Cord and Cord Blood Stem Cell Tissue Engineering for Therapeutic Intervention in Liver Disease

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‘The liver has always been an important symbol in occult physiology. As the largest organ, the one containing the most blood, it was regarded as the darkest, least penetrable part of man's innards. Thus it was considered to contain the secret of fate and was used for fortune-telling. In Plato, and in later physiology, the liver represented the darkest passions, particularly the bloody, smoky ones of wrath, jealousy, and greed which drive men to action. Thus the liver meant the impulsive attachment to life itself ‘

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James Hillman

Abstract

Liver cirrhosis and/or liver malignancies have been nominated as the 5th leading cause of death worldwide. The WHO reported, in 2006, that 20 million people around the globe suffer from some form or other of severe liver illness. The ultimate fate of end-stage liver disorders is hepatic dysfunction and eventually organ failure. The only curative mode of management for liver failure is liver transplantation, which is subject to many limitations. Novel alternatives, such as artificial and bio-artificial support devices only aid in temporary replacement of some liver function until an organ is available for transplantation. These newer modalities also have drawbacks or remain experimental and still demand further controlled trials to allow proof of concept and safety before transferring them to the bedside. Regenerative medicine and stem cell therapy has recently shown promise in the management of various human diseases. Recent reports of stem cell plasticity and its multipotentiality has raised hopes of stem cell therapy offering exciting therapeutic possibilities for patients with chronic liver disease. With the understanding that stem cells might not just be about making organs *ex vivo*, but also regenerating a patients own tissues; a concept is now developing to use stem cells to treat patients with serious disease conditions that are terminal or where conventional modes of treatment are insufficient. There exists a choice of stem cells that have been reported to be capable of self-renewal and differentiation to hepato-biliary cell lineages both *in vitro* and *in vivo.* These include: rodent and human embryonic stem cells, bone marrow haematopoietic stem cells, mesenchymal stem cells, umbilical cord blood stem cells, foetal liver progenitor cell and adult liver progenitor cells. It may, however, be argued that with a global population of 6 billion people and a global birth rate in access of 130 million per year, the products of birth ,umbilical cord and cord blood, possibly provide the most readily accessible and ethically sound alternative source of stem cells. The differentiated stem cells can be potentially exploited for gene therapy, cellular transplant, bio-artificial liver-assisted devices, drug toxicology testing and use as an *in vitro* model to understand the developmental biology of the liver.

In this study UCB-derived nucleated cells and umbilical cord-derived Mesenchymal stem cells were exploited for liver differentiation *ex vivo*. These cells were cultured on extracellular matrix (ECM) protein-coated dishes and inserted into ECM incorporated scaffold 3D culture systems. Stimulation with exogenous mitogens and morphogens to induce hepatic histogenesis was experimented. Immunofluorescence analysis revealed the expression of markers specific for: hepatic stem cells (CK-19), hepatoblasts (AFP) and

mature hepatic and biliary epithelium markers including: albumin (ALB), and cytokeratin-18 (CK-18) and cytokeratin-19 (CK-19) and cytokeratin-7 (CK-7) respectively. The differentiated cells displayed several features of hepatic cell kinetics and metabolic activities, including glycogen synthesis, uptake of Indocyanine green dye and cytochrome P450 activity.

These cells may prove to have potential in developing cellular therapy for various liver disorders for which the current mode of therapy is inadequate and also provide an adequate *in vitro* model of parenchymal liver cells in toxicology and in bioartificial liver research.

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List of Abbreviations Used

ADS- Adult stem cell
ALCAM- Activates leukocyte cell adhesion molecule
APC- Antigen presenting cell
B- B cell
B^{act}- Activated B cell
BCR- B cell receptor
BSE- Bovine spongiform encephalopathy
CAM- Cell adhesion molecule
CB- Cord blood
CD- Cluster of designation
CHO- Carbohydrate moiety
CDCP1- Cub-domain containing protein
CRSF- Cytokine Receptor SuperFamily
DAPI- 4', 6-diamidino-2-phenyllindole dilactate
DC- Dentritic cell
Dexa- Dexamethasone
DIC- Differential interference contrast
EBSS- Earle's basic salt solution
ECM- Extracellular matrix
Endoth- Endothelial cell
ES- Embryonic stem cell
ESC- Embryonic stem cell
    mESC- Mouse embryonic stem cell
    hESC- Human embryonic stem cell
EGF- Epidermal growth factor
FACS- Fluorescent activated cell sorting
FDC- Follicular Dentritic cell
FITC- Flourescein isothiocyanate
FGF- Fibroblast growth factor
    b-FGF- basic- Fibroblast growth factor
    a-FGF- acidic- Fibroblast growth factor
G-CSF- Granulocyte- colony stimulating factor
Gran- Granulocyte
GPI- Glycophosphatidylinositol
H-CAM- Homing-associated cell adhesion molecule
Hex- Haematopoietically expressed homeodomain
HLA- Human leukocyte antigen
HGF- Hepatocyte growth factor
HIV- Human immunodeficiency virus
HSC- Haematopoietic stem cell
HSPC- Haematopoietic stem/progenitor cell
HUCPV- Human umbilical cord perivascular
ICM- Inner cell mass
kD- Kilo Dalton
IL- Interleukin
LBP- LPS-binding protein
Lin- Lineage
LinNeg- Lineage negative
LPS- Lipopolysacharride
LTC-IC- Long-term culture-initiating cell

Lymph- Lymphocyte
IgSF- Immunoglobulin Super Family
MAdCAM- Mucosal laddressin-cell adhesion molecule
MARS- Molecular adsorbent recycling system
MC- mononuclear cell
MHC- Major Histocompatibility complex
MNC-Mononuclear cell
Mono- Monocyte
MPB- mobilized peripheral blood
MSC- Mesenchymal stem cell
MSCGM- Mesenchymal stem cell growth medium
NK- Natural killer cell
Pgp-1- Phagocytic glycoprotein-1
Prox 1- Prospero-related homeobox
RBC- Red blood cell
Rtp- Room temperature
SCF- Stem cell factor
SEPET- Selective plasma filtration
SOP- Standard operational protocol
SPAD- Single-pass albumin dialysis
SSEA- Stage-specific embryonic antigen
T- T cell
T^{act}- Activated T cell
TCR- T cell receptor
TNC- Total nucleated count
TM- Transmembrane
TM4SF- 4-Transmembrane Spanning protein Super Family
TGF- Transforming growth factor
TNC- Total nuclear count
TRA- Tumour rejection antigen
TRITC- Tetramethyl rhodamine isothiocyanate
UCB- Umbilical cord blood
VCAM- Vascular cell adhesion molecule
VLA- Very late antigen
VEGFR- Vascular endothelial growth factor
w/- Without
2-D- 2- dimensional
3-D- 3-Dimensional

SI units

Ng nanograms = $1x10^{-9}$g

μg micrograms = $1x10^{-6}$ g

μl microlotres = $1x10^{-6}$ L

ml milliliters = $1x10^{-3}$ L

g grams

Declaration

I, Saba Habibollah, hereby declare that no portion of the material referred to in this thesis has been previously submitted in support of another degree or qualification at this, or any other University or Institute of Learning. Material generated through joint work has been acknowledged and the appropriate publications cited. In all other cases, material from the work of others has been acknowledged, and quotations and paraphrases suitably indicated.

