CD14), an epithelial cell marker (CD31) and MSC markers (CD44 and CD58).

4.4 Sheep CD34+ cells isolated from frozen, or fresh AF, or BM functionally engraft into NSG mice

In previous sections, I had showed that the sheep amniotic fluid or bone marrow CD34+ cells could be isolated, cultured in the hematopoietic condition, and efficiently transduced with lentivirus. In addition, these CD34+ cells had the same characterization as hematopoietic stem cells. In the following section, I demonstrate their xenogeneic transplantation by delivering sheep CD34+ cells (three groups) to immunocompromised NSG mice.

In Figure 4.5, the experimental design of primary xenogeneic transplantation of sheep CD34+ cells from three sources (fresh, frozen amniotic fluid and adult bone marrow cells) into NSG mice is presented.

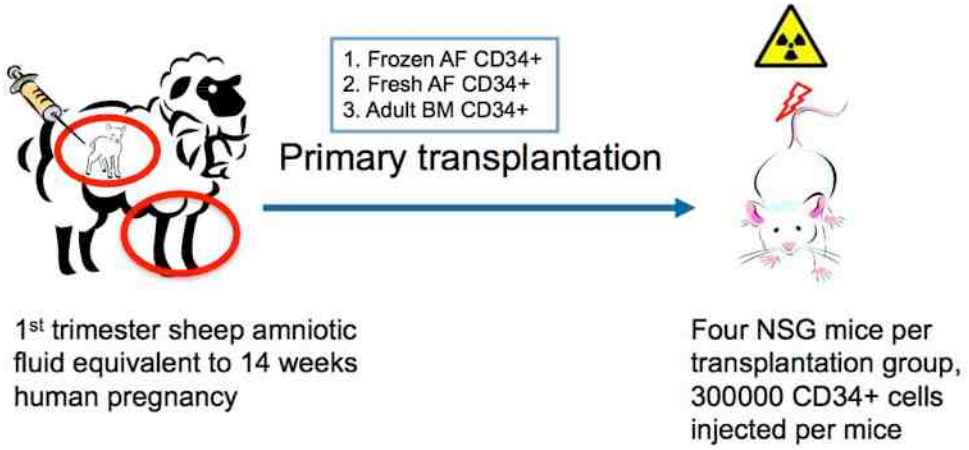


Figure 4.5 The experimental design.
CD34+ cells were selected and transduced from three different cell sources, fresh AF, frozen AF and adult bone marrow (BM). Mice were subjected to sub-lethal irradiation, followed by tail vein injection of prepared transduced sheep CD34+ cells.

Amniotic fluid was collected at the first trimester of sheep pregnancy that was equivalent to 14 weeks of human pregnancy. All the animal experiments were performed by qualified personal licence holders (Mike Blundell and I) under existing project licences. A sub-lethal dose of irradiation (300 Rads for 67 seconds) was given to NSG mice (12 week-old, n=14) 1 hour prior transplantation. Sheep CD34+ cells from three groups were transduced with SFFV-eGFP virus vector for 24 hours before prepared for injection by suspension in PBS. Transduced $3x10^5$ cells (both GFP+ and GFP- cells) in 200µl PBS were injected into each animal via tail vein. The procedures were performed under the laminar flow hood in the SCID animal room at Institute of Child Health, UCL.

Four out of 6 animals injected with frozen sheep AF CD34+ cells survived until the end of the study, but two animals (NSG1, and NSG2) died within 10 days due to poor tolerance to irradiation (Table 4.3). Fresh sheep AF CD34+ cells (NSG7 - 10) or adult bone marrow CD34+ cells (NSG11 -14) were injected into a further 8 animals, 4 per group, and had 100% survival. The overall survival was 85.7% (12 out of 14). In each group, 3 of the 4 surviving animals were culled at 3 months of age for use in secondary transplantation. The remaining three animals (one per group) were sacrificed at 6 months for final analysis.

In summary, I have shown that eGFP transduced CD34+ sheep cells from amniotic fluid or adult bone marrow could engraft into NSG mice after primary transplantion with a good survival rate.

Table 4.3 Summary of xenogeneic transplantation using sheep CD34+cells into NOD SCID gamma (NSG) mice.
Transduced CD34+ cells from frozen AF (n=6), fresh AF (n=4) or adult bone marrow (BM, n=4) cell sources were injected. In each group, 3 of the 4 surviving animals were culled at 3 months of age for use in secondary transplantation.

Animal ID	Injected cells	Number	Virus	Irradiation	Remark
NSG1	Frozen AF CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Died within 10 days
NSG2	Frozen AF CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Died within 10 days
NSG3	Frozen AF CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 3 months
NSG4	Frozen AF CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 3 months
NSG5	Frozen AF CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 3 months
NSG6	Frozen AF CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 6 months
NSG7	Fresh AF CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 3 months
NSG8	Fresh AF CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 3 months
NSG9	Fresh AF CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 3 months
NSG10	Fresh AF CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 6 months
NSG11	BM CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 3 months
NSG12	BM CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 3 months
NSG13	BM CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 3 months
NSG14	BM CD34+	$3X10^5$	SFFV-eGFP	300 Rads	Culled at 6 months

4.5 GFP+ cells could be detected in the peripheral blood of transplanted NSG mice from all three groups

After xenogeneic transplantation, I examined for donor cell engraftment and to detect GFP+ cell levels in the peripheral blood, and the hematopoietic organs.

Blood samples were taken from the tail vein of all primary transplanted NSG mice at 1, and 3 months after transplantation. Samples were analysed for the presence of sheep CD45 antibody using flow cytometry. Blood from yellow fluorescent protein (YFP) transgenic mice was used as a positive control, while blood from uninjected wild type CD1 mice was the negative control. 20 to 25 µl fresh blood could be collected from each mouse. 5µl heparin was added into each microcentrifuge tube before collecting the blood. The details of the animal procedures were described in Chapter 2. Flow cytometry was performed to analyse the percentage of GFP+ cells in the peripheral blood.

The three groups of NSG mice which received frozen, fresh AF, or adult bone marrow cell injections showed GFP+ cells in peripheral blood at 1 month after transplantation (mean GFP+ cells 3.24%, 3.48%, and 4.9%, respectively) and at 3 months (mean GFP+ cells 3.12%, 3.4%, and 5.26%, respectively, Table 4.4 and Figure 4.6,). The percentage of detectable GFP+ cells in peripheral blood was higher in NSG mice injected with adult BM CD34+ cells compared to CD34+ cells from AF sources at 3 months ($p<0.05$, either BM versus frozen AF or BM versus fresh AF). There was no statistically significant difference in the percentage of GFP+ cells detected in the peripheral blood of NSG mice injected with frozen or fresh AF CD34+ cell.

Table 4.4 The percentage of GFP+ cells in the peripheral blood of injected NSG mice from all three groups at 1 and 3 months after transplantation.
SD: standard deviation.

	GFP+ cells in blood (%)			GFP+ cells in blood (%)			GFP+ cells in blood (%)	
Frozen AF	1 Month	3 Months	**Fresh AF**	1 Month	3 Months	**Adult BM**	1 Month	3 Months
NSG3	2.52	3.54	NSG7	3.20	3.65	NSG11	4.30	4.87
NSG4	4.30	3.61	NSG8	3.40	3.23	NSG12	4.50	5.16
NSG5	3.32	2.92	NSG9	4.90	4.12	NSG13	5.20	5.51
NSG6	2.80	2.40	NSG10	2.40	2.60	NSG14	5.60	5.50
Mean	3.24	3.12		3.48	3.40		4.90	5.26
SD	0.78	0.57		1.04	0.65		0.61	0.31

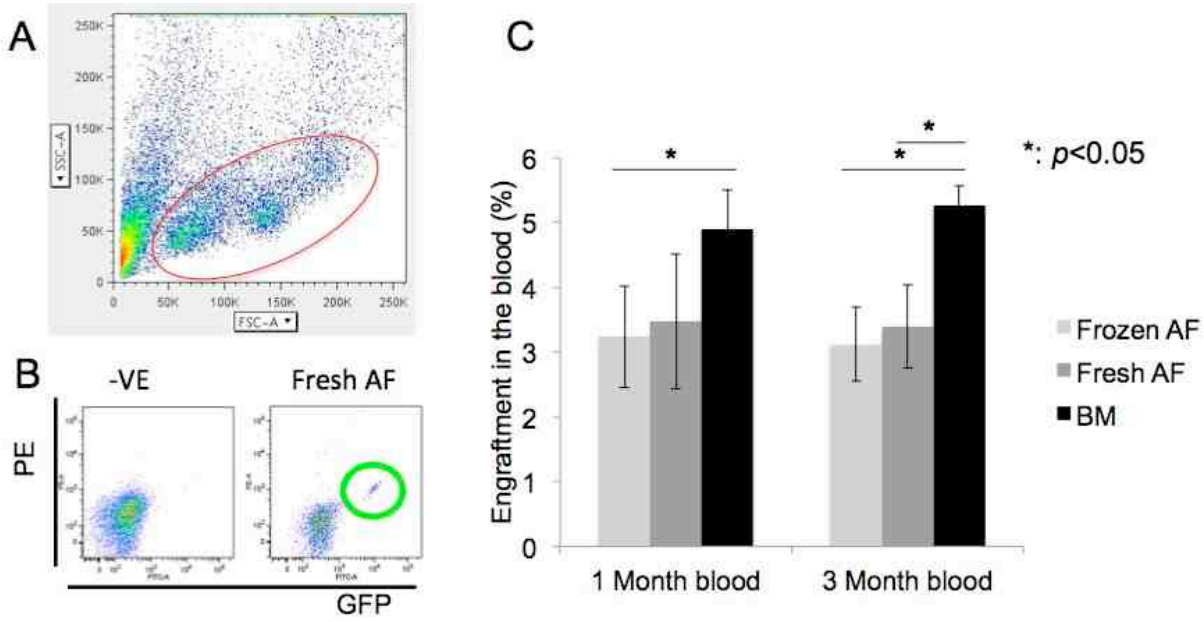


Figure 4.6 Analysis of peripheral blood engraftment of GFP+ CD34+ sheep cells in NSG mice. The panel A showed the initial distribution of total blood cells and the nucleated blood cells were gated in the red circle. Panel B showed an example flow cytometric graph from the blood of one NSG mouse injected with fresh AF cells. The green circle in the panel B showed the positive GFP cells detected in the peripheral blood (3.23%, NSG8). The negative control (-VE) was used as the blood sample from CD1 wild type mouse. The X-axis showed the GFP channel while the Y-axis presented the PE channel. In panel C, the histogram compares blood engraftment after injection of sheep CD34+ GFP transduced from three sources (n=4, each group) at 1 month, and 3 months after injection. There was significantly higher engraftment when the source of cells was adult sheep bone marrow (black bar) compared with cells sourced from frozen AF (grey bar) or fresh AF (light grey bar). Error bar: standard deviation; *: p<0.05; AF: amniotic fluid.

In summary, this experiment showed that GFP+ cells could be found in the peripheral blood of NSG mice after xenogenic transplantation of sheep CD34+ cells at 1, and 3-month time points after transplantation. All transplanted animals had detectable GFP+ cells (2.4 to 5.6%), bone marrow sourced CD34+ cells resulted in higher levels of cells in the peripheral blood when compared with AF sourced CD34+ cells.

4.6 GFP+ cells could be detected using flow cytometry in haematopoietic organs of NSG mice from all three groups 3 months after transplantation

To study engraftment in haematopoietic organs, three animals from each group were culled at 3 months after transplantation. The organs were widely sampled at post mortem examination according to the protocol written in the index. Single cell suspensions from liver (act as the main haematopoietic organ in fetuses and contribute the haematopoiesis in neonatal development), bone marrow, and spleen were prepared for flow cytometry.

4.6.1 GFP+ cells and sheep CD34+ cells are detected in the bone marrow of transplanted NSG mice from all three groups

For bone marrow cells analysis, two femoral bones were collected from each mouse. Bone marrow cells were washed out through 40µm cell strainer and prepared as single cell suspension (described in Chapter 2) for flow cytometry analysis. Antibodies to GFP detection and sheep CD34 were used. The bone marrow cells from transgenic YFP mice were used as the positive control, and bone marrow cells from an uninjected wild type mouse was used as negative control.

Figure 4.7 gives an example of flow cytometry analysis for detection of GFP+ cells in negative/positive control, and frozen or fresh AFS or BM injected NSG mice. The red circle indicated the percentage of GFP positive population out of the total bone marrow cells.

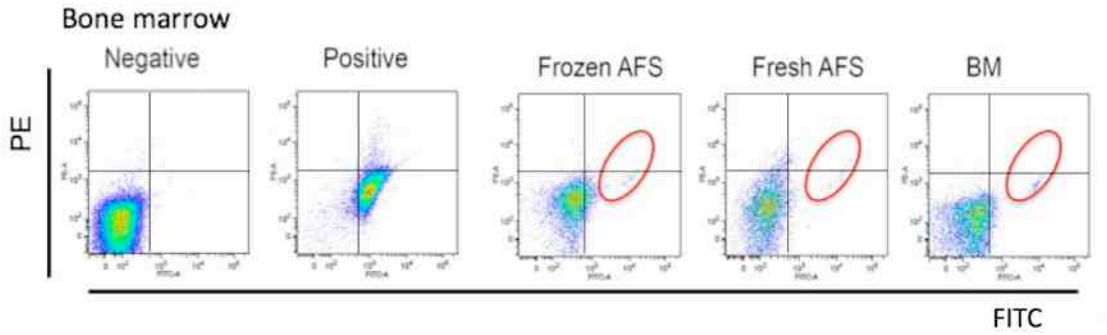


Figure 4.7 Example of flow cytometry to analyse engraftment of GFP+ cells in the bone marrow of injected NSG mice three months after transplantation.
The X-axis for FITC channel presented the GFP cells. The red circle indicated the percentage of GFP positive population out of the total bone marrow cells. Negative control: uninjected wild type mice; positive control: GFP transduced bone marrow cells.

At 3 months after transplantation, GFP positive and sheep CD34+ cells could be detected in the bone marrow of all the injected NSG mice from all three groups (n=3 per group). Sheep GFP+ and CD34+ cells were detected in the bone marrow 3 months after injection with CD34+ frozen and fresh sheep AF, or adult sheep bone marrow cells (mean GFP+ cells 12.03%, 14.87%, and 15.87%, respectively; mean sheep CD34+ cells 2.57%, 3.26%, 3.51%, respectively, Table 4.5 and Figure 4.7).

Table 4.5 The percentage of sheep GFP+ and CD34+ cells in the bone marrow of injected NSG mice from all three groups (frozen AF, fresh AF or adult BM) at 3 months after transplantation. SD: standard deviation; AF: amniotic fluid; BM: bone marrow.

Animal ID	Injected cells	GFP+ cells in BM (%)	Sheep CD34+ cells in BM (%)
NSG3	Frozen AF	11.80	3.12
NSG4	Frozen AF	10.80	3.23
NSG5	Frozen AF	13.50	2.45
Mean		12.03	2.93
SD		1.22	0.42
NSG7	Fresh AF	12.00	3.68
NSG8	Fresh AF	17.20	3.12
NSG9	Fresh AF	15.40	2.98
Mean		14.87	3.26
SD		2.64	0.37
NSG11	Adult BM	13.30	3.10
NSG12	Adult BM	18.10	3.56
NSG13	Adult BM	16.20	3.86
Mean		15.87	3.51
SD		2.42	0.38

The percentage of GFP+ cells in the bone marrow of NSG mice injected with adult BM CD34+ cells was higher than NSG mice injected with frozen or fresh AF CD34+ cells (Figure 4.8). The difference between adult BM and frozen AF group reached the statistic significance by ANOVA (p<0.05).

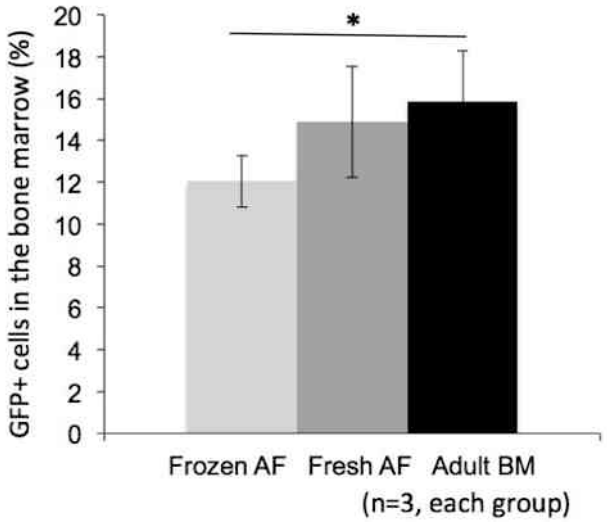


Figure 4.8 The histogram compares the percentage of GFP+ cells in bone marrow of injected NSG mice from three sources (n=3, each group) at 3 months.
There was significantly higher engraftment when the source of cells was adult sheep bone marrow (black bar) compared with cells sourced from frozen AF (grey bar). Error bar: standard deviation; *: p<0.05.

In order to understand the distribution and the differentiation of engrafted sheep CD34+ cells in the mice bone marrow, I sorted the sheep CD34+ cells and stained with markers of T and B cells for flow cytometry analysis. Figure 4.9, I gated the left red circle to indicate the sheep CD34+/GFP- cells (3.68% in the left panel). Then specific sheep antibodies including CD2 (T cells) and CD21 (B cells) were incubated with sorted sheep CD34+/GFP- cells (Figure 4.9, right panel). The ratio of T cells to B cells was approximately 1:3.

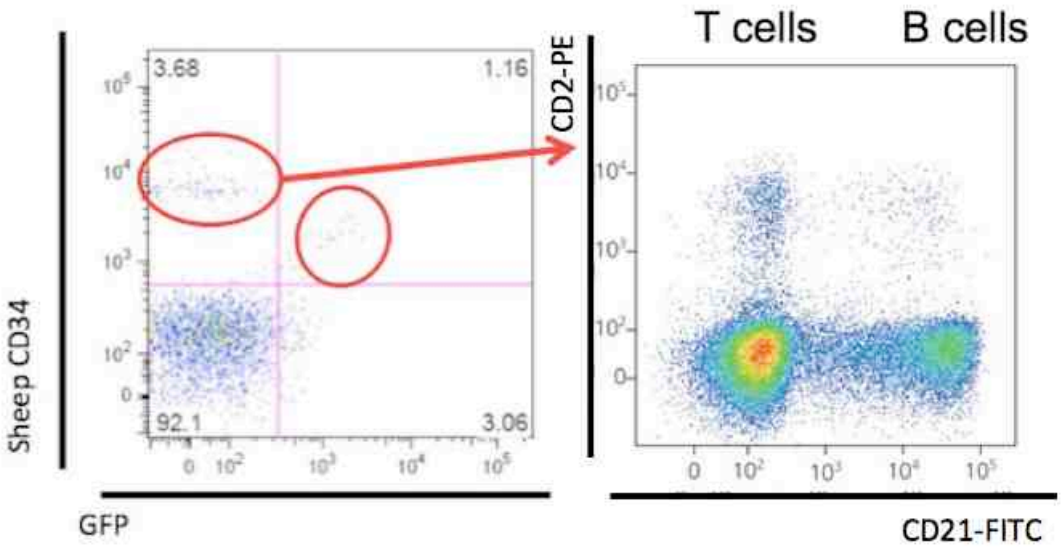


Figure 4.9 The flow cytometry study shows T cell and B cell differentiation of transplanted sheep CD34+ cells in NSG mice.
In the left panel, the left red circle indicates that the sheep CD34+/GFP- cells in the bone marrow was approximately 3.68%. These cells were sorted and gated for T cell (sheep CD2) and B cell (sheep CD21) markers in the right panel.

In summary, both GFP+ and CD34+ sheep cells could be detected in the bone marrow of NSG mice from all three groups (frozen AF, fresh AF or adult BM cells transplantation) at 3 months. The percentage of GFP+ cells in the bone marrow from the adult BM group was higher than frozen AF group. Both T cells and B cells sheep specific markers could be found in sorted CD34+ cells that demonstrated the differentiation of the transplanted CD34+ in the bone marrow of recipients and also showed the evidence of bone marrow engraftment.

4.6.2 GFP+ cells could be detected in the liver and spleen of transplanted NSG mice from all three groups

In the previous results, I had showed that GFP+ cells could be detected in the bone marrow of transplanted animals and further demonstrated their differentiation into T and B cells. I then performed the flow cytometry study in other hematopoietic organs including liver and spleen to detect evidence of GFP+ cells after transplantation of sheep CD34+ GFP transduced cells. The single cell suspension of liver and spleen from the culled animal at 3 months were prepared as described in the methods. YFP transgenic mice were used as positive control, and not injected wild type mice were used as negative control.

GFP+ cells were detected in the spleen and liver at 3 month after injection (Figure 4.10 and Figure 4.11). Transplantation of adult BM resulted in a higher engraftment rate than transplantation with either fresh AF and frozen AF in the spleen (6.7%, 5.8%, and 5.2%), and in the liver (15.6%, 14.3%, and 8.5%; Figure 4.11). The frozen AF cells in the liver showed significant lower engraftment than the other two groups (p<0.05 by ANOVA). There was no statistic significance among three groups in spleen.

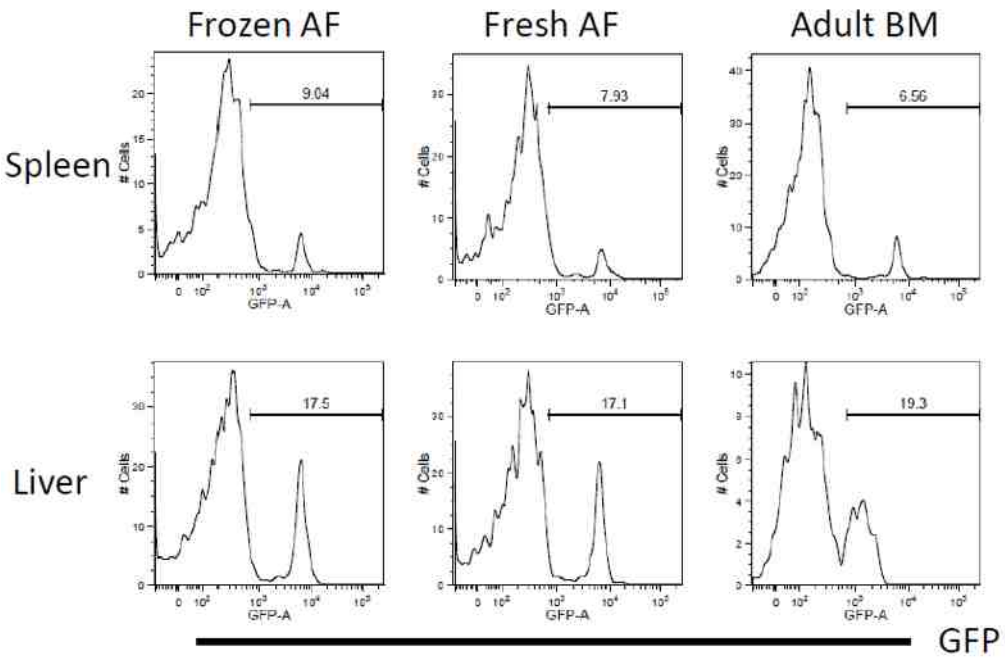


Figure 4.10 Analysis of the spleen and liver three months after primary injection.
The bar in the histograms showed percentage of the GFP+ cells in the spleen or liver presented as the smaller peak area located in the right.

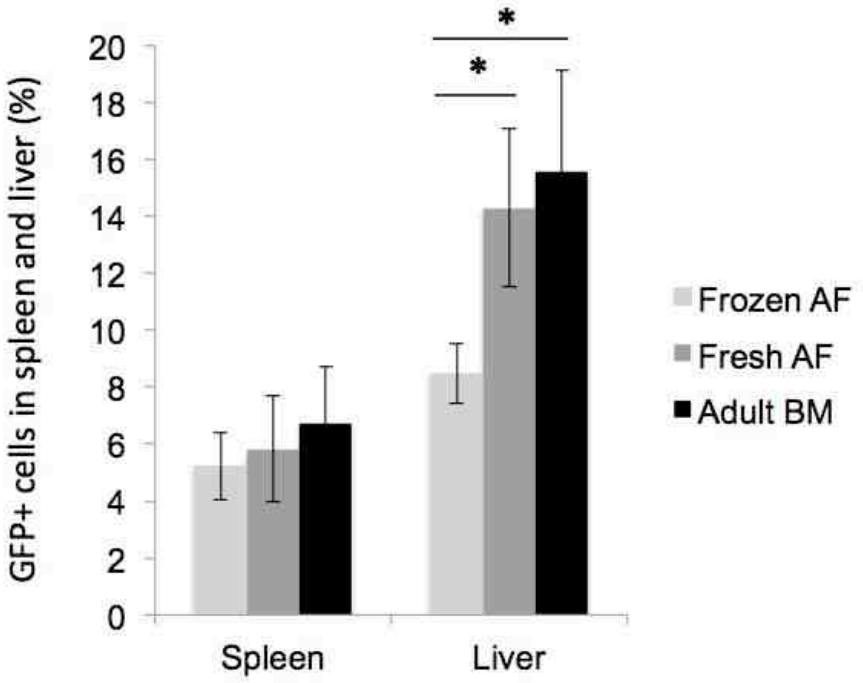


Figure 4.11 Summary of haematopoietic organ analysis 3 months after transplantation of GFP transduced sheep CD34+ cells.
Cell sources were frozen AF (light grey), fresh AF (dark grey) and adult bone marrow (black). In the liver, cells from frozen AF also showed a significantly lower level of engraftment than those fresh AF and BM groups. There was no difference in spleen engraftment. Error bar: standard deviation, *: $p<0.05$.

In summary transplanted GFP+ CD34+ frozen, fresh AF or BM sheep cells could engraft into all haematopoietic organs of NSG mice, but there was lower level engraftment in the spleen when compared with the bone marrow or liver. Injected bone marrow cells had a higher engraftment rate than AF derived cells.

4.7 Bone marrow from NSG mice transplanted with GFP+ sheep CD34+ cells formed GFP positive hematopoietic colonies

In order to demonstrate that the injected cells are really of hematopoietic origin, I cultured samples of bone marrow from NSG transplanted mice to look for evidence of GFP positive colonies. The cells were prepared under hematopoietic conditions and seeded into semi-solid culture medium to form the hematopoietic colonies (described in Chapter 2). A few green colonies were detected in bone marrow from all NSG mice transplanted with frozen, fresh AF, or BM GFP+ CD34+ sheep cells (n=3 per group). The green hematopoietic colonies in each group could be observed directly under

fluorescence microscopy (Figure 4.12). The bone marrow from YFP transgenic mice were used as positive control and uninjected wild type mice were used as negative control.

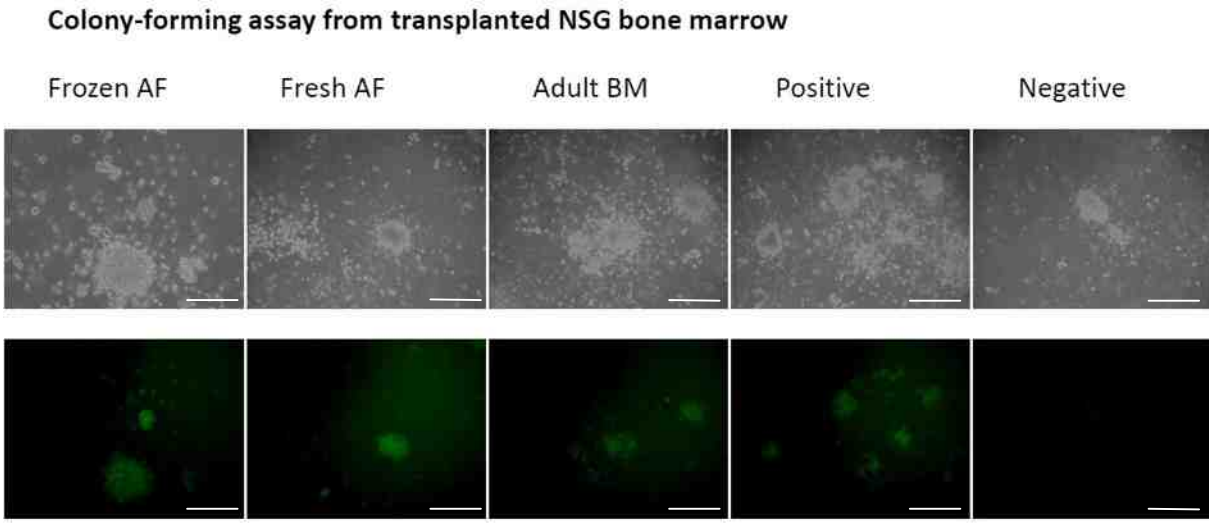


Figure 4.12 Colony-forming assay to detect the presence of GFP+ cells in the bone marrow of NSG mice transplanted with GFP+ sheep CD34+ cells sourced from frozen or fresh AF, or sheep adult bone marrow.
Positive green colonies could be detected. Bone marrow from an YFP mouse was used as a positive control, while bone marrow from an uninjected wild type mouse was used for a negative control. Upper panel: phase contrast field; lower panel: green fluorescence signal. Bar: 100µm.

The mean percentage of GFP positive colonies in the bone marrow of each group was 6.25%, 11.1%, and 13.8% after transplantation of GFP+ sheep CD34+ cells from frozen AF, fresh AF, and bone marrow sources respectively (Table 4.6 and Figure 4.13). Bone marrow cells from three NSG mice per group were counted for green colonies and each NSG mouse had two plates of colonies checked after seeding. Therefore, a total 6 plates per group were analysed. The number of positive colonies was higher in NSG mice transplanted with bone marrow and fresh AF cell sources than frozen AF cell source, but this difference did not reach statistical significance.

Table 4.6 The GFP+ colonies per high power field in the bone marrow of NSG mice transplanted with sheep fresh or frozen CD34+ AF or sheep adult bone marrow CD34+ cells.
6 plated per groups were counted for GFP+ hematopoietic colonies. SD: standard deviation.

	GFP+ colonies per high power field (%)		
	Frozen AF	Fresh AF	Adult BM
plate 1	6.10	15.30	18.90
plate 2	5.70	11.30	17.20
plate 3	0.10	6.30	5.30
plate 4	15.30	13.80	14.90
plate 5	7.40	10.20	9.90
plate 6	2.90	9.40	16.30
Mean	6.25	11.05	13.75
SD	5.15	3.22	5.15

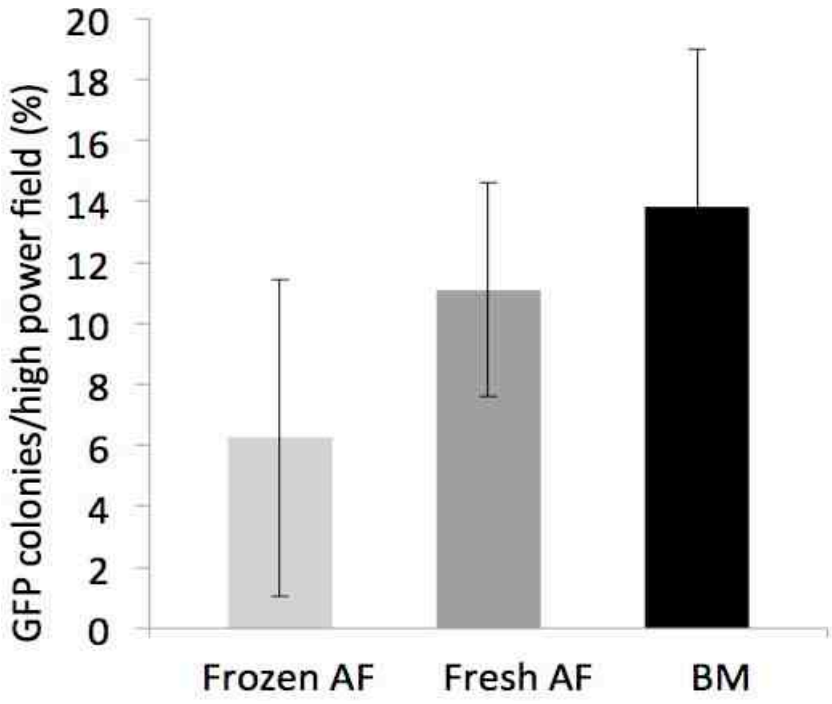


Figure 4.13 GFP positive colonies in the bone marrow of NSG mice 3 months after transplantation with GFP+ sheep CD34+ cells from three different sources.
Cells sourced from adult sheep bone marrow, had a slightly higher percentage of GFP positive colonies than the other two groups, but this did not reach the statistical significance (ANOVA).
Error bar: standard deviation.

The hematopoietic colonies were further divided into three different types which are colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM), colony forming unit-granulocyte, monocyte (CFU-G/M/GM), burst-forming unit/CFU-erythroid (BFU/CFU-E). GFP+ colonies of all three types were seen in the bone marrow of transplanted animals (Figure 4.14). There was no significant difference (ANOVA) in the mean level of the three different types of colonies in bone marrow analysed after transplantation using GFP+ sheep CD34+ frozen AF, or fresh AF or BM cells (Table 4.7, and Figure 4.14).

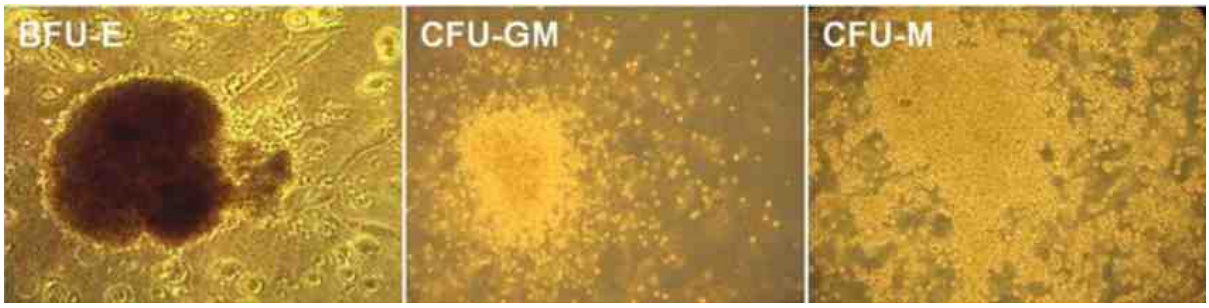


Figure 4.14 The morphology of the three different types of hematopoietic colonies. CFU-GEMM: colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte; CFU-G/M/GM: colony forming unit-granulocyte; BFU/CFU-E: burst-forming unit/colony forming unit-erythroid. Bar: 100µm.

Table 4.7 The percentage of the three different types of colonies counted in the semi-soild culture. CFU-GEMM: colonies forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte; CFU-G/M/GM: colonies forming unit-granulocyte; BFU/CFU-E: burst-forming unit/colonies forming unit-erythroid; AF: amniotic fluid; BM: bone marrow.

	Frozen AF (%)	Fresh AF (%)	BM (%)
BFU/CFU-E	32	33	40
CFU-G/M/GM	46	48	45
CFU-GEMM	22	19	15

In summary, I have shown that bone marrow cells collected from NSG mice transplanted with GFP+ CD34+ sheep cells from frozen and fresh AF, and adult bone marrow sources, could form all types of haematopoietic colonies *in vitro*. Cells sourced from adult sheep bone marrow had a slightly higher percentage of GFP+ colonies than the other two groups, but this difference did not reach statistical significance.

4.8 GFP DNA could be detected by PCR in the liver, spleen, and adrenal gland of transplanted NSG mice

To determine the extent of transplanted cell spread, I examined genomic DNA extracted from organs collected at post mortem examination of all nine transplanted NSG mice, for the presence of GFP DNA. Figure 4.15 shows examples of PCR gels from animals transplanted with frozen AF, fresh AF, and bone marrow sources of GFP+ sheep CD34+ cells. Mouse specific beta-actin was used for the internal control primer.

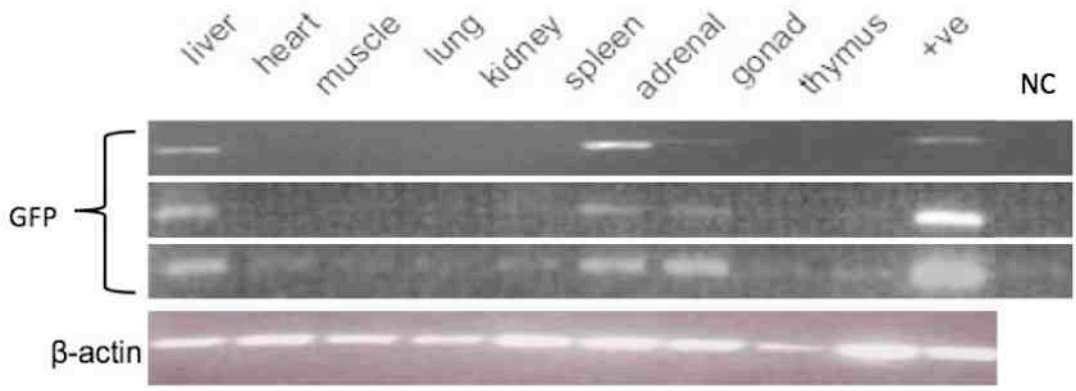


Figure 4.15 An example of a PCR gel for GFP DNA detection.
GFP: 306 base pairs; mouse beta-actin: 251 base pairs. Positive control: transduced GFP amniotic fluid cells. NC: negative control (water).

Table 4.8 summarises the findings from all animals analysed. GFP DNA was detected in the liver in 8 out of 9 transplanted NSG mice. There was no signal detectable in tissue samples from the heart, muscle, lung, kidney and gonad of transplanted NSG mice. Low levels of GFP DNA were detected in the spleen (5 out of 9 animals, 2 out of 3 fresh AF and 2 out of 3 bone marrow group), and the adrenal gland (2 out of 9 animals, 1 out of 3 fresh AF and 1 out of 3 bone marrow group). I could not detect any GFP DNA in another hematopoietic organ (thymus) among all three groups at 3 month after xenogeneic transplantation.

Table 4.8 PCR detection of GFP DNA in the tissues of NSG mice transplanted with GFP+ sheep CD34+ cells.
Eight out of 9 animals showed the positive GFP signal in the liver, while 5 out of 9 and 2 out of 9 were positive in the spleen and adrenal gland respectively.

Animal	CD34+ Cells	Liver	Heart	Muscle	Lung	Kidney	Spleen	Adrenal	Gonad	Thymus
NSG3	Frozen AF	+	-	-	-	-	-	-	-	-
NSG4	Frozen AF	+	-	-	-	-	+	-	-	-
NSG5	Frozen AF	-	-	-	-	-	-	-	-	-
NSG7	Fresh AF	+	-	-	-	-	+	+	-	-
NSG8	Fresh AF	+	-	-	-	-	+	-	-	-
NSG9	Fresh AF	+	-	-	-	-	-	-	-	-
NSG11	BM	+	-	-	-	-	+	+	-	-
NSG12	BM	+	-	-	-	-	-	-	-	-
NSG13	BM	+	-	-	-	-	+	-	-	-

In summary, the results of PCR demonstrated that the transplanted GFP transduced sheep CD34+ amniotic fluid or bone marrow cells spread to most of haematopoietic organs, especially the liver of NSG mice.

4.9 Sheep CD34+ AF or BM cells transduced with GFP could be detected in tissues by immunofluorescence

Previous results demonstrated the GFP+ cells could be found in the liver by flow cytometry and PCR studies. Here I would perform immunofluorescence to confirm the transplanted cells could engraft in the liver and other hematopoietic organs. The methods of preparing the frozen section of organs were described in Chapter 2. Rabbit anti-GFP antibody, goat anti-mouse alpha fetoprotein (AFP), goat anti-mouse albumin, biotin conjugated metallophilic macrophages antibody (MOMA) were used as primary antibodies for liver, adrenal gland and spleen staining respectively.
In Figure 4.16, both transplanted AF (upper panel) and bone marrow (lower panel) CD34+ cells mice showed the presence of GFP positive cells in the liver sections. The GFP+ cells in the liver were presenting round or spindle shape in morphology.

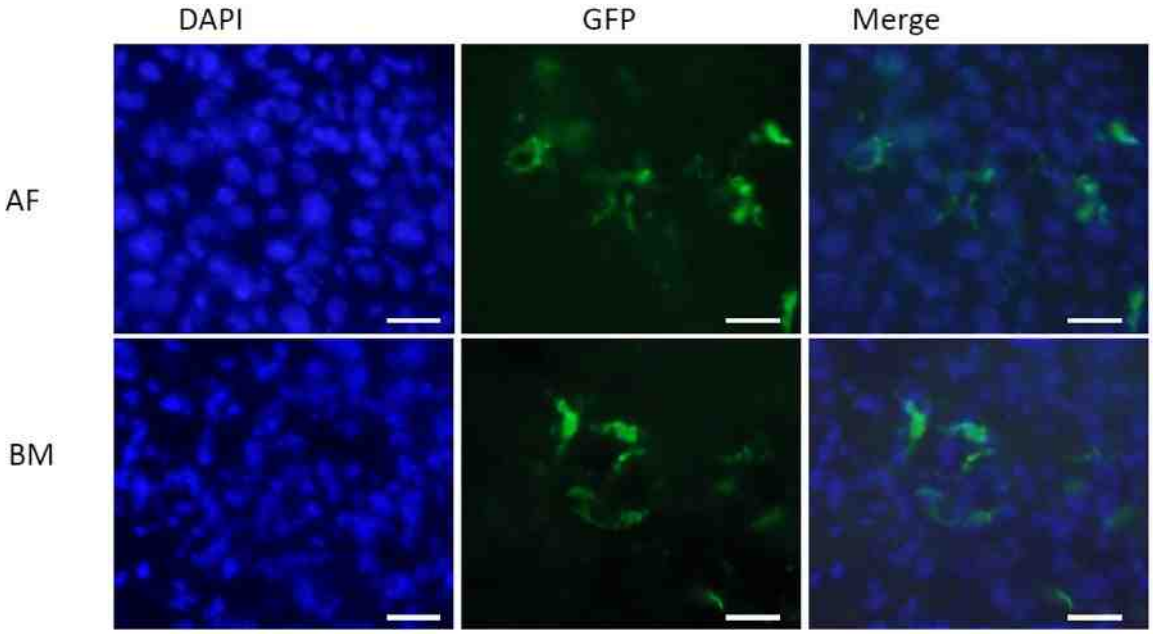


Figure 4.16 Immunofluorescence study on the liver sections of transplanted NSG mice.
In the upper panel, the group transplanted with sheep CD34+ amniotic fluid (AF) cells showed detectable positive GFP cells in the liver; as well as bone marrow (BM) group in the lower panel. DAPI was used for nucleus staining. Bar: 10μm.

The percentage of GFP+ cells in each high power field did not show a significant difference among all three groups (1.2% to 1.8%), although there was a tendency for a higher GFP+ve cell number in the BM group, compared to the fresh AF and frozen AF groups (Figure 4.17).

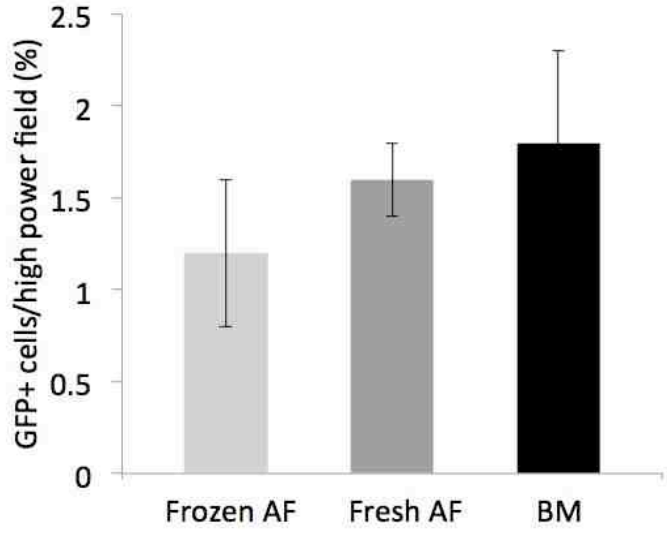


Figure 4.17 The percentage of GFP+ cells in each high power field was counted among frozen AF, fresh AF, and BM cells injected groups.
Counting was performed by three individuals who were blinded to the cell source. There was no statistically significant difference between the groups (ANOVA).