Signature:

Date:

The following section is a brief insight into the biology of stem cells.

1 Introduction

1.1 Definition of stem cell

The concept of stem cell arose from pioneering studies of Till and McCullogh on the haematopoietic stem cell and those of Leblond on spermatogenesis and intestinal crypt (Handbook of Stem Cells, volume1- Embryonic Stem Cells, Lanza 2004).

Stem cells can be defined as single cells that are clonal precursors of both more identical stem cells and a defined set of differentiated progeny (Till and McCulloch 1961) (Weissman, Anderson et al. 2001) (Smith 2001).

More explicitly, stem cells are capable of generating daughter cells identical to their mother (self-renewal) as well as produce progeny with more restricted potential (differentiated cells) (Handbook of Stem Cells , Volume 1, embryonic stem cells, Lanza 2004). This board definition may be acceptable for embryonic and foetal stem cells that do not remain in existence for the life-time of an organism. But this definition proves inadequate in trying to discriminate between transient adult progenitor cells that have a reduced capacity of self-renewal and adult stem cells. Hence, it is important when describing adult stem cells to further restrict this definition to cells that self-renew through-out the life span of the organism (van der Kooy and Weiss 2000) (Handbook of Stem Cells, volume1- Embryonic Stem Cells, 2004).

Parameters that play a pivotal role in defining ‘stemness’ include the following: self renewal (replication capacity), clonality, and potency.

➢ Self renewal

Most *in vitro* cultures of somatic cells demonstrate a finite number of population doubling (less than 80) prior to undergoing senescence or replication arrest, and this can be contrasted to the seemingly unrestricted proliferation capacity of stem cells in culture (Hayflick 1973; Hayflick 1974; Sherr and DePinho 2000). Therefore, if a cell that can undergo more than twice this number of population doublings (160) without tumour transformation may possibly be regarded as ‘capable of extensive proliferation’. (Handbook of Stem Cells, Volume 1, embryonic stem cells, Lanza 2004).

➢ Clonality

Stem cells are referred to as clonal entities: single cells that are capable of creating more stem cells. This phenomenon is essential for any definitive characterisation of self-renewal, potential and lineage (Weissman, Anderson et al. 2001).

➢ Potency

Stem cells can be classified by the extent to which they can differentiate into different cell types, this is defined as potency. A lineage hierarchy classification of stem cells based on potency is as follows:

A. Totipotent stem cell - controversial and changing area

Totipotent stem cells- are capable of differentiating into any cell type in the body in addition to the placenta which nourishes the embryo i.e. both embryonic and extra-embryonic tissues. A fertilized egg (zygote) is by definition the only totipotent stem cell. Embryonic stem (ES) cells which represent a model for investigation of the fundamental aspects of cell stemness are therefore not exactly totipotent. ES cells are derived from the inner cell mass (ICM) of the blastocyst and are equivalent to ICM- pluripotent. Hence, they can generate tissues of all three germ layers: ectoderm, mesoderm and endoderm; but cannot produce the extra-embryonic tissues (De Felici, Farini et al. 2009). There is a substantial debate surrounding this area and concepts of totipotency and ES cells are swiftly changing.

B. Pluripotent stem cell

Pluripotent stem cells- are descendants of the totipotent stem cells of the embryo. These cells can differentiate into any cell type, except for totipotent stem cells and extra-embryonic membranes. A single pluripotent stem cell has the ability to give rise to tissues of all three germ layers (endoderm, mesoderm and ectoderm) from which all the cells of the body arise. Embryonic stem cells (derived from inner cell mass), embryonic germ cells and recently cord and cord blood-derived stem cells (McGuckin, Forraz et al. 2005; McGuckin, Forraz et al. 2006) (Harris and Rogers 2007) are examples.

C. Multipotent stem cell

Oligopotent/multipotent stem cells- are descendants of pluripotent stem cells and antecedents of specialized cells in particular tissues. Adult stem cells are multipotent stem cells with a more constraint differentiation potential and capacity for self-renewal. Examples include haematopoietic stem cells (HSCs) which give rise to all the cells in the blood and

neural stem cells which contribute to nerve cells (neurons) and neural support cells (oligodendrocytes and astrocytes).

D. Unipotent stem cell

Unipotent/Progenitor cells- have a very limited differentiation potential and can produce only one cell type. For example erythroid progenitor cells differentiate into red blood cells only.

1.1.1 Source of stem cells

Stem cells may be sourced from the blastocyst in the developing embryo prior to implantation (embryonic stem cells) or derived from the foetus (foetal stem cells) or, from blood and tissues postnatally ('adult' stem cells), each forming a unique component of the revolution in stem cell research and therapies (Watt and Contreras 2005). Adult Stem Cells (ADS) or somatic stem cells are immature cell groups capable of tissue repair, maintaining tissue homeostasis and repair in a niche-specific controlled microenvironment (Heissig, Ohki et al. 2005) (Li and Xie 2005). Although, ADS have been characterized in many tissues (including neural, muscular, hepatic and cardiovascular tissues), hematopoietic stem cells remain the most characterized ADS population with clinical evidence. General experimental approaches and paradigms applicable to adult or tissue-specific mammalian stem cells have been defined in the haemopoietic system (Till and McCulloch 1961; Jones, Wagner et al. 1990; Morrison, Wright et al. 1997; McCulloch 2003). .

ADS have more therapeutic potential than ES cells. There are no current clinical treatments based on ES cells, and there are in fact only few modest published successes using animal models. On the other had, ADS therapies are well established and in clinical practice for over 3 decades. The most commonly utilized sources of ADS cell is the bone marrow, although most tissues of the body harbour lineage-restricted stem cells. Cord blood derived stem cells enjoy a special niche between ES cells and ADS cells. ES cells are occasionally considered to be totipotent, while ADS cells are more restricted in their potential and more likely to be multipotent. Cord blood-derived stem cells have been characterized to be pluripotent with ES cell-like properties (McGuckin, Forraz et al. 2005; McGuckin, Forraz et al. 2006). These stem cells may safely be regarded as comparable to foetal stem cells, as they are derived from foetal tissues (extra-embryonic membranes).

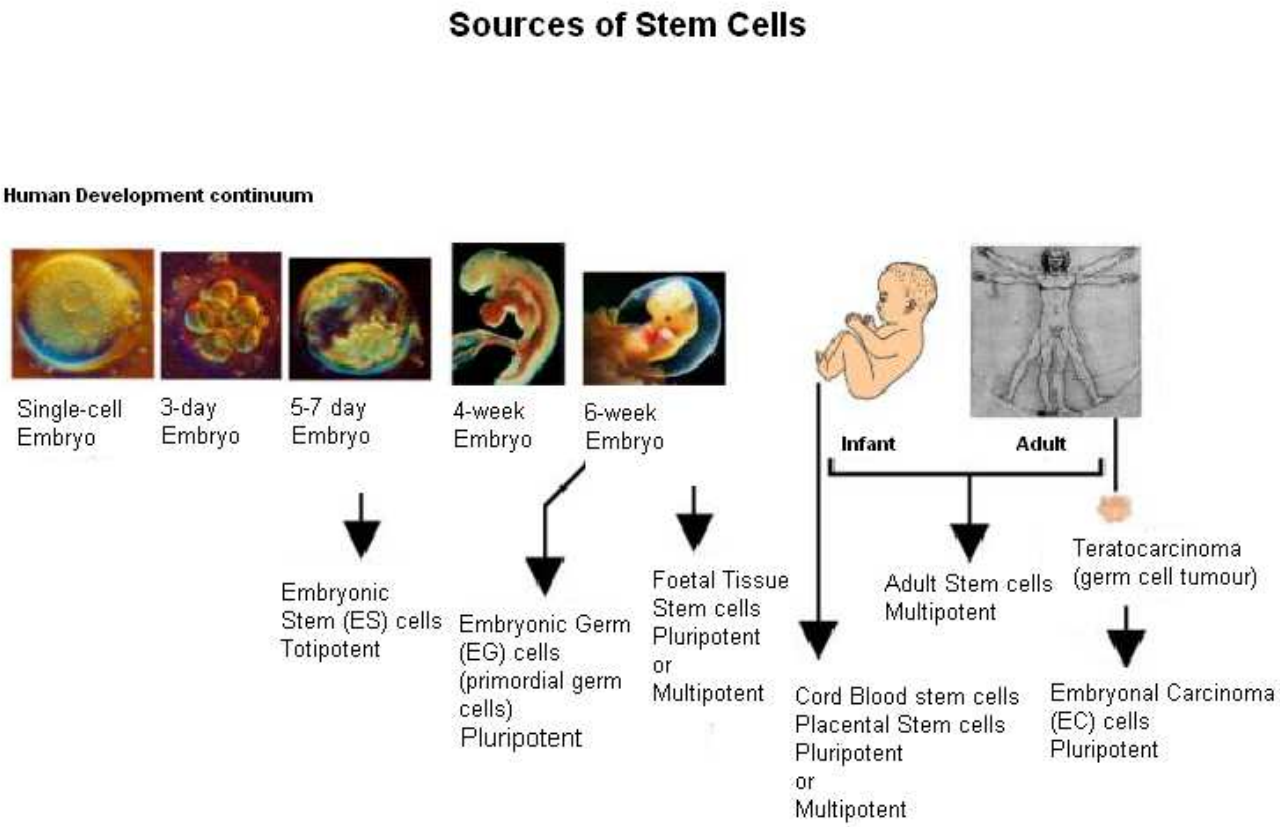


Figure 1. Sources of stem cells. ES cell are derived from the inner cell mass (ICM) of the blastocyst and may be considered totipotent. Primordial germ cells are derived from embryonic germ cells and are pluripotent. Foetal cell stems are derived from the developing foetus and are pluripotent or multipotent. All stem cells derived after birth is defined as adult stem cells (ADS). These cells have restricted potential and are usually multipotent. Cord blood-derived stem cells occupy a niche between ES cell and ADS. These have been characterized to be pluripotent and exhibit some ES cell-like properties. Adapted from www.stemcellresearch.org/testimony/images/20040929prentice.htm

In this study umbilical cord blood was used as the tissue source for primitive haematopoietic stem cells and primitive progenitor cells. The following section elaborates briefly on the identification and characterization of these primitive cells in blood.

1.1.2 Characterization of haematopoietic stem cells

Haematopoietic stem cells (HSCs) resemble lymphocytes in morphology. Examined microscopically they are non-adherent and rounded cells, with a rounded nucleus and low cytoplasm-to-nucleus ration.

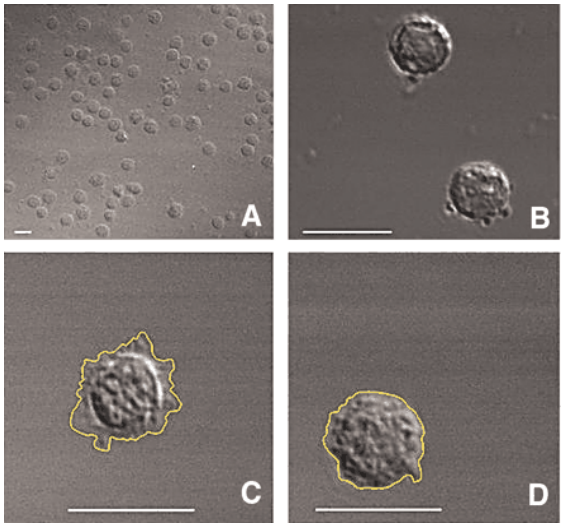


Figure 2. Differential interference contrast (DIC) image of haematopoietic stem cells by laser-scanning confocal microscope. Haematopoietic stem cells derived from cord blood (CB) and bone marrow (BM). (A) Shows a low magnification picture of CB MNCs (x630). (B) Reveals haemopoietic progenitor cells from CB at a medium magnification (x630x6). (C) and (D) are DIC images of haemopoietic progenitor cells from CB and BM respectively, at high magnification (x630x8). Both BM and CB progenitor cells have typical "blast" morphology with a discrete cytoplasm and a large nucleus. Interestingly, BM progenitor cells reveal increased membrane activity with multiple protrusions, reflecting their physiological interacting status in the marrow when compared to normally circulating CB progenitor cells. Membrane contours represented in yellow (computer generated). White scale bar is equivalent to 10 µm. Adapted from *McGuckin et al, 2003b.*

Since haematopoietic stem cells cannot be isolated as a pure population, it is not possible to identify them microscopically. The above description is based on the morphological characteristics of a heterogeneous population, of which haematopoietic stem cells are a component.

The frequency of HSCs in any tissue is extremely low. This quality coupled with the lack of a single molecular marker that is expressed exclusively by HSC has made purification and characterization of these cells a highly challenging goal. After many exhaustive studies researchers have been able to identify several markers whose expression is gained (or lost) at different rates as primitive haematopoietic cells differentiate. It has been possible to subdivide the functionally heterogeneous mixture of cells found in blood into more homogenous subpopulations by targeting various combinations of markers. In the absence of reliable direct markers for HSCs, the better route for the their identification and enumeration depends on functional long-term, multilineage, *in vivo* repopulation assays (Wognum, Eaves et al. 2003).

1.1.2.1 Cell surface markers associated with the haematopoietic system

The cluster of differentiation (cluster of designation) (CD) is a system of cell markers commonly used to identify cells. This protocol allows cells to be defined based on what molecules are present on their surface. CD molecules can act in numerous ways, often acting as receptors or ligands (the molecule that activates a receptor) and associated with cell signaling. Some CD proteins do not play a role in cell signaling, but have other functions, such as cell adhesion (Zola, Swart et al. 2005; Zola, Swart et al. 2007). CD for humans is numbered up to 350 most recently (as of 2009) (HCDM, responsible for HLDA workshop and CD molecules) (Human Cell Differentiation Molecules Council (successor to the HLDA Workshops) (http://www.hcdm.org/MoleculeInformation/tabid/54/Default.aspx. Retrieved 2009-06-01). Cell populations are usually identified by using a combination of CD markers.