In order to determine if the GFP+ cells detected in the liver were truly differentiated into hepatocytes from the originally injected cells, I co-stained the slide with AFP. A few double stained AFP and GFP cells were detected in the liver sections, which showed evidence of hepatocyte differentiation (Figure 4.18, left panel). GFP positive stained cells were also seen in the adrenal gland and spleen (Figure 4.18, middle and right panels). For spleen analysis, MOMA was used for co-staining with GFP. MOMA is a useful marker for the identification of macrophage subpopulations in various organs, mostly characterized by a high level of non-specific esterase expression. Staining is particularly noteworthy with the metallophilic macrophages adjacent to the marginal zone of the spleen. In Figure 4.19, macrophages in the spleen showed red signal uptake, while GFP+ cells was not co-stained with MOMA suggesting that donor cells were present in the spleen, but they had not differentiated into macrophages.

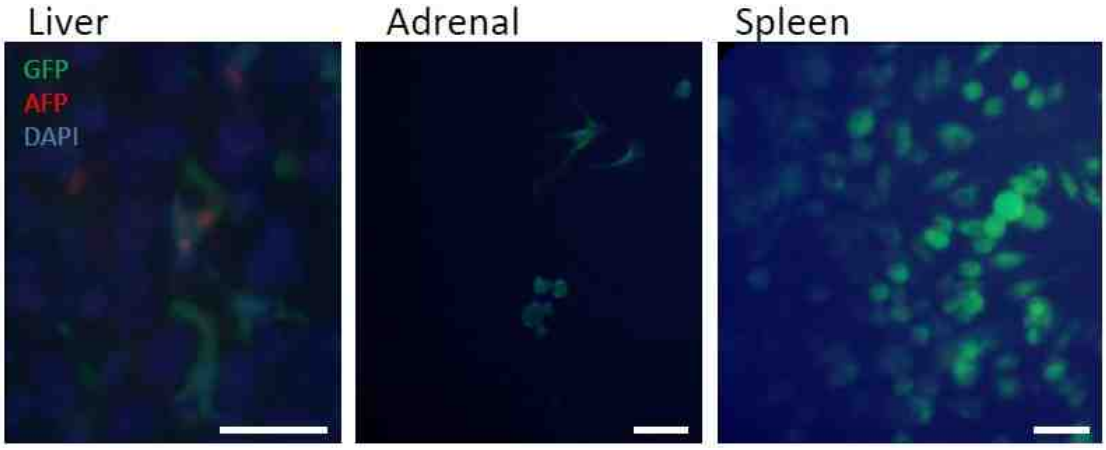


Figure 4.18 Immunofluorescence study on the liver, adrenal gland and spleen section of NSG mice transplanted with fresh amniotic fluid CD34+/GFP+ cells.
In the liver, a few cells co-stained with GFP and alpha-fetoprotein (AFP) were found. In the adrenal gland and spleen, the green positive cells could be also detected but there was no co-staining. Bar: 10µm.

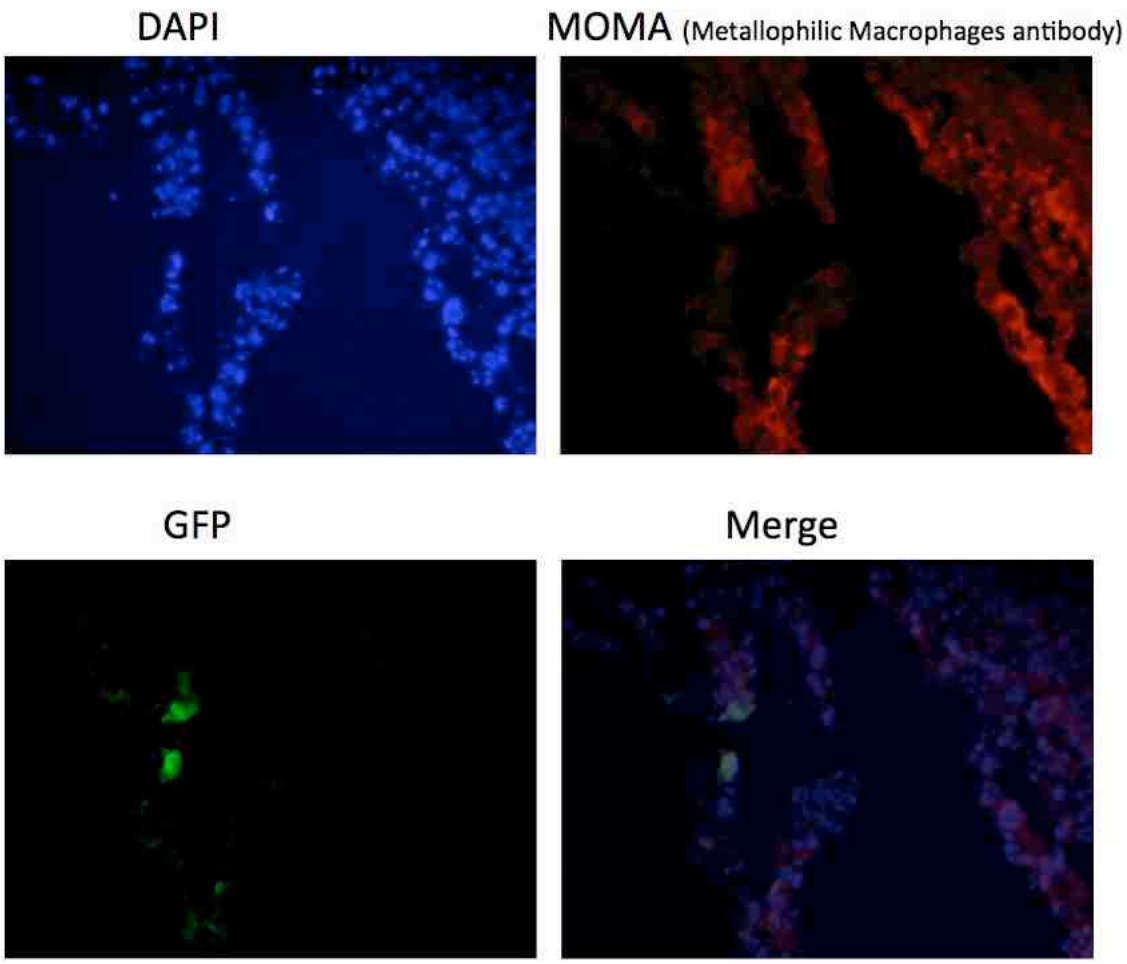


Figure 4.19 Immunofluorescence study in the spleen.
The section showed the metallophilic macrophages antibody (MOMA) staining the splenocyte in the sinusoid area of the spleen, which presented as red. The GFP+ cells were located outside of this area (merge image). Bar: 40μm.

To investigate more details of the cell structure from transplanted animals, confocal microscopy was arranged to study the positive slides from immunofluorescence staining. In Figure 4.20, GFP was expressed in the entire cytoplasm of the GFP+ cells in the liver. DAPI staining for the nucleus was presenting as blue dots. In Figure 4.21, the spleen section further confirmed the positive GFP signal located in the cytoplasm of the spleen cells. Interestingly, some macrophages which were slightly bigger than other cells in the spleen section showed co-staining with MOMA and GFP suggesting that these were donor cells (Figure 4.22).

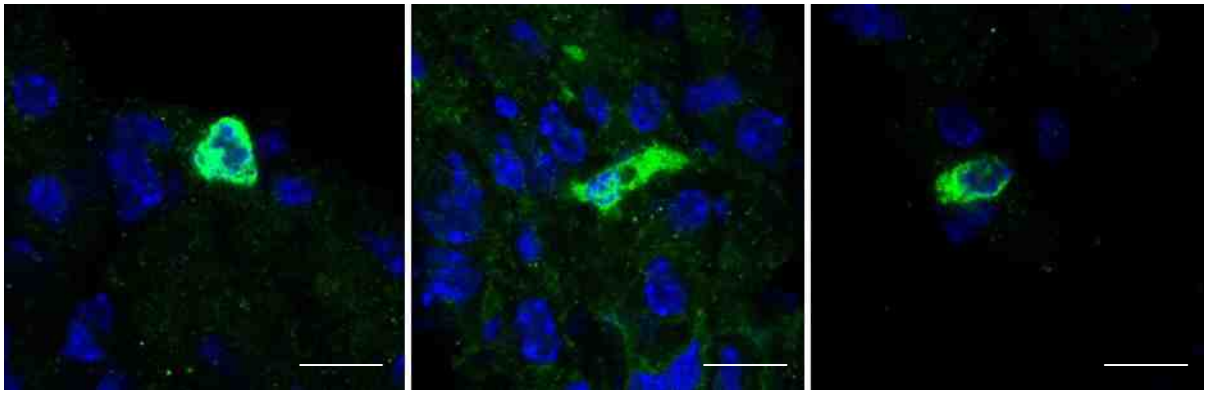


Figure 4.20 Confocal microscopy showed the GFP positive cells in the liver.
Green: GFP in the cytoplasm. Blue: DAPI for the nucleus. Bar: 10µm.

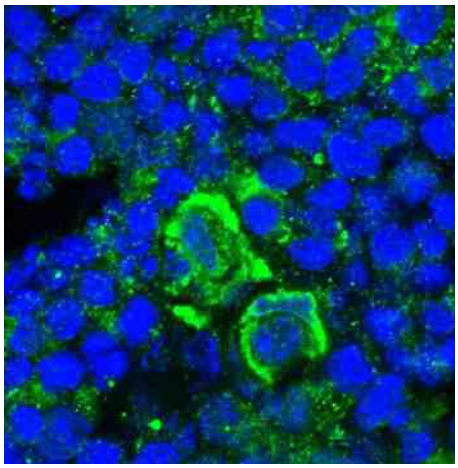


Figure 4.21 Confocal microscopy showed the positive GFP cells in the spleen.

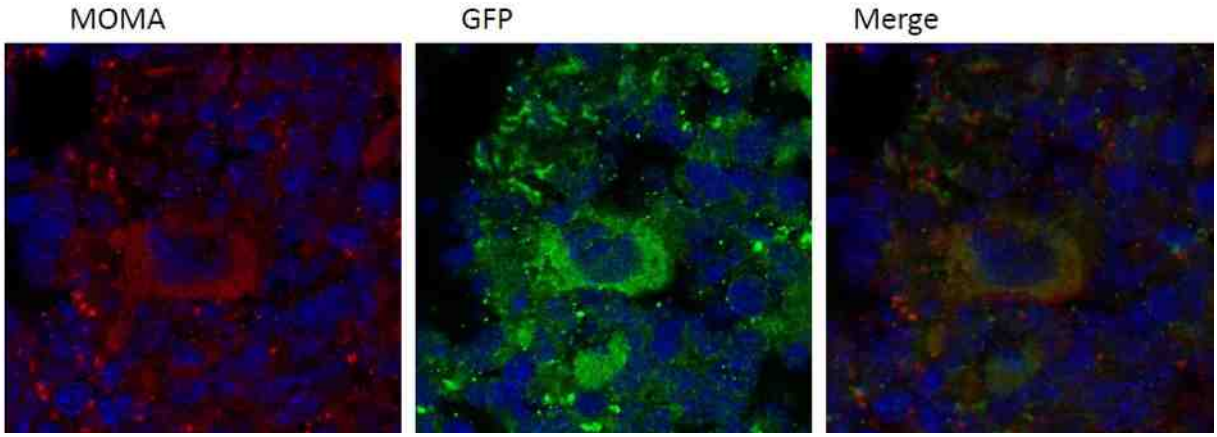


Figure 4.22 Confocal microscopy showed the transplanted donor cells in the spleen.
The spleen presented metallophilic macrophages antibody (MOMA) and GFP double positive cells.

In summary, flowcytometric analysis, PCR, and immunofluorescence showed quite similar results suggesting engraftment in haematopoietic organs. These primary transplanted animals demonstrated haematopoietic migration after transplantation of CD34+ cells (either from BM or AF). Some degree of differentiation of the cells to hepatocytes was also detected, however functional complete differentiation was not demonstrated and was not the aim of my work, which focused on the haematopoietic engraftment.

4.10 GFP+ cells could be detected in the peripheral blood and other hematopoietic organs 3 months after secondary transplantation into NSG mice

To study the nature of hematopoietic stem cells and hematopoietic function from the transplanted GFP+/sheep CD34+ cells from the primary transplanted NSG mice, I further performed secondary transplantation into NSG mice using the bone marrow cells from 3 primary recipients of each transplanted NSG group (frozen AF, fresh AF, and adult BM CD34+ cells) (Figure 4.23). Briefly, I sacrificed the primary transplanted animals to collect bone marrow cells from bilateral femoral bones of each mouse from frozen AF, fresh AF, and adult BM injection groups (n=3, per group). Bone marrow cells were flushed out of the femoral bones with PBS. All the bone marrow cells, without sorting from the primary recipients, were prepared for fresh injection into the secondary recipients (n=8).

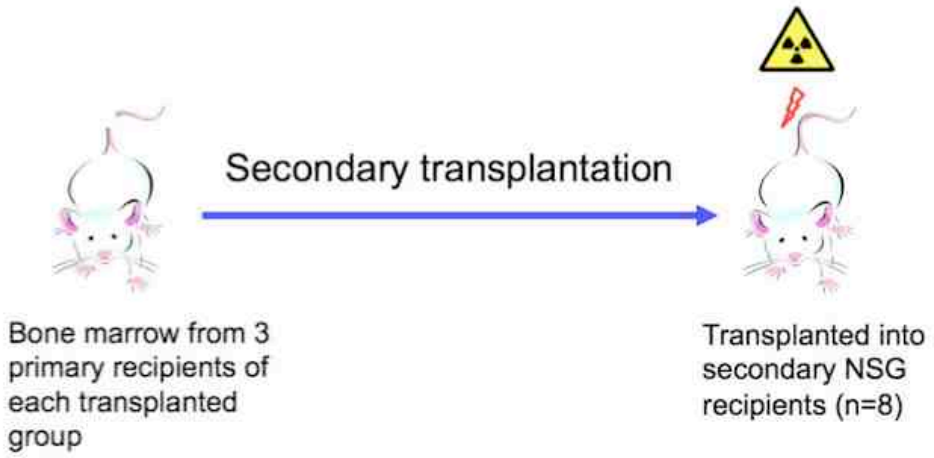


Figure 4.23 Experimental design of secondary NSG transplantation by using whole bone marrow cell suspension from primary NSG recipients.

As for primary transplantation, these mice were treated with sub-lethal dose of irradiation then injected with $3x10^6$ bone marrow cells from primary NSG transplanted mice. Eight NSG mice were injected via tail vein intravascular injection (NSG15 to NSG 22, Table 4.9).

Table 4.9 Summary of secondary transplantation using bone marrow cells from primary transplanted NSG mice.

Animal ID	Injected bone marrow cells	Number	Virus	Irradiation	Remark
NSG15	2nd transplant from frozen	$3X10^6$	-	300 Rads	Culled at 3 months
NSG16	2nd transplant from frozen	$3X10^6$	-	300 Rads	Culled at 3 months
NSG17	2nd transplant from frozen	$3X10^6$	-	300 Rads	Culled at 3 months
NSG18	2nd transplant from fresh	$3X10^6$	-	300 Rads	Culled at 3 months
NSG19	2nd transplant from fresh	$3X10^6$	-	300 Rads	Culled at 3 months
NSG20	2nd transplant from fresh	$3X10^6$	-	300 Rads	Culled at 3 months
NSG21	2nd transplant from BM	$3X10^6$	-	300 Rads	Culled at 3 months
NSG22	2nd transplant from BM	$3X10^6$	-	300 Rads	Culled at 3 months

The first three mice received BM cells from frozen AF group (NSG15-NSG17); three mice received BM cells from the fresh AF group (NSG18-NSG20) and two mice received BM cells from BM group (NSG21, NSG22). I considered only transplanting GFP+ cells after sorting but was concerned about the number of cells that might be lost. Therefore I pooled all cells for secondary transplantation with the aim of demonstrating that some GFP+ cells would engraft in them demonstrating that these cells were stem cells. All the secondary transplanted animals were sacrificed at 3 month after transplantation.

Peripheral blood was taken for analysis from secondary transplanted NSG mice at 1, 2, and 3 months after injection. Figure 4-23 showed that at 1 month post transplant, GFP+ cells could be detected in the peripheral blood of two out three animals in frozen AF group (NSG15 and NSG17, 67%); two out three animals in fresh AF group (NSG 18 and NSG 20, 67%) and all two transplanted from BM group (NSG21 and NSG 22). The level was higher if the cells were transplanted from bone marrow CD34+ cells injected animals (black line); compared with frozen AF (grey line) and fresh AF (black dash line) CD34+ cells (Figure 4.24).

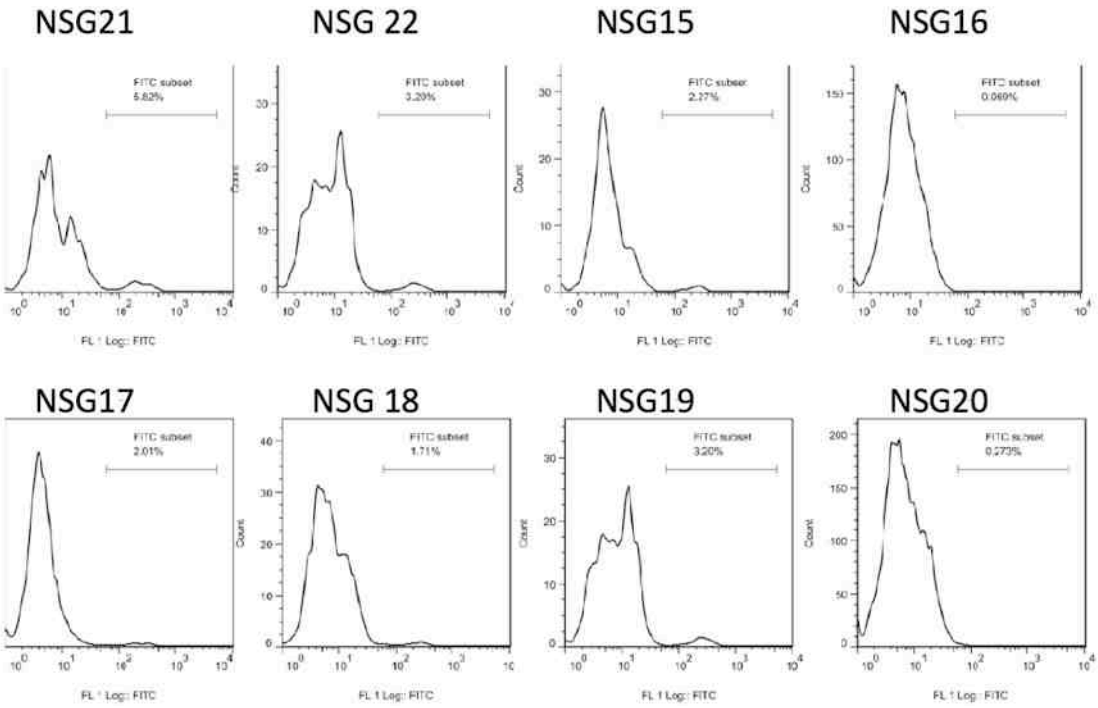


Figure 4.24 First month blood analysis of the secondary transplanted NSG mice.
The histogram showed the results from six animals transplanted with whole bone marrow suspension from NSG primary recipients transplanted with GFP+ sheep CD34+ cells from frozen and fresh AF and adult bone marrow sources. The small peak next to the main peak in the graph indicated the GFP+ cells in the peripheral blood of NSG 15, 17, 18, 19, 21, 22. The shoulders on the main peaks were heterogeneous of the cells because some of the samples were not washed with red blood cell lysis buffer completely. There were no GFP+ cells detected in the blood of two animals (NSG16 and NSG 20). X-axis: green positive cells in the FITC channel. Y-axis: cell counts.

Blood was collected at 2 and 3 months after transplantation, the results of which are shown below (Figure 4.25). Animals that were positive for GFP cells remained positive, but levels fell slightly overall in all groups. Levels were higher in the BM group compared with either AF groups at 1, 2, 3 months time points (p<0.05, ANOVA).

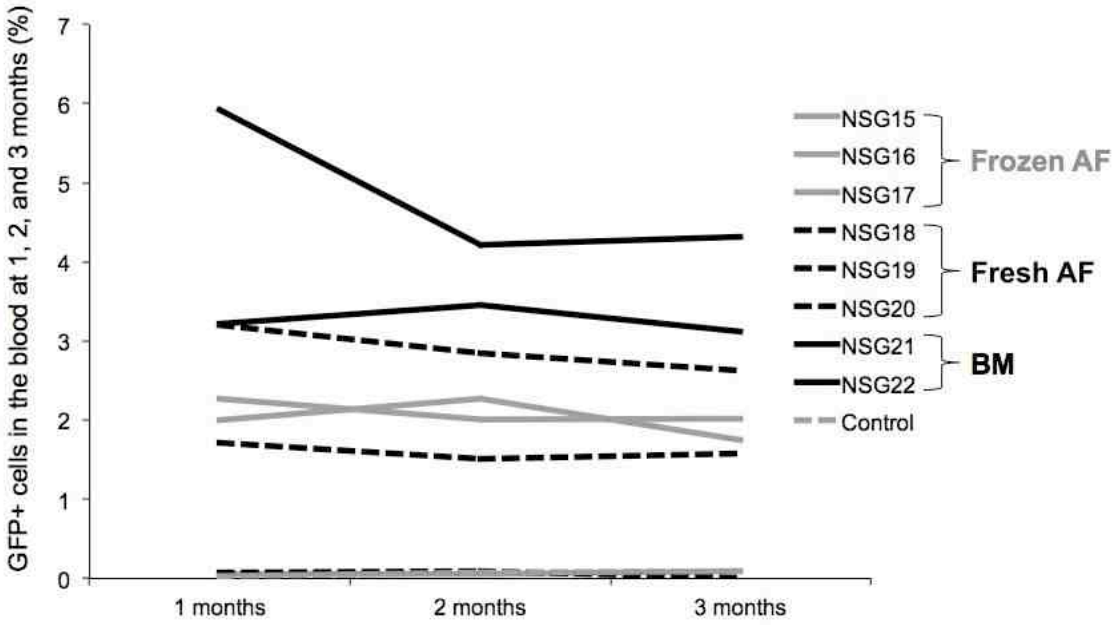


Figure 4.25 The blood analysis of secondary NSG transplantation at 1, 2, and 3 months. The injected BM cells group (NSG21, NSG22 as black line) showed higher level of GFP+ cells in the blood than the injected frozen AF (grey lines) and fresh AF (black dash line) groups. No cells were detected in the blood of two animals injected with frozen or fresh AF (NSG16 and NSG20). Control: uninjected animal (grey dash line); BM: bone marrow; AF: amniotic fluid.

At three months after transplantation animals underwent a scheduled post mortem examination. I analysed hematopoietic organs and could find the GFP+ cells in the liver, spleen, and bone marrow of the secondary transplanted animals that also showed GFP+ detectable cells in the peripheral blood. In those animals that had blood that was negative for GFP (NSG16 and NSG20), the other hematopoietic organs of these two animals were all negative for GFP. This suggested that blood results might be an indicator for haematopoietic system engraftment. There were no positive GFP cells detected in the thymus from all eight animals (Table 4.10).

Table 4.10 The percentage of GFP+ cells in the hematopoietic organs of the secondary transplanted mice.
The blood results were obtained at 1, 2, and 3 months (M). The other haematopoietic organs were analysed at 3 months after injection.

Cells injected	Animal	Blood 1M	Blood 2M	Blood 3M	Liver	Spleen	BM	Thymus
Frozen AF	NSG15	2.27	2.01	2.02	3.15	1.31	2.34	0.43
Frozen AF	NSG16	0.07	0.05	0.09	0.27	0.31	0.41	0.13
Frozen AF	NSG17	2.01	2.28	1.75	2.45	2.12	2.54	0.01
Fresh AF	NSG18	1.71	1.51	1.58	2.69	1.12	1.98	0.32
Fresh AF	NSG19	3.2	2.84	2.62	4.12	2.74	3.12	0.14
Fresh AF	NSG20	0.07	0.09	0.01	0.47	0.21	0.35	0.34
BM	NSG21	5.92	4.21	4.32	4.76	2.55	4.12	0.21
BM	NSG22	3.21	3.45	3.12	4.49	2.34	3.23	0.18
uninjected	Control	0.03	0.08	0.09	0.16	0.24	0.22	0.45

In summary, whole bone marrow cells from NSG mice that underwent primary transplantation with GFP+ sheep CD34+ AF or adult bone marrow, could be isolated and transplanted to secondary NSG mice recipients. The GFP+ cells could be found in the peripheral blood and other hematopoietic organs, that confirmed the evidence of engraftment from the primary injected GFP+/sheep CD34+ AF or BM cells and demonstrated their truly hematopoietic potential.

4.11 Prenatal autologous transplantation using transduced sheep CD34+ AFS cells

Following the results of primary and secondary transplantation using GFP transduced CD34+ sheep amniotic fluid or bone marrow cells, I conducted a study on prenatal autologous transplantation in sheep to determine the possibility that this technique could be applied in clinical practice for the treatment of hematopoietic disorders prenatally in the future. The basic concept was similar to the *in utero* autologous transplantation using mesenchymal stem cells derived from sheep amniotic fluid (Chapter 3). In this section, I instead studied freshly isolated CD34+ sheep amniotic fluid cells and hypothesized that these CD34+ stem cells could display better hematopoietic function and engraftment than mesenchymal stem cells.

4.11.1 Ultrasound-guided in utero transplantation with autologous CD34+ amniotic fluid stem cells is achievable

Five time-mated pregnant Ewes were enrolled in this study. All the animal procedures were performed in the Royal Veterinary College, Camden as described in Chapter 3 and method section in Chapter 2. Briefly, ultrasound-guided amniocentesis for collecting amniotic fluid was performed under general anaesthesia from 59 to 63 days (Term = 145 days). 20ml of clear fluid was withdrawn smoothly from each gestation sac. I sorted the cells using sheep specific CD34 antibody for positive selection. The CD34+ AFS cells were cultured in suspension HSC medium with conditioning cytokine. The same SFFV-eGFP viral vector was used for *in vitro* transduction (MOI=50) for 48 hours before transplantation back into the original donor fetus at 61 to 65 days of gestation. On the day of transplantation cells were washed, counted and resuspended in 1.5ml PBS. The ewe was anaesthetized as before, the abdomen prepared and freshly isolated CD34+ AFS cells ($2X10^4$ in 1.5ml PBS) were injected into the peritoneal cavity under ultrasound-guidance. The experimental design is demonstrated in Figure 4.26.

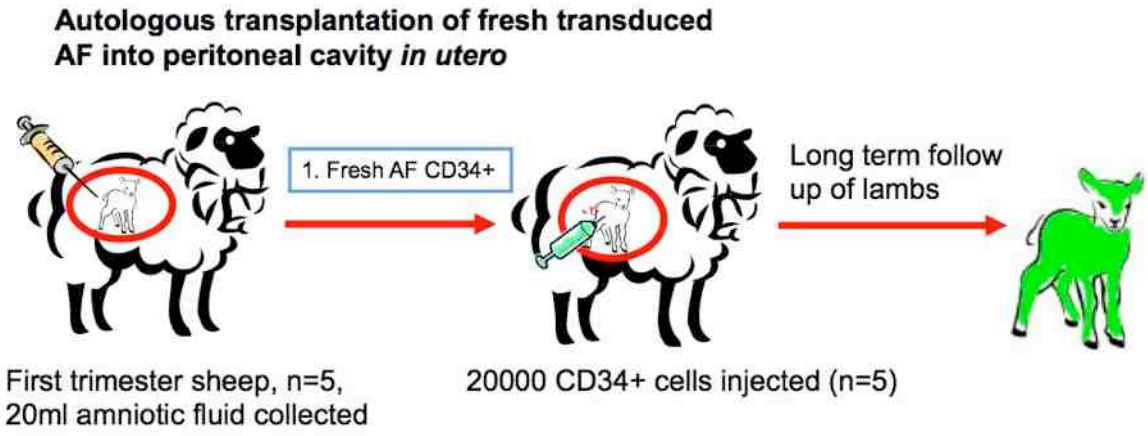


Figure 4.26 Experimental design of prenatal autologous transplantation study with sheep CD34+ AF cells.
The amniotic fluid stem cells were collected under ultrasound guidance, CD34+ cells were isolated, cultured and transduced with lentivirus encoding with eGFP for 2 days. Then 20000 CD34+ AF cells were transplanted back into the peritoneal cavity of the same fetus via ultrasound-guided technique. The lambs underwent a scheduled post mortem examination at 6 months of age for long-term follow-up.

They were all twin pregnancies. The first two injections, ewe X14 was not successful with fetal loss in one fetus 10 days after injection. At post mortem examination haemorrhage was noted in one fetus (Table 4-11). There was intrauterine fetal death of both twins from ewe X15 and preterm delivery at day 126. Maternal endometritis was confirmed by Gram-negative bacteria found in the culture of endometrium. Both fetuses had a slowing of growth velocity approximately 25 days before death (see section below) but no cause for death was found, and there were no structural fetal malformations at post mortem examination.

Table 4.11 The summary of the prenatal autologous transplanted sheep.
X16A, X17B, and X18A in bold indicate those lambs that survived long term.

Ewes	Lambs	Amniocentesis (Days)	Injection (Days)	Birth (Days)	Remark
X14	X14A	59	61	-	No fetal heart beat noted 1 week after injection
	X14B	59	61	-	
X15A	X15A	60	62	126	Two abortus found at Day 126 due to endometrial infection
	X15B				
X16	**X16A**	60	62	145	X16A doing well, X16B died in utero around 90 days
	X16B				
X17	X17A	63	65	145	X17A was culled one day after birth due to dehydration
	X17B				
X18	**X18A**	63	65	145	X18B was culled one day after birth due to dehydration
	X18B				

In one further ewe (X16) one fetus (X16B) was found to have died *in utero* at approximately 90 days of gestation. Five lambs were delivered at term. Initial assessments had been done as soon as the lambs were born at RVC and included birth weight, gender, time to standing, girth at umbilicus, biparietal diameter, height to shoulder, heart rate, respiratory rate, O2 saturation and temperature which were recorded by the staff in RVC or myself.

In each of the born twin pairs, one twin lamb was rejected by the ewe (X17A and X18B) and both lambs became dehydrated within 24 hours of birth. One ewe had obvious mastitis, but the other ewe did not. Despite artificial feeding, the lambs' condition deteriorated and a scheduled post mortem examination was performed two days after birth on the advice of the veterinary surgeons (Table 4.11). There was no evidence of structural abnormality or infection at post mortem examination of these lambs. The remaining three lambs (X16A, X17B, and X18A) did well and survived to scheduled post mortem examination at 6 months of age.

4.11.2 The growth of the sheep fetuses could be regularly monitored by ultrasound

To understand the *in utero* wellbeing of the fetal sheep after injection, I scanned the fetuses by using the ultrasound machine as described in Chapter 3. In addition, the intrauterine episodes including fetal death or growth restriction or hemorrhage could be detected easily by ultrasound.

The ultrasound routine scan for the fetal biometry was carried out approximately every 20 days after transplantation. The data was compared with the study published by Barbera A et al (Barbera et al., 1995a). Basically, occipital snout length (OSL), biparietal diameter (BPD), femur length (FL), and abdominal circumference (AC) were recorded by using sonographic measurement. Fetal growth was normal in all cases without any delay or intrauterine growth retardation except X15A and X15B. These twins showed slow growth around 100 days, and then were found to have died *in utero* at 126 days. Uninjected pregnant sheep was used as the control (Table 4.12 and Figure 4.27).

Table 4.12 Summary of the fetal sheep biometry.
This included occipital snout length (OSL), biparietal diameter (BPD), Femur length (FL), and abdominal circumference (AC) which were measured at 60, 80, 100, 120, and 140 days. X15A and X15B showed slow growth at 100 days, and then ended up with abortion at 126 days. *:first measurement around 60 days before amniocentesis.

OSL (cm)	60 d*	80 d	100 d	120 d	140 d	**BPD** (cm)	60 days*	80 days	100 days	120 days	140 days
X14A	42					X14A	31				
X14B	44					X14B	32				
X15A	44	62	98	105		X15A	33	44	56	60	
X15B	46	64	99	107		X15B	32	43	56	58	
X16A	43	65	100	120	132	X16A	31	46	59	65	76
X17A	45	60	95	121	135	X17A	32	45	58	67	76
X17B	45	59	103	119	136	X17B	30	47	54	64	75
X18A	44	58	96	118	130	X18A	30	42	53	68	75
X18B	43	60	96	119	133	X18B	29	44	54	66	73
control	45	61	98	116	132	control	30	44	58	66	74
FL (cm)	60 d*	80 days	100 days	120 days	140 days	**AC** (cm)	60 days*	80 days	100 days	120 days	140 days
X14A	13					X14A	150				
X14B	13					X14B	154				
X15A	12	22	35	55		X15A	152	200	244	288	
X15B	14	21	34	56		X15B	153	198	246	280	
X16A	14	22	34	63	81	X16A	155	199	251	299	368
X17A	15	20	31	62	81	X17A	158	205	252	302	367
X17B	13	23	33	66	83	X17B	150	195	241	304	378
X18A	13	19	36	65	80	X18A	149	206	246	298	381
X18B	15	22	37	60	77	X18B	155	199	244	300	369
control	13	21	36	63	80	control	152	201	243	302	378

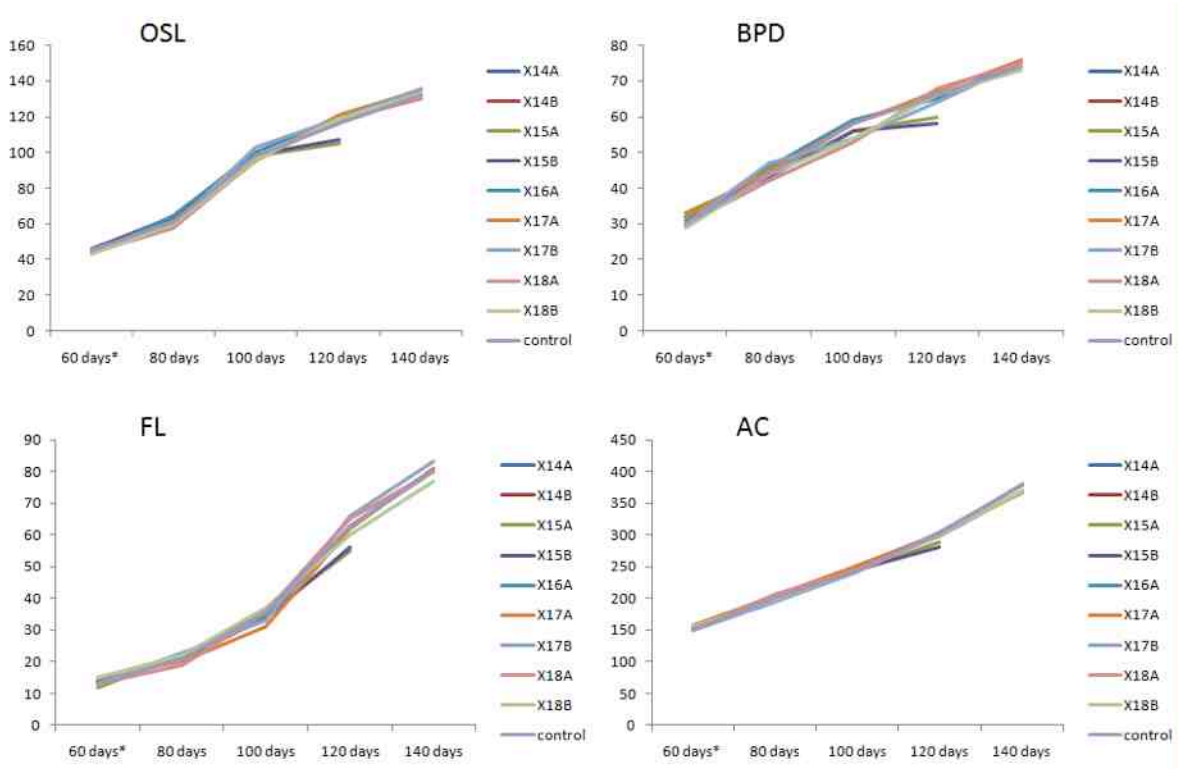


Figure 4.27 Fetal sheep growth for all parameters including occipital snout length (OSL), biparietal diameter (BPD), Femur length (FL), and abdominal circumference (AC). Uninjected animal was for negative control.

4.11.3 Surviving lambs show normal growth velocity after birth up to 6 months old

In order to monitor and understand the growth condition of the surviving lambs, I measured their weight, shoulder height and umbilical girth regularly. The postnatal growth curve of the three surviving lambs Table 4.14 and Figure 4.28 showed. All of them demonstrated a normal growing condition with good feeding. The activities were normal till the last day of the experiment. I sacrificed these lambs at 6 month-old, 9 months after prenatal autologous sheep CD34+ amniotic fluid stem cells transplantation.

Table 4.13 The summary for the postnatal growth of three lambs up to 6 months before post-mortem.
Body weight, shoulder height, and umbilical girth were recorded monthly.

X16A	At birth	1 month	2 months	3 months	4 months	5 months	6 months
Weight (kg)	4.7	16	26	37	41.5	43	45
Shoulder height (cm)	36	47	55	60	63	65	67
Umbilical girth (cm)	38	65	83	98	105	107	112
X17B							
Weight (kg)	4.5	11	25	35	42	44	46
Shoulder height (cm)	36	48.9	51	56	58	59	62
Umbilical girth (cm)	38.2	58	82	97	106	107	111
X18A							
Weight (kg)	5.8	16.5	25	26	31	36	40
Shoulder height (cm)	36	49	50	55	58	59	60
Umbilical girth (cm)	44.5	61	79	86	89	99	102

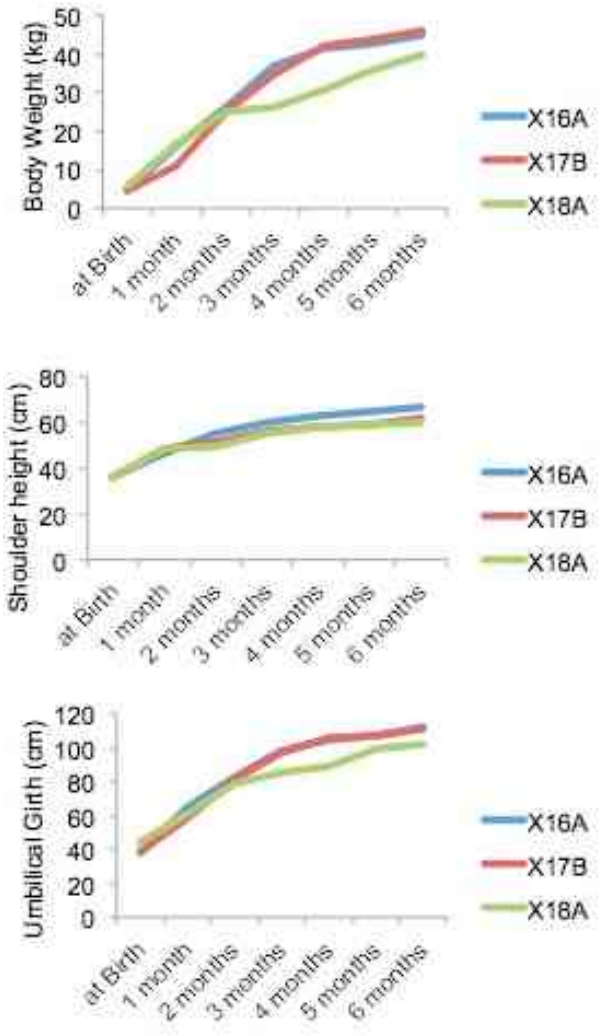


Figure 4.28 Growth curve after birth showing in body weight, shoulder height and umbilical girth.

In summary, survival to birth of lambs that received autologous injection of transduced CD34+ AF stem cells was acceptable. The fetal growth could be monitored by regular ultrasound exam and appeared to be normal in those lambs that were born alive. Three lambs survived long term to 6 months of age without any apparent abnormalities.

4.11.4 GFP+ cells could be detected in the peripheral blood of prenatal autologous transplanted lambs up to 6 months

To investigate the migration or the engraftment of the GFP+ cells in the hematopoietic organs of the transplanted lambs, I took peripheral blood regularly up to 6 months and other hematopoietic organs including liver, bone marrow, and spleen at post-mortem. The flow cytometry was performed for analysis of all these samples.. GFP+ cells were detected in the blood from all 5 lambs at term (1.6% to 4.5%, Table 4.14 and Figure 4.29). Blood from uninjected sheep was used as negative control. For the three long-term lambs, the blood was positive for GFP up to 6 months of age (X16A, X17B, and X18A) although the Figure 4.29 only showed the data till 16 weeks. The strength of the GFP+ signal was starting to decrease after 4 weeks of age (4 months after injection) but kept steady around 2% of GFP+ cells in the blood. The three ewes (X16, X17 and X18) were negative for GFP signal as well as the uninjected control.

For other haematopoietic organs, I analysed the samples taken from two lambs necropsied 48 hours after birth due to neonatal dehydration as described before. In table 4.14, the spleen and thymus were negative for GFP+ cells engraftment in both lambs (X17A, and X18B), but the liver and bone marrow were slightly positive in flow cytometric study (2.9%, 4.3%, respectively in the liver; and 2.2%, 2.5% in bone marrow).

Table 4.14 Summary of the blood engraftment up to 8 weeks and analysis of haematopoietic organs in those animals necropsied at birth.
Uninjected sheep was used as negative control.
IUT: in utero transplantation; BM: bone marrow; w: weeks after birth.

	Blood (%)						Term analysis (%)			
Lambs	before IUT	Day 0	1 w	2 w	4 w	8 w	Liver	Spleen	BM	Thymus
X16A		3.1	3.3	3.4	2.8	2.4				
X17A		1.6					2.9	0.1	2.2	0.2
X17B		2.3	2.5	2.3	2.1	2.2				
X18A		2.1	2.2	2.1	1.9	1.7				
X18B		4.5					4.3	0.2	2.5	0.3
X16	0.3	0.1			0.2	0.3				
X17	0.1	0.1			0.4	0.4				
X18	0.2	0.3					0.1	0.1	0.2	0.1
Control						0.4	0.2	0.2	0.1	0.3

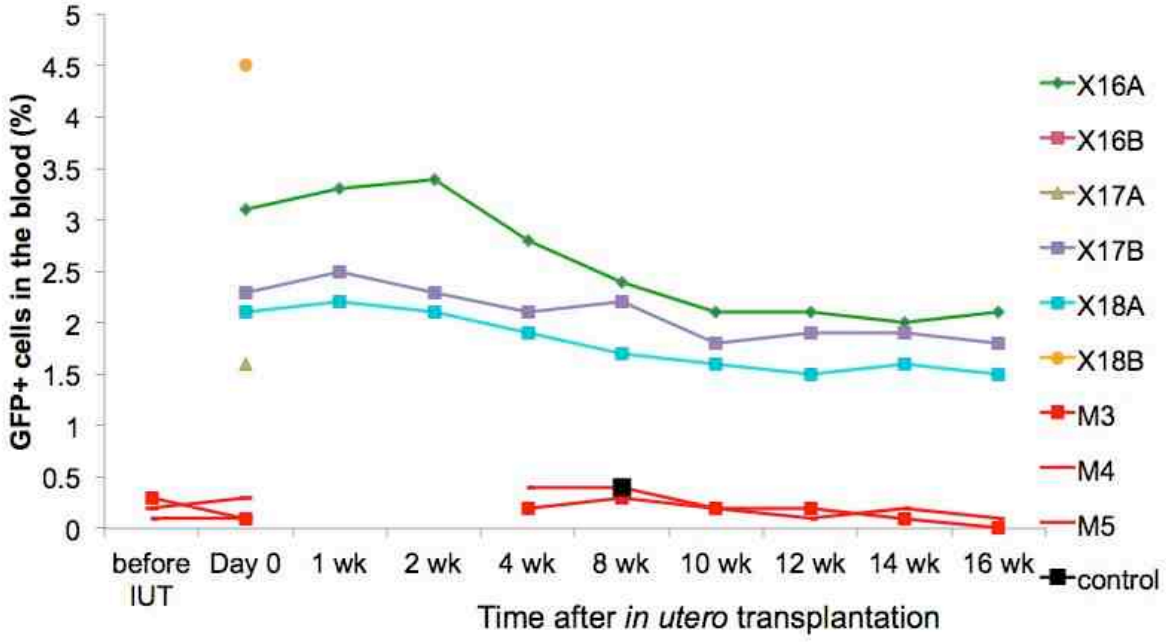


Figure 4.29 The peripheral blood engraftment after prenatal transplantation of autologous sheep CD34+ amniotic fluid stem cells.
All five born lambs showed positive GFP cells in the peripheral blood at birth, and all three survivors revealed persistent level around 2% of engraftment in the peripheral blood up to 16 weeks. Uninjected sheep was used as the negative control.