Terminal differentiation of haematopoietic stem/progenitor cells is associated with the expression of a variety of surface markers. Thus, lack of expression of these lineage (lin) markers, which in combination include all of the mature haematopoietic cell subtypes (erythrocytes, T-cells, B-cells, natural killer cells, monocytes and granulocytes) can be used to distinguish immature and immuno-naive cells from the more abundant differentiated cells in blood. The selected cell population is commonly referred to as Lin^- (Linage negative) cells. The following table highlights some of the more commonly used CD antigens used in

characterization of differentiated blood cells. *In vitro* isolation of haematopoietic primitive stem/progenitor cells involves identification and subsequent removal of these linage committed cells leaving behind the enriched lineage negative cell fraction.

Table 1. Table of human HSC CD cellular antigens. (http://www.ebioscience.com/ebioscience/whatsnew/pdf/HumanCD_Poster.pdf)

Antigen Name	Other Names	Molecular Weight (MW)	Structure	Distribution	Function
CD2	T11, LFA-2, SRBC-R	50kD	IgSF	Thymocytes, T, NK	CD58 ligand, adhesion, T cell activation
CD3γ, CD3δ	T3		IgSF	T, thymocyte subset	w/TCR, TCR surface expression/signal transduction
CD3ε	T3	20kD	IgSF	T, thymocyte subset	w/TCR, TCR surface expression/signal transduction
CD4	T4	55kD	IgSF	Thymocyte subset, T subset, mono, mac	MHC class II coreceptor, HIV receptor, T cell differentiation/activatio-n
CD8a	T8, Leu-2	32-34kD	IgSF	Thymocyte subset, T subset, NK	MHC class I coreceptor, receptor for some mutated HIV-1, T-cell differentiation/activatio-n
CD8b		32-34kD	IgSF	Thymocyte subset, T subset	
CD11c	P150, 95, CR4, integrin αX	150kD	Integrin family	DC, myeloid cells, NK, B, T subset	Binds CD54, fibrinogen and iC3b
CD14	LPS-R	53-55kD	GPI-linked	Mono, mac, langerhans cells, gran^{low}	Receptor for LPS/LBP, LPS recognition
CD15	Lweis-x, Lex		CHO	Neutrophils, eosinophils, mono	Adhesion
CD16a	FcyRIIIA	50-65kD	IgSF	Neutrophils, mac, NK	Component of low affinity Fc receptor, phagocytosis and ADCC
CD16b	FcyRIIIB	48kD	IgSF	Neutrophils	Component of low affinity Fc receptor, phagocytosis and AADCC

Antigen Name	Other Names	Molecular Weight (MW)	Structure	Distribution	Function
CD19	B4	95kD	IgSF	B, FDC	Complex w/CD21 and CD81, BCR coreceptor, B cell activation/differentiatio-n
CD20	B1, Bp35	33-37kD	TM4SF	B, T subset	B cell activation
CD24	BA-1	35-45kD	GPI-linked	Thymocytes, erythrocytes, peripheral lymph, myeloid	Binds P-selectin
CD25	Tac, p55	55kD	Type I TM	T^{act}, B^{act}, Lymph progenitors	IL-2Rα, w/IL-2Rβ and γ to form high affinity complex
CD36	GPIV	88kD		Platelets, mono, mac, endoth, early erythrocytes	ECM receptor, adhesion, phagocytosis
CD38	T10	45kD		Variable levels on majority of haematopoiet i-c cells, high expression on plasma cells, B and T^{act}	Ecto-ADP-ribosyl cyclise, cell activation
CD45	LCA, T200, B220	180-240kD		Haematopoie t-ic cells, multiple isoforms from alternative splicing	Tyrosine phosphatise, enhanced TCR and BCR signals
CD45RA		205-220kD		B, T subset (naïve), mono	Exon A isoform of CD45
CD56	NCAM	175-185kD	IgSF	NK, T subset, neurons, some large granular lymphocyte leukemias, myeloid leukemias	Adhesion
CD66b	CD67, CGM6	95-100kD	IgSF, CEA family	Gran	Cell adhesion, neutrophils activation

Antigen Name	Other Names	Molecular Weight (MW)	Structure	Distribution	Function
CDw123	IL-3R	70kD	CRSF	Lymph subset, basophils, haematopoietic progenitors, Mac, DC, megakaryocyt-es	IL-3Rα, w/CDw131
CD235a	Glycophorin A	36kD		Erythrocytes	
HLA-ABC		45, 11-12kD		Nucleated cells	Cell-mediated immune response and tumour surveillance
HLA-DR				APC, T^{act}	Presentation of peptides to $CD34^{+}$ T lymphocytes

Cell surface profile of human haematopoietic stem cells (HSCs)

- **CD34**

The first identified marker to be recognized on primitive haematopoietic cells was CD34. CD34 is still the most commonly used marker to obtain enriched populations of haematopoietic stem/progenitor cells for both laboratory based research and clinical application (Wognum, Eaves et al. 2003). CD34 is expressed on approximately 1-4% of the nucleated cells in the normal human bone marrow aspirate samples and <0.1% of the nucleated cells in steady-state human peripheral blood (Civin, Strauss et al. 1984; Strauss, Rowley et al. 1986; Andrews, Bryant et al. 1992; Saeland, Duvert et al. 1992).

$CD34^+$ have been shown to be capable of producing multilineage haematopoietic engraftment in myeloablated recipients (Andrews, Bryant et al. 1992; Civin, Trischmann et al. 1996; Larochelle, Vormoor et al. 1996; Zanjani, Almeida-Porada et al. 1997; Vogel, Scheding et al. 2000). However, it is worth noting that even in a highly purified $CD34^+$ cell population (>90% pure) the frequency of cells possessing progenitor activity (Colony forming unit potential) is <20%, the frequency of Long-term culture initiating cells (LTC-IC) and HSCs is <0.1% (Wang, Doedens et al. 1997; Holyoake, Nicolini et al. 1999). $CD34^+$ cell population can be subdivided into HSCs, primitive progenitor cells and lineage committed progenitor cells. The different expression patterns of other markers on these cells allow these cells to be distinguished from one another.

$CD34^-$ primitive haematopoietic cells have also been identified in human cord blood and adult peripheral blood (Goodell, Rosenzweig et al. 1997; Bhatia, Bonnet et al. 1998; Zanjani, Almeida-Porada et al. 1998). These cells are identified by the lack of CD38 expression and the positive expression of CD133. Only 0.2% of lineage negative $CD34^-$ cells in human cord blood were found to express CD133 (Gallacher, Murdoch et al. 2000).

- **CD133**

CD133 (also known as AC133) is expressed on majority of, but not all $CD34^+$ cells. These include repopulating cells, immature progenitors, and monocyte/granulocyte progenitors, but not most erythroid progenitors. As noted previously, CD133 also appears to be expressed on the $CD34^-$ HSCs identified in human cord blood (Miraglia, Godfrey et al. 1997; Yin, Miraglia et al. 1997).