In summary, GFP+ cells could be found in the peripheral blood of prenatal autologous transplanted lambs using CD34+ amniotic fluid cells up to 6 months of age, and nine months after *in utero* transplantation. The hematopoietic organs including the liver and bone marrow were positive for GFP cells in animals analysed at birth and at 6 months after birth. Sheep CD34+ amniotic fluid cells could be isolated freshly and displayed the hematopoietic potential.

Discussion

This chapter showed the capability of isolation the CD34+ cells from sheep amniotic fluid, and further from bone marrow as well. There is no Ckit antibody available that could be used in sheep. Therefore we chose this novel sheep specific antibody provided kindly from Dr. Poroda to test out the function and cell characterization. From the positive fraction of CD34 cells, they also presented the positive CD45 cells, but negative for CD31, CD14, CD44, CD58. This population of CD34+ cells were therefore quite different from the mesenchymal lineage studied in Chapter 3. I tested the haematopoietic potential of these cells by using flow cytometry, PCR, and immunostaining and proved the engraftment in most of the blood producing organs of the injected NSG mice.

After primary transplantation, we further performed secondary transplantation using the whole bone marrow cells collected from the primary injected animals in each group. I found about half level of the engraftment in blood, liver and bone marrow of NSG mice when comparing to primary transplantation, but negative for spleen and thymus. Due to the low number of animals, I used ANOVA to do the statistics on this experiment. But the tendency of the blood engraftment looked like presenting higher GFP level in bone marrow group rather than frozen or fresh AF injected groups.

Finally, I performed autologous prenatal transplantation in fetal sheep as described in Chapter 3, but using sheep CD34+ AF cells. I cultured these cells as haematopoietic stem cells in suspension then transduced them overnight with the same lentivial vector, which drove roughly the same transducation efficiency about 60%. This was not expected since other studies have shown a lower rate of gene transfer, for example in human bone marrow-derived MSC, embryonic stem cell, and HSC of 40% (Zhang

et al., 2004), 14-48% (Gharwan et al., 2007), and 50% (Sutton et al., 1998), respectively. I followed the blood engraftment until 6 months of age equivalent to 9 months after transplantation. Haematopoietic organs were also analysed in two lambs at birth and 3 lambs at 6 months of age. The data showed quite stable low level GFP+ cell engraftment in the blood, the liver and bone marrow but none in the spleen and thymus.

I will focus on immunological response or antibodies produced by the mother to injected cells in the future work. T cells response to GFP would be an important issue to find out the truly reaction from the ewes or lambs. Furthermore, to understand if the engrafted cells have the potential of clonal expansion, we will need to investigate the integrate site of the vector in injected lambs. Finally, I would like to look the large animal model of haematological disease in sheep, and to see if we could cure or improve the phenotype of affected foetuses prenatally. There is a sheep haemophilia model published by Porada at al. and we were starting to contact this collaborator to find out and set up the experiment (Porada et al., 2010b). The idea would be taking amniotic fluid as our description and transducing with therapeutic vector containing FVIII in vitro. Then we could transplant these genetic modified amniotic fluid CD34+ stem cells back into the affected donor foetus.

With the above future plans, there will be lots of hard work need to be carried on in this field. AF derived stem cells are an important source of autologous cells that could have a prenatal therapeutic value in cell therapy or cell based gene therapy in the future.

Chapter 5

Mouse amniotic fluid stem cells can engraft after *in utero* transplantation

5 Chapter 5: Mouse amniotic fluid stem cells can engraft after in utero transplantation

Introduction

I previously showed that transduced AFMSCs and CD34+ amniotic fluid cells could engraft after autologous transplantation in fetal sheep sheep. However, there are few single gene disorders available in the sheep, which also represent a very expensive animal model in which fine mechanisms and pathways are difficult to elucidate. In order to investigate the therapeutic effect of the amniotic fluid stem cells for prenatal transplantation, I therefore used as animal model the mouse.

In humans, successful In utero transplantation (IUT) has so far been limited to fetuses with severe immunologic defects, where there is a survival advantage for donor cells (Shaw et al., 2011b). But the human fetus can mount an alloresponse as early as the second trimester and allogeneic cells injected at that time engraft poorly. The presence of a functionally developed immune system at such an early stage of pregnancy could be overcome by the use of autologous cells. In mice, previous studies had shown that prenatal transplantation was superior when congenic bone marrow HSC injection were used instead of cells of allogeneic origin (Peranteau et al., 2007). In particular this difference started to be noticed when mice were one month old.

Autologous progenitors can be easily derived from amniotic fluid with minimal risk for both the fetus and the mother (De Coppi et al., 2007a). Transplantation of genetically corrected autologous or congenic AFSCs into a fetus could be an alternative therapeutic strategy for the treatment of congenital disease in human or mice. In some conditions IUT may be more effective than postnatal therapy, because the children are already severely affected at birth. For parents faced with the difficult situation of a fetus with a congenital disease, a therapeutic prenatal option would be of great value. This proposal will investigate the potential for combining in utero stem cell transplantation and gene therapy into one therapeutic strategy in a mouse model of human disease.

In this chapter, I investigated the hematopoietic potential of mouse amniotic fluid stem cells in prenatal cell therapy or cell-based gene therapy. Both congenic and allogeneic transplantation models were studied and compared using wild type mice.

Finally, I tested out the therapeutic effect of prenatal amniotic fluid stem cell transplantation in the *th3* mouse model of thalassaemia, to determine whether there might be any therapeutic benefit of *in utero* transplantation with AFS cells.

5.1 Cell therapy model: A pilot study of prenatal allogeneic transplantation using AFS cells of YFP transgenic mice to MF1 mice

To prove the possibility of mice AFS cells could be applied as a cell therapy source in prenatal transplantation, I first aimed to demonstrate transplantation and engraftment evidence in a wild type mouse model. Therefore, I conducted experiments to examine isolation and culture of amniotic fluid cells from pregnant mice, to inject these cells into the fetal mice and to examine for evidence of engraftment after prenatal allogeneic transplantation.

5.1.1 Amniotic fluid stem cells can be freshly isolated from YFP mice and injected prenatally into wild type MF1 mice (allogeneic in utero transplantation)

The first step for this prenatal transplantation experiment was to set up the time-mated mice. I designed the experiment as shown in Figure 5.1. Two YFP and two MF1 female mice were mated on the same day to respective YFP and MF1 males. Then the vaginal plugs were checked the following morning to confirm successful mating. Abdominal palpation at day E12 was used to confirm pregnancy. On day E14 amniotic fluid was collected from the two pregnant YFP transgenic mice as described in the methods section.

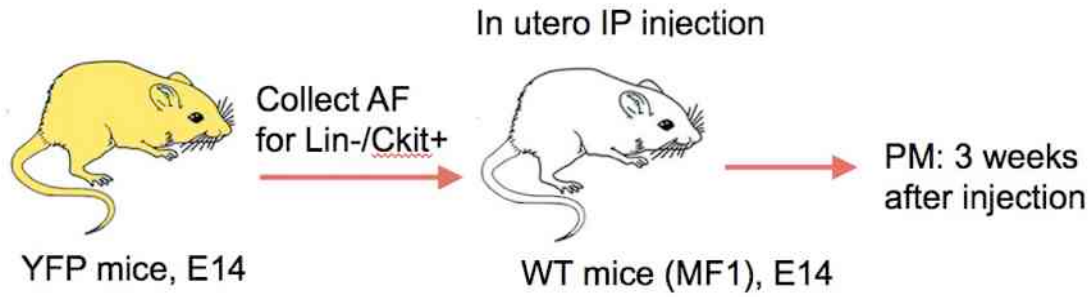


Figure 5.1 The experimental design of allogeneic AFS cell *in utero* transplantation.
YFP transgenic mice were sacrificed for amniotic fluid collection and stem cell isolation at E14. These cells were injected on the same day into the peritoneal cavity of fetal MF1 mice at E14. Post-mortem examination was carried out 3 weeks after injection. WT: wild type; PM: post-mortem.

After fresh collection of the fluid, AFS cells were firstly isolated with mouse Lineage depletion kits for negative selection, then with mouse Ckit microbeads for positive selection. The isolated Ckit+Lin- AFS cells were harvested under HSC conditioning medium and prepared for transplantation straightaway. I collected 100000 Lin-/Ckit+ fresh AFS cells from two YFP transgenic dams with a total 15 pups (8 for the first dam and 9 for the second one) at E14. The percentage of Lin-/Ckit+ cells in both animals was 1.5% of total pooled cells (Table 5.1).

Table 5.1 Characterisation of AFS cells isolated from two YFP transgenic mice at E14. Of the total cells, Lin- cells represented between 3.3 to 4.2% of the total cells, while only 1.5% were Lin-/Ckit+ cells. GA: gestational age

Animal	GA	Pups	Total cells	Lin- cells (number/%)	Lin-/Ckit+ cells (number/%)
YFP1	E14	8	$3.0X10^6$	$1.0X10^5$ (3.3%)	$4.5X10^4$ (1.5%)
YFP2	E14	9	$3.6X10^6$	$1.5X10^5$ (4.2%)	$5.5X10^4$ (1.5%)
Total		17	$6.6X10^6$	$2.5X10^5$ (3.8%)	$1.0X10^5$ (1.5%)

Then Lin-/Ckit+/YFP+ mouse AFS cells (10000 cells in 20µ PBS) were injected intraperitoneally into each MF1 fetal mouse (n=9) at E14. Dr Simon Waddington performed these injections since at the time I was learning the technique. Four pups of the first MF1 dam and 5 pups of the second MF1 dam were injected smoothly. Dams delivered pups (n=19) normally at E20. Pup survival after birth was 68 % (13 out of 19 born). All pups were culled for the analysis 3 weeks after birth using the post-mortem protocol as described in the index.

5.1.2 AFS cells maintained a high purity of Ckit+ population after magnetic sorting

In order to prove the injected amniotic fluid stem cells were Ckit+, I examined the purity of Ckit+ and Ckit- populations after magnetic sorting using the cells surplus to *in utero* injection 24 hours after *in utero* transplantation (90000 cells used for injection with 10000 cells left).

The cells were cultured for 24 hours in HSC conditioning medium with all essential cytokines in the 24 well flat-bottom ultra-low attachment plates as described. Anti-mouse CD117 antibody conjugated with PE was used for cell selection. Both positive and negative population of cells were incubated with this antibody following by

standard protocol described in the methods section. I performed flow cytometry to detect the percentage of Ckit+ marked cells in these two groups of cells.
Nearly all (99.9%) Ckit- cells were negative for Ckit-PE antibody, which meant the Ckit- cells after magnetic sorting was truly negative for Ckit (Figure 5.2). There was a 97.3% Ckit+ rate in the PE channel (Figure 5.2), thus the cells I injected were able to maintain Ckit+ cell markers after 24 hours culturing. Furthermore, the magnetic microbead conjugated CD117 antibody could be used for cell isolation.

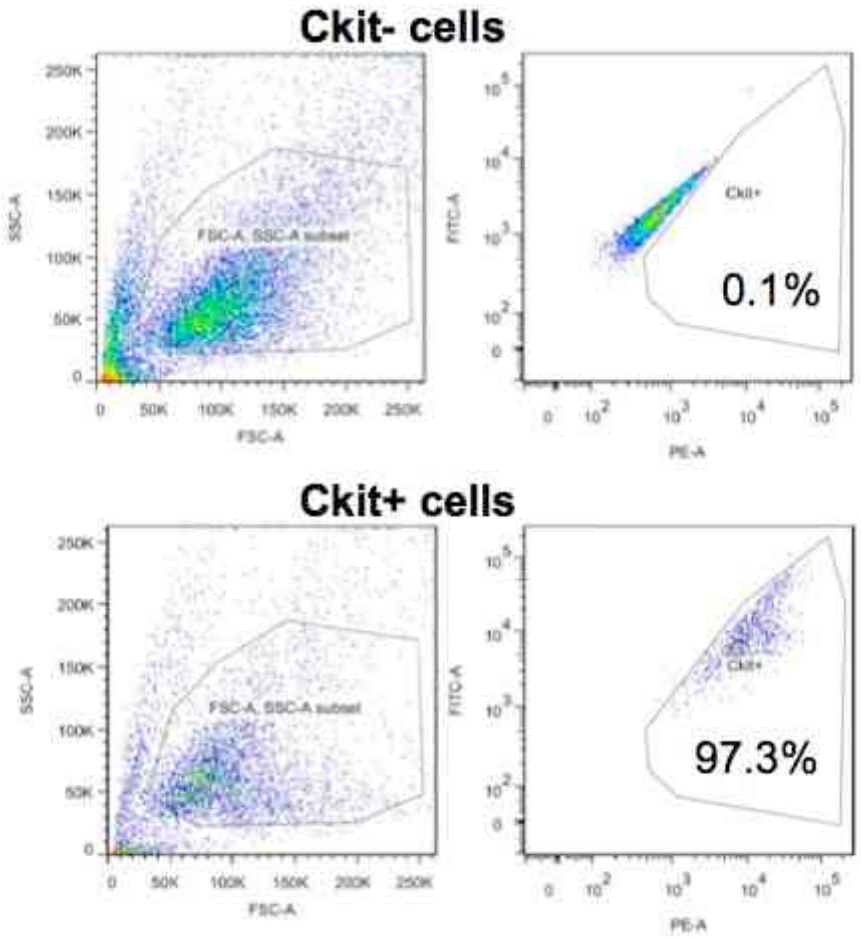


Figure 5.2 The purity of the magnetic sorted Ckit+ and Ckit- cells.
Amniotic fluid cells were gated first in the left graph of each cell type, then the triangle area in the right graph was gated for PE stained Ckit+ cells. Upper panel (sorted Ckit- cells) showed nearly all the cells were negative for Ckit, while lower panel (sorted Ckit+ cells) showed 97.3% were Ckit+.

In summary, magnetic sorting demonstrated that after 24 hours of culture, the sorted mouse AFS cells could maintain a very high Ckit+ rate, similar to findings in sheep AFS cells after CD34 selection.

5.1.3 The AFS cells collected from YFP transgenic mice demonstrate YFP positive signal in PCR and flow cytometry analysis

To prove that the amniotic fluid stem cells isolated from YFP transgenic mice were truly YFP+ cells, I performed PCR to demonstrate YFP DNA in the cells, and flow cytometry analysis to confirm these cells expressed YFP signal.
Genomic DNA was extracted from YFP+ AFS cells using a standard kit. For a positive control I used DNA extracted from the liver of YFP transgenic mouse. DNA extracted from AFS cells of wild type MF1 mice was used for the negative control. The AFS cells from YFP+ mice showed the same sized band of 307 base pairs as the positive control (Figure 5.3).

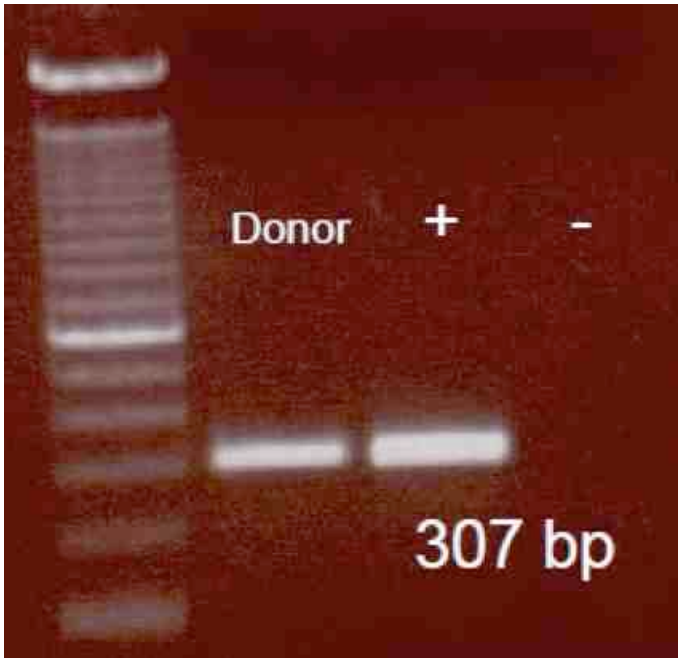


Figure 5.3 PCR gel to confirm the presence of DNA for the YFP gene in AFS cells from YFP transgenic mice. DNA from YFP AFS cells and from wild type AFS cells transduced with an SFFV eGFP lentivirus vector (+ve control) was examined in a PCR reaction for the presence of YFP DNA. A 307 bp band was demonstrated in both types of cells. DNA from wild type AFS cells was used for the negative control. Ladder size: 100bp.

Furthermore, the flow cytometric study showed that 95.6% of YFP AFS cells were positive in the FITC channel for YFP expression compared to 0.2% of wild type AFS cells (Figure 5.4). All the cells were naturally isolated and underwent flow cytometry analysis directly without viral transduction.

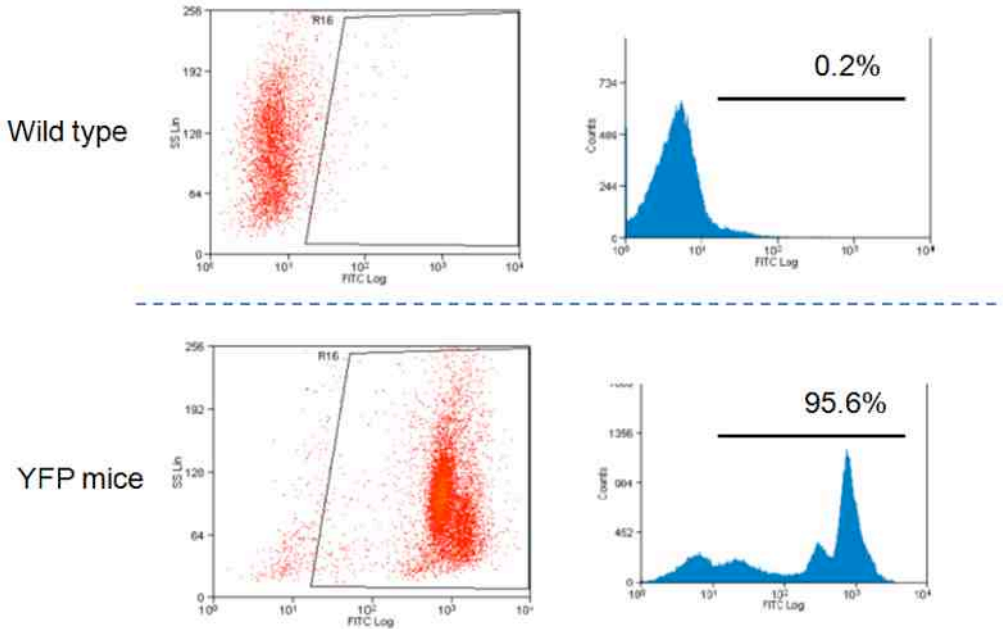


Figure 5.4 Flow cytometry to detect YFP expression.
YFP expression is seen in 95.6% of AFS cells obtained from YFP mice but in only 0.2% of AFS cells from wild type mice.

In summary, the isolated Lin-/Ckit+/YFP+ amniotic fluid cells presented the strong YFP signal that could be detected by PCR and flow cytometry. In this cell therapy model with prenatal allogeneic transplantation, amniotic fluid from YFP transgenic mice could be a good source of donor stem cells.

5.1.4 Prenatal transplantation of allogeneic YFP+/Ckit+/Lin-AFS cells by intraperitoneal injection in wild type mice results in detection of transplanted cells mainly in haematopoietic organs at three weeks after birth

In order to understand the migration and behaviour of transplanted AFS cells in wild type mouse recipients, I performed studies to trace IP injected YFP+/Lin-/cKit+ AFS cells 3 weeks after birth by PCR and flow cytometry.

Briefly, pup survival after injection to birth and up to the time of scheduled post mortem examination at 3 weeks of age was 100%. Nine injected mice from two litters were analysed using PCR with YFP specific primers and by using the positive gated FITC channel in flow cytometry analysis. Two dams were sacrificed in addition to determine the possibility of feto-maternal trafficking. Four non-injected aged-matched mice were used as negative control. All the animals were culled for the

comprehensive post-mortem examination. The organs were also extracted for analysis of stem cell spread and stored for further study.

Two out of 9 injected pups showed evidence of transplanted cells in the blood (22%), and in 7 pups there was evidence of liver spread (78%) when analysed using flow cytometry (Figure 5.5 and Table 5.2). In Figure 5-5, it can be seen that the percentage of YFP positive cells in the peripheral blood was 3.3±1.1% (n=2, mean ± standard deviation), and 2.9±0.8% in the liver (n=7).

Table 5.2 Frequency of detection of YFP+/Lin-/cKit+ AFS cells in hematopoietic organs, 3 weeks after IP injection into wild type mice for allogeneic transplantation.
BM: bone marrow; -ve: negative; Mat.: maternal.

FACS	Litter 1 (N=4)	Litter 2 (N=5)	Total (N=9)	-ve control (N=4)	Mat. Sample (N=2)
Blood	1 (25%)	1 (20%)	2 (22%)	0	0
Liver	4 (100%)	3 (60%)	7 (78%)	0	0
Spleen	1 (25%)	0	1 (11%)	0	0
BM	0	0	0	0	0

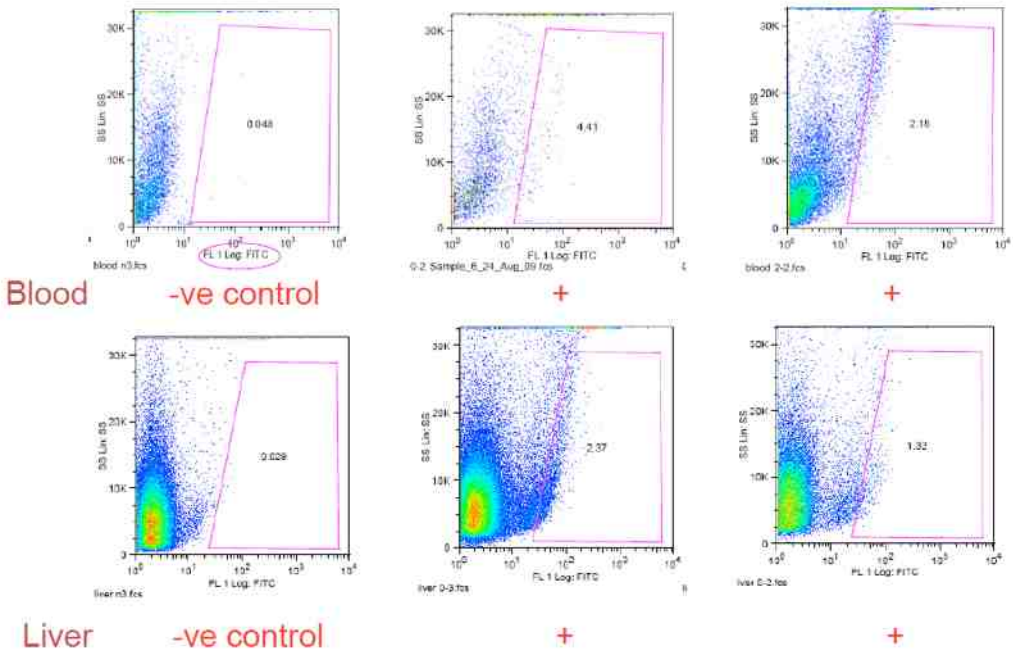


Figure 5.5 Graphs of flow cytometry show YFP+ cells could be detected in the peripheral blood, and in the liver.
Uninjected animals were set as the negative control. From the two example cases, the FITC gated channel (X axis) demonstrated 4.4 and 2.2% of YFP+ cells in the blood, and 2.3% and 1.3% YFP+ cells in the liver. –ve: negative.

One of the nine pups had 4.2% YFP+ cells in the spleen (Figure 5.6).

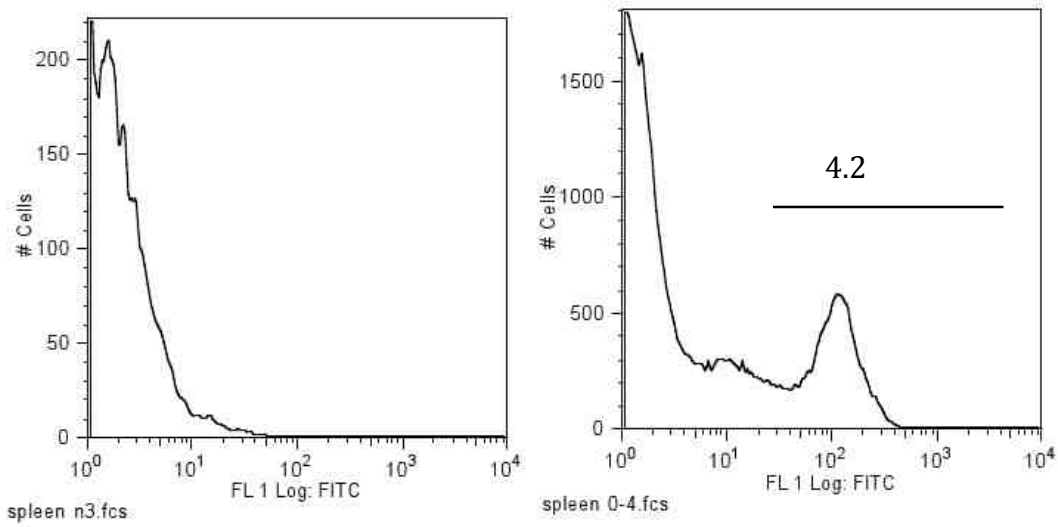


Figure 5.6 Flow cytometric analysis of spleen 3 weeks after birth in a pup transplanted *in utero* with YFP+/Lin-/Ckit+ AFS cells by intraperitoneal injection.
4.2% YFP+ cells are present in the spleen (Right panel). Left panel: negative control.

PCR analysis of the bone marrow showed the presence of YFP+ cells in the blood of one pup from Litter 1 and one pup from Litter 2, giving a total of 22% positive bone marrow for the 9 pups. These pups were those that had YFP+ cells in the peripheral blood. YFP DNA could also be detected by PCR in adrenal gland, gonad, and muscle (Figure 5.7).

Figure 5.7 PCR gels showing analysis for the presence of YFP+ DNA in the organs of wild type mice at 3 weeks of age after intraperitoneal injection of YFP+/Lin-/Ckit+ AFS cells.
In two pups a YFP+ signal (307 bp) was detected in the bone marrow. There is widespread migration of the injected AFS cells into the organs of recipients after prenatal allogeneic transplantation. YFP DNA positive bands could be detected in muscle, adrenal gland, gonad and spleen. H: heart, M: muscle, Sk: skin, L: liver, K: kidney, AU: lung, A: adrenal gland, S: spleen, G: gonad, +: positive control, *: repeated samples. P: positive control of GFP, BM: bone marrow.

In summary, these data demonstrate that injected YFP+/Lin-/Ckit+ amniotic fluid stem cells derived from YFP transgenic mice could be transplanted *in utero* and are detected three weeks after both present mainly in the hematopoietic organs of wild type mice after prenatal allogeneic transplantation.

5.1.5 Summary

Allogeneic sourced amniotic fluid stem cells from YFP+ transgenic mice, which have been isolated/sorted with Lin-/Ckit+ antibodies, can be transplanted *in utero* and are detected in the peripheral blood and other hematologic organs of wild type recipients 3 weeks after birth. The sorted Lin-/Ckit+ cells contribute around 1.5% of total amniotic fluid cells and maintained the Ckit+ purity over 97%. YFP+ injected cells could be found mainly in the liver (78%) compared to bone marrow (22%), blood (22%) and spleen (11%) of injected pups. This study confirmed the hematopoietic potential of the mice amniotic fluid stem cells.

5.2 Cell based gene therapy model: Prenatal allogeneic transplantation of the transduced AFS cells of MF1 mice to MF1 mice

Following from the previous section of the results, I hypothesised that mouse amniotic fluid stem cells could be transduced with vectors and had the capability of engraftment after *in utero* transplantation, and might therefore be useful for prenatal cell based gene therapy. Once these cells could be genetically modified, I would then be able to use the therapeutic gene transduced AFS cells to investigate the treatment in any disease model of mice. In this section, I therefore investigated *in utero* allogeneic transplantation using sorted and luciferase transduced mice AFS cells.

5.2.1 Lin-/Ckit+ amniotic fluid stem cells can be freshly isolated from MF1 mice, transduced with lentivirus and delivered by in utero intravascular injection into MF1 mice

In the previous section, I showed the total number and the percentage of Lin-/Ckit+ AFS cells from YFP transgenic mice from a C57BL/6 background. In these studies, I aimed to demonstrate the isolation of these cells from amniotic fluid of MF1 wild type mice. Compared to B6 background mice, MF1 mice have the advantage of a bigger litter size that can reach up 18 to 20 pups per pregnant dam and an improved neonatal survival rate.

In these experiments, two groups of MF1 mice were time-mated, one donor and one recipient (n=2 each group) (Figure 5.8). In the donor group, after sacrifice at E13, amniotic fluid was collected from each pup and pooled. Lin-/Ckit+ cells were isolated, cultured in suspension with HSC conditioning medium and essential cytokines in flat bottom ultra-low attachment plates to try to maintain all cells in suspension (Figure 5.9). Transplantation from MF1 mice to MF1 mice is an allogeneic graft study due to the MF1 mice are outbred.

In total 30 pups from two dams provided the amniotic fluid collected at E13 (Table 5.3). Of the total amniotic fluid cells collected, Lin- cells contributed on average 3.3%, while the Lin-/Ckit+ cells contributed an average 1.6%. In total, $4X10^5$ cells were isolated from two dams and used for prenatal allogeneic cell based gene therapy.

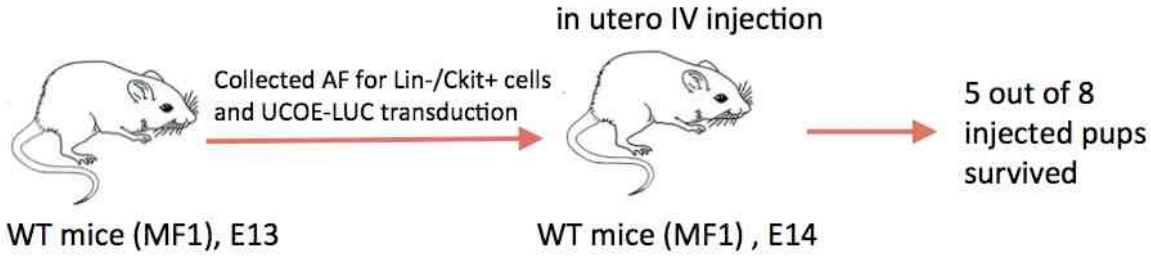


Figure 5.8 The experimental design of prenatal cell based gene therapy model in MF1 mice.
MF1 mice were time-mated and sacrificed at E13 for Lin-/Ckit+ cell isolation and transduction with UCO-E luciferase lentivirus for 24 hours. 50000 transduced cells were transplanted prenatally into each fetal mouse of MF1 at E14 via intravascular route. Five out 8 injected mice were survival for long-term follow-up. WT: wild type; UCOE-LUC: UCO-E promoter encoding luciferase; IV: intravascular injection.

Table 5.3 Amniotic fluid stem cell collection from MF1 mice.
The Lin- cells contributed 3.2-3.5% of the total cell number, and Lin-/Ckit+ cells contributed 1.6% of the total. WT: wild type MF1 mouse.

Animal	Date	Pups	Total cells	Lin- cells (number/%)	Lin-/Ckit+ cells (number/%)
WT1	E13	12	$1X10^7$	$3.5X10^5$ (3.5%)	$1.5X10^5$ (1.5%)
WT2	E13	18	$1.5X10^7$	$4.8X10^5$ (3.2%)	$2.5X10^5$ (1.25%)
Total		30	$2.5X10^7$	$8.3X10^5$ (3.3%)	$4X10^5$ (1.6%)

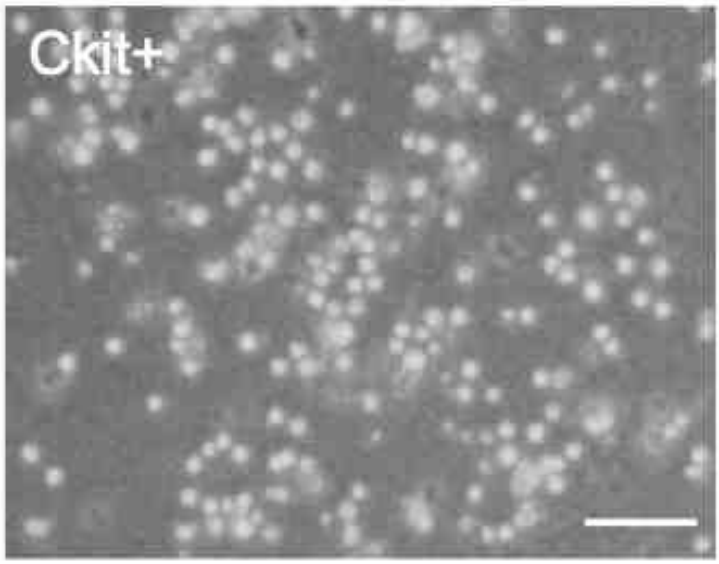


Figure 5.9 The MF1 mouse AFS cells were cultured in flat bottom ultra-low attachment plates to keep all the cells growing in suspension.
These cells were round shape under HSC specific conditions. Bar: 100µm.

Cells were transduced for 24 hours with lentivirus vector containing the UCO-E promoter driving the luciferase reporter gene, which was applied to the ultra-low attachment plates (MOI=50). The next day 50000 transduced cells were sorted and prepared in 20µl PBS and injected intravenously via the yolk sac vessel into MF1 fetal mouse pups from two recipient MF1 mothers, at E14 by Simon Waddington (n=8, total $4X10^5$ cells). Eight fetal injections of the AF cells were successfully delivered via the intravascular route with no complications (four pups per dam). In order to distinguish uninjected from injected animals, the injected fetal mice were marked by injection with colloidal carbon (10 µl) in the hind limbs after intravascular injection of the donor cells.
Out of eight marked and injected pups, five survived to term and delivered alive at E20 (62.5% survival rate, 5 out of 8). A further nine uninjected unmarked pups delivered from the two dams and were used as negative controls.

In summary, AFS cells could be derived from MF1 wild type mouse amniotic fluid, in slightly higher cell numbers than could be obtained with B6 background YFP+ mice. These data are comparable to the findings from YFP mice described in the previous section and also to published literature (De Coppi et al., 2007a, Ditadi et al., 2009). I also demonstrated the successful surgical method for *in utero* intravascular injection of transduced AFSCs with over 60% survival rate.

5.2.2 Luciferase expression is detected in MF1 mice using an in vivo imaging system up to 18 months after receiving in utero intravascular injection of Lin-/Ckit+amniotic fluid derived stem cells that have been transduced with lentivirus luciferase vector.

To determine cell engraftment and transgenic protein expression, luciferase uptake was measured in all injected mice (n=14) using the in vivo imaging system (IVIS), by collection of peripheral blood using the in vitro luciferase assay system at 16 weeks old (n=14) and in the liver of one animal at 4 months of age.
Whole body bioimaging was performed at 6 weeks after fetal injection (Figure 5.10 A) and showed strong luciferase expression in the area of the liver of all five injected animals (F1, F2, F5, F6, M1). There were also slightly positive signals around the bone marrow of lower extremities of the animal F5, F6, and M1. The other nine

uninjected animals were used as the negative controls and did not show any luciferase signal detected by IVIS. Repeated IVIS study at 12 weeks after injection (Figure 5.10 B) found that strong liver luciferase uptake could still be detected in all five injected animals, but the previous signal in lower extremities from the three mice was no longer detectable. The negative control remained the zero uptake of luciferase. At four months of age, one of the injected mice was arranged for partial hepatectomy but unfortunately the animal died after surgery due to haemorrhage. Apart from this mouse, the remaining four injected mice survived well and showing persistent luciferase expression in the region of the liver up to 18 months of age (Figure 5.10 C). The strength of the luciferase signal had decreased dramatically compared with previous imaging studies.

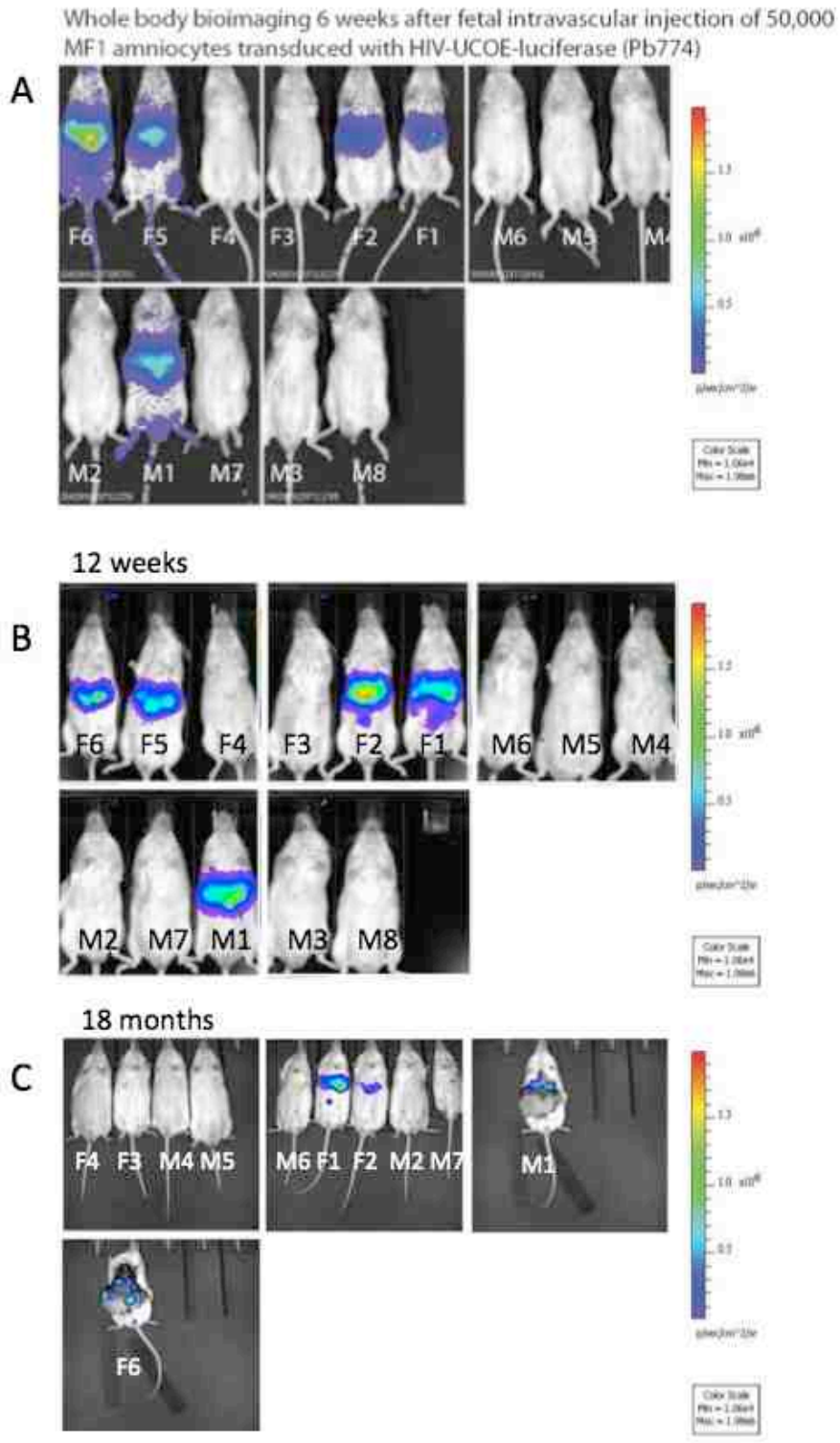


Figure 5.10 Measurement of luciferase expression by in vivo imaging system (IVIS) in MF1 mice that received *in utero* intravascular injection of Lin-/Ckit+ amniotic fluid derived stem cells transduced with luciferase vector.
(A) IVIS at 6 weeks of age shows that all five injected mice (F1, F2, F5, F6, M1) have strong luciferase signal in the liver and three also presented luciferase signal in the lower limbs (F5, F6, M1). The other nine uninjected animals were negative for luciferase. (B) Repeated IVIS at 12 weeks of age demonstrated all five injected mice still having the strong luciferase signal in the liver, but not in lower extremities. The other 9 uninjected animals remained negative for luciferase. (C) At 18 months of age, the positive luciferase signals were still shown in previous positive animals including F1, F2, F6, and M1.

In summary, IVIS showed persistent luciferase expression in the region of the liver of *in utero* transplanted mice suggesting that transduced mice AFS cells could engraft in the liver as long as 18 months after prenatal allogeneic transplantation. The fall in luciferase expression at 18 months could indicate silencing of transduced cell expression, which is known to be a problem with lentivirus vectors.

5.2.3 Luciferase expression is detected in the peripheral blood and liver of MF1 mice after in utero intravascular injection of Lin-/Ckit+ amniotic fluid derived stem cells transduced with lentivirus luciferase vector.

The *in vivo* study had suggested some evidence of liver engraftment, and I therefore explored this further by analysis of the haematopoietic organs in particular the peripheral blood and liver. *In vitro* luciferase assay was performed to detect the level of luciferase expression in the peripheral blood and liver. The relative light units (RLU) were recorded by luminometer. Fourteen mice (5 injected and 9 uninjected) were bled via tail vein direct puncture at 16 weeks of age and one year old (Table 5.4). Open surgery with liver lobectomy was also preformed in one of the injected mice at 16 weeks of age. Fresh liver samples were collected and prepared as single cell suspensions, then the RLU was measured by luminometer three times. Other parts of the liver were stored for immunohistochemistry staining and results are reported in the following section.

Table 5.4 *In vitro* luciferase assay in peripheral blood at 16 weeks and one year of age. The relative light units (RLU) were detected and measured by luminometer in the peripheral blood and liver at 16 week-old. Two out of 5 injected mice were positive for luciferase in the peripheral blood. All the three liver sections from injected mouse (F5) showed strong positive signal compared with the peripheral blood. Water was used as the negative control.

Test tube No.	Sex	Ear mark	Luminometer at 16 week (RLU)	Luminometer at 1 year (RLU)
5	M, injected	1	264	275
10	F, injected	1	244	214
11	F, injected	2	78	50
13	F, injected	6	50	55
14 (lobectomy)	F, injected	5	73	
1	M	5	61	59
2	M	4	33	56
3	M	6	25	39
4	M	3	65	53
6	M	7	47	36
7	M	8	29	51
8	M	2	38	48
9	F	8	41	34
12	F	4	60	64
Liver section 1			1210	
Liver section 2			1178	
Liver section 3			1305	
Blank (Water)			60	58

Luciferase assay reagent was made by adding buffer to the lyophilized luciferase assay substrate. The amount of100 µl reagent was added to each well for reaction and further measurement. Of the five injected mice, only two (M1 and F1) had positive luciferase expression detected in the peripheral blood at 16 weeks of age (Figure 5.11). In the remaining three injected mice, luciferase expression was similar to negative control. All uninjected mice had levels of luciferase expression comparable to water sample (negative control). The findings remained the same at one year of age (Table 5.4 and Figure 5.11). In the liver samples, there was a strong positive signal from all three sections of liver lobe of mouse F5 (1178, 1210 and 1305 RLU) that were analysed, which was far higher than results from peripheral blood (244 and 264 RLU).

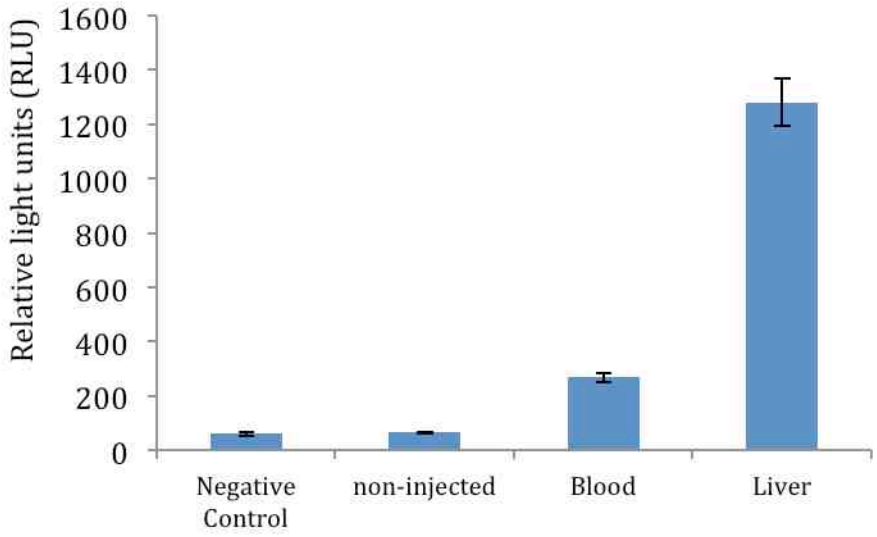


Figure 5.11 The results of in vitro luciferase assay at 16 weeks after transplantation.
This histogram showed the level of luciferase signal at 16 week-old. In those animals that tested positive in the blood, the relative light units (RLU) detected by luminometer was on average 250 in the peripheral blood. The RLU detected by luminometer of liver sections from one animals was on average 1231. The luciferase level of the uninjected animals was as low as negative control (water).

In summary, MF1 mice that received *in utero* injection of Lin-/Ckit+ amniotic fluid derived stem cells transduced with lentivirus luciferase vector showed evidence of transgenic luciferase expression in the peripheral blood up to one year after birth. Most importantly, the liver of one of the transplanted animals showed a strongly positive signal of luciferase expression at four months after birth, comparable to the results achieved from *in vivo* bioimaging study described in the previous section.

5.2.4 Immunohistochemistry staining confirmed the engraftment of luciferase transduced AFS cells in the liver after in utero allogeneic transplantation

In order to understand the results presenting strong liver signals detected by both IVIS and in vitro luciferase assay, I analysed the liver of one transplanted animal using immunohistochemistry. One of the injected mice (F5) was selected for partial hepatectomy at 16 weeks of age in order to determine if this would increase expression of luciferase. Unfortunately the animal was found dead one day after surgery due to massive blood loss during the operation. Liver samples were collected for *in vitro* luciferase assay and for immunohistochemistry staining. Immunohistochemistry using anti-luciferase antibody (Figure 5.12) showed brown coloured cells positive for luciferase uptake located mainly in the area where

hepatocytes are located and in the stromal area. The percentage of the positive cells per high power field was recorded from 5 different sections and counted by a blinded research colleague. The average positive rate was 38±5%. Unfortunately we missed the negative control image here. The background signal that could limit the conclusions made cannot be ruled out.

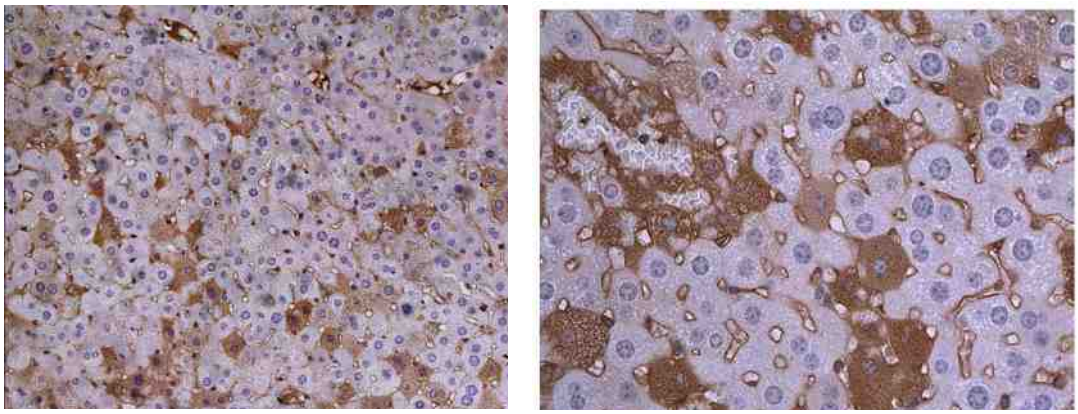


Figure 5.12 Immunohistochemical staining for anti-luciferase of the liver from an MF1 mouse that received an *in utero* intravascular injection of Lin-/Ckit+ amniotic fluid derived stem cells transduced with lentivirus luciferase vector.
The liver was collected 16 weeks after birth. The brown round shape cells are the hepatocytes staining positively with luciferase. There were also some small positive cells in the stromal area. Left panel: 10X; right panel: 40X.

5.2.5 Summary

In this section, I demonstrated that MF1 mouse Lin-/Ckit+ amniotic fluid stem cells could be selected and transduced with viral vectors encoding marker genes as a model of cell-based gene therapy. *In utero* allogeneic transplantation with these cells showed engraftment in the liver and peripheral blood. Luciferase was a good marking gene for amniotic fluid stem cells allowing them to be traced either *in vivo* or *in vitro* studies short and long term up to one year after birth, but did not lend itself to detailed analysis by FACS of peripheral blood or organ suspensions.

5.3 A comparison study of engraftment after in utero transplantation of congenic or allogeneic sourced Lin-/Ckit+ AFS cells in the mouse

In the two previous sections, I showed that mouse Lin-/Ckit+ amniotic fluid stem cells could be selected and transduced with lentivirus vectors. The sorted allogeneic mouse AFS cells could be transplanted *in utero* achieving engraftment long term into haematopoietic organs.

Previous work by Peranteau et al. regarding *in utero* transplantation with bone marrow derived HSCs in mice demonstrated higher levels of and more long term engraftment after transplantation with congenic sourced cells when compared with allogeneic sources (Peranteau et al., 2007). More recent studies have found that that the mother can produce antibodies and T cells to allogeneic sourced donor HSCs after *in utero* transplantation in the mouse, which can either cross the placenta, or are detected in the breast milk (Merianos et al., 2009, Peng et al., 2009, Nijagal et al., 2011b). These immune responses limit the engraftment of transplanted cells.

In this section, I investigated whether there was a difference between *in utero* transplantation with congenic or allogeneic sourced mouse amniotic fluid stem cells. In the literatures, most of the researchers were using bone marrow cells as the source of HSCs. This would be the first study investigating amniotic fluid stem cells for *in utero* transplantation.

5.3.1 Congenic or allogeneic in utero transplantation of amniotic fluid stem cells freshly isolated from YFP transgenic mice has a good survival rate

My previous work showed that Lin-/Ckit+ AFS cells derived from YFP transgenic mice could engraft in the haematopoietic organs of wild type mice after *in utero* allogeneic transplantation (Section 5.1). In these experiments, I compared engraftment after *in utero* allogeneic and congenic transplantation in mice.

The experimental design is shown in Figure 5.13. Three groups of mice were time-mated at the same time, YFP transgenic mice on a C57Bl6 background, normal C57BL/6 background mice, and CD1 background mice. For one *in utero* congenic or allogeneic transplantation, three YFP transgenic mice were sacrificed for amniotic fluid collection at E13.5, and the fluid was pooled from all pups in the three litters (Table 5.5). The Lin-/Ckit+/YFP+ AFS cells were isolated as previously described.

1.0% to 1.5% of Lin-/Ckit+ cells out of total amniotic fluid cells were collected from YFP mice, which was comparable to the results of my previous two sections (Table 5.5). All the AFS cells were freshly isolated without further culturing, prepared in PBS (20000 cells), and injected *in utero* into the peritoneal cavity of each recipient fetal mouse the same day as isolation (Figure 5.14). I performed all procedures and injections. No viral vector was used for transduction as the natural colour of yellow fluorescence protein could be detected after prenatal transplantation as reported in the previous section. For congenic transplantation, Lin-/Ckit+ AFS cells from YFP transgenic mice were transplanted *in utero* at E13.5 into the same background (C57BL/6) mice. For allogeneic transplantation, Lin-/Ckit+ AFS cells from YFP transgenic mice were transplanted *in utero* at E13.5 into CD1 mice (different background). All fetal mice in each litter were injected.

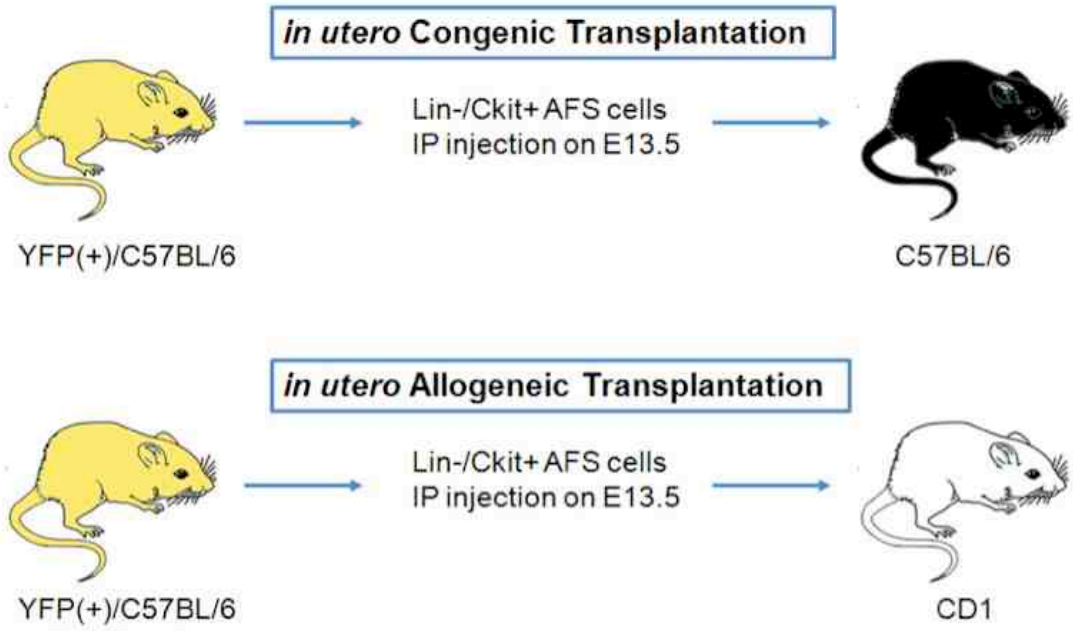


Figure 5.13 Experimental design to compare *in utero* allogeneic and congenic transplantation of amniotic fluid derived stem cells.
YFP transgenic mice have the same background as black mice (C57BL/6) but different background from the white mice (CD1). The Lin-/Ckit+ amniotic fluid stem cells were collected and isolated freshly for the same day injection at E13.5. IP: intraperitoneal.

Table 5.5 Results of YFP Lin-/Ckit+ AFS cells collected from YFP transgenic mice after magnetic sorting.
Amniotic fluid from all pups from three YFP transgenic mice was collected at E13.5 and pooled for isolation. The amniotic fluid stem cells were then injected into indicated congenic (C1-C3) or allogeneic (A1-A3) transplantation at E13.5.

Animal	GA	Pups	Total AF cells	Lin- cells (number/%)	Lin-/Ckit+ cells (number/%)	Injected animal
YFP1	E13.5	8				
YFP2	E13.5	9	$2X10^7$	$6.5X10^5$ (3.3%)	$2.6X10^5$ (1.3%)	A1
YFP3	E13.5	7				
YFP4	E13.5	8				
YFP5	E13.5	6	$2.5X10^7$	$8X10^5$ (3.2%)	$3X10^5$ (1.2%)	A2
YFP6	E13.5	6				
YFP7	E13.5	5				
YFP8	E13.5	8	$2X10^7$	$9X10^5$ (4.5%)	$3X10^5$ (1.5%)	A3
YFP9	E13.5	9				
YFP10	E13.5	5				
YFP11	E13.5	6	$1.5X10^7$	$6X10^5$ (4%)	$1.6X10^5$ (1.0%)	C1
YFP12	E13.5	5				
YFP13	E13.5	7				
YFP14	E13.5	4	$1.5X10^7$	$6X10^5$ (4%)	$2X10^5$ (1.3%)	C2
YFP15	E13.5	8				
YFP16	E13.5	4				
YFP17	E13.5	8	$2X10^7$	$7X10^5$ (3.5%)	$2.5X10^5$ (1.2%)	C3
YFP18	E13.5	8				

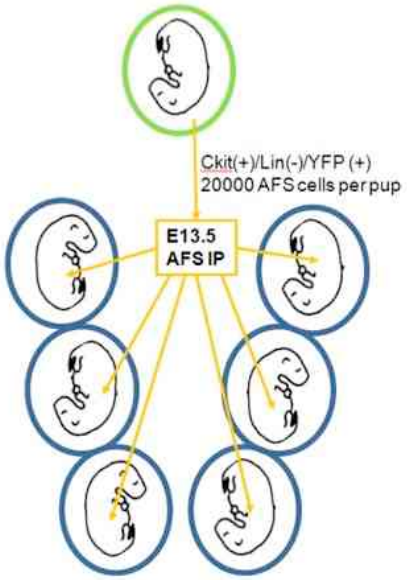


Figure 5.14The scheme of the prenatal amniotic fluid stem cells transplantation.
The graph was showing amniotic fluid stem (AFS) cells were collected for in utero transplantation into the peritoneal cavity. 20000 Lin-/Ckit+/YFP+ amniotic fluid stem cells were transplanted intraperitoneally per pup. IP: intraperitoneal.

Six litters of mice (three congenic and another three for allogeneic transplantation) were delivered at E20 to E21. Pup survival rate was 62.5% (10 pups survived out of 16 injections) in congenic transplantation and 65.5% (21 pups survived out of 32 injections) in allogeneic group (Table 5.6).

Table 5.6 The survival rates of congenic and allogeneic prenatal transplantation. Three dams of each study arm were injected with amniotic fluid stem cells. There was no statistic difference in the survival rate between these two groups. A1 to A3: allogeneic transplantation; C1 to C2: congenic transplantation.

Animal ID	Pups injected	Pups born alive	Survival rate to birth
A1	10	6	
A2	12	7	
A3	10	8	
Total	32	21	65.50%
C1	6	3	
C2	6	4	
C3	4	3	
Total	16	10	62.50%

In summary, Lin-/Ckit+ AFS cells could be freshly isolated from YFP transgenic mice and injected into the peritoneal cavity of fetal CD1 mice (allogeneic transplantation), and C57/BL6 mice (congenic transplantation) with a survival rate of 62 to 65%. The percentage of sorted Lin-/Ckit+ AFS cells was 1.0 to 1.5% of total AF cells, similar to previous results.

5.3.2 Mice injected in utero with YFP Lin-/Ckit+AFS cells had a higher level of YFP positive cells in the peripheral blood after congenic transplantation than after allogeneic transplantation

Following successful *in utero* congenic and allogeneic transplantation, I bled all the mice pups at 1, 3, and 6 months after birth to measure the level of YFP+ AFS cells in the peripheral blood. All the blood samples were prepared as previously described using RBC lysis buffer. The positive YFP signal was detected by flow cytometry in FITC channel. Blood samples from transgenic YFP mice were used as positive control; age-mated blood pellets from wild type uninjected mice were used as

negative control. The mean of YFP+ cells detected in negative control was less than 1.0%; and over 95% in the positive control (Figure 5.15). Figure 5.15 shows an example of flow cytometry study in the peripheral blood after congenic transplantation and allogeneic transplantation, with positive and negative control. Only a level of higher than 1% YFP cells counted as a positive signal in the peripheral blood of animals.

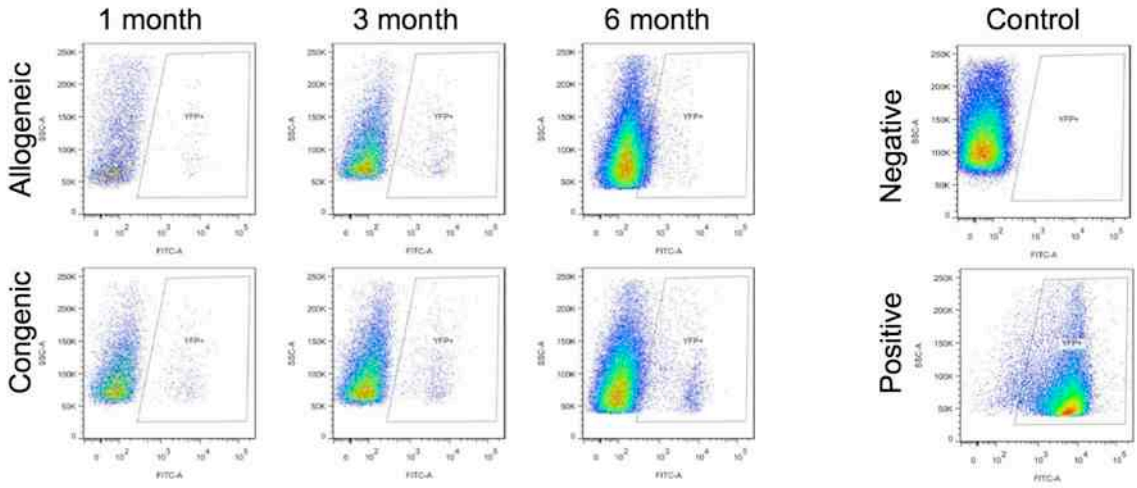


Figure 5.15 Example of flow cytometric analysis for peripheral blood samples at 1, 3, and 6 months after transplantation.
The gating area demonstrated the percentage of positive YFP cells in negative control, positive control, allogeneic or congenic transplantation. Positive control: peripheral blood of YFP transgenic mice. Negative control: peripheral blood of uninjected animals.

Table 5.7 shows the flow cytometry analysis of peripheral blood of congenic or allogeneic transplanted animals at 1, 3, and 6 months after injection. After congenic transplantation all 10 injected animals showed positive YFP signals in the peripheral blood at 1 and 3 months of age (Figure 5.16). At 6 months after birth, all but one animal had YFP positive signal in the peripheral blood (90%, 9 out 10 mice). In the allogeneic transplanted animals there was a steep fall in the number of animals having YFP signal in their peripheral blood from 86% (18 out of 21) at 1 month old to 29% (6 positive out of 21) at 6 months of age ($p<0.05$, ANOVA).
The mean level of YFP positive cells in the peripheral blood was significantly higher after congenic compared to allogeneic transplantation (3.59, 4.42, 4.03% at 1, 3, and 6 months of age compared with 1.51, 1.54, 1.20% respectively, $p<0.05$, ANOVA).

Table 5.7 The percentage of positive YFP cells in the peripheral blood of *in utero* congenic or allogeneic transplanted mice at 1, 3, and 6 months after birth.
Congenic transplantation gave significantly higher levels of YFP positive cells than allogeneic transplantation and more animals remained with a positive signal. (SD: standard deviation; *:$p<0.05$)

Congenic transplantation (%)				*Allogeneic transplantation (%)*			
Animal ID	1 Month	3 Month	6 Month	Animal ID	1 Month	3 Month	6 Month
C1-1	2.9	3.3	3.5	A1-1	1.1	1.1	0.4
C1-2	3.8	5	4.9	A1-2	2.1	2.4	2.9
C1-3	4.1	6.6	5.7	A1-3	2.3	2.8	2.9
C2-1	3	2.1	0.8	A1-4	1.1	0.6	0.4
C2-2	4.3	4.5	4.6	A1-5	2	2.3	0.9
C2-3	3.7	4.5	4.2	A1-6	2	1.5	0.6
C2-4	5.2	4.9	4.3	A2-1	1.6	1.4	0.5
C3-1	2.1	3.8	3.9	A2-2	2.6	1.5	0.7
C3-2	3.3	4.2	3.8	A2-3	0.7	0.4	0.6
C3-3	3.5	5.3	4.6	A2-4	1.1	0.8	0.4
				A2-5	1.5	0.5	0.8
				A2-6	2.1	2.2	2.5
				A2-7	1.5	2.3	2.8
				A3-1	0.2	0.2	0.6
				A3-2	1	0.5	0.6
				A3-3	1.3	1.8	0.4
				A3-4	1.4	1.5	0.3
				A3-5	0.3	0.2	0.6
				A3-6	1.6	1.8	0.7
				A3-7	2.2	3.2	3
				A3-8	2	3.3	2.7
Mean (n=10)	3.59	4.42	4.03	Mean (n=21)	1.51*	1.54*	1.20*
SD	0.85	1.21	1.29	SD	0.65	0.96	1.05
Animals with a positive YFP signal (number)	100% (10/10)	100% (10/10)	90% (9/10)	Animals with a positive YFP signal (number)	86% (18/21)	67% (14/21)	29% (6/21)

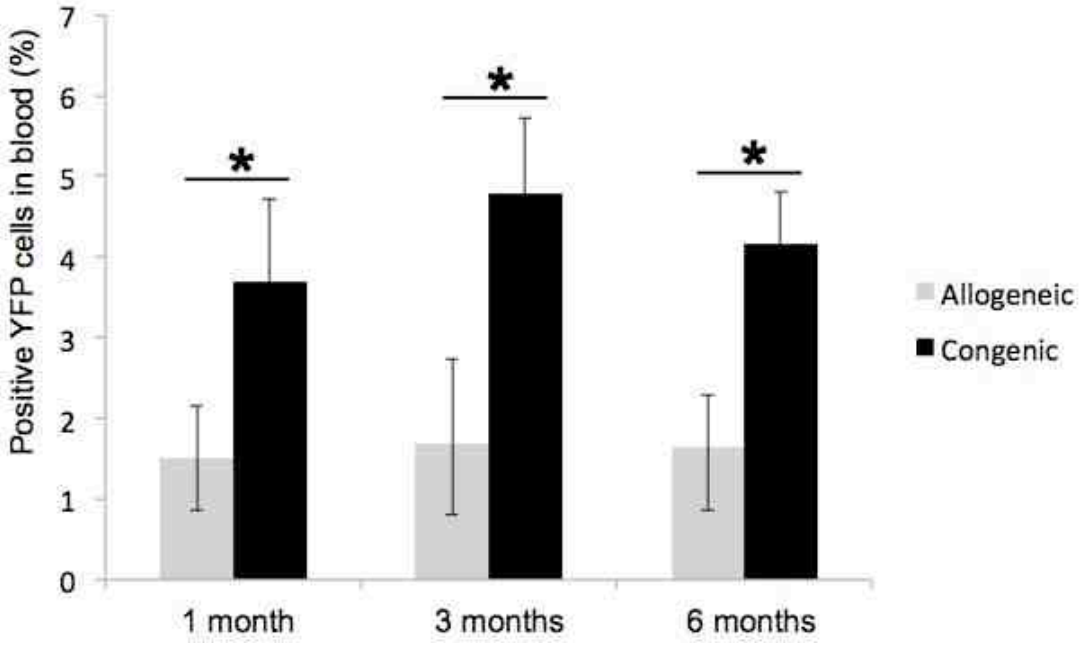


Figure 5.16 Flow cytometric study for positive YFP signal in the peripheral blood engraftment after allogeneic or congenic prenatal transplantation at 1, 3, and 6 months after birth. Congenic transplantation (black bar) resulted in a significantly higher % of positive YFP cells in peripheral blood than allogeneic transplantation (gray bar) at all three time points ($p<0.05$).

In summary, Lin-/Ckit+ amniotic fluid stem cells derived from transgenic YFP mice were detectable in the peripheral blood detected by flow cytometry analysis after both congenic and allogeneic *in utero* transplantation up to 6 months after birth. However, congenic transplantation showed significantly higher levels of YFP positive cells in the blood than allogeneic transplantation at all three time points (1, 3, and 6 months after birth). In addition, the number of animals showing some YFP positive signal was higher after congenic transplantation (100% at 1 and 3 months and falling to 90% at 6 months after birth) compared to allogeneic transplantation where it decreased dramatically from 86% at 1 month to 29% after 6 months of age.

5.3.3 Levels of YFP positive cells in the hematopoietic organs of injected animals is also higher after congenic transplantation than after allogeneic transplantation at 6 months old

To study the haematopoietic organs of transplanted animals, I sacrificed six congenic and six allogeneic transplanted mice at 6 months after birth, all of which were showing positive YFP blood signal. The allogeneic mice analysed were A1-2, A1-3, A2-6, A2-7, A3-7, A3-8, which showed persistent positive YFP signal detectable in the peripheral blood at 1, 3, and 6 months postnatal time points. Another six congenic transplanted mice were selected randomly from three dams C1, C2 and C3 (2 mice from each dam). These 6 congenic mice were all presenting positive YFP blood signal.

A comprehensive post mortem examination with widespread tissue sampling was performed according to that described in the Appendix. Single cell suspensions of the liver, BM and spleen were prepared for flow cytometry analysis as previous described.

In Table 5.8 and Figure 5.17, flow cytometry analysis showed the different levels of YFP+ cells in the liver, bone marrow, or spleen of the six animals of each study arm at six months after birth. The levels of YFP+ cells in the liver and spleen were significantly higher in congenic compared with allogeneic transplanted mice (7.16% vs 3.5% in liver and 7.65% vs 3.08 in spleen, both $p<0.05$). In the bone marrow, although levels of YFP+ cells were higher in congenic compared with allogeneic transplanted animals although the difference was not significant (4.89% vs 3.27%).

Table 5.8 The percentage of positive YFP cells in the hematopoietic organs of *in utero* congenic or allogeneic transplanted mice.
The levels of YFP+ cell in the liver and spleen were significantly higher after congenic compared with allogeneic transplantation. There was no significantly difference in the bone marrow. SD: standard deviation; *:$p<0.05$.

	Congenic Transplantation (%)				Allogeneic transplantation (%)		
ID	Liver	BM	Spleen	ID	Liver	BM	Spleen
C1-2	9.07	5.01	10.30	A1-2	4.65	2.68	4.27
C1-3	8.57	6.11	6.43	A1-3	4.12	4.57	2.12
C2-2	6.32	4.23	8.76	A2-6	2.43	2.12	2.32
C2-3	6.54	5.61	6.43	A2-7	5.43	3.27	4.67
C3-1	7.21	3.23	5.32	A3-7	2.22	2.10	3.89
C3-2	5.23	5.12	8.65	A3-8	2.12	4.89	1.23
Mean (n=6)	7.16	4.89	7.65	Mean (n=6)	3.50*	3.27	3.08*
SD	1.45	1.03	1.88	SD	1.42	1.21	1.38

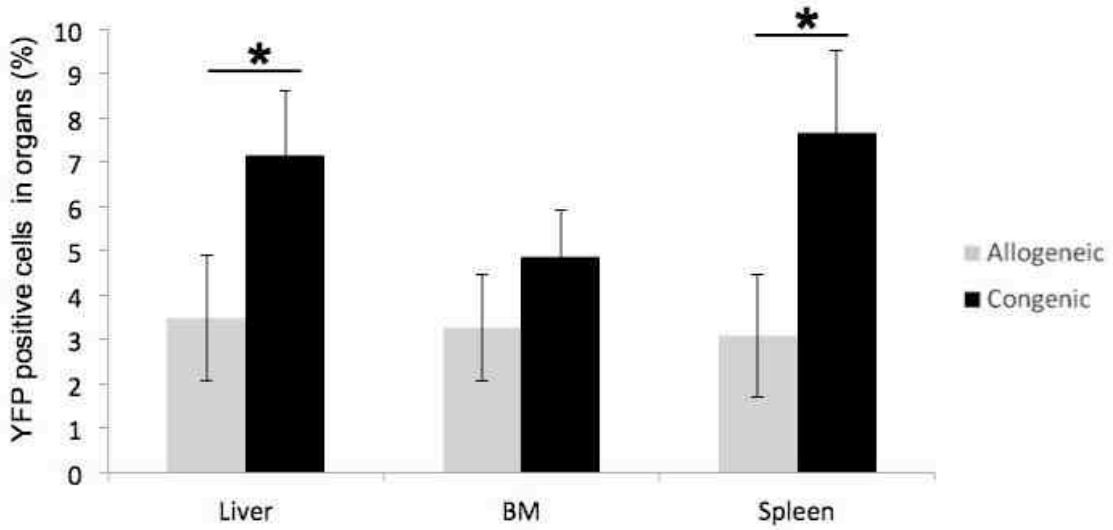


Figure 5.17 Flow cytometric study showing the level of YFP +ve cells in the hematopoietic organs of allogeneic or congenic *in utero* transplanted animals at 6 months after birth (n=6, each arm). Congenic transplantation (black bar) resulted in significantly higher levels of YFP+ cells in liver and spleen than allogeneic model (gray bar) (*:$p<0.05$).

In summary, not only the peripheral blood, but also the hematopoietic organs (liver, bone marrow and spleen) had evidence of YFP+ cells detectable by flow cytometry, with higher levels seen after congenic compared with allogeneic transplantation.

5.3.4 Mouse Lin-Ckit+/YFP+ amniotic fluid stem cells engraft in the liver after in utero transplantation

To determine whether the injected cells truly engrafted in the liver after *in utero* transplantation, I performed immunofluorescent histochemistry for YFP and albumin, a marker of hepatocytes in sections of liver taken from congenic and allogeneic transplanted mice (n=2, each arm). Two example liver sections examined with immunofluorescent microscopy are shown in Figure 5.18. The green signal indicates cells positive for YFP antibody, and the blue signal indicates the cell nuclei (DAPI staining). On gross observation, more green cells were present after congenic compared with allogeneic transplantation. The number of YFP positive cells per high power field was derived. I assigned two colleagues to count the positive green cells in high power fields from the liver sections, who were blind to the source of the stem cells (congenic of allogeneic). The number of positive cells was then compared statistically, and levels were significantly higher after congenic versus allogeneic transplantation (35.1 vs 18.8, $p<0.05$, Figure 5.19).

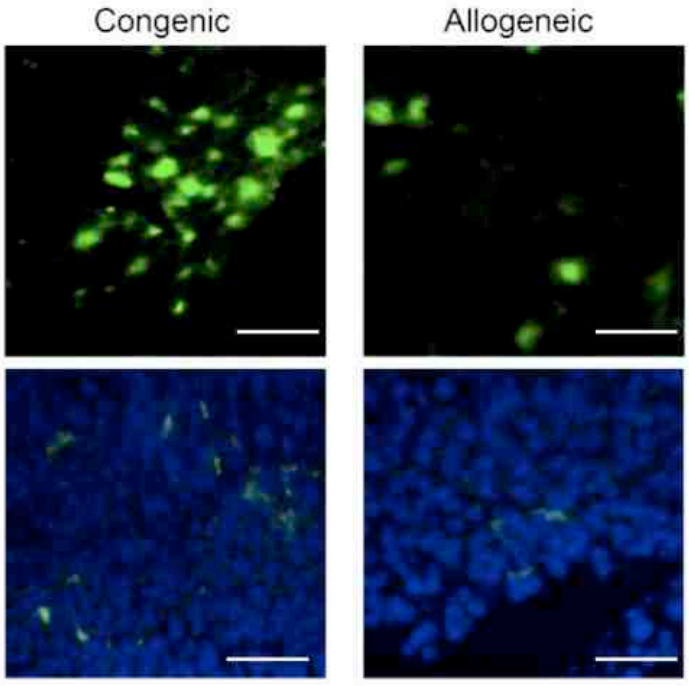


Figure 5.18 Immunofluorescence microscopy showed the YFP+ cells (green cells) in the liver of congenic and allogeneic transplanted animals.
In the left four images, the upper panel presented the green cells observed in the dark background; the lower panel showed the merged images with DAPI. Magnification bar = 50μm.

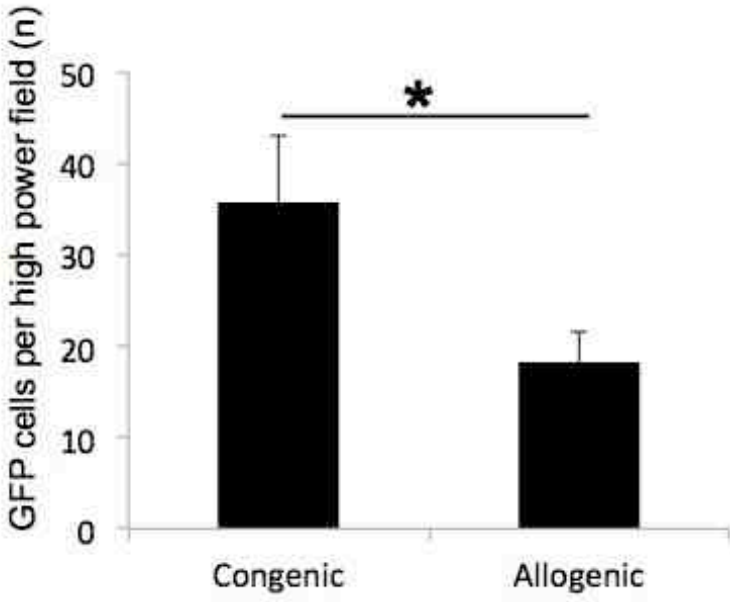


Figure 5.19 Histogram showing the number of YFP+ cells per high power field.
In general, congenic transplantation mice had higher positive cell number counted per high power field than allogeneic transplantation (histogram). *:p<0.05.

In order to understand if the YFP positive cells in the liver were truly showing hepatocyte differentiation, I co-stained the liver sections with YFP antibody and mouse albumin antibody (Figure 5.20). Albumin is a specific marker of hepatocytes. Confocal microscopy was performed to determine the liver structure after incubation with these two antibodies. After either allogeneic or congenic transplantation, YFP+ cells could be detected and co-stained with albumin. In the merged images, some transplanted YFP+ AFS cells showed the double staining.

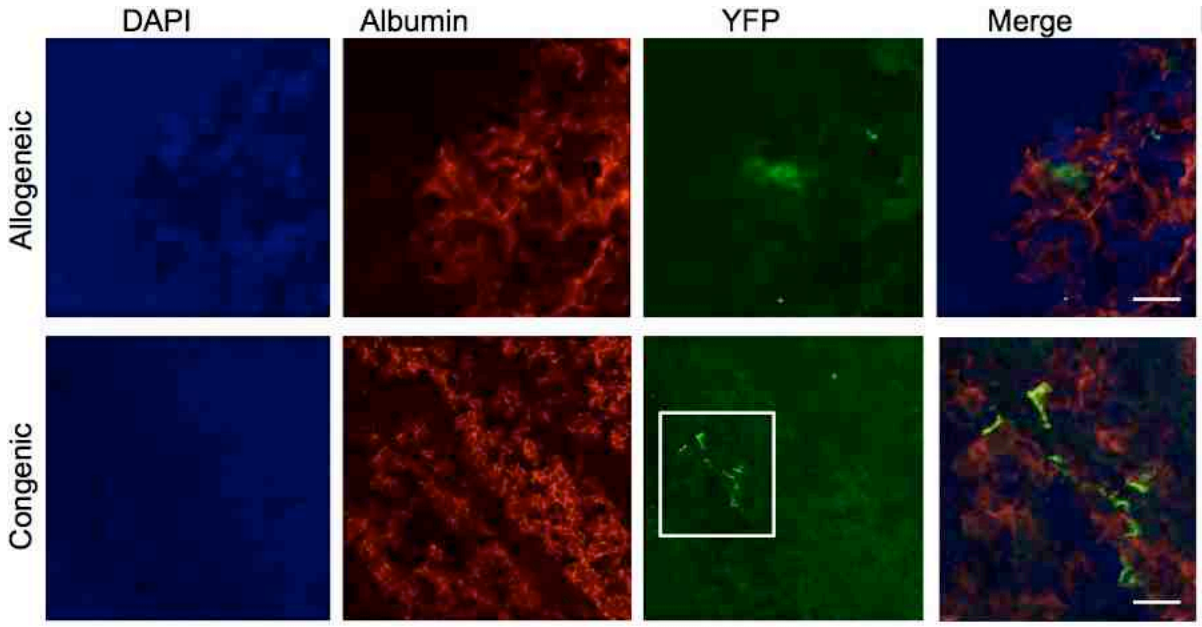


Figure 5.20 Confocal microscopy showed the YFP+ (green) cells in the liver of allogeneic or congenic transplantation mice co-staining with albumin (red).
After the merging of images, the double staining of the hepatocytes of transplanted mice could be observed. Bar: 10 μm.

In conclusion, I observed that mice injected *in utero* with YFP+/Lin-/Ckit+ AFS cells showed evidence of cell engraftment in the liver after allogeneic or congenic transplantation. Furthermore, the frequency of positively stained engrafted cells in the liver appeared to be higher after congenic compared to allogeneic transplantation.

5.3.5 *YFP DNA could be detected by sensitive PCR detection in the liver, spleen, and adrenal gland at 6 months after in utero congenic or allogeneic transplantation*

To detect spread of the injected YFP+/Lin-/CKit+ cells, I performed PCR analysis to detect the YFP DNA on a wide range of tissues collected at post mortem examination at 6 months of age. Genomic DNA was extracted from each organ for the PCR study. Six mice from each group (same animals as previous section) were taken for this analysis. The liver of transgenic YFP mice was used as positive control. The liver of uninjected wild type mice was used as negative control.

All congenic transplanted animals had YFP DNA detected in the liver (100%, 6/6, Table 5.9) compared to 67% (4 out of 6) after allogeneic transplantation. The adrenal gland was positive for YFP DNA from three out of six animals (50%) after congenic, but only one out of 6 (17%, 1/6) after allogeneic transplantation. There were 4 cases with positive YFP DNA in the spleen after congenic model, but 2 cases after allogeneic transplantation. YFP DNA could not be detected in the kidney, lung, heart, muscle, and gonad of transplanted mice in either group. Generally, the frequency of YFP positive organs (liver, adrenal and spleen) was higher in congenic mice compared to allogeneic mice. Figure 5.21 demonstrates an example of a PCR gel from an animal that underwent congenic transplantation showing positive YFP bands in the organs.

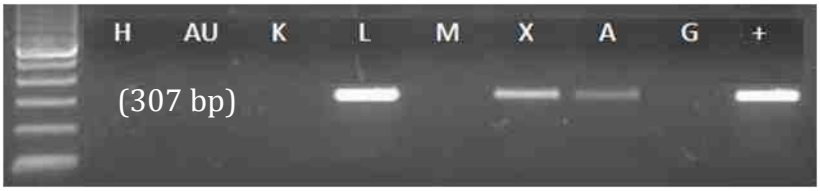


Figure 5.21 An example of a PCR gel detecting YFP DNA in systemic organs of congenic transplanted mice.
The single band (307bp) represented the PCR product of YFP gene, which could be found in the liver, spleen, adrenal gland and positive control. H: heart; AU: lung; K: kidney; L: liver; M: muscle; X: spleen; A: adrenal gland; G: gonad; +: positive control, DNA from YFP transgenic mouse liver. Ladder: 100bp

Table 5.9 PCR analysis for YFP DNA detected in the systemic organs of congenic (C1-C3) or allogeneic transplanted (A1-A3) mice.
Genomic DNA was extracted from six animals per study group; a positive signal was only seen in the liver, adrenal gland, and spleen of transplanted animals. YFP DNA was more commonly detected in tissues from congenic compared with allogeneic transplanted mice.

	Liver	Kidney	Lung	Heart	Adrenal	Spleen	Muscle	Gonad
C1-2	+	-	-	-	+	+	-	-
C1-3	+	-	-	-	+	+	-	-
C2-2	+	-	-	-	-	+	-	-
C2-3	+	-	-	-	+	+	-	-
C3-1	+	-	-	-	-	-	-	-
C3-2	+	-	-	-	-	-	-	-
Frequency of YFP+ DNA	100%	0%	0%	0%	50%	67%	0%	0%
A1-2	-	-	-	-	-	-	-	-
A1-3	+	-	-	-	-	-	-	-
A2-6	+	-	-	-	+	+	-	-
A2-7	+	-	-	-	-	+	-	-
A3-7	-	-	-	-	-	-	-	-
A3-8	+	-	-	-	-	-	-	-
Frequency of YFP+ DNA	67%	0%	0%	0%	17%%	33%	0%	0%

In summary, the liver appears to be the main organ targeting after *in utero* intraperitoneal transplantation of YFP+/Lin-/Ckit+ amniotic fluid stem cells, which could be detected by YFP PCR. The adrenal gland and spleen showed positive YFP signal in animals from both groups. Congenic transplantation always showed higher frequency of YFP DNA in organs than allogeneic transplantation according to the PCR results.

5.3.6 Bone marrow cells of congenic but not allogeneic transplanted mice can form YFP+ haematopoietic colonies

To further prove the amniotic fluid stem cells having and maintaining the hematopoietic potential in the mice after *in utero* congenic or allogeneic transplantation, I performed colony forming assays in semi-solid methylcellulose culture on bone marrow cells collected from the YFP+ AFS cells injected animals at 6 months of age

Bone marrow cells were harvested as previously described in single cells suspension and conditioning medium specific for haematopoietic stem cells. Six mice per group from Table 5.8 were sacrificed for bone marrow collection; all were positive for YFP cells in the bone marrow, liver and blood. The different types of colony were recorded and followed the recommended guideline. Bone marrow from transgenic YFP mice were used as positive control and from uninjected wild type mice as negative control. As shown in Figure 5.22, the bone marrow cells from YFP+ mice (first row) could form all three kinds of haematopoietic green colonies, while the bone marrow cells from wild type did not form any YFP positive colonies (2^{nd} row). After congenic transplantation, a few green YFP positive cells (approximately1% out of total) could be observed in every kind of colony formed including CFU-E, CFU-GEMM, and CFU-G/M/GM (3^{rd} row). Similar to negative control, there were no green colony forming cells from the bone marrow cells after allogeneic transplantation (4^{th} row).

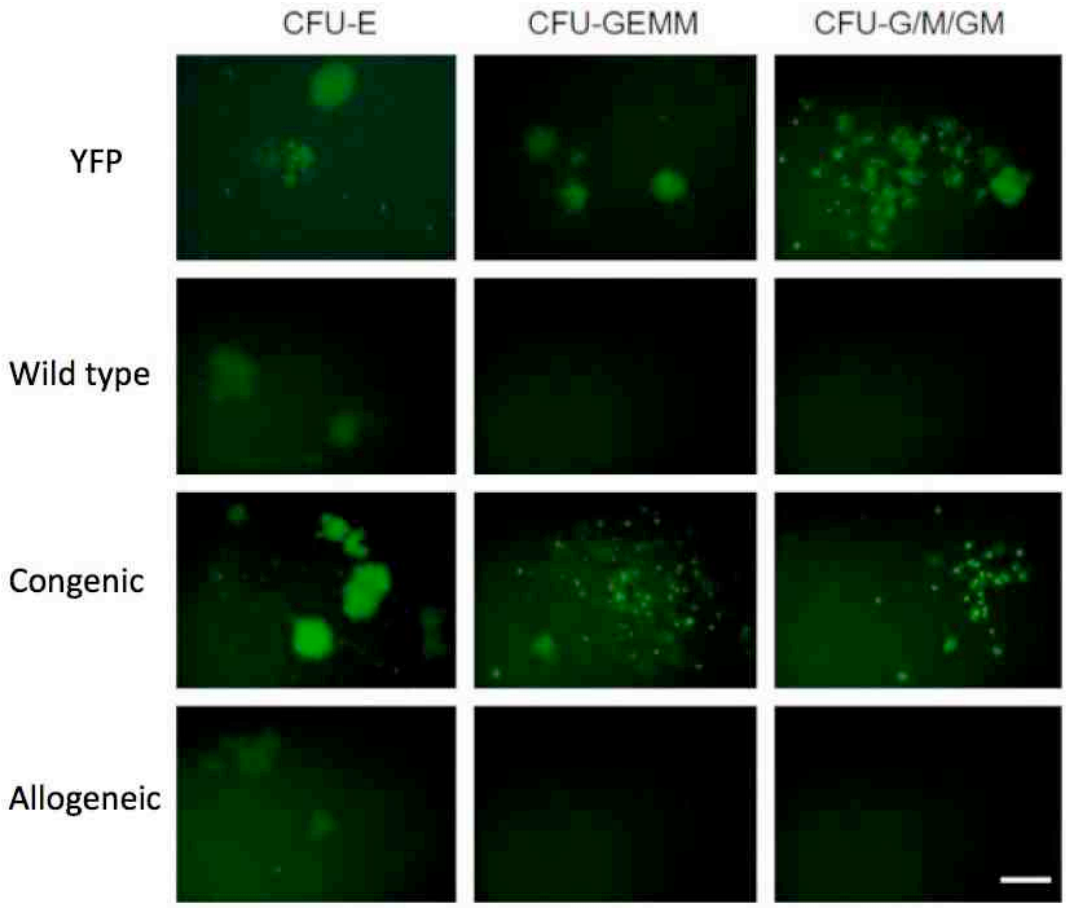


Figure 5.22 Methylcellulose colony forming assay on bone marrow cells from YFP transgenic mice, wild type, or mice transplanted using congenic or allogeneic sourced YFP+/Lin-/Ckit+ AFSCs.
YFP+ colonies could be observed under fluorescence microscopy in YFP transgenic mice (positive control) and a few colonies in congenic transplanted bone marrow. There were no YFP colonies (green cells) in negative control or allogeneic transplantated bone marrow. Bar: 100 μm.