CD34 and CD133 are very frequently used for the isolation and enrichment of HSCs. Other markers that have also been applied include: **CD90** (also known as **Thy1**) which is a

member of the immunoglobulin superfamily and is expressed on $CD34^+$ cell populations; **CDCP1** (Cub-domain containing protein) which has a pattern of expression on adult human bone marrow cells similar to that of CD133 (Conze, Lammers et al. 2003); Stem cell factor receptor (**C-KIT or CD117**) expressed on approximately 2/3 of $CD34^+$ cells including most lineage-committed progenitor cells, and is selectively up regulated in terminally differentiating erythroid cells but is absent from virtually all circulating mature blood cells (Kawashima, Zanjani et al. 1996; Wognum, de Jong et al. 1996); Vascular endothelial growth factor receptor 1 (**VEGFR-1**) expressed on approximately 5% of $CD34^+$ cells in human cord blood and fœtal liver (Hattori, Heissig et al. 2002). 0.1-0.5% of $CD34^+$ cells in adult bone marrow express vascular endothelial growth factor 2 (**VEGFR-2**) also known as **KDR** (Ziegler, Valtieri et al. 1999).

A combination of cell surface markers noted above are usually used to identify and isolate rare population of primitive haematopoietic stem/progenitor cells, This, however, does not equate to a definitive HSC phenotype, which must identify only cells with HSC function (long-term, multi-lineage repopulation). It is this lack of a definitive phenotype that poses significant challenges to the development of techniques to isolate sufficiently pure yields of HSCs reproducibly for research and clinical transplantation purposes. Interestingly, even the purest human HSC-containing populations are likely to be heterogeneous. The frequency of human cord blood cells capable of repopulating NOD/SCID mice has been estimated at 1 per 600–1,000 $lin^-CD34^+CD38^-$ cells after intravenous injection and 1 per 44 $CD34^+CD38^-$ cells after direct intra-bone marrow injection (Wognum, Eaves et al. 2003).

Considerable work has been carried out to characterize and classify primitive cells in the haematopoietic system. One such work is by Gotze *et al* who demonstrated that CD34 expression is acquired at a later stage in the development of the haematopoietic system and is preceded by CD133 (Gallacher, Murdoch et al. 2000; Gotze, Schiemann et al. 2007). Based on similar reported work (McGuckin, Pearce et al. 2003; Christina Basford 2010), HSCs may be classified as:

- Early-stage HSC ($CD45^+/CD34^-/CD133^+$)
- Mid-stage HSC ($CD45^+/CD34^+/CD133^+$)
- Late-stage HSC ($CD45^+/CD34^+$)

This classification was used for the analysis of HSCs in this study.

In addition to haematopoietic stem cells, non-haematopoietic stem cells have also been identified in cord blood. A well known population of non-haematopoietic stem cells are the **Mesenchymal stem cells**. These progenitor cells are multipotent in their differentaitive capacity and are identified and isolated based on a distinct profile. These cells are negative for haematopoietic cell markers: CD45, CD34 and HLA-DR. They test positive for the various markers elaborated in the table 2. It should be noted, however, that the isolation of MSCs from UCB is not associated with 100% success; previous studies have demonstrated that only in 63% of UCB units could MSCs be isolated in culture with a frequency of MSC-like cells ranging from 0 to 2.3 clones per 1x 10^8 MNC cells (Bieback, Kern et al. 2004), and the isolation is very much dependent on the quality and volume of the cord blood. Lineage negative cells, on the other hand, could prove to be an easier alternative to MSCs, as irrespective of the size of the cord blood units these cells can be isolated from nearly every cord blood specimens processed, albeit at very low yields.

Irrespective of their source, all MSCs share a core MSC profile distinct from that of other foetal organs. These cells have the same morphology, similar population doubling time, all are negative for CD34, CD45 and HLA-DR, all are positive for CD29, CD44 (adhesion markers), CD105, CD73 (Mesenchymal markers) and HLA-A-, B-, and C- (Class I histocompatibility complex). Some MSC sources have been shown to express stem cell markers, including c-kit, Nanog, Oct4 and Sox2 (Mitchell, Weiss et al. 2003; Weiss, Medicetty et al. 2006). Regulation of extracellular matrix (ECM) and adhesion is a prominent feature of all MSCs. This feature is one of the main determinants of differentiation of stromal cells to somatic Mesenchymal cells.

Although all MSCs are considered multipotent, disparate differentiation tendencies of MSCs from different sources should be considered (Chang, Shih et al. 2006). This phenomenon may result from the function of the MSCs in their prime location of isolation. For example, the most prominent functions of amniotic fluid MSCs may be involvement in uterine contraction and its related signaling transduction pathways. Amniotic membrane MSCs play a role in homeostasis of fluid and electrolytes. Cord blood MSCs are an important part of the innate immune system against viruses and bacteria. One major difference between UCB MSCs and other MSC sources is their reported down-regulation of some genes including homeobox genes, probably because cord blood is taken from term babies. Other genes are up regulated in amniotic fluid and BM MSCs, related to the important feature of frequent renewal (Tsai, Hwang et al. 2007). Furthermore, BM MSCs participate in skeletal development, BMP signaling, ossification, bone mineralization, dopamine biosynthesis, synaptic transmission and regulation of neurotransmitter secretion. The BM has been

proposed to be a source of neural stem cells because some bone marrow stroma is innervated and contains nerve terminal which maybe responsible for the synapse-related protein (Safford, Safford et al. 2004).

Table 2. Mesenchymal stem cell markers.

Antigen Name	Other Name	Molecular Weight	Structure	Distribution	Function
CD29	Integrin β1	130kD	Integrin family	Lymph, mono, gran, platelets, mast cells, fibroblasts, endoth	w/CD49a (VLA-1) receptor for VCAM-1, MAdCAM-1 and ECM
CD44	H-CAM, Pgp-1	80-95kD	Hyaladherin family	Haematopoietic and non-haematopoitic cells, except platelets, hepatocytes, testis	Binds hyaluronic acid, adhesion `
CD73		69kD	GPI-linked	B subset, T subset, FDC, epith	Ecto-5'-nucleotidase, nucleoside uptake, T costimulation, lymph adhesion
CD105	Endoglin	95kD	Homodimer	Endoth, bone marrowsubset, activated mac	Cellular response to TGF-β1)
CD106	VCAM-1	110kD	IgSF	Activated endoth, FDC	VLA-4 (CD49d/Cd29) receptor, leukocyte
CD166	ALCAM	105kD	IgSF	Neurons, mono, epith, fibroblast, T^{act}	CD6 ligand, adhesion

1.1.2.2 Molecular definition of stem cells

Stem cells have the unique ability to self-renew and to differentiate into specialized cell types. This is one of the most important characteristics of stem cells and a part of their molecular definition. For a long time embryos or germ-cell derived cells were thought to be the only source of pluripotency- a dogma that has been challenged in the last decade. The unique stem cell machinery that drives the molecular mechanisms underlying pluripotency and self-renewal is governed by a set of transcription factors centered on the triumvirate of Oct4, Sox2 and Nanog. All three proteins are co-localized in the chromatin and are associated with other transcriptional modulators that have been more recently identified to play a role in the transcriptional control of pluripotency. Expression levels of these pivotal transcription factors impact the eventual developmental potential of stem cells (Bosnali, Munst et al. 2009). Mechanisms of action of this core transcriptional network regulating stem cell machniery are illustrated in Figure 3 and 4.

Table 3. Key transcription factors of pluripotency.

Name of Transcription Factor	Explanation	Gene Family
Oct4	Octamer binding transcription factor 4	POU (Pit, Oct, Unc) domain TF
Sox2	SRY (sex determining region Y)-related HMG box2 transcription factor	HMG (High Mobility Group) of DNA-binding proteins
Nanog	Named after 'Tir na nOg' (celtic mythology= land of the ever young)	Homeodomain transcription factor

(Bosnali, Munst et al. 2009)

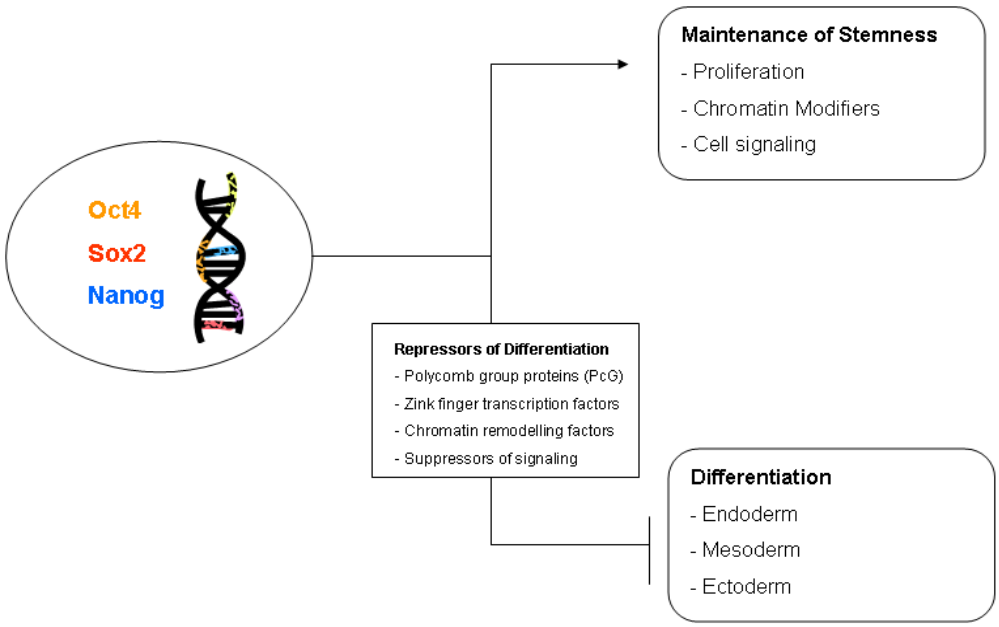


Figure 3. Oct4, Sox2 and Nonog form the core transcriptional network regulating stem cell machinery. Several hundred downstream genes are associated with this core network. These can be classified into two groups of genes exerting opposing functions. One group includes genes associated with maintenance of stemness while the other group activates pathways that lead to inhibition of differentiation. Adapted from (Bosnali, Munst et al. 2009)

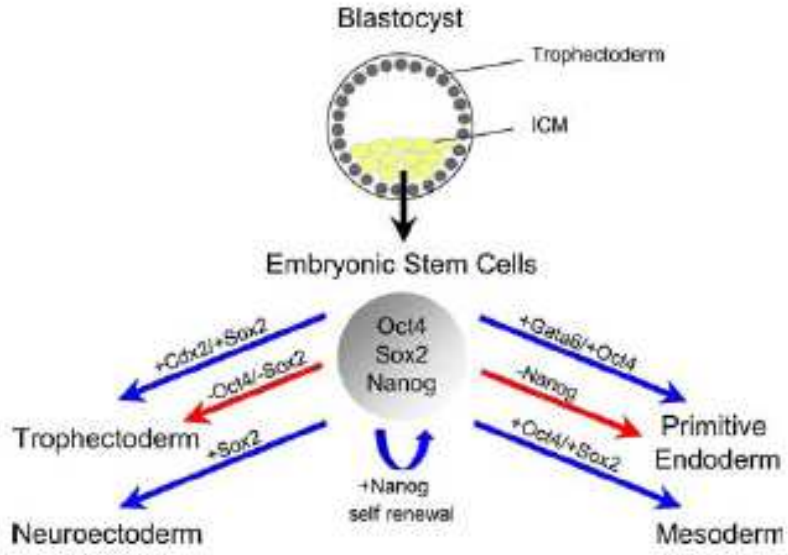


Figure 4. Expression levels of key transcription factors inpact on the developmental potential of embryonic stem cells. ES cells exhibit pluripotent differentiation potential that is regulated by three main transcription factors: Oct4, Sox2 and Nanog. Alterations in the expression levels of these factor *in vitro* (over expression indicated by blue arrows, and loss of function indicated by red arrows) results in differentiation of ES cells toward the indicated lineage (Bosnali, Munst et al. 2009).

These pluripotency determining factors have also been shown to induce pluripotency in somatic cells. Takahashi and Yamanka demonstrated for the first time that over expression of Oct4 and Sox2, together with two other transcription factors c-Myc and Klf4 was sufficient to reprogramme fibroblast cells into ES-like cells. These cells have been referrred to induced pluripotent stem cells (iPS) (Takahashi and Yamanaka 2006).

1.1.3 Haemopoiesis and ontology of haemopoiesis

Much of our current knowledge and scientific concepts about stem cells comes from haematology**.** The following section sheds light on the key concepts of haemopoiesis and haematopoietic cell hierarchy.

Blood production is a complex task. The normal human body generates 200 billion erythrocytes and 70 billion granulocytes per kilogram of body weight daily (Ogawa 1993; Weissman, Anderson et al. 2001). Blood production is achieved by means of a process termed 'haemopoiesis'

Haemopoiesis (from the Greek ' haima': blood; and ' poiein': to make) provides a reliable and efficient blood cell system that consists of a dynamic and complex developmental process capable of continuous blood cell generation and also rapid cell proliferation in response to haemorrhage, infection or hypoxia (Ogawa 1993; Weissman, Anderson et al. 2001) (*Weissman, 2000*).

1.1.3.1 Ontogeny of Haemopoiesis

In mammalian development haemopoiesis occurs in two distinct phases: primitive and definitive phases (Figure 5). The two stages occur at different anatomical sites and generate different cell types, probably through divergent genetic programs (Tavassoli 1991).

Primitive haemopoiesis (also termed as mesoblastic phase) instigates during embryonic life within yolk sac blood islands at approximately embryonic day 15-28 (E 15-18) (Figure 6). These blood island cells originate from the ventral mesoderm. Erythrocytes and myeloid lineage cells are produced during this stage of development (Palis, Robertson et al. 1999).