The number and type of YFP positive colonies (Figure 5.23) were recorded in six animals from each group by two other colleagues blindly. All these experiments were duplicated (two plates for each BM sample). Congenic colonies were classified as CFU-E, CFU-GEMM, and CFU-G/M/GM with the percentage of total colonies 1.6%, 0.6%, and 0.4%, respectively. Bone marrow cells from allogeneic transplanted mice (gray bar) however, had far fewer YFP cells of CFU-E type (0.4%) compared to congenic transplantation ($p<0.05$), and no colonies of type CFU-GEMM or CFU-G/M/GM were formed.

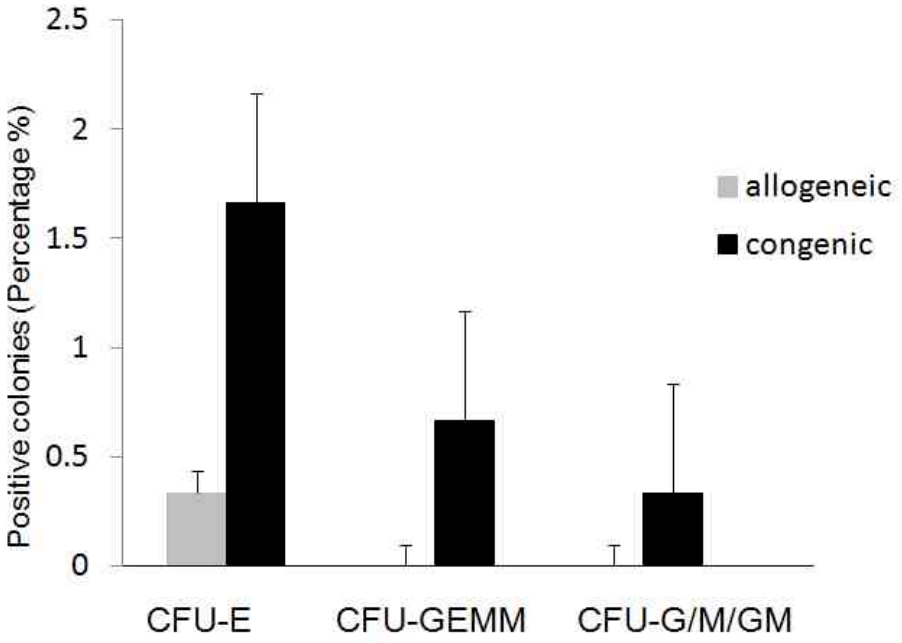


Figure 5.23 The percentage of positive YFP haematopoietic colonies found in semi-solid culture from the bone marrow cells of congenic or allogeneic transplantated animals.
All three YFP+ colony types were seen in congenic transplanted bone marrow. There were no YFP positive colonies of CFU-GEMM and CFU-G/M/GM in allogeneic transplanted animals. The percentage of YFP+ haematopoietic CFU-E colonies was higher after congenic transplantation compared with allogeneic transplantation (1.6% vs 0.4%, $p<0.05$).

In summary, I have shown that donor bone marrow cells collected six months after intraperitoneal *in utero* transplantation in mice can form haematopoietic stem cell colonies. After congenic transplantation, YFP+ cells from the bone marrow of injected mice could be found as green colonies in all three types of differentiation, with the percentage of YFP+ colonies about 1.5% of total colonies. After allogeneic transplantation, only one YFP positive colony could be found in the CFU-E, but not in CFU-GEMM or CFU-G/M/GM. The percentage of YFP+ colonies in semi-solid culturing was higher after congenic transplantation than allogeneic transplantation.

5.3.7 Summary

In this section, I compared *in utero* transplantation of congenic versus allogeneic sourced Lin-/Ckit+/YFP+ amniotic fluid stem cells in the mouse. The survival rate after *in utero* congenic or allogeneic transplantation was good (62%) with no difference observed between the two stem cell sources. By using flow cytometry, PCR and immunofluorescence studies, I showed that the transplanted AFS cells mainly engrafted in the haematopoietic organs of recipient mice including liver, spleen, bone marrow and peripheral blood. In general, the congenic sourced injected cells showed a higher and more sustained engraftment when compared to allogeneic sourced cells. Furthermore, these donor cells could differentiate into hepatocytes in the recipient liver showing the double staining of albumin and YFP. YFP positive bone marrow cells obtained from the recipient mice demonstrated their haematopoietic potential by forming all three colonies types (congenic sourced cells) in methylcellulose culture.

5.4 In utero congenic transplantation of transduced AFS cells in a mouse model of disease: the th3 thalassaemia mouse

In the previous three sections I showed that amniotic fluid stem cells could be freshly obtained from pregnant mice with around 1.5% of Lin-/Ckit+ population of total cells after sorting. These cells could be transduced with a lentivirus vector encoding luciferase for cell marking. All the results from mice (Lin-/Ckit+ AFS cells, this chapter) and sheep (CD34+ AFS cells, chapter 4) revealed the haematopoietic potential of amniotic fluid stem cells with engraftment in the peripheral blood, liver, spleen and bone marrow after *in utero* transplantation. The transduced AFS cells could not only engraft in the bone marrow, but, in the colony-forming assays, also differentiate into all types of haematopoietic colonies. After both congenic and allogeneic *in utero* transplantation, AFS cells had shown the ability to engraft, with an advantage of more frequent and a higher level of engraftment seen in cells from congenic sources compared to allogeneic sources.

Using this information I hypothesized that amniotic fluid derived haematopoietic stem cells from autologous sources could be used for prenatal therapy of haematological disease. Thalassemia for example is a very common autosomal recessive disease of the haematological system. As described in the introduction (Chapter 1), thalassaemia are particularly associated with people of Mediterranean origin, Arabs, and Asians (Ortuno et al., 1990). I am interested in this disease also because thalassemia is the second most common inherited disease in Taiwan (Chern and Chen, 2000, Ko et al., 1993, Lin et al., 1991). A commonly used thalassaemia mouse model, the *th3* mouse on a BL6 background (Vadolas et al., 2005) was available at Imperial College London in collaboration with Dr Simon Waddington. I therefore conducted some pilot experiments exploring an AFS cell-based prenatal gene therapy approach using congenic transplantation in thalassaemia mice.

Generally homozygous *th3* mice die *in utero* late in gestation or are too sick to survive after birth, a situation similar to human alpha thalassemia. Heterozygous *th3* mice have a very severe phenotype of anaemia similar to human beta thalassaemia major. My hypothesis was that *in utero* transplantation of congenic derived Lin-/Ckit+ amniotic fluid stem cells could treat thalassaemia prenatally.

In this section, I present some pilot experiments in which I used amniotic fluid stem cells derived from wild type YFP transgenic mice to try to correct the phenotype of heterozygous mice or rescue the homozygous animals. In parallel some other experiments performed in this mouse model by Dr Citra Mattar, a visiting PhD student from National University of Singapore, explore phenotypic correction after *in utero* transplantation with human fetal liver derived HSCs.

5.4.1 Experiments in the th3 thalassaemia mouse model are limited by the low conception rate in this mouse model

Early in my PhD I set up phenotyping the *th3* mouse colony by direct blood smear staining and genotyping by PCR following the protocol in the literature (Vadolas et al., 2005). Initially there were 5 cages of thalassaemia mice including 12 female and 8 male that had not been genotyped. After confirmation of genotype, 10 female and 6 male heterozygous thalassaemia mice were mated to expand the colony. Immediately after birth pups were geneotyped and wild type, unaffected mice were culled. Further colony expansion was performed to make sure there were sufficient heterozygous mice for prenatal transplantation.

Eight breeding pairs of thalassemia heterozygous female cross-mated with heterozygous male (het x het) were set up. Table 5.10 shows the monthly results of mating. From December 2009 to October 2010, the breeding cages were mated twice or three times per month. A few pairs were set up for time-mating with YFP mice on the same day for congenic transplantation. The conception rate for *th3* het x het was 7.11% which is low in comparison with other mouse models I was using at the time (over 40% for CD1 mice mating rate). During this period, I performed four congenic *in utero* AFSC injections (Table 5.10).

Due to the poor conception rate, I decided to mate thalassemia heterozygous male back crossed with a CCR2 wild type female. From November 2010 to April 2011, the average conception rate was still low (7.77%, Table 5.10). Two injections were performed here in December 2010 and February 2011 (Table 5.11, injection group 4 and 5). There were also pinworm and mouse parvovirus infections in the animal house at the time which meant that all the mice had to be transferred into a new environment within individually ventilated cages (IVC). Finally, the *th3* thalassemia colony was moved from Imperial College to UCL after April 2011 when Dr Simon

Waddington moved offices, and no further experiments could be performed due to setting up of the colony.

Table 5.10 Mating history and conception rate of *th3* thalassemia mice.
th3: thalassemia mice; CCR2: wild type mice. The conception rate of th3Xth3 was 7.11%, which was similar to th3XCCR2 conception rate, 7.77%. Some of the pregnant dams were selected for *in utero* transplantation (see comment). No.: number.

Month-Year	Mating (Male X female)	No. Pairs	No. of mating	No. of pregnancy	Conception rate	Comment
Dec-09	th3 X th3	8	18	1	5.56%	
Jan-10	th3 X th3	8	24	1	4.17%	
Feb-10	th3 X th3	9	18	1	5.56%	
Mar-10	th3 X th3	9	18	0	0.00%	
Apr-10	th3 X th3	9	18	2	11.11%	for 2 prenatal injections
May-10	th3 X th3	9	18	3	16.67%	for 2 prenatal injections
Jun-10	th3 X th3	9	27	2	7.41%	for 1 prenatal injection
Jul-10	th3 X th3	9	18	2	11.11%	
Aug-10	th3 X th3	8	8	0	0.00%	
Sep-10	th3 X th3	8	16	0	0.00%	
Oct-10	th3 X th3	7	14	2	14.29%	for 2 prenatal injections
Total		93	197	14	7.11%	
Nov-10	th3 X CCR2	7	21	3	14.29%	for 3 prenatal injections
Dec-10	th3 X CCR2	7	21	1	4.76%	
Jan-11	th3 X CCR2	7	21	2	9.52%	for 2 prenatal injections
Feb-11	th3 X CCR2	7	14	0	0.00%	
Mar-11	th3 X CCR2	6	12	1	8.33%	
Apr-11	th3 X CCR2	7	14	1	7.14%	Moved the colony to UCL
Total		41	103	8	7.77%	

5.4.2 Genotype and phenotype can differentiate the unaffected mice from heterozygous mice

In order to make distinguish between homozygous, heterozygous or unaffected animals, I established a PCR genotyping method and a quick blood smear method. The thalassaemia in *th3* thalassemia mice is a very severe form of anaemia that mimics human alpha major thalassemia. In heterozygous animals, one of the mouse β-globin is replaced by one HPRT gene as described in the introduction. In homozygous animals, both two internal β-globin genes are replaced by two HPRT genes so that these animals cannot produce the normal red blood cells and maintain haematopoietic function.

For blood smears, blood was collected from the tail vein of mice and one small drop was placed with a drop of heparin (5ul) immediately onto a slide and the blood drop was smeared along the slide to spread it. All the blood smears were checked by myself and Dr. Anna David. In Figure 5.24, an example of blood smears after Giemsa May-Grünwald staining is shown from a heterozygous *th3* mouse (Figure 5.24A) and a homozygous normal mouse (Figure 5.24B). The abnormal fragmented and misshapen red blood cells (light grey with round shape) can be clearly seen in the heterozygous blood smear, whereas all red blood cells from the homozygous wild type smear are of consistent size and shape. This method took only one hour to have the initial results; therefore I used this technique as the first step for identification of mouse phenotype.

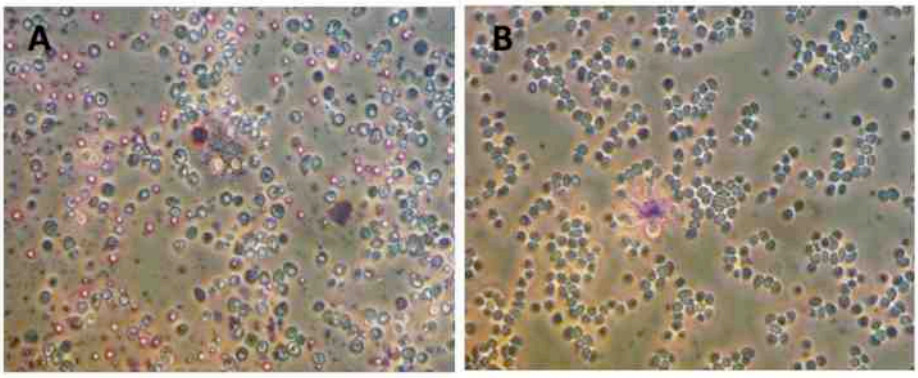


Figure 5.24An example of Giemsa May-Grünwald staining in the peripheral blood of *th3* thalassemia mice.
(A) the blood film of heterozygous *th3* thalassemia mice shows uneven shape, color, and size of red blood cells. The small fragments of cells indicated the cell rupture. (B) A normal blood smear with red blood cells that look uniform without rupture. The purple stained cells were while blood cells, which could be found in both images. 400X

For multiplex PCR gel, I collected the ear of mice after labelling them using an ear marking system. A small piece around 2 to 3 mm of ear was prepared for DNA extraction as described in the material and methods. All the PCR and primer conditions followed a protocol (Vadolas et al., 2005) for quick genotyping. Wild type *th3* mice had only one thick band of mouse beta-globin, at 249 base pairs. Heterozygous mice had 2 bands seen. The mouse beta-globin band (249 bp) was lighter in intensity in heterozygous animals as they have only one copy of this gene. These mice also had a HPRT gene band at 315 base pairs. .Homozygous *th3* mice had only one band of the HPRT gene (315bp) and no mouse beta-globin gene (249bp). Results from the multiplex PCR are shown in figure 5.25. The results were the same as those found by blood smears.

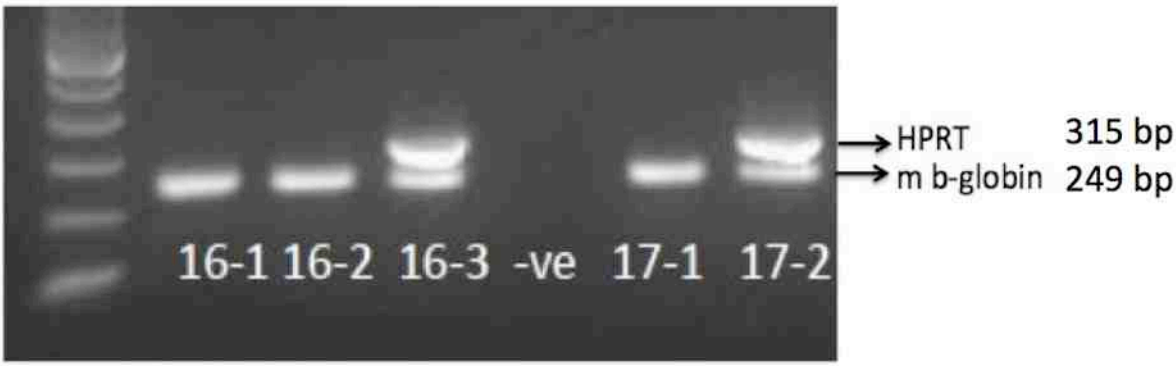


Figure 5.25 An example of PCR genotyping gel of thalassemia *th3* mice.
16-1, 16-2, and17-1 had only one band of mouse beta-globin (249 base pairs) that indicated these animals were unaffected, wild type mice. 16-3 and 17-2 had 2 bands, one beta-globin band and an additional band for the HPRT gene (315 base pairs) indicating that these were heterozygous thalassemia mice. No homozygous *th3* thalassaemia mice were detected. Ladder: 100bp

Gross examination of *th3* thalassemia pups showed heterozygous pups to look very pale either as fetuses or after birth (Figure 5.26). *Th3* mice had the same genetic background as B6 wild type; therefore it became difficult to tell the colour difference after the growth of the black hair. Not only the white skin colour was different, but also the body size and body weight were also smaller in thalassemia heterozygous pups than in wild type newborns.

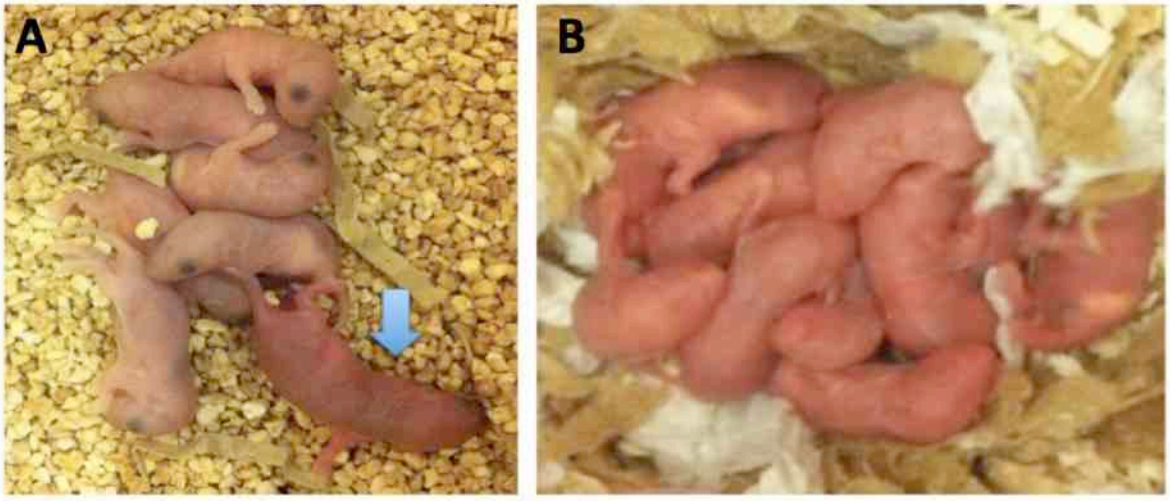


Figure 5.26 Phenotype of the thalassemia mice.
(A) pups from th3Xth3 matings show two different types of newborns. One of them presented the red color of skin that are homozygous wild type (arrow), but the others are very pale with white skin, which are the heterozygous pups. (B) Normal pups of CD1 mouse showed uniform and pink color of the skin.

In summary, I demonstrated that the genotype in *th3* thalassaemia mice can be determined using blood film and multiplex PCR. Also it was possible to distinguish either the diseased mice or normal mice by gross observation either in fetal stage or early postnatal period.

5.4.3 In utero injections of stem cells in th3 mice give a very low pup survival rate

Because of the low conception rate it was not possible to do prenatal transplantation experiments every month as some of the pregnant dams were for colony maintenance and expansion, or donor YFP transgenic mice were not pregnant for amniotic fluid collection. In Table 5.11, I list all the prenatal injections performed in the thalassemia colonies. Some experiments were performed using human fetal liver derived HSCs (hflHSCs) and were part of a collaboration with Dr Citra Mattar, National University of Singapore. Five groups of injections were performed during this time period, performed by Dr Simon Waddington who is experienced in the technique, and most of them showed a high mortality after birth due to spontaneous miscarriage or because of cannibalization by dams. In each case injection was via the intraperitoneal or intravascular route. The first two injections in two animals in June 2010 used transduced GFP AFS cells derived from MF1 mice but gave no pup survival. The second group of injections was using the amniotic fluid stem cells from YFP

transgenic mice, which had been transduced with HIV-UCOE-GFP at E14. This is the only experiment where there was long term postnatal survival (three pups) after prenatal amniotic fluid stem cell transplantation in this thalassemia colony.

Table 5.11 Summary of *in utero* stem cell transplantation in *th3* thalassemia mice. hflHSC: human fetal liver haematopoietic stem cells. PN: postnatal; GA: gestational age at injection.

Injection group	Date	Cell/Gene	Recipient	Virus	Cell no.	No. Pups, GA	Outcome
1	June 2010	MF1 AFS	th3 x th3	UCOE-GFP	20000	7, E11	Miscarriage
1	June 2010	MF1 AFS	th3 x th3	UCOE-GFP	20000	8, E13	1 alive, but died day 1PN
2	July 2010	YFP AFS	th3 x th3	UCOE-LUC	10000	10, E14	3 survived (1 WT, 2 Het)
2	July 2010	YFP AFS	th3 x th3	UCOE-LUC	10000	6, E14	Miscarriage
3	November 2010	hflHSC	th3 x th3	-	200000	6, E15	3 alive but died day 1PN
3	November 2010	hflHSC	th3 x th3	-	200000	5, E12	Miscarriage
4	December 2010	hflHSC	th3 X CCR2	-	200000	12, E15	8 alive
4	December 2010	hflHSC	th3 X CCR2	-	200000	10, E15	7 alive
4	December 2010	hflHSC	th3 X CCR2	-	200000	9, E15	8 alive
5	February 2011	YFP AFS	th3 X CCR2	-	50000	10, E15	Miscarriage

In summary, *th3* thalassemia heterozygous mice could be time-mated but with a very low conception rate either in *th3*hetX*th3*het or *th3*hetXCCR2. After five groups of prenatal injections, the overall mortality was too high to complete the experimental number required due to miscarriages or cannibalization of the pups.

5.4.4 *In utero congenic transplantation using luciferase transduced Lin-/Ckit+ AFS cells derived from YFP transgenic mice can be achieved in th3 thalassaemic mice*

Most of the experiments for the thalassemia mice prenatal injection were not successful due to high fetal mortality rate. Here I present the example of *in utero* congenic transplantation by transduced amniotic fluid stem cells (Table 5.12). I chose to use AFSCs collected from YFP transgenic mice, and to transduce them with lentivirus vectors to enable us to use FACS analysis for YFP level and IVIS for luciferase expression in the same animal.

The *th3* female mice were time-mated with *th3* male one day after YFP female cross YFP male mating (Figure 5.27). The YFP female mice were sacrificed at E14 for amniotic fluid collection and cell sorting. Around 1.5% of YFP+/Lin-/Ckit+ fresh AFS cells were isolated for fetal injection. A total of 100000 YFP+/Lin-/Ckit+ AFS cells were obtained for injection into ten pups. These cells were cultured in HSC conditioning medium with cytokines overnight. HIV lentivirus vector containing the UCO-E promoter driving the luciferase transgene was used to infect the cells for 24 hours at an MOI=50. This was the same viral vector as used for experiments described in Chapter 5.2. The next day I prepared 10000 cells in 20μl PBS per pup for fetal intraperitoneal injection at E14 carried out by Dr. Simon Waddington.

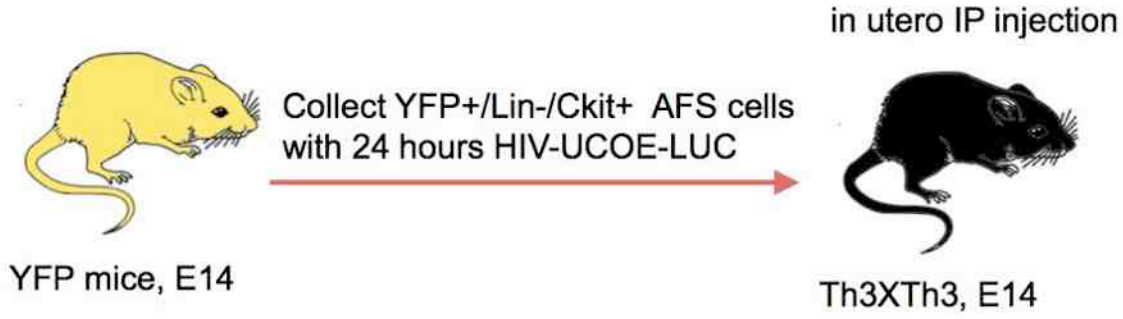


Figure 5.27 Experimental design of the *congenic in utero* transplantation with mice AFS cells. Lin-/Ckit+ amniotic fluid stem cells were collected and isolated from YFP transgenic mice. These cells were then transduced for 24 hours with HIV-UCOE luciferase vector. These transduced cells were injected intraperitoneally into *th3* thalassemia fetal mice (th3Xth3).

Three pups were delivered alive (30% survival rate, 3 out of 10 pups). Two were presenting an anaemic white appearance over the whole body and skin. The other one looked normal and larger in size with pink skin (Figure 5.28A). IVIS scanning for the luciferase activity was performed at Day 1 after delivery, which showed strong luciferase signal uptake among all three mice (Figure 5.28 B).

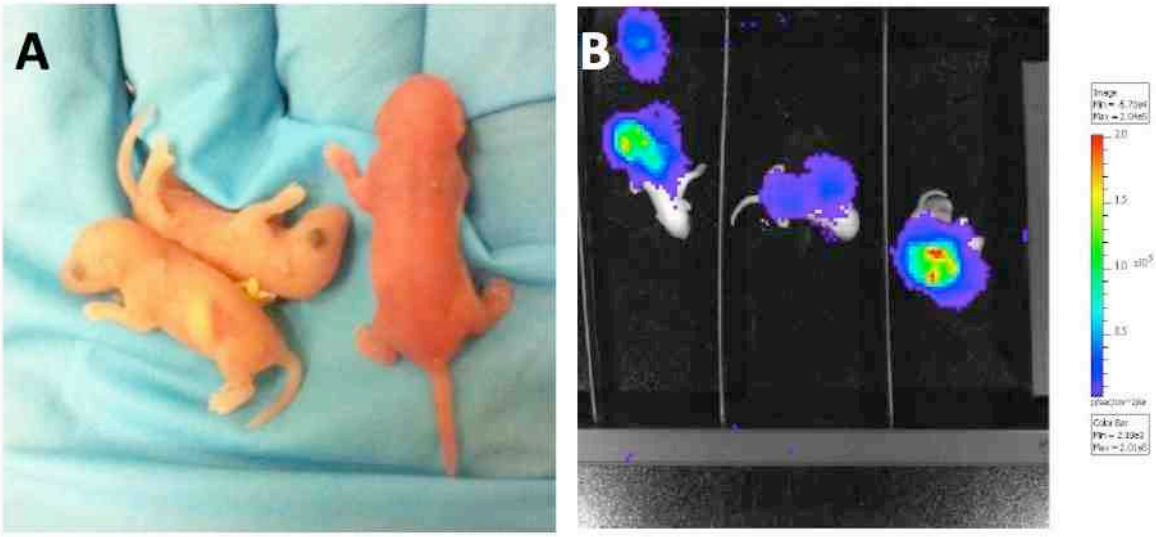


Figure 5.28 Three pups were born after *in utero* congenic transplantation with luciferase transduced YFP+/Lin-/Ckit+ amniotic fluid stem cells.
Those cells were from a *th3 thalassemia* heterozygous mother and father mating (A) Of the three pups born, two appeared to be anaemic and were smaller mice compared to the pink and larger pup. Genotyping confirmed the smaller ones were heterozygous *th3* and the larger one was wild type unaffected mouse. (B) IVIS image showing that all three injected pups had a positive luciferase signal in the body one day after birth.

Unfortunately, one of the pale heterozygous mice died spontaneously two days after birth. The growth velocity of the remaining two mice was very different from Day1 (Figure 5.29).

Due to poor nutrition and food intake of the pale mouse, this mouse became very sick and stopped growing and after two weeks of age it was decided to cull the pup. Blood and organs were taken for further engraftment analysis.

Figure 5.29 The growth curve of the thalassemia heterozygous mouse compared with wild type animal.
The red line presented the wild type while the blue line indicated the heterozygous thalassemia mouse.

5.4.5 Transplanted AFS cells showed low level of YFP+ cells in the blood of heterozygous mouse but without correction of the phenotype

In order to understand and confirm the genotype of the delivered mice as described in the previous section, blood smear and PCR on an ear clip marking were performed. Further peripheral blood was collected for whole blood analysis and counting, and to determine the cell engraftment level in the peripheral blood by using flow cytometry. The heterogeneous red blood cell morphology in the stained blood smears (Figure 5.30A and B), indicated the paler smaller animals were heterozygous *th3* thalassemia mice. On the other hand, the blood film from the pinker larger animal showed the homogeneous and uniform red blood cells indicating homozygous wild type (Figure 5.30).

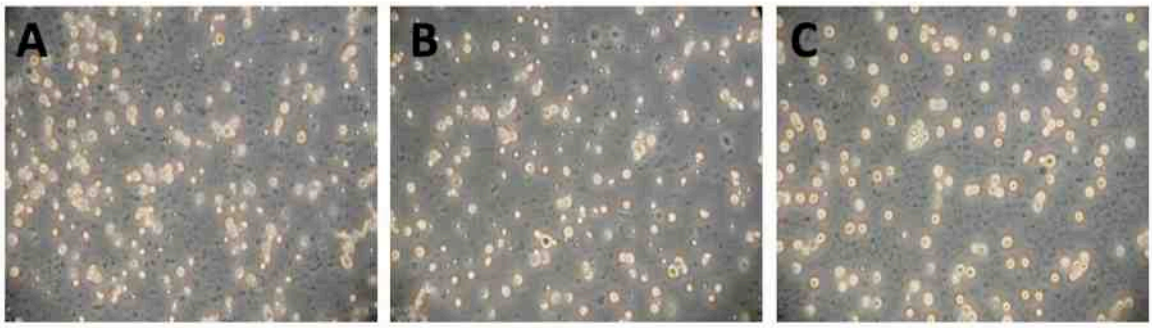


Figure 5.30 The blood smear of the three injected mice delivered by thalassemia dam. (A) and (B) blood films show a heterogeneous morphology of red blood cells with lots of fragments that indicate the peripheral blood of heterozygous animals. (C) The appearance of red blood cells was uniform and homogeneous showing the normal result of wild type mouse. 40X

The multiplex PCR gel (Figure 5.31), showed the genotyping of the two thalassaemia mouse pups that survived up to two weeks of age. The wild type mouse had only one thick band of mouse β-globin, but the heterozygous animal had two bands including one thinner mouse β-globin band and an HPRT band. The results were also compatible with blood smear study.

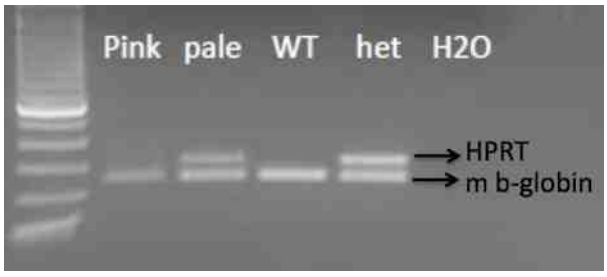


Figure 5.31 The PCR genotyping of the AFS cells transplanted mice after delivery. There was only one band of mouse β-globin in injected wild type mouse (pink). In injected heterozygous thalassemia mouse (pale), the PCR product had two bands (mouse β-globin and HPRT). WT: wild type control; het: heterozygous control. Ladder: 100bp

Peripheral blood was taken on Day 14 when I sacrificed the animals for haematological study. All the parameters including hematocrit (Hct), red blood cells (RBC), mean corpuscular volume (MCV), red cell distribution width (RDW), and hemoglobin (Hb) were compared with those from similar aged known heterozygous and wild type animals (Table 5.12 and Figure 5.32). The blood count in the injected thalassemia heterozygous mouse looked very similar compared with heterozygous uninjected control as published in the literature (Vadolas et al., 2005). The transplanted wild type mice also had showed the compatible haematologic data to the uninjected wild type mice.

Table 5.12 Haematologic values of injected wild type and heterozygous thalassemia mice alongside with uninjected control mice at the same age.
Het: heterozygous; WT: wild type; Hct: hematocrit; RBC: red blood cells count; MCV: mean corpuscular volume; RDW: red cell distribution width; Hb: hemoglobin. Uninjected-WT: data from uninjected colony; Uninjected-Het: from (Vadolas et al., 2005)

	In utero injected Heterozygous	Uninjected-Het	*In utero injected* Wild Type	Uninjected-WT
RBC (10^6/mm^3)	5.4	6.6	7.29	9.5
Hb (g/dl)	9.8	10.9	15.2	16.5
Hct (%)	27.3	30.5	43.2	45.7
MCV (fl)	44.9	43	56.2	51.1
RDW	48.2	46.1	19.3	17.4

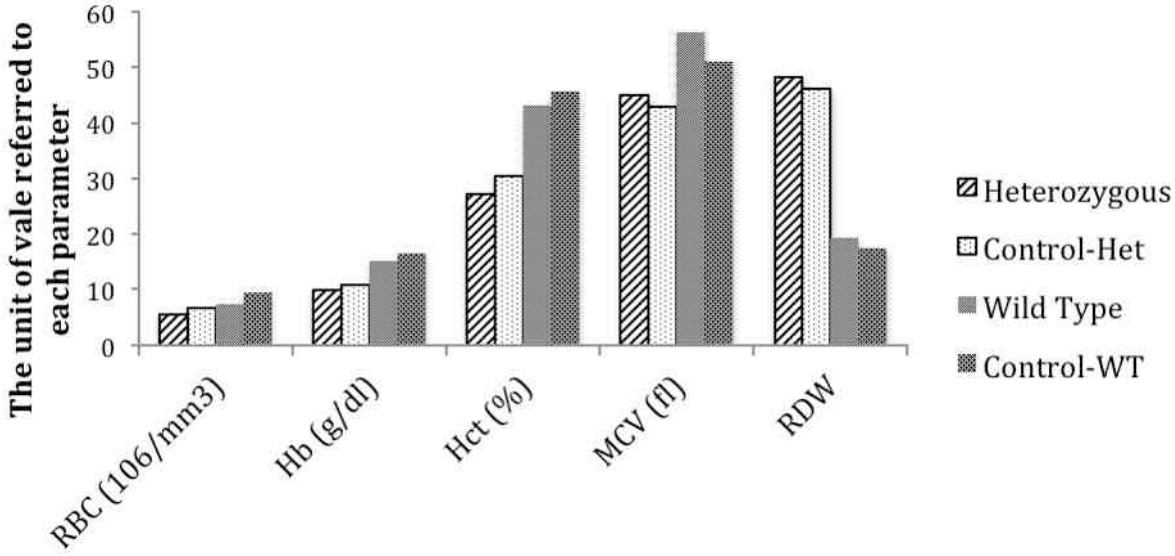


Figure 5.32 The histogram of all the parameters in transplanted animal either unaffected or heterozygous mouse compared with the uninjected control value.
Het: heterozygous; WT: wild type; Hct: hematocrit; RBC: red blood cells count; MCV: mean corpuscular volume; RDW: red cell distribution width; Hb: hemoglobin.

I performed the flow cytometry to analyze the blood engraftment status of these two animals on Day 14 after birth. The cells I injected were luciferase transduced YFP+/Lin-/Ckit+ amniotic fluid stem cells, so I chose the FITC/GFP channel to detect the YFP+ cells after gating the blood cells. Blood from uninjected wild type animals were used as negative control and from transgenic YFP+ mice were set as the positive control. The areas of P1, P2, and Q4 indicated the positive YFP cell counted

by flow cytometry (Figure 5.33). In positive control, 99.1% of the cells were inside the positive gate; but only 0.2% in negative control. The *in utero* transplanted wild type animal had only around 0.3% positive cells. The *in utero* injected heterozygous *th3* thalassemia mouse had slightly more YFP positive cells 1.2% than unaffected mouse.

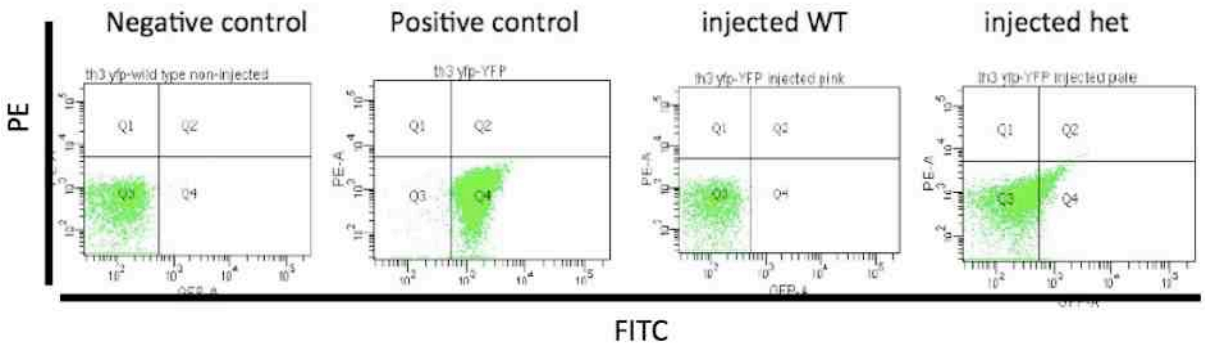


Figure 5-33. Flow cytometry studies of the transplanted animals versus control animals. The gate Q4 indicated the percentage of positive cells in each animal. Negative control: uninjected wild type (WT), positive control: uninjected YFP mouse

Interestingly, both congenic transplantation animals showed very low level of YFP+ cells in the peripheral blood compared to results in congenic and allogeneic transplanted Bl6 and CD1 mouse experiments. The same numbers of Lin-/Ckit+ YFP cells were injected *in utero* in each case. We hypothesized that heterozygous *th3* mice might have a larger niche available for engraftment of normal cells since they were anaemic. Because of the small number of pups surviving and examined it is not possible to determine whether there is a genuine difference in engraftment between the two experiments, or whether this occurred by chance alone. The poor clinical appearance and haematologic data of the *in utero* injected *th3* heterozygous mouse suggested that the level of cell engraftment was insufficient to correct the phenotype or to improve the severity of thalassemia.

In summary, the phenotype and genotype of transplanted heterozygous and wild type mice could be determined using blood smear and multiplex PCR. The clinical progress and data in this one pup did not suggest any benefit on thalassemia severity after prenatal congenic transplantation in this single experiment. The low level of transplanted cells in the peripheral blood of both *in utero* injected animals may be

responsible for the lack of a clear clinical effect, but further experiments are needed to investigate.

5.4.5 Summary and future plans

I had shown the first model of prenatal congenic transplantation by using transduced amniotic fluid stem cells in thalassemia mice. The aim of this project was trying to treat or cure the affected foetuses, or improve the phenotype of heterozygous mice. However, due to low pregnant rate and high mortality of in utero injection in this model, I can only show one dam with successful transplantation that had been transplanted with luciferase transduced YFP+ AFS cells. The initial in vivo luciferase imaging system detected the luciferase signal in the liver area among all three injected pups. Then the flow cytometry presented that the engraftment of peripheral blood looked very low in heterozygous mouse, but did not show any evidence of engraftment in unaffected mouse. During the 2010 to 2011, the thalassaemia colony in Imperial College had experienced pinworm and parvovirus infection, all the animals were transferred back to University College London in summer time of 2011. Currently the entire colony was moved to UCL with 5 breeding pairs and some individuals. Considering the severity of this disease model, we started to look another thalassemia model that demonstrated a humanized beta-thalassemia model published by US group (Huo et al., 2009b). The future collaboration with the new mouse would be very important to complete this project.

In previous sections and chapters, I had shown the potential of the amniotic fluid stem cells that could be used in the prenatal transplantation with good level of engraftment, especially for congenic (mice) or autologous (sheep and human) transplantation. Although the thalassemia model I tested in the final section was not working well, this concept using AFS cells for prenatal transplantation could be still applied in another animal model of thalassemia or other diseases in mice.

Chapter 6

Human amniotic fluid stem cells can be transduced and xenotransplanted *in utero* in mice

6 *Chapter 6: Human amniotic fluid stem cells can be transduced and xenotransplanted in utero in mice*

Introduction

Human amniotic fluid and the cells within the fluid has been used for prenatal diagnosis such as fetal karyotyping or DNA analysis for decades. The main aim of amniocentesis in fetal medicine is to diagnose chromosomal abnormalities or genetic defects prenatally and the reasons why couples decide to undergo this invasive procedure include elevated risk for Down's syndrome after screening tests, certain markers or structural fetal abnormalities noted on ultrasound which make chromosomal aneuploidy or genetic disease suspicious, and previous history of an affected child (Mujezinovic and Alfirevic, 2007). Due to the widespread availability of prenatal screening and diagnosis, amniocentesis is performed routinely in clinic and the amniotic fluid obtained not only facilitates cytogenetic and molecular diagnosis but also provides a promising source of MSCs or other pluripotent stem cells (Delo et al., 2006) without the ethical controversy of ESCs (Prusa and Hengstschlager, 2002).

It was confirmed in 2003 that MSCs exist in human AF (In 't Anker et al., 2003). MSCs can be retrieved from human second-trimester AF with good efficiency (around 0.9-1.5%) (Roubelakis et al., 2007) that is superior to human bone marrow and cord blood. Several pluripotent markers (Oct-4, NANOG and SSEA-4) are always positive on human AF MSCs (Roubelakis et al., 2007). Distinguishing them from other amniotic fluid cell types is simple because they adhere to plates during cultivation. These MSCs are pluripotent in differentiation. For example, they may differentiate *ex vivo* into cardiomyocyte-like cells and engraft in rat heart with cardiomyocyte-like characteristics (Zhao et al., 2005). Although the majority of stem cells from AF are more phenotypically similar to MSCs than to HSCs (Roubelakis et al., 2007), currently cells with haematopoietic potential have been identified in a subpopulation (Lin-/Ckit+ stem cells) of human and murine AF stem cells (Ditadi et al., 2009). In Ditadi's research, both human and murine Lin-/Ckit+ AF stem cells were capable of differentiating into erythroid, myeloid and lymphoid cells in vitro. Pre-clinical experiments on *in utero* transplantation (IUT) of human HSCs into animals has been ongoing for decades, in rodents by Pallavicini MG in 1992

(Pallavicini et al., 1992), in sheep by Zanjani ED in 1992 (Zanjani et al., 1992). In Pallavicini's research the transplanted human HSCs engrafted successfully and haematopoietic chimerism in rodents was demonstrated. In Zanjani's study the transplanted human HSCs engrafted in 13 out of 33 recipients and all 13 sheep were positive on bone marrow. Some engraftment was even confirmed to persist until 2 years after delivery.

Before considering IUT of AFS cells in humans it is important to observe transplanted human AFS cells in experimental animals by xenogenic IUT. Currently there are no studies using xenogenic IUT of stem cells from human AF, but there are several published studies of xenogenic IUT using human MSCs from other origins. IUT of human fetal blood MSCs in mice with osteogenesis imperfecta type III was demonstrated in 2008 (Guillot et al., 2008) and these MSCs were sourced from women undergoing surgical termination of pregnancy in the first trimester. In addition to the engraftment confirmed in recipient mice, this IUT achieved the therapeutic effect where there was a two-thirds reduction in long bone fractures.

Chou proved that the MSCs from human bone marrow engrafted successfully in wild type fetal mice after IUT (Chou et al., 2006b). At one month after delivery, the frequency of detection by PCR was 56% and the engraftment was seen in all 18 examined organs/tissues that originated from the three embryonic layers. The engraftment in blood became undetectable at 3 months after delivery and by 5 months all evidence of engraftment had disappeared.

Several IUTs of human stem cells (for example, HSCs and MSCs from fetal blood, MSCs from bone marrow) in mice have been published and successful engraftment has been confirmed. In this chapter, I aimed to demonstrate that human amniotic fluid stem cells could be isolated, cultured under MSC or HSC condition, transduced with lentivirus, expanded, and transplanted into fetal mice. The amniotic fluid was collected from second trimester amniocentesis or third trimester amniodrainage and a comparison between second or third trimester amniotic fluid sources was made..

Once the IUT of human AFS cells in mice is successful and human AFS cells are proved to be pluripotent *in vivo*, this experimental model may serve as a platform whereby the prerequisite for fetal therapies by autologous human AFS cells may move on.

6.1 Human amniotic fluid can be collected for research purposes from women undergoing second trimester amniocentesis or third trimester amniodrainage

At the start of this project I successfully applied for ethical approval from the Joint UCLH/UCL Committee on the Ethics of Human Research to collect human amniotic fluid for research purposes, from women undergoing amniocentesis or amniodrainage for clinical purposes (REC Ref 08/H0714/87). All the samples were collected in the Fetal Medicine Unit at the EGA and Obstetric Wing, UCLH once women had given signed informed consent. Trained physicians carefully consulted pregnant women individually while they were waiting for their FMU appointment by providing them with a patient information leaflet. Before performing the procedure (amniocentesis or amniodrainage) women were asked whether they would agree to use the sample for research purposes after clinical work had been completed or on any surplus fluid to clinical use.

From February 2009 to April 2011 (Table 6.1), I collected 47 cases of human amniotic fluid including 33 cases of second trimester amniocentesis (15 to 27 weeks of gestation), 11 cases of third trimester amniodrainage (28 to 34 weeks of gestation), and three cases of term pregnancy (37 to 39 weeks of gestation) amniotic fluid which were collected during Caesarean section and cultured by another research group. All fluid samples were sent to The Doctor's Laboratory (TDL, Whitfield Street, London) for prenatal genetic diagnosis and traditional chromosome study. Once a fetal karyotype or genetic diagnosis had been issued by the lab, TDL informed me so that I could collect the harvested or cultured amniotic fluid cells prepared in their laboratory. The methodology of culturing these two kinds of cells was described in the chapter 2. Fresh amniotic fluid was only available if the amniocentesis was performed after 16 weeks of gestation when surplus 1-2ml could be collected over and above the usual collection of 15ml for genetic testing.

Table 6.1 Summary of the cases of human amniotic fluid collection.
Down's risk: increased risk from screening test; maternal request: mother requested amniocentesis in the absence of an increased risk for Down's syndrome from screening tests; ultrasound finding: abnormal findings on ultrasound increased the risk of Down's syndrome or a genetic disease; polyhydramnios: amniodrainage performed to reduce amniotic fluid volume.

ID	Gestational age (week)	Maternal age (years)	Reason	Procedure date
H001	17	37	Down's risk	05/02/2009
H002	17	37	Down's risk	20/02/2009
H003	18	38	Down's risk	20/02/2009
H004	17	39	Down's risk	11/03/2009
H005	18	18	Down's risk	11/03/2009
H006	17	34	Down's risk	25/03/2009
H007	15	34	Down's risk	02/04/2009
H008	15	23	Down's risk	30/04/2009
H009	15	45	maternal request	30/04/2009
H010	23	38	maternal request	06/05/2009
H011	16	40	maternal request	06/05/2009
H012	16	39	maternal request	11/06/2009
H013	15	35	Down's risk	12/06/2009
H014	18	32	Down's risk	12/06/2009
H015	19	26	Down's risk	25/06/2009
H016	24	32	polyhydramnios	22/07/2009
H017	24	32	polyhydramnios	29/07/2009
H018	20	26	ultrasound finding	02/09/2009
H019	28	37	polyhydramnios	08/09/2009
H020	29	37	polyhydramnios	15/09/2009
H021	20	33	ultrasound finding	07/10/2009
H022	23	33	ultrasound finding	07/10/2009
H023	15	43	Maternal request	04/11/2009
H024	15	36	Down's risk	04/11/2009
H025	28	38	polyhydramnios	06/11/2009
H026	16	38	ultrasound finding	20/01/2010
H027	18	31	Down's risk	10/02/2010
H028	28	25	polyhydramnios	10/02/2010
H029	15	37	maternal request	07/04/2010
H030	15	23	Down's risk	28/04/2010
H031	22	21	ultrasound finding	28/04/2010
H032	16	40	Down's risk	05/05/2010
H033	16	37	Down's risk	12/05/2010
H034	17	26	Down's risk	12/05/2010
H035	30	33	polyhydramnios	26/05/2010
H036	23	26	ultrasound finding	09/06/2010
H037	19	40	polyhydramnios	16/06/2010
H038	39	36	term	01/06/2010
H039	37	32	term	01/06/2010
H040	38	28	term	01/06/2010
H041	19	32	ultrasound finding	13/08/2010
H042	34	44	polyhydramnios	22/09/2010

H043	15	26	Down's risk	02/11/2010
H044	18	37	Down's risk	10/11/2010
H050	31	33	polyhydramnios	07/03/2011
H051	34	36	polyhydramnios	29/03/2011
H052	20	19	ultrasound finding	06/04/2011

The main reason for amniocentesis or amniodrainage was high risk screening results (19 out of total 47 cases, 40.43%, Table 6.2) which included either first trimester combined test or second trimester Down syndrome screening tests such as the quadruple or Integrated tests. Usually women having the combined screening test opted to have a chorionic villus sampling test but occasionally they decided to wait until 15 weeks for an amniocentesis. There were 8 cases (17.02%) of amniocentesis performed because of an ultrasound finding including congenital heart disease, cleft lip/palate and limb abnormalities. Two women chose to have amniocentesis electively because of a previous Down's syndrome birth. There were another 4 pregnant women (8.51%) with low risk for Down's syndrome on screening who requested invasive procedure electively (Table 6.2, and Figure 6.1). For the third trimester pregnancy, 11 cases (23.4%) were collected due to polyhydramnios because of fetal anomaly or twin to twin transfusion syndrome.

Table 6.2 Summary of the reasons for invasive amniocentesis and amniodrainage.

Reason for the procedure	Number	%
Increased risk of Down's syndrome on screening test	19	40.4
Abnormal fetal ultrasound finding	8	17.0
Previous Down's, maternal request	2	4.3
Maternal request	4	8.51
Polyhydramnios (3nd trimester)	11	23.4
Term (3rd trimester)	3	6.4
Total	47	100.0

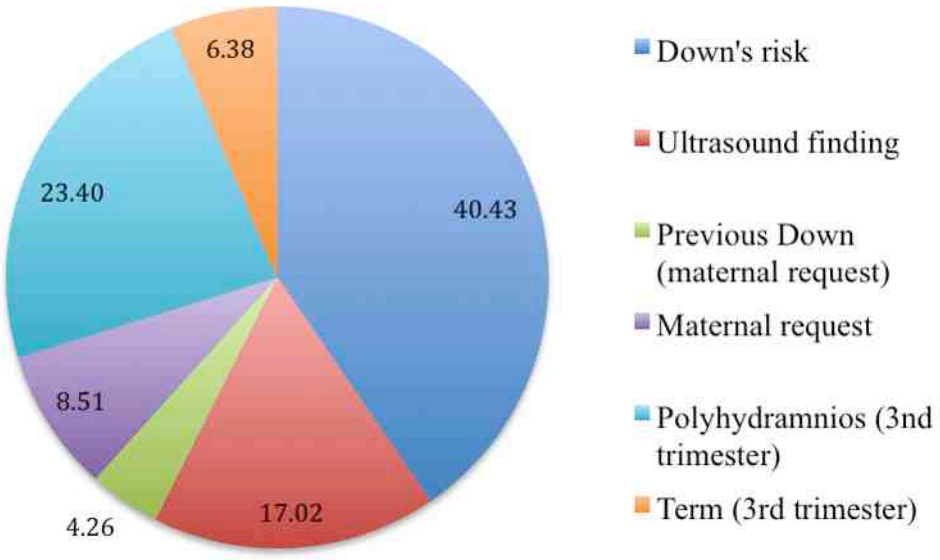


Figure 6.1 The distribution of the different reasons for either amniocentesis or amniodrainage. (%)

There was no statistically significant difference in maternal age between women having second or third trimester amniocentesis/amniodrainage (Table 6.3). Second trimester samples were all sampled within 15 to 27 weeks, compared with third trimester (28 to 34 weeks). Figure 6.2 showed the distribution of amniotic fluid case numbers in different gestational weeks. The mean gestational age of amniocentesis was (17.55 ± 2.53), which was significantly earlier than the mean gestational age of amniodrainage (28.09 ± 4.46) (Table 6.3).

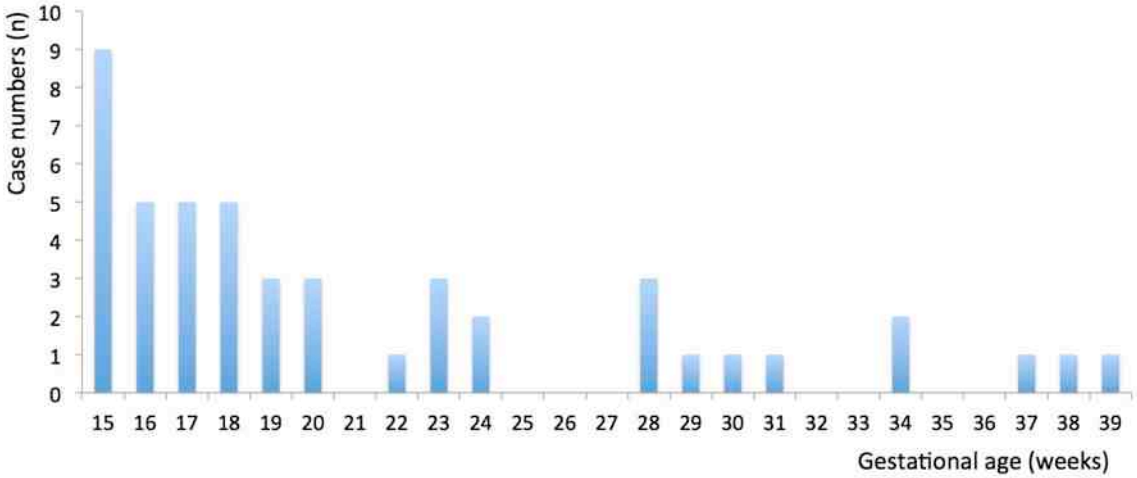


Figure 6.2 The distribution of amniotic fluid case numbers in different gestational weeks.

Table 6.3 Comparison of the maternal age and gestational age between second and third trimester invasive procedures.

	Second trimester amniocentesis (N=33)	Third trimester amniodrainage (N=11)
Mean maternal age (year)	32.67 ± 7.13	35.18 ± 5.00
Mean gestational age (week)	17.55 ± 2.53	31.09 ± 4.46

In summary, human amniotic fluid could be collected in the hospital from women undergoing amniocentesis or amniodrainage. The main reason for amniocentesis was an increased risk of Down's syndrome on screening, followed by abnormal ultrasound finding and maternal request. There was no difference in the maternal age of women undergoing amniocentesis and amniodrainage.

6.2 Human amniotic fluid Ckit+ cells from second or third trimester gestations can be isolated and cultured in adherent plates

The standard methods of isolation and culturing human Ckit+ AF cells have been published (De Coppi et al., 2007a). In the beginning of this project, I isolated the Ckit+ cells from freshly collected amniotic fluid or from cultured unselected cells. Then I seeded the cells in adherent plates with mesenchymal stem cell conditioning medium to show that I could grow and maintain the human amniotic fluid cells in culture.

The percentage of Ckit+ cells was studied in cases of amniocentesis from second or third trimester of pregnancy (n=6 per group, total=12) using flow cytometry. The six cases from second trimester were all collected freshly and with harvested, cultured cells provided from TDL (all three types of cells: fresh, cultured, or harvested as mentioned in Chapter 2). The average gestational age was these 6 second-trimester amniotic fluid samples were 18.1 weeks. Another 6 samples were collected from third trimester amniodrainage had either fresh or fresh frozen (fresh cells were frozen down immediately after collection and thaw again few months later) cell types. The average gestational age was 31.4 weeks.

Figure 6.3 and Table 6.4 show that, in samples the same woman, the percentage of Ckit+ cells was higher in fresh amniotic fluid (5.2%), before any culturing than in harvested (1.2%) or cultured (1.6%) Ckit+ cells ($p<0.05$). There was also a higher

Ckit+ percentage in second trimester AF when compared to third trimester AF, whether fresh (1.5%) or fresh frozen (all cells from amniotic fluid samples without selection) (1.2%) AF cells cultured in adherent plates (p<0.05, ANOVA test).

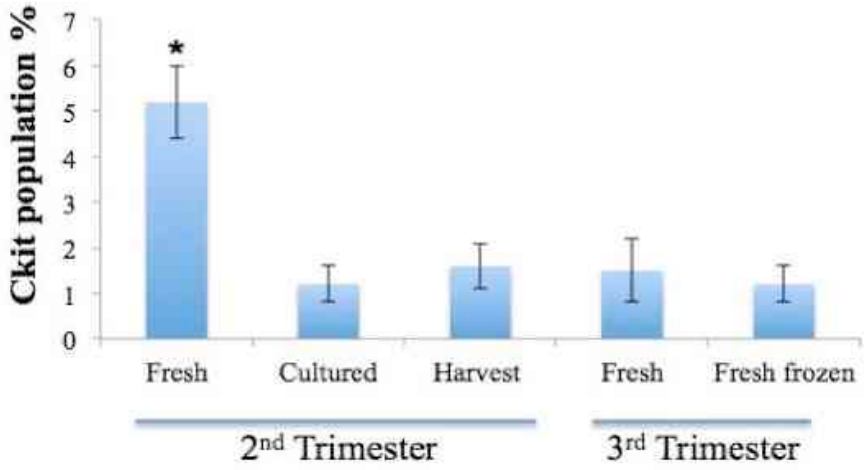


Figure 6.3 The percentage of Ckit+ cells in AF samples from second or third trimester amniotic fluid handled in different ways.
The percentage of Ckit+ cells in freshly isolated second trimester AF was highest when compared to other handling methods or third trimester sourced AF. (*p<0.05, error bar: standard deviation)

Table 6.4 Characteristics of human amniotic fluid stem cells derived from second or third trimester amniotic fluid samples.

	2nd trimester (n=6, 16-19weeks)			3rd trimester (n=6, 28-34 weeks)	
	Fresh	Cultured	Harvest	Fresh	Fresh frozen
Cell size (um)	12.1±3.4	15.8±2.6	15.2±4.1	15.6±2.9	16.1±3.8
Cell density (cell/ml)	68000	-	-	24000	-
Doubling time (hours)	30±4.9	36±4.6	37±4.7	35±2.7	38±5.3
Ckit+ population (%)	5.2±0.9	1.2±0.4	1.6±0.4	1.5±0.6	1.2±0.4

The cell size was measured as described in the methods section. I found freshly isolated second trimester Ckit+ cells had the smallest diameter, compared to other type of cells isolated from second or third trimester AF (Table 6.4, and Figure 6.4, p<0.05, ANOVA). The doubling time of cell growth in adherent plates was calculated for all Ckit+ cells types and results indicated that freshly isolated Ckit+ cells from second trimester AF had the shortest doubling time, suggesting that these cells had the greater stem cell potential. (Table 6.4 and Figure 6.5, p<0.05, ANOVA)

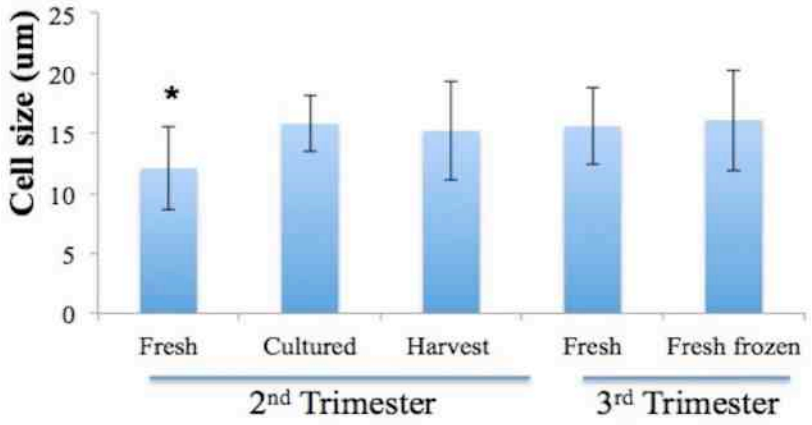


Figure 6.4 The cell size of Ckit+cells collected from second or third trimester amniotic fluid handled in different ways.
The cell size in freshly isolated second trimester sampled AF is smaller than other samples. (*p<0.05, error bar: standard deviation)

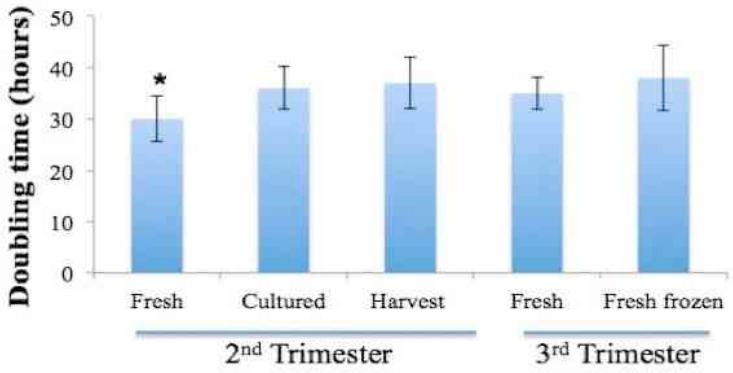


Figure 6.5 The Ckit+ cell doubling time of the different culturing samples from second or third trimester amniotic fluid.
The doubling time in fresh sample of 2nd trimester is faster than other samples. (*p<0.05, error bar: standard deviation)

The amount of fresh amniotic fluid available from second trimester samples was very little ranging from 1 to 5 ml, because of the need to make most of the sample available for clinical analysis purposes. Third trimester amniodrainage samples had volumes ranging from 500ml to 2400ml. The second trimester amniotic fluid was clear and yellow coloured. Some cases of amniodrainage were either blood-tinged or contained a lot of debris and dead cells. I calculated the cell density (N=6, each group) between second and third trimester amniotic fluid. The cell density in third trimester amniotic fluid from polyhydramios pregnancies was more dilute (24000 cells/ml) than second trimester amniocentesis (68000 cells/ml), but perhaps not as

much as expected since the volume of amniodrainage was nearly 100 times that of the volume of amniocentesis.

In summary, I showed that the character of Ckit+ AF cells cultured in adherent plates depended on the trimester of source and their handling after isolation. The percentage of Ckit+ out of total cells was approximately 5% in fresh second trimester AF, compared to approximately 1.5% of Ckit+ cells in other cell types including second trimester cultured or harvested AF cells and third trimester fresh or fresh frozen AF cells. After Ckit sorting, the second trimester fresh Ckit+ AF cells had the smallest cell size, shortest doubling time, and higher cell density than all other Ckit+ cell sources.

6.3 Human amniotic fluid Ckit+ cells express markers of pluripotent stem cells

In order to understand whether the Ckit+ cells derived from second and third trimester human amniotic fluid have stem cell pluripotency, I performed RT-PCR analysis for the expression of common pluripotent stem cells markers including Oct-4, Sox-3, Klf-4, c-myc, and NANOG. β-actin was used as internal control.

Three cases of each Ckit+ cell type were selected from the above experiments (second and third trimester fresh, harvested, cultured or fresh frozen). RNA was extracted from the cell pellets and reverse transcribed to cDNA before PCR was performed for pluripotent gene expression. Table 6.5 shows that pluripotent markers NANOG and Oct-4 were expressed in nearly all Ckit+ cell types. Only in one sample of cultured second trimester cells was expression of Oct-4 not detected. The c-myc positive expression rate in second trimester was 100% compared with 66.7% (two out of three) third trimester AF samples. Klf-4 expression was most common in second trimester fresh AF samples (2 out of 3). There was only one sample of fresh 2nd trimester Ckit+ AF cells that showed Sox-2 positive, while all other samples were negative.

Table 6.5 The summary of PCR results in different types of amniotic fluid (AF) Ckit+ cells (N=3, each arm).
The number indicated the positive samples out of 3.

(Number of positive samples)	**2nd trimester Ckit+ AF cells (n=3)**			**3rd trimester Ckit+ AF cells (n=3)**	
	Fresh	Cultured	Harvest	Fresh	Fresh frozen
NANOG	3	3	3	3	3
Oct-4	3	2	3	3	3
c-myc	3	3	3	2	2
Sox-2	1	0	0	0	0
Klf-4	2	1	1	1	0
β-actin	3	3	3	3	3

In summary, the freshly isolated second trimester human amniotic fluid Ckit+ cells were most likely to express pluripotent stem cell markers.

6.4 Human amniotic fluid Ckit+ cells from the second or third trimester cultured in plates could be efficiently transduced with lentivirus encoding eGFP

In previous sections, I have shown that human amniotic fluid Ckit+ cells could be isolated using magnetic sorting and cultured under conditioning medium in adherent plates. Also, these cells expressed pluripotent stem cells markers. In general, the fresh amniotic fluid cells from second trimester showed more evidence of pluripotency compared to other kinds of Ckit+ cells. In this section, I used lentivirus SFFV-eGFP vector to test the viral transduction efficiency. This virus vector was the same one I used in Chapter 3 and 4.

The samples were taken from the same sources as the previous section that investigated for cell size and doubling time (n=6, each arm). Essentially, 100000 cells per 24 well non-culture dish were seeded with vector (MOI=50) for 48 hours transduction. Flow cytometry was performed to measure the percentage of GFP+ cells and the results are shown in Table 6.6. The mean transduction efficiency varied in second trimester cells from 58% in those that were freshly sourced to 56.5% in harvested cells, and 54.5% in cultured cells, but this difference was not significant. Transduction efficiency was lower in third trimester cells (45.5% and 44.5% in fresh and fresh frozen) that were significantly different to the transduction efficiency of fresh and fresh frozen second trimester sourced cells (ANOVA test, $p<0.05$).

Table 6.6 Viral vector transduction efficiency for human amniotic fluid stem cells from second and third trimester.

GFP+ 2nd trimester Ckit+ AF cells (% of total)				GFP+ 3rd trimester Ckit+ AF cells (% of total)		
Sample No.	Fresh	Cultured	Harvest	Sample No.	Fresh	Fresh frozen
1	63.0	60.0	58.0	1	43.0	47.0
2	54.0	49.0	44.0	2	46.0	51.0
3	55.0	61.0	63.0	3	55.0	47.0
4	68.0	66.0	59.0	4	36.0	39.0
5	42.0	46.0	49.0	5	51.0	44.0
6	66.0	57.0	54.0	6	42.0	39.0
Mean	58.0	56.5	54.5	Mean	45.5	44.5
SD	9.7	7.6	7.0	SD	6.8	4.8

In summary, human amniotic fluid Ckit+ cells either from second or third trimester could be transduced efficiently with lentivirus. In fresh cells, the transduction efficiency was higher in second trimester than third trimester.

6.5 Human amniotic fluid Ckit+ cells could be cultured, expanded and maintain their purity in suspension and form haematopoietic colonies

In this section I aimed to demonstrate the hematopoietic potential of human amniotic fluid Ckit+ cells. Ditadi et al. had previously shown that fresh uncultured human and mice AF Ckit+ cells could display haematopoietic stem cell functions either *in vivo* or *in vitro* (Ditadi et al., 2009). However, they could not show the ability to expand these cells *ex vivo* and maintain their haematopoietic stem cell function. I therefore investigated culture and expansion of these cells in different ways to see if haematopoietic potential could be demonstrated.

Results from the previous section suggested that the highest Ckit+ cells percentage (around 5%) was found in fresh human amniotic fluid stem cells from second trimester collection. I hypothesized that adherent culturing techniques would reduce the proportion of Ckit+ cells and could affect their ability to maintain haematopoietic stem cell characteristics after culture. Therefore I studied the percentage of Ckit+ cells before and after adherent culturing of cells taken from the same amniotic fluid sample in 4 cases from the second trimester of pregnancy. The Ckit+ percentage of fresh AF

cells was tested using flowcytometry immediately after collecting the fluid samples. Then I seeded the cells into petri dish for 2 weeks until the cells reached 70% confluence in 150mm culture plate. The percentage of Ckit+ cell after culturing was tested again. The Ckit+ population was significantly higher before culturing than after (5.5 % versus 1.5%, Figure 6.6, $p<0.05$) showing that culturing in adherent conditions reduced the Ckit+ population.

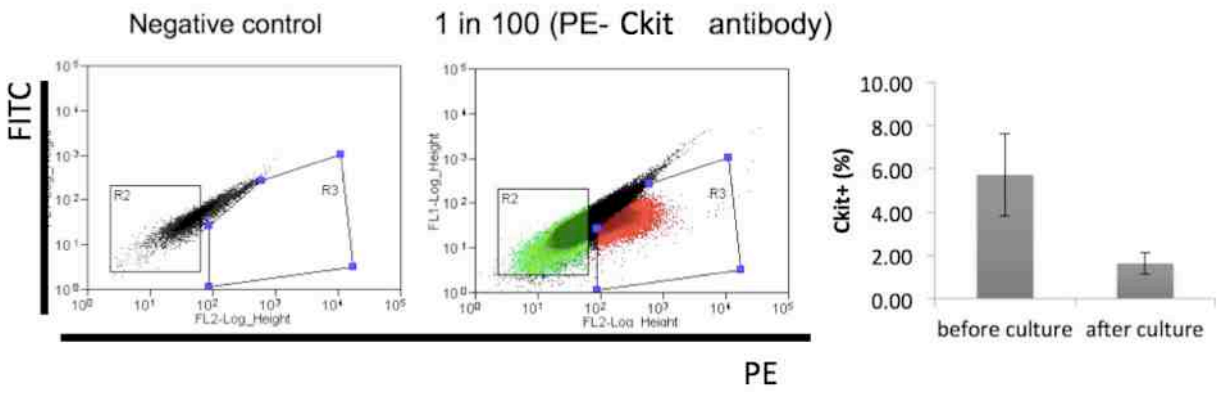


Figure 6.6 The effect of culturing in adherent plates on the percentage of Ckit+ cells in second trimester amniotic fluid samples (n = 4).
The flow cytometry images from one case show the negative control in the left panel, and fresh human amniotic fluid cells conjugated with CD117 antibody in the middle panel. Gate R3 indicates the Ckit+ population (PE positive), and Gate R2 indicated the most of dead cells. In the histogram of right panel, the percentage Ckit+ cells decreased from a mean of 5.5% down to 1.5% after culturing the cells in adherent plates ($p<0.05$, ANOVA test). Error bar: standard deviation.

As an alternative to adherent plates I investigated culturing in suspension with HSC conditioning medium containing all essential cytokines including IL3, IL6, TPO, SCF and FL in 24-well ultra low attachment plates, which is the standard culturing methods for cord blood CD34+ cells. I studied freshly isolated Ckit+ cells from second and third trimester amniotic fluid, and harvested / cultured AF Ckit+ cells that had been selected after clinical diagnostic testing was complete. All the concentration of cytokine and culturing condition were described in the Chapter 2. All the second trimester harvested or cultured cells (n=6 cases) ceased growth within 5 days. There was no evidence of cell expansion after 5 days under the liquid culture in low attachment plates when I studied second trimester harvested or cultured cells. These cells had been cultured in MSC conditions in the clinical diagnostic laboratory (TDL) before sending back to our stem cell lab. I also cultured fresh amniotic fluid stem cells

collected at third trimester (n=3 cases) in ultra low attachment plates but again there did not appear to be any growth after 7 days.

Unlike harvested or cultured AFS cell, second trimester fresh AFS cells could be cultured in liquid suspension as HSC condition. I tested 10 cases, and demonstrated *in vitro* expansion in four of these cases. I was able to demonstrate the cells growing in suspension until at least 42 days when I terminated the experiment to analyse the cells. The characteristics of the cases in which I successfully expanded cells are shown in Table 6.7. Cell expansion did not appear to be related to amniotic fluid volume, maternal age or gestational age. Contamination appeared (within 5 days of culturing) to be a problem in those cases that failed to expand. I trashed the cells as soon as contamination noted.

Fresh amniotic fluid contains many dead epithelial cells that could not be removed completely in the low attachment plates. In addition, I did not use antibiotics for these haematopoietic conditioning culture for human amniotic fluid stem cells. These could be the reasons why the liquid culturing got high percentage of contamination.

Table 6.7 The summary of the second trimester fresh amniotic fluid cells culturing in the HSC suspension condition.

Cells	Amniotic fluid	Volume (ml)	Gestational age (year)	Maternal age (year)	Culturing days	Outcome
H005	Clear	2	18	18	42	Expansion
H006	Clear	3	17	34	3	Contamination
H007	Clear	3	15	34	5	Contamination
H008	Clear	2	15	23	4	Contamination
H009	Clear	4	15	45	42	Expansion
H011	Clear	3	16	40	42	Expansion
H012	Clear	3	16	39	5	Contamination
H013	Clear	4	15	35	5	Contamination
H014	Clear	5	18	32	42	Expansion
H015	Clear	5	19	26	14	Not expansion

Cell growth and density increased over time from 14 days (Figure 6.7). The alive cell number was too few to be counted before 14 days as most of the cells in the culture were dead cells. All four cases had a similar growth curve from day 14 to 42 (Figure 6.7).

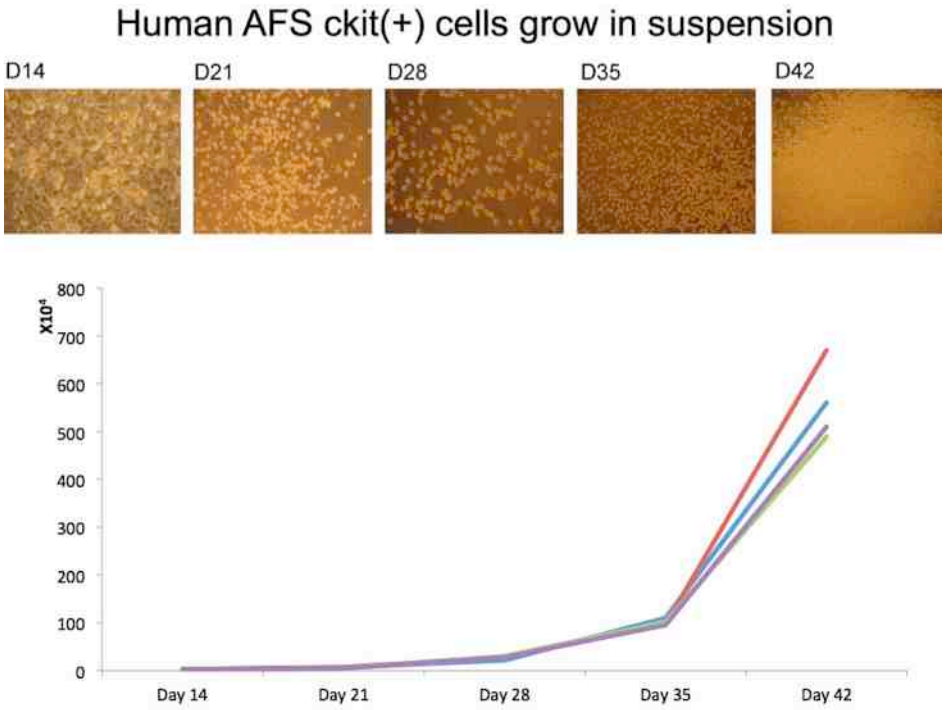


Figure 6.7 The *in vitro* expansion in liquid suspension conditions of human amniotic fluid Ckit+ cells from freshly collected second trimester amniotic fluid.
The upper panel shows the gross morphology and cell growth over time in one well of a 24 well low-attachment plate. In the lower panel, the growth curves of the four different samples are shown as different coloured lines. The growth curves followed a similar pattern.

To test for purity I performed experiments on all four samples that had been cultured in the suspension for 42 days. The fresh amniotic fluid sample contained about 5% of Ckit+ cells initially and after MACS sorting, I seeded only Ckit+ cells in the suspension condition. Even after 42 days culturing in suspension, samples in the low-attachment plates could maintain up to 95% Ckit+ cells (Figure 6.8).

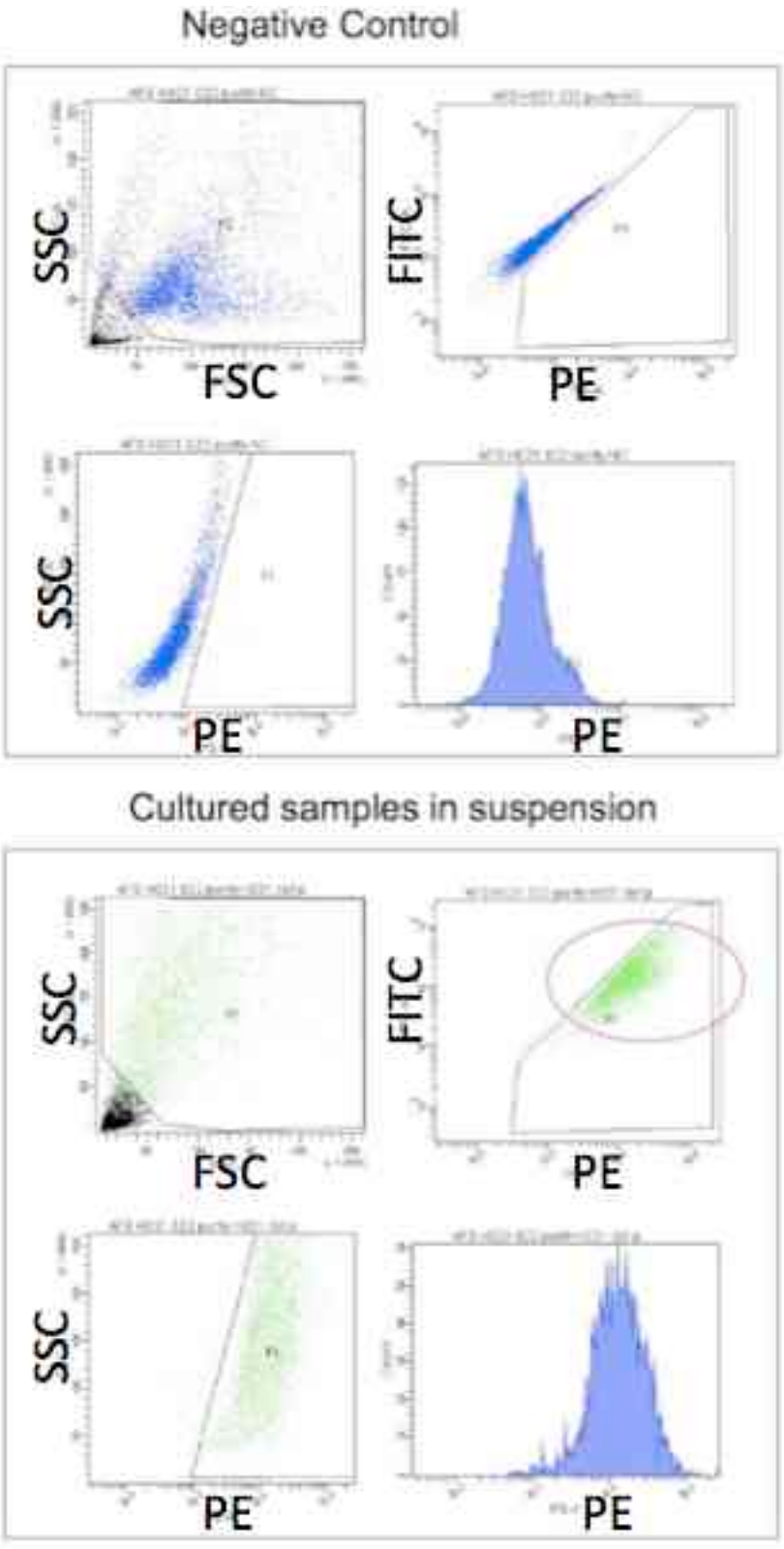


Figure 6.8 Flow cytometry analysis of Ckit+ cells after 42 days of culture in suspension in one case of second trimester amniotic fluid.
In the upper panel, cultured Ckit- cells (staining with PE) were used as negative control. The gate in the right upper corner (P3) indicated the Ckit+ percentage. In the lower panel, over 95% cells could be detected in the Ckit+ gate (pink circle).

To detect haematopoietic colonies the pure Ckit+ cells selected using FACS after culturing in suspension were seeded in semi-solid conditions. The colony-forming unit assay (StemCell Technologies, UK) was performed after 15 days culture and demonstrated all three lineages kinds of haematopoietic colonies (Figure 6.9).

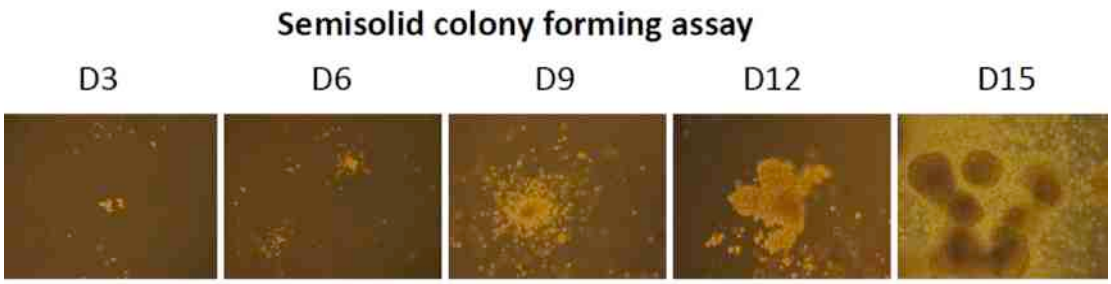


Figure 6.9 Formation of haematopoietic colonies was studied in Ckit+ cells cultured in suspension for 42 days from second trimester fresh amniotic fluid samples. Haematopoietic colonies could be observed from day 9 up to day 15.

In summary, I have demonstrated that the human amniotic fluid Ckit+ cells from fresh second trimester amniotic fluid samples could be cultured, expanded and maintain the purity in suspension and form haematopoietic colonies. The percentage of Ckit+ cells was reduced after culture for 2 weeks in adherent plates. In only 40% of second trimester amniotic fluid samples was it possible to expand Ckit+ cells in suspension. Characteristics of successfully expanded samples could maintain the Ckit+ purity up to 42 days.

6.6 Second trimester fresh human amniotic fluid Ckit+ stem cells can form the embryoid body

In order to study the pluripotent characteristics of human amniotic fluid Ckit+ cells, I performed another experiment to detect the embryoid body (EB) formation, following the EB formation protocol as described in the literature (Valli et al., 2009). The sorted Ckit+ cells (10,000 to 20,000 cells) from freshly collected second trimester amniotic fluid (n=2) were cultured in 96-well low-attachment plates to form embryoid bodies. The gross appearance of the EB from two different amniotic fluid samples (H005 and H009) after 7 days culturing is shown in Figure 6.10. In the left panel, the EB was floating in the liquid suspension. In the right panel, the EB was picked up from the

liquid suspension after 48 hours culturing and plated onto the gelatin-coating plate for preparation for the immuno-staining.

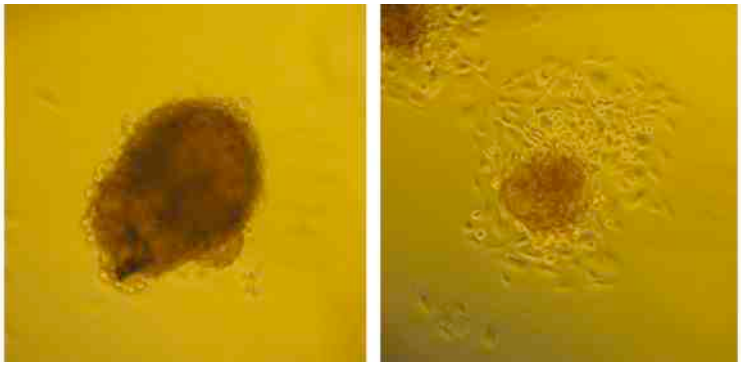


Figure 6.10 The grossly appearance of the embryoid body (EB) from two different samples. The human amniotic fluid Ckit+ cells were seeded using hanging drop or in the 96-well plate to generate the EB. In the left panel, one of the EB was floating on the liquid suspension. In the right panel, the EB was seeded onto the gelatin-coated plated for further immune-staining.

The EB was then stained using three germ cell layer antibodies (Figure 6.11) and shown to be positive for alpha fetoprotein (endoderm), alpha actinin (mesoderm), and beta-tubulin (ectoderm).

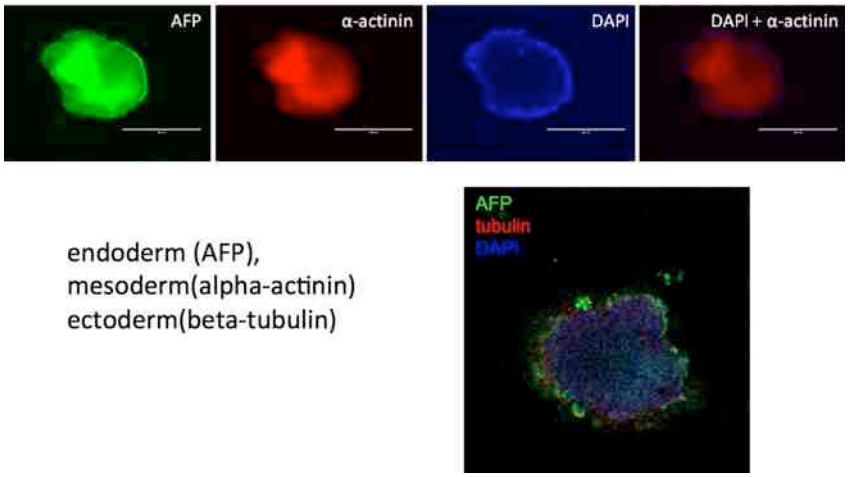


Figure 6.11 Immune-staining of an EB formed from Ckit+ cells isolated from freshly collected second trimester amniotic fluid.
All the three germ layer makers including alpha-fetaprotein (AFP), alpha-actinin and beta-tubulin were positive for EB staining. Bar: 100 um.

In summary, I have shown that fresh human second trimester amniotic fluid Ckit+ cells have similar pluripotential characteristics as embryonic stem cells. The EB could be formed easily following a standard protocol and positively stained with all three germ cell layer markers.

6.7 Prenatal xenotransplantation in a mouse cell therapy model by using human cultured or fresh frozen amniotic fluid stem cells

I conducted an experiment to demonstrate the possibility of using human AFS cells in prenatal cell therapy application by examining transplantation of human amniotic fluid stem cells into fetal mice. This is the first investigation to inject human AFS cells into fetal mice using intraperitoneal injection. Because of the difficulty coordinating collection of fresh second trimester amniotic fluid with pregnant mice at the appropriate gestation and additionally having a sufficiently large number of cells, I examined engraftment after *in utero* transplantation of cultured and fresh frozen AFS cells derived from third trimester pregnancies where a large volume of amniotic fluid had been collected from amniodrainage.

6.7.1 Animal procedure and experimental design

Two related experiments were performed using either cultured or fresh frozen human amniotic fluid Ckit+ cells. Cultured third trimester AF cells were available from a case of amniodrainage at 34 weeks of gestation (Table 6.8 and Figure 6.12). Fresh frozen third trimester AF cells were available from a case of amniodrainage at 35 weeks of gestation. After primary culturing or defrosting of the frozen cells, Ckit+ amniotic fluid cells were selected using Ckit microbead before injection.

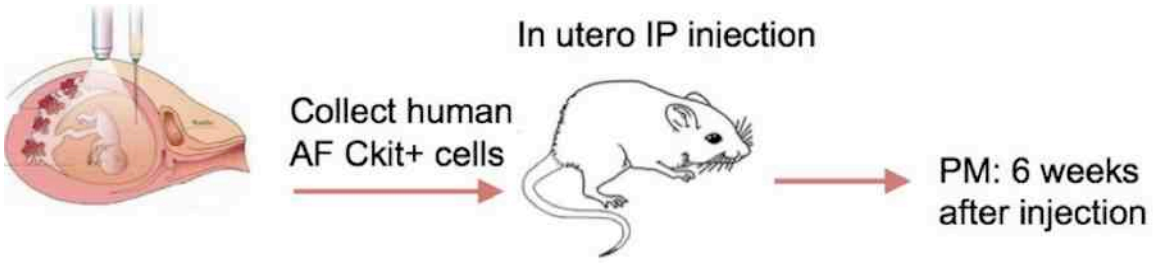


Figure 6.12 The experimental design of the prenatal xenotransplantation using third trimester human amniotic fluid stem cells.

The cultured AF cells were collected then seeded immediately after CKit selection of fresh amniotic fluid. All the operation procedures were performed as previously described. After laparotomy 12 fetuses were identified in the first mother (HM1), 12

fetuses in the second mother (HM2) and 15 fetuses in the third mother (HM3). The total 60,000 cultured human AFS cells (in 20µl PBS) were injected into the peritoneal cavity of each pup at E14.

Table 6.8 Summary of experiments: amniotic fluid samples and intrauterine transplantation (IUT).

	Cultured human AFS cells	Fresh frozen human AFS cells
Gestational age	34 weeks	35 weeks
Date of IUT	2/6/2011	7/7/2011
Gestational age of fetal mice	E14	E14
Injected AFS cell number	60,000 cells in 20µl PBS	10,000 cells in 20µl PBS
Number of pregnant mice	3 (HM1, HM2, HM3)	3 (HM4, HM5, HM6)
Numbers of recipient fetuses	39	45
Quantity of live progeny	21	19
Delivery rate in 2 arms	54 % (21/39)	42 % (19/45)

In the fresh frozen group, the AFS cells were frozen 3 months before use and then they were thawed for CKit sorting before IUT. Following the CKit sorting, the cell suspension was checked for its concentration (approximately 500 cells/µl). After laparotomy 13 fetuses were identified in the first mother mouse (HM4), 17 in the second mother (HM5) and 15 in the third mother (HM6). Because of the lower cell concentration, only 10,000 fresh frozen human AFS cells (in PBS 20µl) were injected into the peritoneal cavity of each pup.

Two animals from each group (HM1 and HM4) suffered complete miscarriages. The overall delivery rate of live pups was 54% (21/39, Table 6.8) after injection of cultured cells and 42% (19/45) after injection of fresh frozen cells. The average delivery rate of live pups in the two groups was 48% (40/84).
In summary, the third trimester human amniotic fluid stem cells could be cultured or frozen, sorted with Ckit and then injected *in utero* with reasonable survival at birth.