Primitive haemopoietic cells and endothelial cells are believed to have a common precursor cell, termed hemangioblasts (from the Greek 'haima': blood and 'angio': blood vessel) (Choi 1998). This theory of common origin is supported by several lines of evidence. First, primitive haemopoietic and endothelial cells express similar markers, such as CD34 (Wood, May et al. 1997) and vascular endothelial growth factor receptor VEGFR2, also known as Flk-1 (mouse) and KDR (human) (Labastie, Cortes et al. 1998; Lugus, Park et al. 2005) . Moreover, *in vitro* studies have shown that haemopoietic cells and endothelial cells can be cultured from the same bi-potential cell type, termed colony forming blast cell (Choi 1998).

Some controversy, however, surrounds the origin of cells responsible for the definitive phase of haemopoiesis. It was originally believed that cells migrated from the yolk sac into the blood stream and colonized the foetal liver. This long held view was founded upon *in vitro* proof which demonstrated that yolk sac progenitors contained spleen colony forming

units (CFU-S), progenitor cells capable of generating all haemopoietic lineages (Hollands 1987) . However, there are arguments that surround the contribution of the yolk sac to haemopoiesis. It is questionable whether there is a long term contribution from the yolk sac or whether the progenitors found there have a transient role and then migrate to intra-embryonic regions of haemopoiesis and the foetal liver (Orkin and Zon 2002). These views have been proven to be overly simplified through experiments involving grafting of quail embryos into chick yolk sac which demonstrated that the embryo is itself a chief independent source of definitive haematopoietic stem cells (Dieterlen-Lievre 1975). Studies have demonstrated the presence of lympho-myeloid precursors in the embryo proper at time before the establishment of circulation in the yolk sac. These precursors have been demonstrated to originate from a paraoartic region within the embryo termed, aorto-gonad-mesonephros (AGM) (Cumano, Furlonger et al. 1993).

The definitive stage of haemopoiesis consists of two phases:

The **hepatic phase**: occurs at week 6 in the foetal liver. At this stage the spleen also starts to contribute towards granulopoiesis and primitive lymphopoiesis begins in the foetal thymus and lymphoid tissues until birth.

The **myeloid (medullary) phase**: occurs from week 22 of gestation, in the bone marrow and eventually becomes the major site of life-long haemopoiesis.

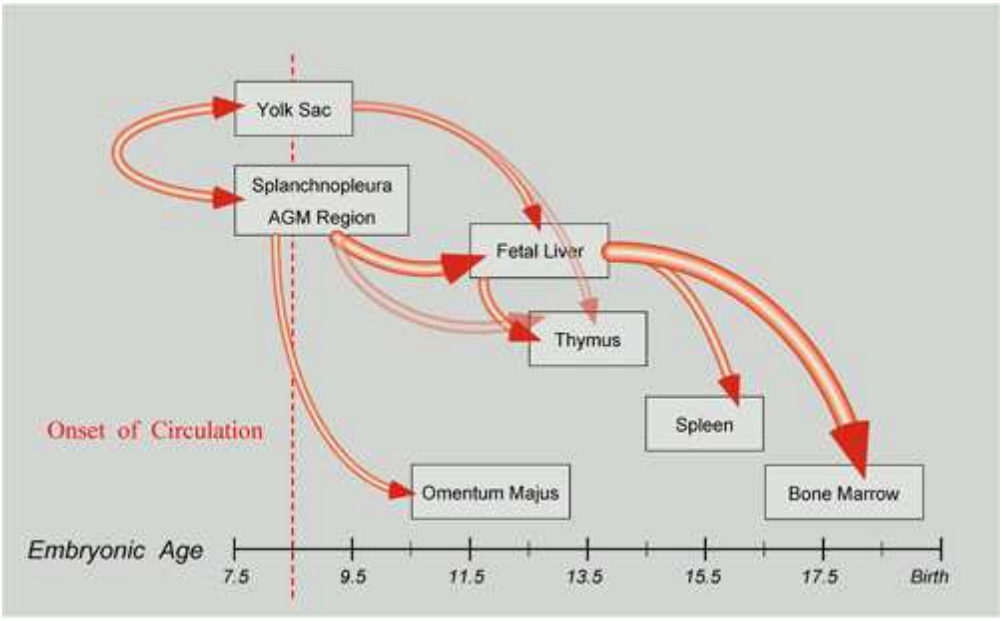


Figure 5. Graphical representation of the different phases of haemopoiesis. The y-axis illustrates the contribution of different tissues towards haemopoiesis during the various stages of gestation and then eventually after birth (postpartum). The Primitive (mesoblastic) phase is predominantly dependent on the input of the yolk sac and AGM region. The Definitive phase initially involves mainly the liver and to some extend the spleen (Hepatic phase) and then the bone marrow which continues to be a source of life-long haemopoiesis (myeloid or Medullary phase).

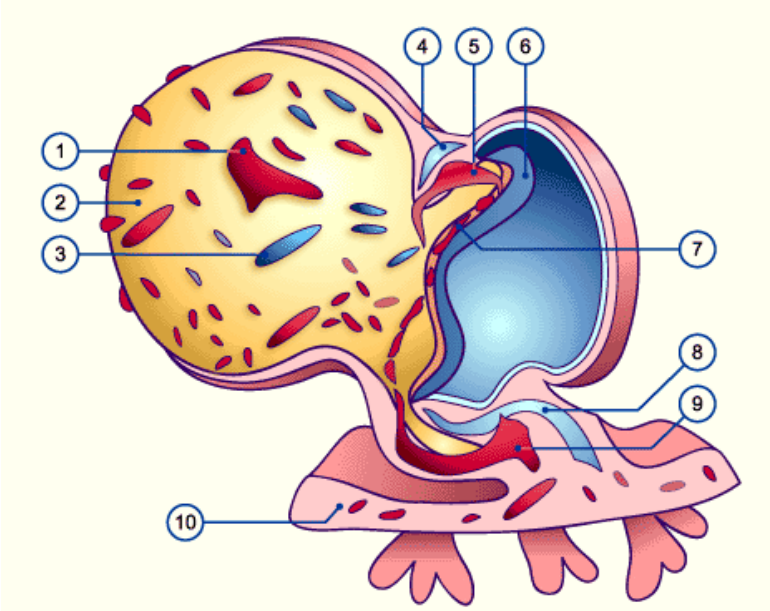


Figure 6. Saggital section of an early embryonic development (E18). Embryo (6) lying in the amnion and connected to the yolk sac (2). Blood islands are visible in the yolk sac at this stage of development (1 and 3). These islands constitute bi-potent stem cells that eventually contribute to the formation of the haemopoietic and vascular system. Primitive blood vessels in the yolk sac converge to form the umbilical vessels; umbilical vein (8) and umbilical arteries (9) that allow exchange of nutrients and gases in the placenta (10). The figure also illustrates the contribution of the aorto-gonad-mesonephros (AGM) (7) paraortic regions towards haemopoiesis. Cardiogenic mesoderm (5), (4).

1.1.3.2 Haematopoietic stem cells

The haemopoietic system comprises a hierarchy of cells with different potentials. At its zenith are rare heterogeneous populations of immature haemopoietic precursor cells, occurring at a frequency of approximately 1 in 10^4–1 in 10^5 cells postnatally (Watt and Contreras 2005). These are multipotent haematopoietic stem cells (Figure 7). HSCs were discovered in the early sixties because of their ability to regenerate blood cells in animals suffering from bone marrow suppression. HSCs are capable of constant movement from the bone marrow to the blood stream and vice versa, a process known as 'Homing' (Orkin and Zon 2002).