6.7.2 Characterisation of stem cells from human amniotic fluids

To understand and confirm that the third trimester amniotic fluid cells had stem cell characteristics and pluripotency, we analysed their expression of the stem cell markers before and after Ckit sorting using RT-PCR.

Except CKit- cells, the unsorted and CKit+ cells in both groups (cultured or fresh frozen cells) expressed Oct-4 (Figure 6.13). The CKit+ cells in both groups were positive for C-Myc and NANOG expression, but were negative for Klf4 expression. For the expression of Sox-2, all three cell types (Ckit-, Ckit+, and unsorted) in the cultured group were positive, but all the cells in the fresh frozen group were negative. No expression of Klf4 was seen in cells from any group.

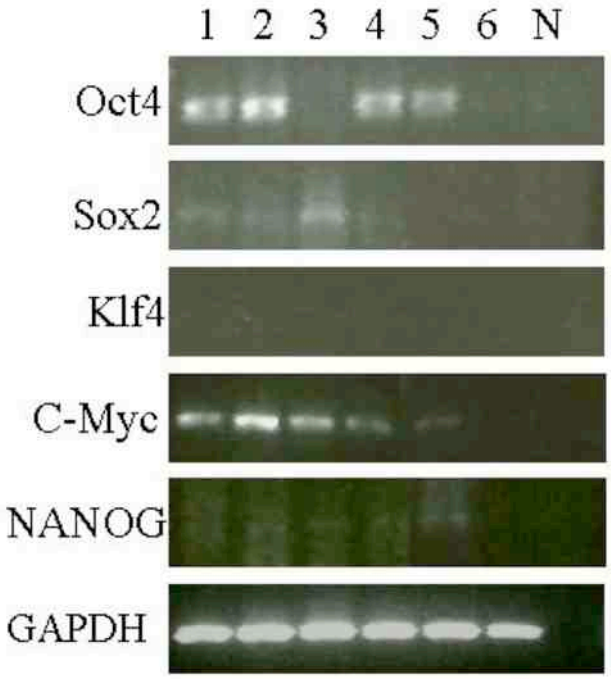


Figure 6.13 RT-PCR of RNA extracted from third trimester fresh frozen and cultured human amniotic fluid stem cells.
N: negative control (water). 1, 2, and 3 were from cultured amniotic fluid stem cells group; while 4, 5, and 6 were from fresh frozen amniotic fluid stem cells. 1 and 4 were unsorted cells, 2 and 5 were Ckit+. 3 and 6 were Ckit- cells.

In summary, the Ckit+ cells in third trimester fresh frozen or cultured AF expressed stem cell markers including Oct-4, C-Myc and NANOG; cultured cells also expressed Sox-2.

6.7.3 Human cells could be detected in the haematopoietic organs of injected mice at 4 and 6 weeks after birth by flow cytometry analysis

To investigate the engraftment status after xenotransplantation prenatally, I analysed for human cells in the peripheral blood, spleen, liver and bone marrow by using flow cytometry. The rate of positive cells was also compared between cultured or fresh frozen amniotic fluid stem cells. In order to observe the engraftment in recipient mice before scheduled post-mortem at 6 weeks of age, peripheral blood form the tail was obtained at 4 weeks after birth and flowcytometry analysis was performed. The blood samples were prepared with RBC lysis buffer as previously described.

Of the 21 recipient mice born from after injection of cultured human AFS cells, 4 were weakly positive for human β2 microglobulin on peripheral blood giving a positive rate of 19% (4/21) (Table 6.9 and Figure 6.14). Of the 19 recipient mice born after injection of fresh frozen human AFS cells, 8 were positive on peripheral blood giving a positive rate of 42% (8/19). The blood from uninjected mice were used as negative control.

At 6 weeks-old, all the transplanted animals were culled for final analysis, the haematopoietic organs were collected and prepared as a single cell suspension for flow cytometry analysis to detect human β2 microglobulin. Other organs were dissected for DNA extraction and long-term storage. Among the 21 recipient mice born in the group using cultured human AFS cells, 17 were sacrificed when aged 6 weeks. Of these sacrificed, 3 (17%) were weakly positive on blood and 4 (23%) were positive on liver (Table 6.9 and Figure 6.14). In all 17 mice there was no detectable human β2 microglobulin positive cells in the spleen and bone marrow.

Table 6.9 Positive rate (human β2 microglobulin) of each organs of flow cytometry analysis in both injection groups.
(Positive animal number / total animal number)

Transplanted cell types	4 week blood	6 week blood	Liver	Spleen	Bone marrow
Cultured AFS cells	19% (4/21)	17% (3/17)	23% (4/17)	0 (0/17)	0 (0/17)
Fresh frozen AFS cells	42% (8/19)	42% (6/14)	35% (5/14)	14% (2/14)	35% (5/14)

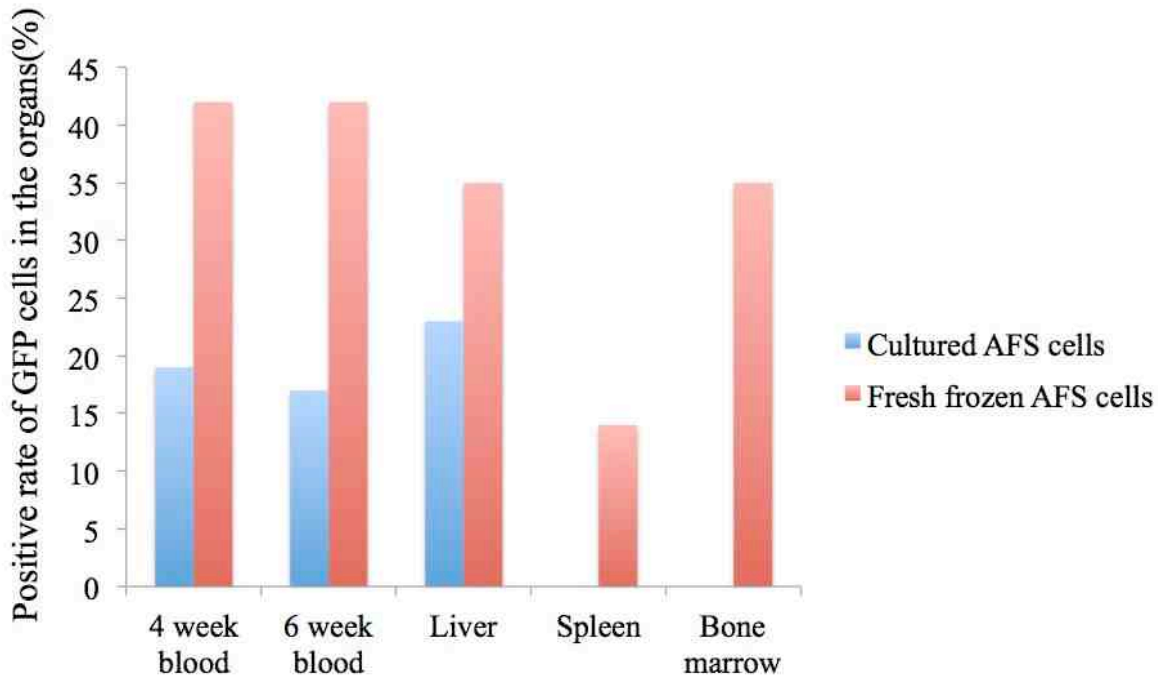


Figure 6.14 The histogram showed the positive rate of detectable GFP cells in the organs of transplanted mice with cultured AFS cells (blue) or fresh frozen AFS cells (red).

In mice receiving injection of fresh frozen AFS cells, 14 out of 19 animals were sacrificed at 6 weeks of age. Of these 14 mice, 6 (42%) had positive blood, 5 (35%) had positive liver, 2 (14%) had positive spleen and 5 (35%) had positive bone marrow samples (Table 6.9). In both groups, with the exception of two mice (HM3-5 and HM3-11), if the recipient mouse tested positive for engraftment in the liver, spleen or bone marrow, it tested positive in blood either 4 weeks or 6 weeks after birth (Table 6.10).

In summary, positive human cells could be detected in the peripheral blood and liver after *in utero* transplantation of cultured or fresh frozen AFS cell. Positive cells could only be found in the spleen or bone marrow of animals transplanted with fresh frozen AFS cells, despite the lower number of cells injected.

Table 6.10 The recipients mice detected positive for human β2 microglobulin by flow cytometry analysis.
The data indicated the percentage of FITC signal in positive channel, FITC labelled on anti-human β2 microglobulin. Written in red plus cross meant the positive results. *: Kept alive for future studies. NC: negative control.

Cultured AFS cells transplantation						Fresh frozen AFS cells transplantation					
Mice ID	4 week blood	6 week blood	Liver	Spleen	BM	Mice ID	4 week blood	6 week blood	Liver	Spleen	BM
HM2-3	+2.33	+2.43	1.01	0.48	0.15	HM5-1	+3.45	+4.14	+4.53	0.54	+2.21
HM2-4	+1.96	+2.12	+4.51	0.93	0.08	HM5-4	+4.22	+4.82	+4.21	0.58	+2.56
HM2-8	+1.72	*	*	*	*	HM5-5	+6.54	+6.34	0.67	0.13	+2.22
HM3-4	+2.16	+2.45	+3.98	0.35	0.45	HM5-11	+4.53	*	*	*	*
HM3-5	1.01	1.32	+4.83	0.87	0.34	HM5-12	+4.35	*	*	*	*
HM3-11	1.22	1.63	+2.63	0.03	0.07	HM6-3	+3.98	+4.58	+5.64	0.63	0.98
						HM6-5	+8.12	+ 9.7	+4.87	+2.32	+2.98
						HM6-6	+5.56	+6.34	+6.01	+3.01	+3.04
NC	0.67	0.94	0.76	0.12	0.44	NC	0.59	0.49	0.81	0.69	0.54
Mean	1.73	1.99	3.39	0.53	0.22	Mean	5.09	5.99	4.32	1.20	2.33
SD	0.53	0.50	1.57	0.37	0.17	SD	1.56	2.04	1.91	1.17	0.75

6.7.4 Engraftment rate of fresh frozen AFS cells is higher than cultured cells after in utero transplantation

In order to compare the engraftment status of these two injection groups, we analysed the flow cytometry data from all of the haematopoietic organs of the transplanted mice. I hypothesized that the cells that had been cultured would start to lose their haematopoietic stem cell potential before transplantation.

Positive cells were seen in the spleen and bone marrow on flow cytometry only in mice transplanted with fresh frozen human AFS cells (Table 6.9, Table 6.10). There were two mice in this group (HM6-5 and HM6-6) in which all samples analysed

(peripheral blood at 4 or 6 weeks, liver, spleen and BM) were positive. This is despite the far lower number of cells injected when compared with injection of cultured cells. Although the number of recipient mice in either group is few, it was clear that the positive rate of each item of FACS analysis in the group using fresh frozen human AFS cells is greater than its counterpart in the group using cultured human AFS cells (Table 6.9).

6.7.5 The level of engraftment after in utero transplantation of third trimester human amniotic fluid stem cells is higher when the AF is fresh frozen when compared to cultured AF

In the blood of recipient mice at 6 weeks after birth, we compared the flow cytometry data between 2 groups using FlowJo version 5.7.1 software. The range of positive channel was gated according to negative control and positive control. Of the positive recipient mice, the percentage of cells presenting FITC signal (labelled on anti-human β2 microglobulin) in positive channel in the group using fresh frozen human AFS cells was obviously greater than the counterpart in the group using cultured human AFS cells (Figure 6.15 and Table 6.10). The raw data and indicated mice ID were showed in the Table 6.10.

From the liver at 6 weeks of age (post-mortem) of the positive recipient mice, the percentage of cells presenting FITC signal (labelled on anti-human β2 microglobulin) in positive channel in the group using fresh frozen human AFS cells was also greater than the counterpart in the group using cultured human AFS cells (Figure 6.16 and Table 6.10).

In the spleen and bone marrow at 6 weeks of age, all the recipient mice in the group using cultured human AFS cells were negative by flow cytometry analysis (Figure 6.17 and Figure 6.18).

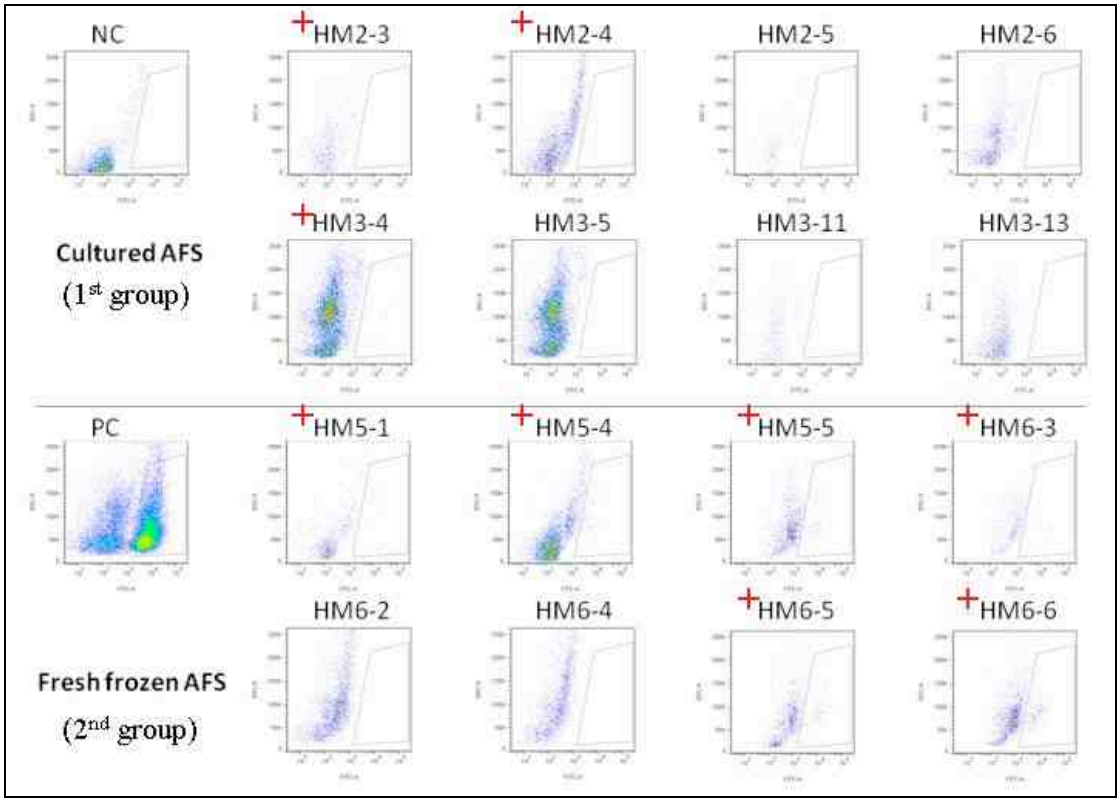


Figure 6.15 FACS analysis in the blood of recipient mice at 6 weeks after birth.
NC: negative control is the blood of an uninjected mouse. PC: positive control is human blood. +: positive for human β2 microglobulin by analysis. In comparison with the 2nd group, of the positive recipient mice, the percentage of cells in positive channel is obviously less in the 1st group. The positive cells were gated in the right hand side. X-axis: FITC, Y-axis: SSC.

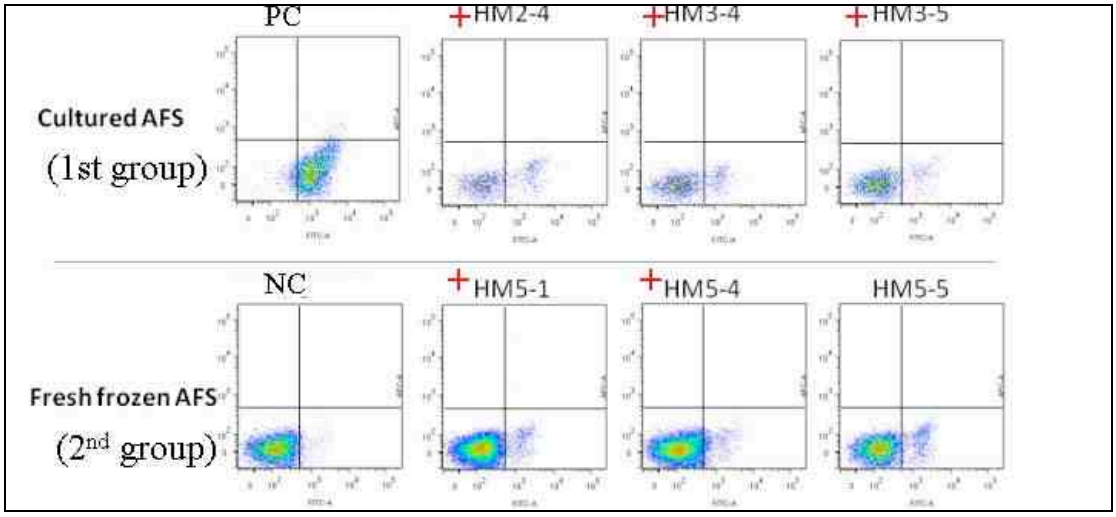


Figure 6.16 Flow cytometry analysis in the liver of recipient mice at 6 weeks after birth.
PC: positive control. NC: negative control. +: positive for human β2 microglobulin by analysis. The positive cells were in the right lower quarter. In comparison with the 1st group, of the positive recipient mice, the percentage of cells in positive channel is greater in the 2nd group. X-axis: FITC, Y-axis: PE.

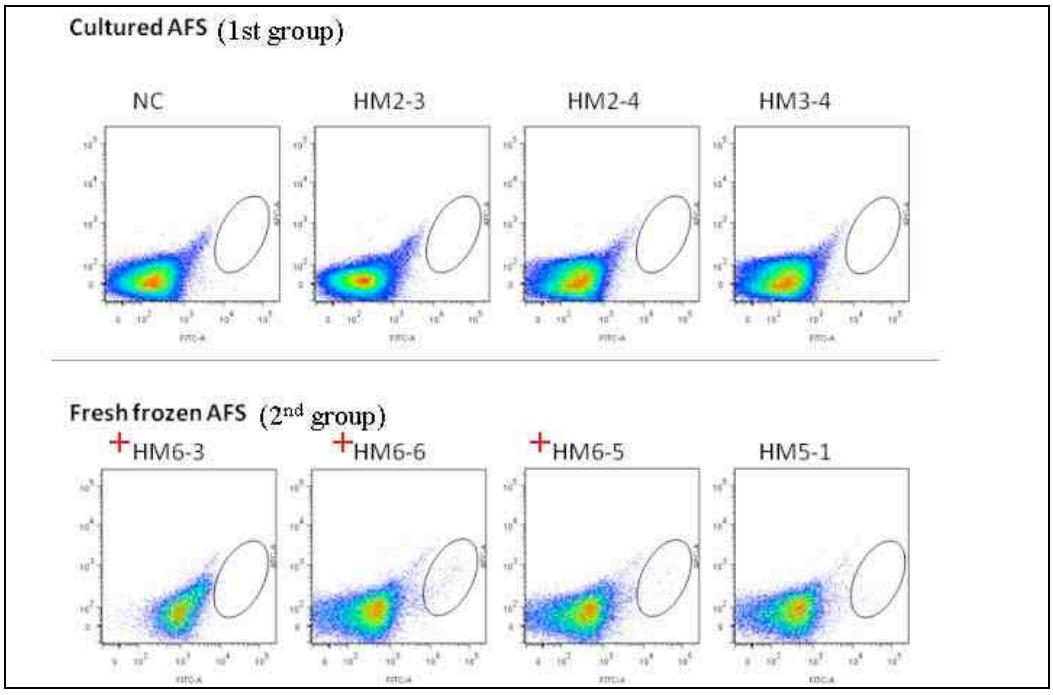


Figure 6.17 Flow cytometry analysis in the spleen of recipient mice at 6 weeks after birth. NC: negative control. +: positive for human β2 microglobulin by analysis. All the recipient mice in the 1st group were negative. The positive cells were gated in the eclipse area. X-axis: FITC, Y-axis: PE.

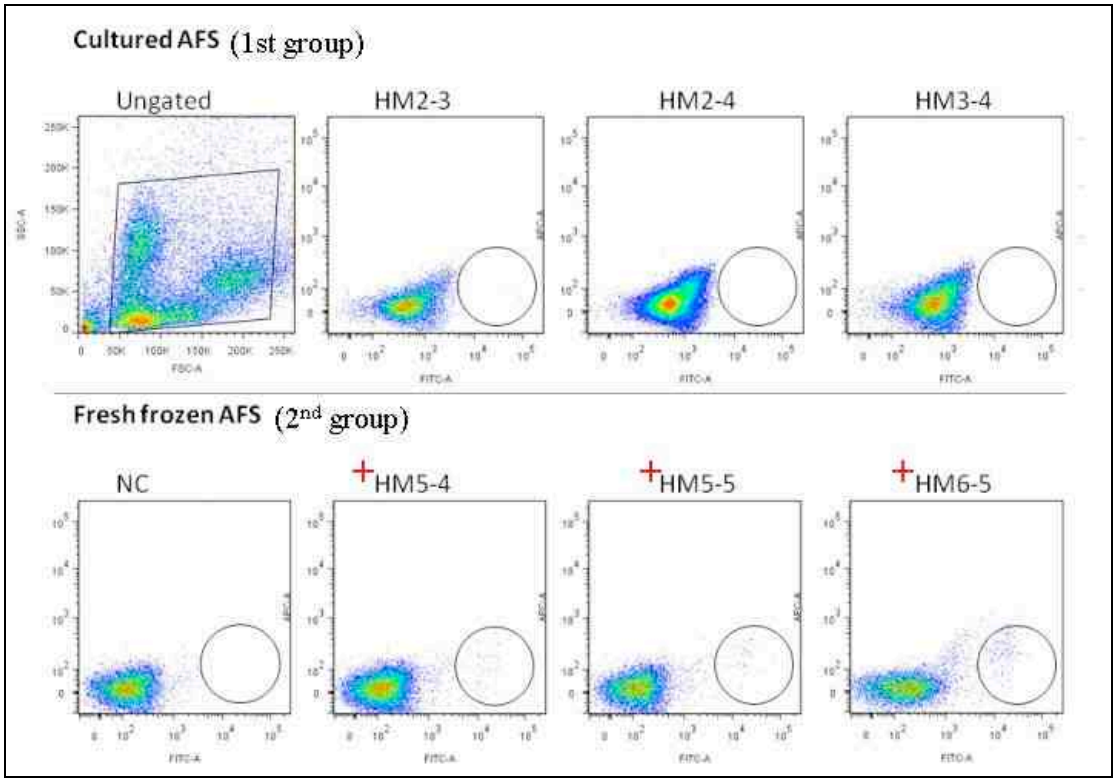


Figure 6.18 Flow cytometry analysis in the bone marrow of recipient mice at 6 weeks after birth. NC: negative control. +: positive for human β2 microglobulin by analysis. All the recipient mice in the 1st group were negative. The positive cells were gated in the eclipse area. X-axis: FITC, Y-axis: PE.

In order to compare between the 2 groups, we studied the recipient mice of which at least one item of flow cytometry analysis was positive and analysed the data of each item in both groups (Table 6.9). The mean level of positive signal by flow cytometry analysis in the second group is higher than its counterpart in the first group, for example, the mean (percentage of FITC signal in positive channel) of the blood at 6 weeks in the second group is 5.99 that is higher than 1.99 (its counterpart in the first group) (Table 6.10).

ANOVA test was used to calculate whether there was any statistically significant difference in the flow cytometry analysis between groups of mice transplanted with culture or fresh frozen human AFS cells. Statistically significant differences ($p \leq 0.05$) were noted on the blood at 4 weeks, the blood at 6 weeks and the bone marrow at 6 weeks (three of them showed $p<0.05$) (Figure 6.19).

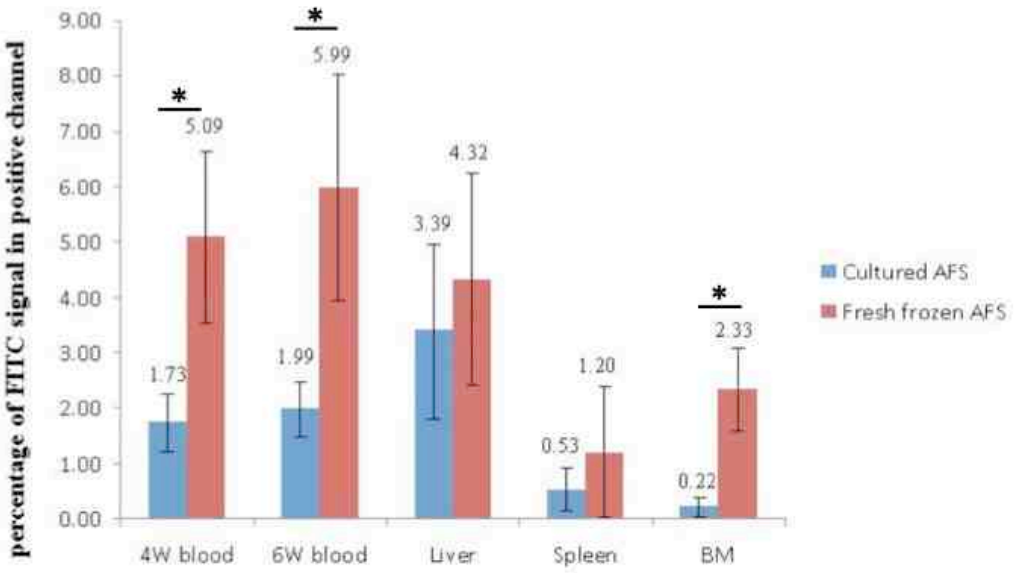


Figure 6.19 A comparison of the level of engraftment in the blood, liver, spleen and bone marrow after *in utero* transplantation of human third trimester fresh frozen or cultured cells in mice. BM: bone marrow. *:$p<0.05$.

In summary, there was a higher level of engraftment after *in utero* injection of fresh frozen when compared to cultured cells. This was despite the much lower cell number injected (10,000 compared to 60,000).

6.7.6 Positive human specific DNA could be detected in the liver of both injection groups by PCR

In the previous section, I showed that injected fresh or fresh frozen human cells could be detected in prenatally transplanted animals by flow cytometry, especially in the haematopoietic organs. I now investigated whether human DNA from injected human cells migrating to the somatic organs after transplantation could be detected using PCR.

I selected all the recipient mice of which at least one item of flow cytometry analysis was positive and detected the frequency of positive human cells in 8 organs/tissues by PCR. The primer I used was anti-human β2 microglobulin (sequence details showed in the material and methods). Among the 5 recipient mice in the group using cultured human AFS cells, human DNA could be detected in four animals (HM2-4, HM3-4, HM3-5, and HM3-11). Three recipient mice were positive in liver alone, the other one (HM2-4) was positive in liver and also weakly positive in the kidney (Figure 6.20, Table 6.11). DNA extracted from human blood was used as positive control.

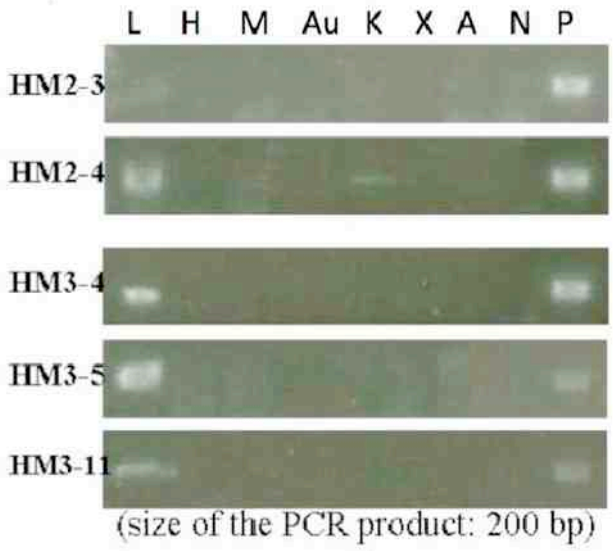


Figure 6.20 PCR results for human β2 microglobulin in the transplanted group using cultured human amniotic fluid cells.
One mouse was negative for all the organs. Liver was positive in four animals, and the kidney was positive in one animal. L: liver; H: heart; M: muscle; Au: lung; K: kidney; X: spleen; A: adrenal gland; N: negative control (water); P: positive control (human blood). Ladder: 100bp.

Among the six recipient mice in the group using fresh frozen human AFS cells for prenatal transplantation, the positive human cells were detected from five (HM5-1, HM5-4, HM6-3, HM6-5, and HM6-6). Two recipient mice were positive in the liver alone, the other two were positive in both liver and spleen, another one was positive in the liver and weakly positive in the adrenal gland (Table 6.11, and Figure 6.21).

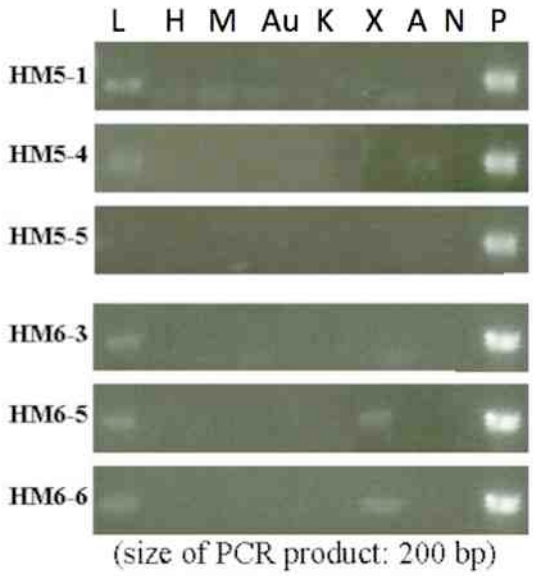


Figure 6.21 PCR results for human β2 microglobulin in the transplanted group using fresh frozen human amniotic fluid cells.
HM5-5 was negative for all the organs. Liver was positive in five animals, spleen was positive in two animals, L: liver; H: heart; M: muscle; Au: lung; K: kidney; X: spleen; A: adrenal gland; N: negative control (water); P: positive control.

A summary of the results is shown below (Table 6.11).

Table 6.11 Frequency of positive human DNA detected in the organs of mice injected *in utero,* using PCR to human β2 microglobulin.

Injected cell types (positive / total mice)	Liver	Heart	Muscle	Lung	Kidney	Spleen	Adrenal	Gonad
Cultured AFS cells	4/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5
Fresh frozen AFS cells	5/6	0/6	0/6	0/6	0/6	2/6	1/6	0/6

In summary, human DNA could be detected in both groups of prenatally transplanted animals by PCR, mainly in the liver. There did not appear to be any particular pattern but this could be due to the limited number of animals studied.

6.7.7 Bone marrow from transplanted mice can form haematopoietic colonies in a human cell Colony-Forming Unit (CFU) Assay

In order to further explore the haematopoietic potential of fresh frozen human AFS cells, we collected the bone marrow of six mice in the group injected with fresh frozen AFS cells, all of which had positive flow cytometry analysis in the blood at six weeks of age. We randomly assigned three of the six to be cultured in methylcellulose medium for human CFU and the other three were cultured in the methylcellulose medium for mouse CFU. After cultivation for seven to 14 days, colonies were present in all of the mouse CFU assays but there was only one human CFU assay in which colonies were present (Figure 6.22).

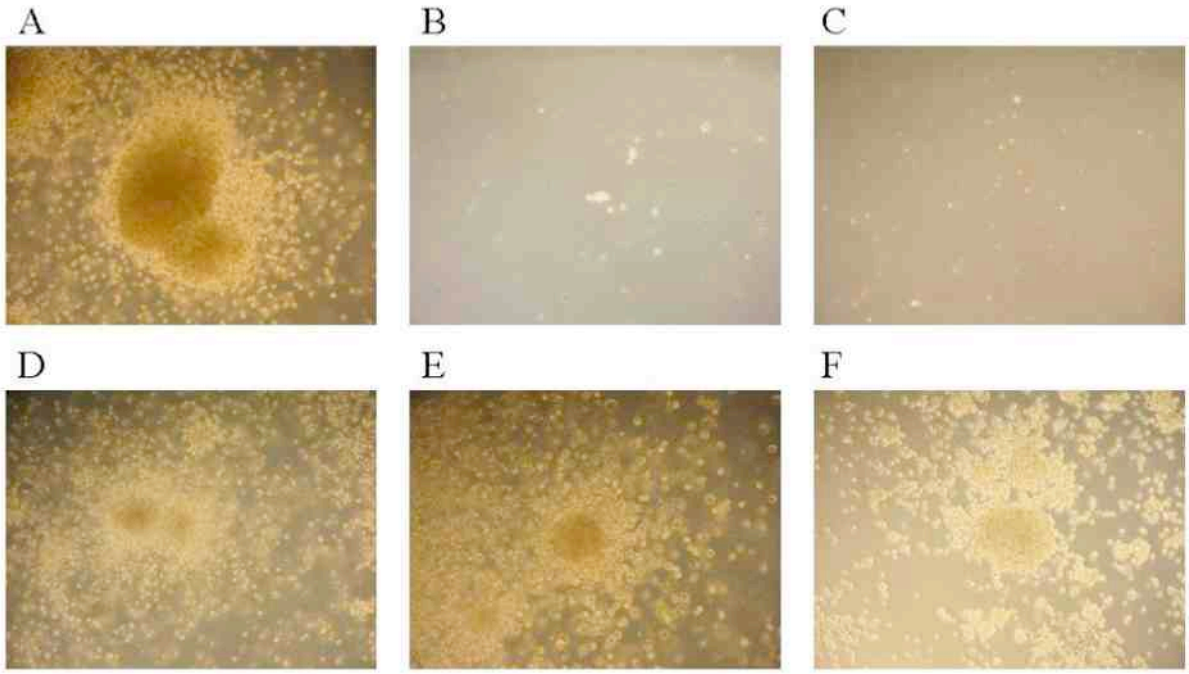


Figure 6.22 Bone marrow from the transplanted mice of the group using fresh frozen human AFS cells in CFU assay.
Panel A, B, and C: Human CFU assay with conditioning human media. Panel D, E, and F: Examples of mice CFU assay with mouse conditioning media. All of the mouse conditioning CFU assays showed the presence of haematopoietic colonies in mouse bone marrow cells. There was only one haematopoietic colony that could be found in the human conditioning media (Panel A).

Cells within the colonies in the methylcellulose medium for human CFU (HM6-3) and for mouse CFC (HM5-5) were also collected and DNA was extracted. PCR was performed to detect the amplified human DNA sequence (human β2 microglobulin). The electrophoresis of PCR products showed a positive signal in the extracted DNA

from the colonies in the medium for human CFU but negative in the extracted DNA from the colonies in the medium for mouse CFU (Figure 6.23).

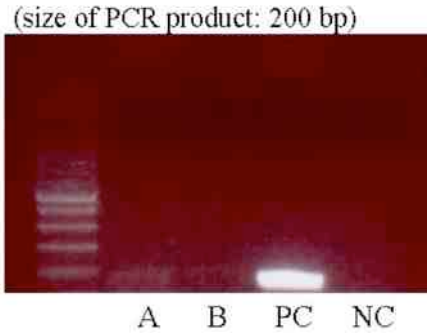


Figure 6.23 The PCR gel for human β2 microglobulin of bone marrow cells from the transplanted mouse. The DNA was extracted from the colonies growing in the semi-solid culture of CFU. A: the colony in the human condition media, HM6-3. B: the colony in the mice condition media of CFU, HM5-5. PC: positive control (human DNA extracted from blood), NC: negative control by using DNA of uninjected mouse. Ladder: 100bp

The finding suggested that the transplanted fresh frozen human AFS cells not only engrafted in the bone marrow of this recipient mouse (HM6-3) but also differentiated into haematopoietic progeny that were compatible with the methylcellulose medium in human CFU assay. Only one hematopoietic colony could be observed in Figure 6.22 (Panel A), the remaining bone marrow cells did not show any colonies in the human conditioning medium of CFU. Thus further studies with more animals will be needed to confirm this finding.

6.7.8 Immunofluorescence staining shows the presence of transplanted human cells in the liver that co-stain with antibodies to mouse albumin

The results in previous sections of PCR and flow cytometry and from other sheep and mouse experiments in this thesis show that the liver is an important site of engraftment after *in utero* transplantation. In this section, I used immunofluorescence for antibodies to human mitochondria to detect engraftment of transplanted human cells into the liver and other organs of prenatally injected mice (n=3, each arm).
A few GFP positively stained cells were identified in mouse liver (Figure 6.24, Panel A). I also performed immunofluorescence for albumin and could identify some positively stained cells (red fluorescent positive, Figure 6.24, Panel B). When images A and B were merged to detect human mitochondria and albumin co-staining (Figure 6.24, Panel D and F) these GFP+ signals were found among red fluorescent signals, showing the presence of albumin in these engrafted human cells. This finding

provides the evidence that the transplanted human AFS cells engrafted among the hepatocytes of mouse liver.

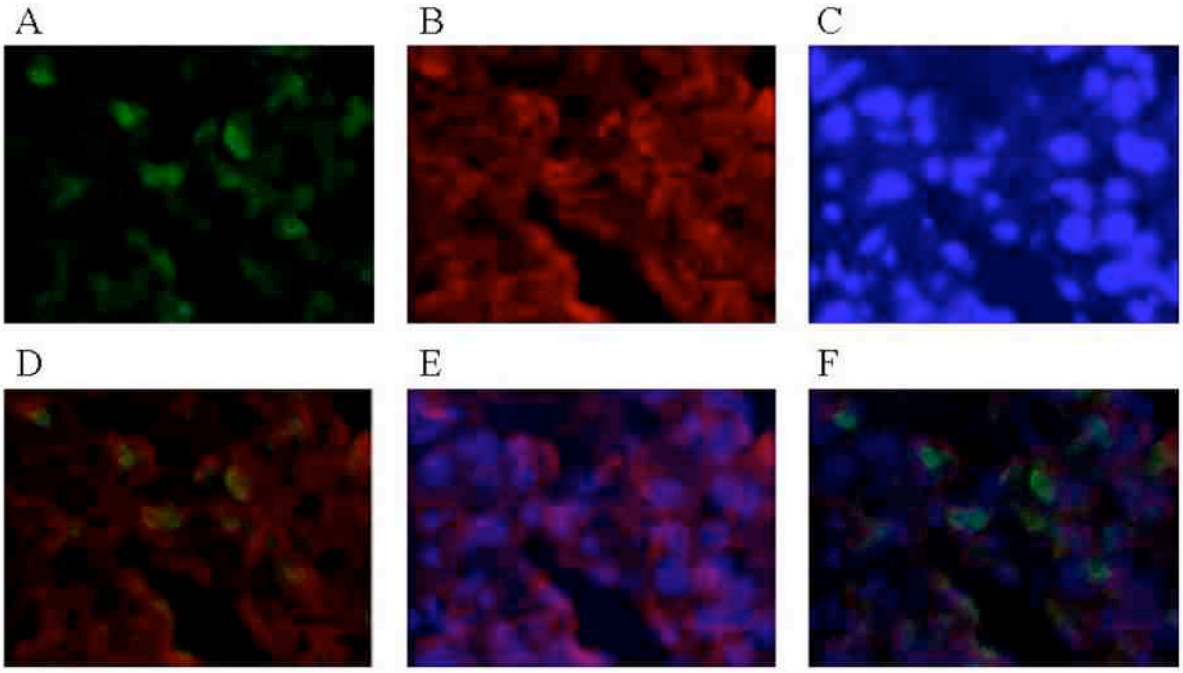


Figure 6.24 Immunofluorescence staining of the liver from a mouse (HM3-4) injected at E14 with fresh frozen AF cells.
(A) GFP+ cells stain positive for anti-human mitochondria showing these cells are of human origin; (B) Red fluorescent protein expressing cells stain positive for anti-mice albumin; (C) Blue stained cells are positive for DAPI. (D) A merged with B: some cells are co-stained with green and red fluorescent protein. (E) B merged with C. (F) All three colours images merged (A, B and C): A few cells co-stained positive for anti-human mitochondria and albumin.

In summary, the liver section from fresh frozen AFS cells transplanted mice showed double staining with anti-human mitochondrial antibody and anti-mice albumin. This finding suggested that the human cells we injected into the peritoneal cavity of the fetal mice had differentiated into liver cells.

Discussion

In this chapter, I carefully studied the characteristics of human amniotic fluid stem cells and their engraftment into fetal mice. Firstly, human amniotic fluid could be collected either from amniocentesis or amniodrainage at different gestational ages and human amniotic fluid stem cells could be isolated via standard MACS isolation into Ckit+ or Ckit- cells. The culturing method could be split into two distinct populations of cells, mesenchymal stem cells that grew on the adherent plates with conditioning medium, and haematopoietic stem cells growing in the non-adherent plate with HSC

medium. Amniotic fluid stem cells have been detected in both second and third trimester amniotic fluid samples, but until now there has been no study on amniodrainage samples until now. My results reveal for the first time using amniotic fluid stem cells from second or third trimester amniodrainage cases. Furthermore I showed that amniotic fluid stem cells could be used in the prenatal xenotransplantation into fetal mice.

Amniotic fluid stem cells could be cultured from second trimester AF or third trimester amniodrainage AF and Ckit+ cells could be isolated from all types of amniotic fluid samples including fresh, cultured or harvested cells. However, the percentage of Ckit+ cells was highest in the fresh samples derived from 2nd trimester compared to others. In addition, second trimester fresh amniotic fluid Ckit+ cells had the smallest cells size, shortest doubling time, and most pluripotent stem cell markers by PCR.

Transduction efficiency with lentivirus vectors was higher among all second trimester samples when compared with third trimester amniodrainage samples (55% versus 45%). I used the same virus to transduce the sheep AFMSCs and CD34+ cells with very similar transduction efficiency (around 50-60%) in Chapter 3, and Chapter 4.

Second trimester freshly isolated Ckit+ cells could be cultured in suspension under HSC-like condition. I also demonstrated their *in vitro* expansion for up to 42 days from 4 out of 10 cases. Furthermore, these cells could maintain the Ckit+ purity above 95% after liquid culturing, and displayed all types of haematopoietic colonies when seeded onto the semi-solid CFU assay after 15 days culturing.

Finally, these Ckit+ AFS cells could form embryoid bodies that stained positive for all three germ layer markers. I concluded that culturing the Ckit+ cells under HSC condition could maintain their haematopoietic potential.

In the last part of this chapter I demonstrated successful *in utero* xenotransplantation using human AFS cells injected into fetal mice. The survival rate of both groups, using cultured or fresh frozen cells was 49%. This acceptable survival rate could be attributed to the immune tolerance to the transplanted human AFS cells by fetal mice (Nijagal et al., 2011b). First time I demonstrated that IUT in mice was a feasible model to evaluate the *in vivo* characteristics of human AFS and their engraftment. In the group using cultured human AFS cells, the DNA sequence specific for human β2 microglobulin was detected in liver (endoderm), kidney (mesoderm) and adrenal

gland (mesoderm and ectoderm) of the recipient mice by PCR. The quantity of AFS cells being transplanted to each fetal mouse in the second group using fresh frozen human AFS cells (10,000 cells per pup) is far less than the previously published IUT of human bone marrow MSCs (50,000 cells per pup) (Chou et al., 2006b). In this study, the transplanted human AFS cells did engraft and persist in various mouse organs until 6 weeks after delivery at least.

It is of interest that though the quantity of the cells implanted to each fetal mouse in the fresh frozen transplanted group (10,000 cells per pup) is far less than that in the cultured transplanted group (60,000 cells per pup), the fresh frozen human AFS cells engrafted in 5 out of the 6 recipient mice and distributed in more organs than cultured human AFS cells by PCR analysis. This finding reminded us that the key factor determining engraftment might be their ability to engraft but not the quantity of the transplanted stem cells. In most of the researches of human AFS, their differentiating potentials were mainly studied by ex vivo experiments on cultured human AFS cells rather than fresh AFS cells. Although cultured human AFS cells were proved to be pluripotent ex vivo, the fresh human AFS cells might possess even more potent capability than cultured ones so that they could engraft more frequently and more broadly in the recipients. On the other hand, the potentials of human AFS cells might deteriorate during cultivation in spite of numerical expansion.

In addition to flow cytometry analysis, the findings from the CFU assay also suggested the existence of haematopoietic progeny from transplanted fresh frozen human AFS cells in mouse bone marrow. Colony formation was noted in the methylcellulose medium for human CFU assay where only human haematopoietic lineage cells may proliferate and form colonies, and these colonies originated from the bone marrow of a recipient mouse after in utero transplantation of fresh human AFS. PCR confirmed that human cells existed in the colonies and human CFC assay demonstrated their haematopoietic differentiation. Previous work on human AF (Ditadi et al., 2009) demonstrated the *ex vivo* haematopoietic potential of C-Kit+Lin- stem cells ($1X10^6$ cells per animal) from human AF by their differentiation into erythroid, myeloid and lymphoid cells. Further work in that study demonstrated that *in vivo,* transplanted mouse AFS C-Kit+Lin- cells were able to engraft in a SCID mouse model and resulted in restoration of the haematopoietic system of SCID mice. Here I further demonstrated the *in vivo* haematopoietic potential of fresh frozen

human AFS C-Kit+Lin- cells by confirming their engraftment and differentiation in the bone marrow of recipient mice after *in utero* transplantation.

In conclusion, human amniotic fluid stem cells sorted with Ckit+ from second trimester had the greatest stem cell potential compared to cells sourced from third trimester amniodrainages. Both fresh frozen and cultured human AFS cells were proved to engraft in mice after *in utero* transplantation, but fresh frozen human AFS cells were more pluripotent than cultured human AFS cells after transplantation. Certain physiological potential like haematopoiesis may decline or even vanish while culturing the human AFS cells ex vivo so that the cultured human AFS cells express only the characteristics of MSCs but not HSCs. Further studies using larger numbers of injected cells and in more animals are required to confirm these initial findings.

Chapter 7

General discussion

7 Chapter 7: General Discussion

7.1 Autologous in utero transplantation is feasible in sheep

The main finding in my first part of thesis is that prenatal autologous stem cell transplantation is feasible in sheep fetuses, either using amniotic fluid mesenchymal stem cells, or amniotic fluid CD34+ stem cells. The experiments, which were used for both short term (3 weeks) and long term (6 months) observations, described for the first time the *in utero* autologous transplantation and engraftment of amniotic fluid derived cells. We showed that using this system, it is possible to obtain a widespread engraftment of transduced autologous cells, with evidence of expression of transgenic protein in major fetal organs, bone marrow and blood. Tissue analysis by PCR, western blot, immunofluorescence and cytofluorimetric assay revealed that AFMSCs injected into the peritoneal cavity preferentially localized in fetal liver, muscle and heart. Moreover, a small number of cells expressing transgenic protein in the liver co-stained with markers of hepatocyte differentiation. When GFP+ CD34+ cells were injected, the transduced cells could be found mainly in the haematopoietic organs including peripheral blood, liver, bone marrow and spleen by using flow cytometry, PCR, colony forming assay and immunofluorescence. The transgenic protein could be detected in long-term lambs followed for up to 6 months of age.

A previous study using sheep fetal liver-derived MSC for prenatal IP transplantation showed widespread but low level engraftment of cells in spleen (1.1%), liver (0.75%), bone marrow (0.2%), blood (0.1%), and thymus (0.5%) by flow cytometry and four female recipients showing the male-specific SRY gene detected by PCR (Schoeberlein et al., 2004). Our results showed that also sheep AFMSCs had a low level, multiorgan engraftment at 2 weeks time post injection. When AF derived CD34+ cells were transplanted, about 2% of GFP positive cells in the peripheral blood of autologous transplanted lambs were found for up to 6 months after injection. CFU assay further confirmed the haematopoietic potential of the injected cells: GFP+ haematopoietic colonies could be seen when bone marrow cells of transplanted NSG mice and lambs were seeded for culture. Moreover, liver engraftment was demonstrated by immunofluorescence in liver section of both lambs (CD34+ cells) and sheep fetuses (AFMSC) showed the co-staining of GFP and CK18 or liver specific AFP.

7.2 The miscarriage rate is low using ultrasound guided techniques for in utero stem cell transplantation in sheep

Using ultrasound-guided amniocentesis, a common clinical procedure with a known fetal loss rate of approximately 1% (Tabor et al., 2009), we were able to isolate AFMSCs and CD34+ cells in 100% of the animals sampled. A miscarriage occurred in one ewe carrying twin pregnancies despite a straightforward ultrasound guided amniocentesis procedure (AFMSC), and in two ewes, that were part of the series of AF derived CD34+ experiments, giving a miscarriage rate of 22% for this specific procedure. This compares favourably to the higher rate of fetal loss (73%) observed in one other study where MSCs were derived from the fetal liver and then used for *in utero* transplantation (Schoeberlein et al., 2004). The reason of the relative lower miscarriage rate in our study could be the amniocentesis is less traumatic compared to the autologous liver cells retrieval.

For in utero stem cell transplantation (IUSCT) in fetal sheep, transuterine ultrasound-guided injection has been shown to achieve a higher rate of engraftment when compared to an open delivery procedure at hysterotomy (70 versus 20%) (Nagao et al., 2009). In both cases cells were delivered intraperitoneally, a route of delivery for fetal blood transfusion in clinical practice for early gestations although it is less commonly performed than cordocentesis (Tongsong et al., 2001).

In our study, we only observed two miscarriages related to the transplantation procedure. Our miscarriage rate was similar to that we have previously reported for both intra-amniotic and intraperitoneal gene transfer to fetal sheep (David et al., 2003a). In comparison, another study of xeno-transplantation of human cord blood and fetal bone marrow MSC into fetal sheep had an overall 37.5% fetal loss rate (Noia et al., 2008).

7.3 Mesenchymal stem cells can be derived from sheep amniotic fluid

In our experimental setting, mesenchymal progenitors were derived in 100% of the animals. In keeping with previous studies in sheep, (Fuchs et al., 2004) and other species (De Coppi et al., 2007b), the AFMSCs were maintained in feeder-free cultures, had a doubling time of approximately 36 to 48 hours, and displayed mesenchymal stem cell markers (CD44 and CD58), while being negative for

hematopoietic and endothelial markers (CD45 and CD31). When cultured in conditional medium (Tsai et al., 2004) these cells could differentiate into adipogenic and osteogenic lineages.

7.4 CD34+ cells can be derived from sheep amniotic fluid and are similar to those derived from sheep bone marrow

The experimental results in Chapter 4 showed that CD34+ cells could be isolated from sheep amniotic fluid as early as 60 days of gestation. Demonstration of sheep AF CD34+ cells haematopoietic potential was based on their ability to form all lineages of haematopoietic colonies. Furthermore there was evidence of engraftment after *in utero* autologous transplantation and postnatal xenotransplantation in mice when compared with sheep bone marrow CD34+ cells. The amniotic fluid cells could be freshly isolated or freshly frozen for storage and future use. I used a novel sheep specific CD34 antibody provided by Dr Porada, a collaborator on this work, who had previously demonstrated that this antibody selected for sheep bone marrow cells. The selected CD34+ cells (positive for CD45, and negative for CD44, CD58) were different from previously selected sheep AFMSCs (positive for CD44, CD58 and negative for CD34, CD45), and could be transduced efficiently using the lentivirus encoding GFP. The overall percentage of CD34+ cells in the amniotic fluid or bone marrow were similar (around 5%) (Porada et al., 2008a). The GFP transduced sheep CD34+ AF or BM cells could be detected in the peripheral blood, liver, bone marrow, and spleen of the transplanted animals. Experimental results further showed the sheep AF CD34+ cells displaying the same haematopoietic potential as the bone marrow CD34+ cells.

7.5 Lentivirus vectors provide good viral transduction efficiency to sheep AF derived MSCs or CD34+ cells

The overall result of the experiments described in this thesis show that lentivirus vectors are able to efficiently transduce sheep AF derived MSCs and CD34+ cells. The efficiency of gene transfer was similar in both groups (63% and 58% on average respectively) and compares favourably with other cell types such as human BM (40%) (Papanikolaou et al., 2012).

After being transduced, expanded undifferentiated cells were auto-transplanted without sorting for two reasons. Firstly, about 60% of gene transfer was achieved in both AFMSCs and CD34+ AF or BM cells. From the literature, the viral transduction rate in human bone marrow-derived MSC, embryonic stem cell, and HSC were 40% (Zhang et al., 2004), 14-48% (Gharwan et al., 2007), and 50% (Sutton et al., 1998), respectively. Thus we were concerned that if only sorted transduced cells were injected, this would result in a low number of engrafted cells. Secondly, we wished to avoid clonal selection of the cells and to observe what could happen with minimal manipulation.

Whilst it has been previously shown that AFS cells can be efficiently transduced using adenoviral vectors (Grisafi et al., 2008), lentivirus vectors have the advantage of allowing sustained transgenic protein expression (Philippe et al., 2006). This is particularly relevant when considering that various progenitors could be isolated from the amniotic fluid, and can differentiate into myocardium, lung epithelial cell, smooth muscle, and neuron cells (De Coppi et al., 2007a, In 't Anker et al., 2003, Bossolasco et al., 2006). More recently another group have reported that ckit(+)/LIN(-) selected mouse and human AF cells have hematopoietic potential in vitro and mouse AF cells are hematopoietic *in vivo* (Ditadi et al., 2009). As a consequence, autologous AF cells could be engineered and used to engraft a fetus affected by congenital disorders in order to provide for expression of the damaged/defective gene.

7.6 Engraftment is observed after in utero transplantation of AF derived cells in fetal sheep

Engraftment of injected AF derived cells was seen after *in utero* transplantation. The level of tissue engraftment I observed was admittedly low in both AFMSC and CD34+ cell experiments. However, I only injected 300000 to few millions of transduced cells prenatally at gestational age around 60-70 days and ultimately, for some congenital diseases, such as severe haemophilia for example, only 1% levels of protein expression are required to ameliorate the severe phenotype (Waddington et al., 2004). The level of GFP positive cells in the peripheral blood of CD34+ cells transplanted lambs was up to 2%, even after 6 months of long-term follow up. Furthermore, the GFP+ cells could also be found in the haematopoietic organs

including liver and bone marrow. Thus for many congenital diseases this may be sufficient to improve the phenotype or even effect a cure.
In order to increase the engraftment rate, liver or organ damage is an option, since this could further create a stem cell niche and improve the homing effect for repairing the damage. One group concluded that the intrahepatic route of prenatal human MSC injection into fetal sheep could lead to a more efficient generation of hepatocytes as compared to the intraperitoneal route (Chamberlain et al., 2007). It is possible therefore that intrahepatic injection might have improved the engraftment rate. The survival rate after intrahepatic and intraperitoneal injection of viral vectors were 81% and 77% respectively (David et al., 2003a). This compares to the overall survival rate of sheep experiments here using intraperitoneal injection which was 75%. Ultrasound-guided injection into the peritoneal cavity was easier than performing local injection into the muscle. Intrahepatic injection is feasible, but rarely used in clinical practice except for liver sampling in the case of congenital liver disorders, most of which are diagnosed by DNA analysis of the amniotic fluid collected by amniocentesis.

7.7 Limitations of sheep study

Performing experiments using sheep as the animal species has some limitations. There were only a few anti-sheep antibodies available which could be used in our study. As far as I know, selection with Ckit+ cells would give the maximal potential and ability to differentiate and engraft. The standard MSC surface markers CD73, CD90, CD105 were not available in sheep and did not cross over with human antibodies either. Therefore CD44 and CD58 were used as alternative surface markers of MSC. For the HSC markers, the standard sheep CD45 antibody was available for experiments. A novel CD34+ antibody was also available and gave good results.

Disease models are also lacking in large animals such as the sheep when compared with other species such as the mouse. A sheep model of haemophilia is being set up in United States by our collaborator who provided the sheep specific CD34 antibody (Porada et al., 2010a, Porada et al., 2011). With my work I have shown that in principle both sheep AFMSC and CD34+ cells can engraft after autologous *in utero* transplantation.

The strengths of sheep studies was that we demonstrated the first large animal model of prenatal autologous amniotic fluid stem cell transplantation similar to the human setting, also with high survival and successful rate, and engraftment in the liver or haematopoietic systems. The weakness was the relative small number of experimental animals. However, the cost of large animals is high, especially their long term follow up and all the surgery costing including anesthesia and recovery. The grants covered the research was not enough at the time to maintain the initial short term AFMSC transplanted sheep so I sacrificed the transplanted animals after three weeks.
All the setting of ultrasound guided technique injection into sheep fetuses was just the same as human ones. The fetal loss rate was 25% (4 out of 16), which is higher than the fetal loss rate after amniocentesis or intraperitoneal fetal blood transfusion separately, but is likely to represent the cumulative fetal loss rate after two procedures. While comparing to the other studies using sheep fetuses, our results showed even better survival and fewer fetal loss rate than others and it is likely that with refinement, the overall fetal loss rate would be lower when applied in clinical practice.

7.8 Further research questions in sheep model

The question of whether autologous IUSCT is superior to allogeneic cells is as yet unresolved and further research needs to be done using both small and large animals to compare the two techniques. In a previous study in sheep, fetal transplantation of MSC derived from fetal liver, the level of engraftment in the bone marrow was not significantly different to that observed after allogeneic derived MSCs (0.16% vs 0.56%). However, engraftment in the fetal liver was higher (0.65 versus 0.23%) after autologous transplantation (Schoeberlein et al., 2004). Similarly we observed a good level of engraftment in the fetal liver, which was confirmed by the appearance of rare cells co-expressing GFP and CK18. This finding could be particularly relevant for future studies since hepatocyte differentiation may lead to the treatment of congenital metabolic disorders (Muraca et al., 2007). Our results could perhaps be improved by preconditioning the cells in hepatocyte differentiation media (Wu and Gupta, 2009) and by injection into the umbilical vein (Soriano et al., 1993). The latter in particular might preferentially target the liver when compared with the widespread tissue engraftment observed after peritoneal injection in this study.

7.9 The amniotic fluid stem cells could be isolated from pregnant mice

Differently from the sheep study, our main finding in the mice projects was that Ckit+/Lin- cells could be isolated from pregnant dams from E12 to E14 and injected in utero maintain their haematopoietic potential. The average percentage of Ckit+/Lin- cells was about 1 to 2% among total cells, which was comparable to previous study (Ditadi et al., 2009), and also could be transplanted into the fetal mice via intraperitoneal or intravascular routes. The freshly isolated mice amniotic fluid stem cells could be used in prenatal cell therapy or cell-based gene therapy as mice to mice transplantation models. The purity of mice AFS cells could maintain the purity and Ckit+ potential when growing in the suspension. These cells could also form all linages of haematopoietic colonies in CFU assay in vitro. Compared to mice bone marrow stem cells, the amniotic fluid stem cells was easily to obtained and collected after peeling off the amniotic membrane of each mouse fetus. Therefore, the cells collected from amniotic fluid could be a good stem cell source for regenerative medicine.

7.10 Congenic in utero transplantation of amniotic fluid stem cells shows higher engraftment than allogeneic in utero transplantation

The main findings in Chapter 5 compared *in utero* congenic and allogeneic transplantation of mouse amniotic fluid stem cells into embryonic mice. I found that congenic transplantation lead to an overall higher engraftment rate than allogeneic transplantation, in peripheral blood, liver, spleen and bone marrow of injected animals. This finding was very similar to a previous study using the same comparison groups where the authors injected mouse bone marrow stem cells. Initially there were no differences in engraftment in the peripheral blood in either group. However by 6 months after birth, engraftment in the allogeneic group had fallen to 19% compared to engraftment after congenic transplantation that remained at 100% (Peranteau et al., 2007).

7.11 Attempts to treat a mouse model of thalassaemia using in utero transplantation of AFS cells are unsuccessful

In Chapter 5.4, I attempted to use prenatal cell therapy with wild type amniotic fluid stem cells to treat thalassaemia fetal mice at E14. Due to the high mortality rate of fetal mice after transplantation and the very poor conception rate in the *th3* transgenic mouse model, I only had two short term thalassaemia survivors from these experiments. These showed low level YFP signals in the peripheral blood and positive luciferase signal in the liver suggesting some engraftment after *in utero* transplantation. However, the blood count of the transplanted animals did not show correction of anemia status of heterozygous mouse that meant the very low level of positive YFP cells in the peripheral blood did not reach the therapeutic threshold for engraftment.

7.12 Future work in mouse models of disease

Future work will need to focus on prenatal transplantation of AFS cells into mouse disease models to treat or correct the disease phenotype. For thalassaemia. alternative less severely affected models such as the humanized thalassaemia mouse model (Huo et al., 2009a), may be a better alternative because of less severity of phenotype prenatally or postnatally. I am also planning to collaborate with a colleague from National Taiwan University who holds the colonies of spinal muscular atrophy (SMA) model of mice (Tsai, 2012, Tsai et al., 2009, Tsai et al., 2008a, Tsai et al., 2008b, Tsai et al., 2007, Tsai et al., 2006a). Tsai et al. has developed the therapeutic method to treat adult SMA mice. As this disease model could occur and progress prenatally, we all agreed to start work in the fetal mice with transplantation of bone marrow MSC and amniotic fluid stem cells.

7.13 Characterisation of stem cells from human amniotic fluid

In most research on characterisation of human AFS cells, FACS analysis revealed many positive surface markers that include HLA-ABC, CD29, CD44, CD73, CD90 and CD105 (De Coppi et al., 2007a). There has been little discussion about the genes or transcription factors that maintain the capability of self-renewal and differentiation in human AFS cells. I performed RT-PCR to confirm transcription of the genes that are closely related to stem cell potencies, and induction of these genes in somatic cells

may even achieve iPS cells (Takahashi and Yamanaka, 2006). According to my results Oct4 was expressed in unsorted cells and C-Kit (+) cells in both groups. Oct4 plays a role in maintaining the undifferentiated status of stem cells (Montemurro et al., 2008) and because previous work revealed that Oct4 is transcribed into mRNA to maintain pluripotency of a certain portion of stem cells from AF (Prusa et al., 2003), we may conclude that through Oct4 transcription, the human AFS cells (C-Kit positive cells from human AF) are capable of differentiating into multilineages.

In addition to Oct4, the C-Kit(+) cells in both groups positively expressed C-Myc and NANOG, so these findings also explain how human AFS cells maintain their proliferating and differentiating potentials. A recent study revealed that NANOG may express when Klf4 is knocked down in ESCs and the expressed NANOG prevents ESCs from differentiating and maintains their ability of proliferation (Zhang et al., 2010). The author also described Klf-4 as an upstream regulator of NANOG and this inference may explain why Klf-4 expression was negative in all the stem cells from human AF in our experiment. The characterisation in this research demonstrated that C-Kit sorting is a practical method to isolate the primitive and potent stem cells from human AF.

7.14 Isolation of AFS from human amniotic fluid

It is quite challenging to get fresh human AFS cells of the similar quantity as cultured human AFS cells for IUT. The literature reveals that there are at least 10,000 amniocytes in 1ml human AF in the second trimester (Prusa and Hengstschläger, 2002) and about 80% of them are viable (Delo et al., 2006). After freezing and thawing approximately 75% of cells remain alive and about 1% of the alive cells are C-kit(+) cells (Delo et al., 2006).

The volume of human AF increases rapidly in the 2nd trimester and reaches approximately 500ml at 20 weeks of gestation, 700 ml in early 3rd trimester and 1,000ml in mid-3rd trimester, then it declines to 800~900 ml at term (Gramellini et al., 2004). Therefore it is not feasible to get 1,000 ml AF from a normal pregnancy unless from a patient with polyhydramnios by amniodrainage. In the two previously published studies on IUT of human stem cells into mice, Chou transplanted 50,000 human bone marrow MSCs to each pup (Chou et al., 2006) and Guillot transplanted 1

x 10^6 human fetal blood MSCs to each pup (Guillot et al., 2008). It is reasonable to get a higher engraftment rate by transplanting more stem cells to each fetal mouse. This would be achievable by ex vivo expanding of cultured cells, but not for fresh frozen human AFS cells.

After amniodrainage, there is frequently at least 2,500ml donated human AF giving at most 2,500 x 10,000 x 0.8 x 0.75 x 0.01=150,000 AFS cells after freezing, thawing and C-kit selection. Usually a pregnant mouse conceives about 15 fetuses and it is reasonable to perform IUT in all the sibling fetuses and detect engraftment after delivery, thus IUT using only 10,000 fresh frozen human AFS cells in each fetal mouse was feasible.

After amniodrainage at 35 weeks of gestation the amniotic fluid cells were observed on Neubauer haemocytometer by 0.4% trypan blue exclusion assay where the percentage of viable cells was far less than expected. One study on human AF in the literature suggested that in 26 samples of between 14 and 15 weeks of gestation (Wahlstrom, 1974), the concentration of amniocytes ranged from 600 to 15,300 per ml and the percentage of viable cells ranged from 4 to 47%. In four samples of between 20 to 21 weeks of gestation, the concentration of amniocytes ranged from 2,900 to 32,600 per ml and the percentage of viable cells ranged from 5 to 36%. In a further study, the percentage of viable cells in human AF was around 18% between 15 and 20 weeks of gestational age (Weise et al., 1984). The data about the percentage of viable amniocytes in the 2nd trimester is far less than what we knew (80%) before.

Amniodrainage is mainly performed to prevent preterm labour in polyhydramnios and to improve survival rate in twin-to-twin transfusion syndrome (Hubinont et al., 2000). There is less concern about aneuploidy in TTTS because the etiology of TTTS is the vascular anastomosis within the sharing placenta. In contrast, aneuploidy should be considered in the fetus with polyhydramnios because polyhydramnios could be attributed to certain congenital anomalies (for example, tracheoesophageal fistula and esophageal atresia) and malfunctions (for example, swallowing disorder) that are combined with aneuploidies (Harman, 2008). It is critical to perform karyotyping before transplantation if the sample came from amniodrainage of polyhydramnios and freezing is necessary until the report is available if implantation of fresh stem cells

from AF is planned. There is less worry about implanting the stem cells with chromosomal abnormality from amniocentesis because most amniocentesis are performed for the purpose of karyotyping and most often the results are released during cultivation of stem cells before implantation.

7.15 Possible deterioration in haematopoietic potential of human AFS cells during expansion

I observed that even though the quantity of the fresh frozen cells transplanted to fetal mice (10,000 cells per pup) was far less than the quantity of cultured cells (60,000 cells per pup), the fresh frozen human AFS cells engrafted in 5 out of the 6 recipient mice and distributed in more organs than cultured human AFS cells by PCR analysis. This finding supports a key factor determining engraftment, that is the quality of the cells and not their quantity. Most research on human AFS cells has studied their differentiating potential by ex vivo experiments on cultured human AFS cells rather than fresh AFS cells. Although cultured human AFS cells were proved to be pluripotent *ex vivo*, fresh human AFS cells might possess even more potent capability than cultured ones so that they could engraft more frequently and more broadly in the recipients. On the other hand, the potential of human AFS cells might deteriorate during cultivation in spite of numerical expansion.

7.16 In utero transplantation of human AFS cells in mice

In this research I demonstrated that IUT of human AFS cells in mice was a feasible model to evaluate the *in vivo* characteristics of human AFS and the physiological sequelae after implantation. After IUT, the delivery rate was 77% in the group using cultured human AFS cells and 59% for the group using fresh frozen human AFS cells, with an average delivery rate of both groups of 67%. This acceptable delivery rate could be attributed to the immune tolerance to the transplanted human AFS cells by fetal mice (Nijagal et al., 2011a).

In this study the flow cytometry by FACS analysis was performed on liver and the other three organs in the haematopoietic system (blood, spleen and bone marrow) so that the haematopoietic potential in the two groups could be compared. Most of the recipient mice were either negative on all items or positive on more than one item.

The finding accords with the haematological and haematopoietic roles shared by liver, blood, spleen and bone marrow. The transplanted human AFS cells may dwell in more than one organ of the haematopoietic system once they engraft successfully with tolerance from their host.

It is of note that in mice transplanted with fresh frozen human AFS cells, 14% of the recipient mice were positive for human β2 microglobulin in spleen by FACS analysis at 6 weeks after birth and 35% were positive in bone marrow. In contrast, in the mice transplanted with cultured human AFS cells all the recipient mice were negative in spleen and bone marrow by FACS analysis at 6 weeks after birth.
In addition to FACS analysis, the finding from CFC assay also proved the existence of haematopoietic progeny from fresh frozen human AFS cells in mouse bone marrow. Colony formation was noted in the methylcellulose medium for human CFC assay where only human haematopoietic lineage cells may proliferate and form colonies. These colonies originated form the bone marrow of a recipient mouse in which the fresh human AFS was transplanted via IUT (HM6-3). PCR confirmed that human cells existed in the colonies.
The *ex vivo* haematopoietic potential of C-Kit+Lin- stem cells from human AF has been demonstrated by their *ex vivo* differentiation into erythroid, myeloid and lymphoid cells (Ditadi et al., 2009). Here I have further proved the *in vivo* haematopoietic potential of fresh frozen human AFS cells by confirming their engraftment and differentiation in the BM of recipient mice.

In mice transplanted with cultured human AFS cells, the DNA sequence specific for human β2 microglobulin was detected in liver (endoderm) and kidney (mesoderm) of the recipient mice by PCR. After transplantation of fresh frozen human AFS cells the human DNA sequence was still detected in liver (endoderm), spleen (mesoderm) and adrenal gland (mesoderm and ectoderm) of the recipient mice by PCR. In comparison, the quantity of fresh frozen AFS cells being transplanted to each fetal mouse (10,000 cells per pup) is far less than in a previously published study on IUT of human bone marrow MSCs (50,000 cells per pup) (Chou et al., 2006). Here, engraftment was confirmed by PCR of 16 analysed organs that originated from the three different embryonic layers. Although I only dissected eight organs from the recipient mice and engraftment was only identified in four organs (liver, spleen, kidney and adrenal

gland), mutilineage differentiation from human AFS cells was still demonstrated because liver is from endoderm, spleen and kidney are from mesoderm, and adrenal gland is from mesoderm and ectoderm.

The frequency of detection of donor cells (MSCs from human BM) in the study by Chou et al (Chou et al., 2006) was 56% at 1 month after birth, 44% at 2 months, 19% at 3 months, 13% at 4 months and 0% at 5 months by PCR of human β2 microglobulin. In my study, recipient mice were sacrificed only at 6 weeks after birth because of the limited number of animals. The frequency of detection of donor cells in mice transplanted with cultured human AFS cells was 4/5 by PCR of human β2 microglobulin and in mice transplanted with fresh frozen human AFS cells was 5/6 by PCR. However, the examined mice had been selected by FACS analysis and the data does not represent the whole sacrificed recipient mice at 6 weeks after birth. Thus my data is not comparable to Chou's study and further work with higher numbers of injected animals is needed to enable comparison.

7.17 Future work on engraftment of human AFS cells in mice

Although engraftment was confirmed in recipient fetal mice at 6 weeks after birth, it is unclear how well the implanted AFS cells engrafted before 6 weeks of age and how long engraftment persisted after 6 weeks of age. Future experiments will need to sacrifice recipient mice at intervals of 4 weeks from 1 month old mice to get the comparable data.

The final objective of this research is to apply AFS cells in fetal therapies, so more understanding of their *in vivo* potential in differentiation is definitely necessary. Although multilineage engraftment was demonstrated in this study, only 8 organs were evaluated. More organs will need to be collected and examined in future experiments, especially the organs in which congenital diseases are involved. For example, evaluate the bone for congenital skeletal disease, the liver for congenital liver disease and evaluate brain or spinal cord for congenital CNS disease. It is imperative to dissect all the organs of therapeutic interests during postmortem at different ages of the recipient mice in the following studies.

Although this IUT model has been proved to be feasible and the transplanted human AFS cells were confirmed to engraft in fetal mice, it is not clear whether engrafted cells have differentiated into the same lineage as the organ where they engraft, for example, have they become hepatocytes in mouse liver. Because the aim of stem cell therapy is to repair the damaged organs or to replace impaired function, there is no point to perform IUT of human AFS cells if they just dwell there but not exert the proper function specific for the organ in which they engraft. It will be important to extract RNA from recipient mice after IUT for RT-PCR to detect the expression of organ-associated genes specific for human, for example, human αFP gene and human albumin gene for liver. In immunofluorescence stain, besides the antibody to prove the existence of the transplanted human cells, the antibody specific to the function of respective lineage is required, for example, anti-human albumin antibody to prove the function of human hepatocytes.

7.18 Translation into clinical practice

The final aim of my research is to apply the human amniotic fluid stem cells into the human fetus for clinical gain. There are a number of steps that will be needed to achieve this goal.

Firstly it will be important to demonstrate that this type of prenatal therapy results in a therapeutic effect.

Secondly it will be necessary to demonstrate whether autologous cells are needed or whether allogeneic cell sources have the same therapeutic effect. This could be achieved using a mouse model of disease, and further in a sheep model of disease.

Thirdly the number and types of cells needed for transplantation will need to be decided, and also whether the cells can be expanded prior to transplantation.

Fourthly safety and toxicology evaluation will be required. Ensuring that the collection and storage of amniotic fluid stem cells meets GMP standards is a priority.

The candidate diseases in clinical practice will need to be selected, for instance SCID, osteogenesis imperfecta, thalassaemia or metabolic liver disease are all those that cause irreversible damages in fetal stages.

7.19 Conclusion

Amniotic fluid derived stem cells isolated from sheep, mice or human are an important source of autologous cells that could have a prenatal therapeutic value in cell therapy or cell based gene therapy in the future.

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Appendix I: Standard of operation for perinatal care of stem cell lambs

Data Collection

- 24-hour cover for lambing to be covered by MB, SS, and AD.
- The aim of this SOP is to outline the data collection
- The baseline data record [BDR] sheet serves as a simple tool to collect the minimum data set expected from all deliveries (maternal, neonatal and placental) – it is deliberately set out in a circling of options / tickbox format to make it easy to complete "on shift"
- The lamb review record [LRR] sheet by contrast is designed to contemporaneously record more detailed, individual information about each lamb's neonatal course
- As a minimum this will be 72h of routine checks, weights ± feeds for uncomplicated neonates (4-hourly for first 24 hours then 6-hourly for a further 48 hours)
- MB to be aware so can easily intervene if any issues arise

Management of Delivery

- If spontaneous delivery witnessed then document the time of birth (24-hour clock) and indicate "present at birth" on BDR sheet
- If not present at delivery then time of birth to be estimated as closely as possible (24-hour clock)
- Once lamb delivered then dry and stimulate
- Immediately carry out initial assessment of neonatal condition (as detailed below)
- Once happy with lamb's wellbeing
- Measure total colostrum yield and record volume on BDR sheet
- Weigh placenta and snap freeze sample (eg one cotyledon 3x 3cm) and put similar sized sample into 4% paraformaldehyde. Sample will need to go into 70% ethanol 24 hours later.
- Record total placental and membrane weights and any abnormalities

Initial Assessment of Lamb

- This should take place immediately if present at delivery (around 1 minute of life) or at first encounter with the lamb if absent at time of birth
- Documentation to follow principle of "ABC" (airway, breathing, circulation) as per human neonatal resuscitation and to partially replicate "APGAR" score of neonatal condition at birth
- Assess breathing and document whether appears normal or abnormally fast, slow or absent
- Assess heart rate and document if sounds / feels normal, slow (e.g. less than 100 bpm) or absent
- Assess tone and document if normal, decreased (floppy) or increased (stiff neck, head back etc.)

Early Neonatal Care / Data Collection

- Once happy with lamb's wellbeing, weigh and record birth weight and sex on BDR sheet
- Measure and record biparietal head diameter using callipers
- Measure and record abdominal girth at level of umbilicus using tape measure
- Take 5ml blood sample and put into purple tube and yellow tube and then into fridge.
- Iodine navel
- Allocate lamb number and record on BDR and LRR sheets
- At around 1 hour of age measure baseline vital signs – oxygen saturation using pulse oximeter, heart rate (manually or using stethoscope), and respiratory rate (count over 15 sec and multiply by 4)– no further measurements to be taken unless clinically indicated
- Add the time of next check to the running day sheet
- Record height to shoulder at 24 hours of age using ruler.

Ongoing Neonatal Care / Documentation

- Review 4-hourly, or more frequently if concerns (if so, document indication on LRR sheet)
- Measure lamb weight twice a day for the first week and record on LRR sheet
- Document all complications or interventions required (and why) on LRR sheet, for example:
    - Hypothermia (v. unlikely)
    - Hypoglycaemia (v. unlikely)
    - Abnormal behaviour e.g. lack of stretching response when "got up" from sleeping
    - Failure to gain weight as expected between initial checks
    - Use of ambient oxygen
    - Use of heating lamp (prophylactic or in response to hypothermia)
    - Supplementary feeding (colostrum, artificial milk) or fluids (e.g. IV/IP dextrose)
    - Use of any additional antibiotics

Blood Samples

- 5ml only to be taken at birth for:
    - Engraftment analysis, in purple tubes for FACS analysis on the same day.
    - Serum to be centrifuged at 3,000rpm and supernatant stored frozen.
- Further blood to be taken at 1, 2, 4, 8, and 12 week of age
    - Put into purple bottle and it will need FACS analysis on the same day.
    - Serum to be centrifuged at 3,000rpm and supernatant stored frozen.
- Further blood sampling to be carried out at regular intervals, plasma and serum to be stored and available for any future analysis

Neonatal Growth

- Measure weight, abdominal girth and shoulder height at birth and 1, 2, 4, 8 and 12 weeks of age.
- Record on neonatal growth record [NGR] sheet

Baseline Data Record Sheet

Ewe No ……...… Pen No …….. Lamb No ……...

Date of birth .....…… Time of birth .....…….....
Present? Y / N

Mode of delivery (please circle) **Spontaneous vaginal / Assisted vaginal / Caesarean**

If not spontaneous rate difficulty of delivery: Easy / Average / Hard

Details

……..
.....
……..……

Total placental weight ……..…… g Membrane weight …… g

Initial Asssessment of Lamb (at delivery or at first encounter) – *please circle one option per row*

AIRWAY	Patent (no action)	Routine suction needed		Extensive suction needed
BREATHING	Normal	Fast	Slow	Absent
HEART RATE	Normal	Slow (e.g. less than 100 bpm)		Absent
MUSCLE TONE	Normal	Floppy	Stiff (neck back etc.)	Absent

Birthweight ……....g Sex
Male / Female

Time to standing ….……min Heart Rate (at 1h) ……bpm

Girth at umbilicus ……..……...cm Resp. Rate (at 1h) ……... /min

Biparietal diameter ….…….……....mm O2 Sats (at 1h) ……....%

Height to shoulder (at 24h) ……....cm Temperature (at 1h) ……....°C

Lamb Review Record Sheet

Sheet No ……

Lamb No ……..… Pen No ……..….... Date of Birth …….……

Date / Time	Date / Time
Date / Time	Date / Time
Date / Time	Date / Time
Date / Time	Date / Time
Date / Time	Date / Time

Neonatal Growth Record Sheet

Lamb No ……..… Pen No ……..… Date of Birth …….……

Date	Postnatal Age	Weight	Shoulder height	Umbilical girth

Appendix II: Sheep AFMSC project PM form

Date:

Animal No:

Fetal measurements:

Right fetus IM injected X3

Fetal Weight:

Crown Rump Length: Femur Length:

Biparietal Diameter: Abdominal Circumference:

Occipito-snout Length:

Left fetus IP injected X4

Fetal Weight:

Crown Rump Length: Femur Length:

Biparietal Diameter: Abdominal Circumference:

Occipito-snout Length:

Post mortem findings:

Photographs taken

1. _____ **5.** _____
2. _____ **6.** _____
3. _____ **7.** _____
4. _____ **8.** _____

Amniotic fluid taken

□ R. fetus AF (culture characteristics) 40mls □ L. fetus AF (culture characteristics) 40ml
□ R. fetus AF (culture for GFP) 20mls □ L. fetus AF (culture for GFP) 20mls

Bloods taken:

For analysis –frozen-

□ Maternal plasma	□ R. Fetal plasma	□ L. Fetal plasma
□ Maternal serum	□ R. Fetal serum	□ L. Fetal serum

For FACS

□ Maternal blood	□ R. Fetal blood	□ L. Fetal blood
	□ R. Fetal spleen	□ L. Fetal spleen
	□ R. Fetal liver	□ L. Fetal liver
	□ R. Fetal BM	□ L. Fetal BM

MATERNAL sampling

For PCR -2 samples for each tissue-

	code	**N1 N2**
Heart	E-H	
Lung	E-AU	
Ovary	E-G	
Liver	E-L	
Adrenal	E-D	
Kidney	E-K	
Spleen	E-X	

For GFP detection -1 sample for each tissue-

	code
Heart	E-H
Lung	E-AU
Ovary	E-G
Liver	E-L
Adrenal	E-D
Kidney	E-K
Spleen	E-X

RIGHT FETUS (X3) Sampling
For PCR
2 samples for each tissue
3 sample for the injected muscle (two edges and the middle part)
3 samples for the liver in three different sites

For GFP
1 sample for each tissue

For Buffer proteins
Samples from muscle and liver

number	organ	Code	PCR		GFP	
			1	2	1	
0	Uterus	**UT**				
	Placenta	**P**				
	Umbilical cord	**U**				
	Amniotic membrane	**F**				
1	**Bone Marrow**	**W**				
2	**Airways and Neighbouring Organs**					
	Thyroid	**Y**				
	Thymus	**T**				
	Lung WT:	**AU**				
3	**Heart** **WT:**	**H**				
4	**Gastro Intestinal System and Neighbouring Organs**					
	Small bowel	**C-S**				
	Rectum	**C-R**				
	Spleen	**X**				
5	**Liver** **WT:**					Buffer protein
	1 right	**L-R**				
	2 left	**L-L**				
	3 central	**L-C**				
6	**Gonads** **WT**	**G**				
7	**Urinary Tract & Neighbouring Organs**					
	Adrenal gland WT:	**A**				
	Kidney WT:	**K**				

Number	Organ	Code	PCR		GFP	
			1	2	1	
8	**Non injected muscle** **As control**					Buffer protein
	Upper extremity	**I-U-C**				
	Lower extremity	**I-L-C**				
	Middle part	**I-M-C**				
9	**Injected muscle**					Buffer protein
	Upper extremity	**I-U**				
	Lower extremity	**I-L**				
	Middle part	**I-M**				
10	**Skin**	**S**				
11	**Brain & Neighbouring Organs**					
	Neurocortex	**N-C**				
	Cerebellum	**N-B**				
	Spinal cord	**N-S**				
	Eye	**N-E**				

LEFT FETUS X4 Sampling IP injected
For PCR
2 samples for each tissue
3 samples for the liver in three different sites

For GFP
1 sample for each tissue

For Buffer proteins
Samples from muscle and liver

number	organ	Code	PCR		GFP	
			1	2	1	
0	Uterus	**UT**				
	Placenta	**P**				
	Umbilical cord	**U**				
	Amniotic membrane	**F**				
1	**Bone Marrow**	**W**				
2	**Airways and Neighbouring Organs**					
	Thyroid	**Y**				
	Thymus	**T**				
	Lung WT:	**AU**				
3	**Heart** **WT:**	**H**				
4	**Gastro Intestinal System and Neighbouring Organs**					
	Small bowel	**C-S**				
	Rectum	**C-R**				
	Spleen	**X**				
5	**Liver** **WT:**					Buffer protein
	left lobe	**LL**				
	right lobe	**LR**				
	central lobe	**LC**				
6	**Gonads** **WT**	**G**				
7	**Urinary Tract & Neighbouring Organs**					
	Adrenal gland WT:	**A**				
	Kidney WT:	**K**				

Number	Organ	Code	P		GFP	
			1	2	1	
8	**Muscle in general**	**M**				Buffer protein
9	**Skin**	**S**				
10	**Brain & Neighbouring Organs**					
	Neurocortex	**N-C**				
	Cerebellum	**N-B**				
	Spinal cord	**N-S**				
	Eye	**N-E**				

4% PFA for immuno/histology cassettes

Cassettes	**Tissues**
1	Maternal ovary, liver, spleen
2	Maternal kidney, adrenal, heart, lung
3	Right fetus amniotic membranes, placenta, umbilical cord, uterus
4	Right fetus gonad, central liver, heart, injected muscle (middle part)
5	Right fetus spleen, adrenal, kidney, non injected muscle(proximal part)
6	Right fetus thyroid, lung, distal injected muscle, thymus
7	Right fetus liver left lobe, small bowel
8	Right fetus liver right lobe, proximal injected muscle, rectum, skin
9	Right fetus bone marrow, bone
10	Right fetus neurocortex, cerebellum, spinal cord, eye
11	Left fetus amniotic membranes, placenta, umbilical cord, uterus
12	Left fetus gonad, central liver, heart
13	Left fetus spleen, adrenal, kidney
14	Left fetus thyroid, lung, thymus
15	Left fetus muscle, left lobe of liver, small bowel
16	Left fetus liver right lobe, rectum, skin
17	Left fetus bone marrow, bone
18	Left fetus neurocortex, cerebellum, spinal cord, eye
19	Right fetus non injected muscle (middle part)
20	Right fetus non injected muscle (distal part)

Appendix III: sheep CD34 autologous project PM form

Date:

Animal No:

Bloods taken:
For analysis –frozen-
□ Maternal serum X4 □ Whole blood X2

For FACS

□ Maternal blood
□ Maternal BM
□ Maternal Liver
□ Maternal Spleen
□ Maternal BM

MATERNAL sampling

1: PCR , 2: frozen section

	code	1	2
Heart	E-H		
Lung	E-AU		
Ovary	E-G		
Liver	E-L		
Adrenal	E-D		
Kidney	E-K		
Spleen	E-X		
BM	E-B		

PM Protocol: sheep CD34 autologous project-lamb PM at 6 month-old

Date:

Animal No:

Sex of lamb:

Lamb measurements:

Weight:

Girth (measure with string at level of the umbilicus):

Standing height (measure shoulder height while lamb is standing up)

Bloods taken:
For analysis –frozen-
□ Lamb serum (5 red tubes)

For hematological and biochemical studies
□ Lamb blood (purple and red tubes)

For FACS (Falcon 50ml tubes + PBS for collection)

□ Lamb blood (5 purple tubes with EDTA)
□ lamb Spleen (3 samples each)
□ lamb liver (central lobe, right lobe, left lobe, 3 samples each)
□ lamb BM (Sternum, Humerus, Femurs, 3 samples each)
□ lamb thymus

For Semi-solid culture
□ lamb BM

For 2nd transplantation to NSG mice, 3 mice for each lamb
□ lamb BM

Things to be prepared:
Labelling
Cryotubes
Falcon tubes
Eppendorf
FACS tubes
4% PFA
liquid nitrogen
24 well plates
autoclave instruments
sterile solution, PBS
ice box

LAMB Sampling
For PCR
2 samples for each tissue

For IHC
1 sample for each tissue

number	organ	Code	PCR		IHC
			1	2	3
1	**Bone Marrow (Sternum, Humerus, Femurs)**	**BS** **BH** **BF**			
2	**Airways / chest Organs**				
	Thyroid	**Y**			
	Thymus	**T**			
	Lung	**AU**			
	Heart	**H**			
3	**Gastro Intestinal System**	**I**			
4	Spleen	**X**			
5	**Liver (central, right, left lobe)**	**LC** **LR** **LL**			
6	**Gonads**	**G**			
7	Adrenal gland	**A**			
8	Kidney	**K**			
9	**muscle**	**M**			

Appendix IV: In utero stem cell injection into mice PM form

Date:
Animal No:

For FACS

- □ Maternal blood
- □ Maternal BM
- □ Fetal blood
- □ Fetal spleen
- □ Fetal liver
- □ Fetal BM

MATERNAL sampling

For PCR+ frozen sampling -2 samples for each tissue-

	code	N1 N2
Heart	E-H	
Lung	E-AU	
Ovary	E-G	
Liver	E-L	
Adrenal	E-D	
Kidney	E-K	
Spleen	E-X	
BM	E-B	

FETUS Sampling
For PCR and frozen section
2 samples for each tissue

number	organ	Code	PCR	
			1	2
0	Placenta	P		
1	Bone Marrow	BM		
2	Lung	AU		
3	Heart	H		
4	Spleen	X		
5	Liver	L		
6	Gonads	G		
7	Adrenal gland	A		
8	Kidney	K		
9	muscle	M		
10	Skin	S		

PM Protocol: **NSG mice**

Date:
Animal No:

For 2nd transplantation
□BM + RBC lysis buffer

For FACS

□peripheral blood
□BM
□Spleen
□Liver
□thymus

Tissue Sampling
For PCR and IHC
3 samples for each tissue (1: DNA, 2: RNA, 3: IHC, frozen section)

number	organ	Code			
			1	2	3
1	Bone Marrow	BM			
2	Lung	AU			
3	Heart	H			
4	Spleen	X			
5	Liver	L			
6	Gonads	G			
7	Adrenal gland	A			
8	Kidney	K			
9	muscle	M			
10	thymus	T			