HSCs are characterized by self-renewal, proliferation and differentiation into all the cells of the haematopoietic system (Wagers, Sherwood et al. 2002). This balance between self-renewal and differentiation has to be maintained at a steady state but must be capable of swift adaptation upon stress. Their genetic and phenotypic heterogeneity resides in their different proliferative and differentaitng abilities, and activation of cell cycle states. HSCs are multipotent, with the ability to commit to one of 10 or 11 functional haemopoietic lineages (Watt and Contreras 2005). The 'true' HSC has probably not been identified to date, however, HSC are typically described as blast cells, with a large nucleus surrounded by a thin cytoplasm (*McGuckin et al, 2003b*) (Figure 2). Their multipotent and long-term repopulating ability enables these cells to populate the whole haemopoietic system at least once over an individual's lifespan (Martin-Rendon and Watt 2003; Martin-Rendon and Watt 2003). Self renewal and quiescence are two specific cell cycle features that enable HSC to continuously replenish the haemopoietic system. Self renewal consists of HSC division giving rise to identical daughter cells, hence maintaining a sufficient pool of HSC to sustain continuous haemopoiesis throughout life (Lajtha 1975; Ogawa 1993). Quiescence was first defined by Lajtha and he suggested that HSCs were at a steady state in quiescence (G0 state). The genetic and pluripotent integrity of HSCs is maintained in this dominant stage thus allowing cell growth and DNA-repair prior to the entry of the cells for self-renewal or differentiation (Metcalf 1999).

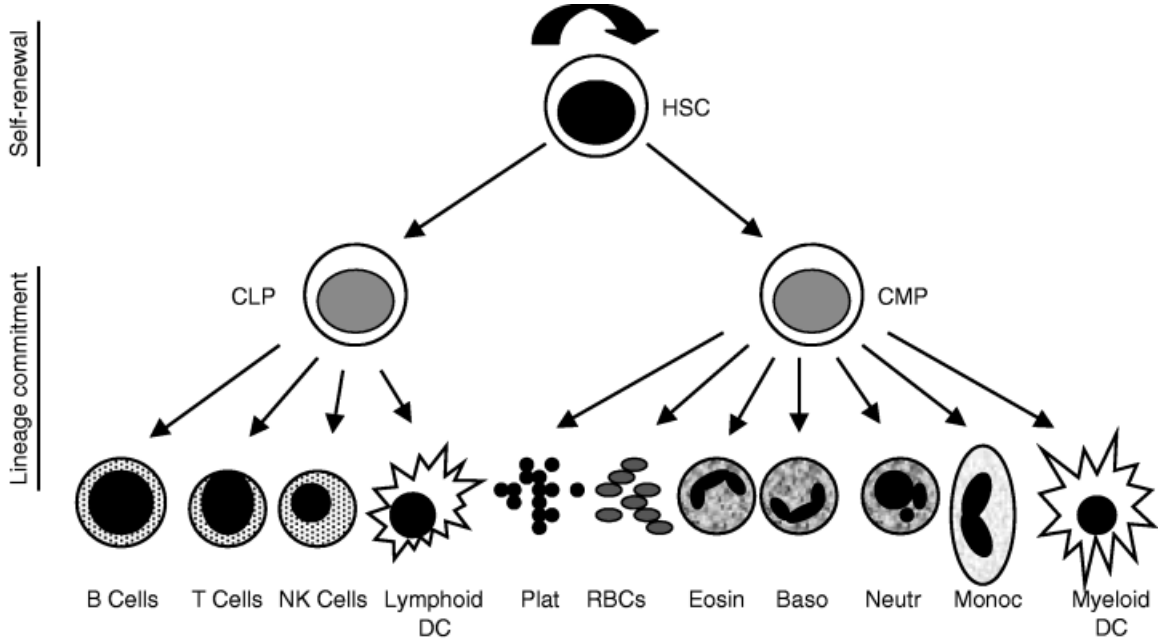


Figure 7. Schematic representation of the hierarchy involved in the development of the haemopoietic system. A haemopoietic stem cell (HSC) balances self-renewal with lineage commitment and differentiation, giving rise to more HSCs and differentiated blood components, that is, myeloid and lymphoid lineages. CLP (common lymphoid progenitor); CMP (common myeloid progenitor); B cells (B lymphocytes); T cells (T lymphocytes); NK cells (natural killer cells); Lymphoid DC (lymphoid dendritic cell); Plat (platelets); RBCs (red blood cells); Eosin (eosinophils); Baso (basophils); Neutr (neutrophils); Monoc (monocytes) and Myeloid DC (myeloid dendritic cells) (Martin-Rendon and Watt, 2003).

In the late 90's and early 2000 an increasing body of evidence suggested the phenomenon of plasticity in HSCs. In most mammalian species the HSC compartment contains a continuum of stem cell types and these cells are believed to be 'plastic' in their proliferative and differentiation capacities. Current studies on haematopoietic and other tissue derived stem cells have challenged one of the main dogmas in biology, namely that mammalian cell differentiation follows established programmes in a hierarchical fashion, and once committed to a particular somatic cell lineage cells do not change into another somatic lineage. The proposed newer concepts of stem cell plasticity or potentiality (Martin-Rendon and Watt 2003) facilitate a better understanding of plasticity, especially for HSCs which seem to have extraordinary abilities to generate or switch between haematopoietic and non-haematopoietic lineages (Orkin and Zon 2002; Poulsom, Alison et al. 2002).

1.1.4 Stem cell plasticity

Plasticity is defined as the ability of stem cells to adopt to new fates. Environmental factors (microenvironment), to which the stem cells are exposed to, have a pivotal role in cell fate decisions. This strengthens the case that stem cell fate is not completely intrinsically determined and emphasizes the importance of the microenvironment.

Embryonic stem (ES) cells are defined as being totipotent and are able of generating all or most tissue types in an organism (Smith 2001; Wilmut 2002). ES cells may be regarded as being truly 'plastic' in their development potential. However, the requirement of human embryos to obtain these stem cells has resulted in many ethical concerns and debate surrounding this area of research. In contrast, ADS are a lot more ethically accepted worldwide and have been applied in clinical practice for many years (Lemischka 1999; Metcalf 1999; McCulloch 2003). As HSC is the best-characterized adult mammalian ADS type it has been used to define 'stemness' of cells in other mammalian adult tissues (Martin-Rendon and Watt 2003).

The exact mechanisms via which cell fate reprogramming occurs are still not fully understood, however, five pathways have been suggested (Martin-Rendon and Watt 2003) (Figure 8):

- Dedifferentiation of mature or lineage-restricted cell to a more immature or precursor cell.
- Transdetermination whereby stem cell potential is redirected giving rise to cell types of a different stem/precursor cell.
- Transdifferentiation where a differentiated cell gains the phenotypic characteristics of another differentiated cell.
- Cell fusion the physical union of two cells
- The presence of very primitive or multiple stem cell types in a tissue whose potential is expressed when the stem cell enters and interacts with the appropriate microenvironment.

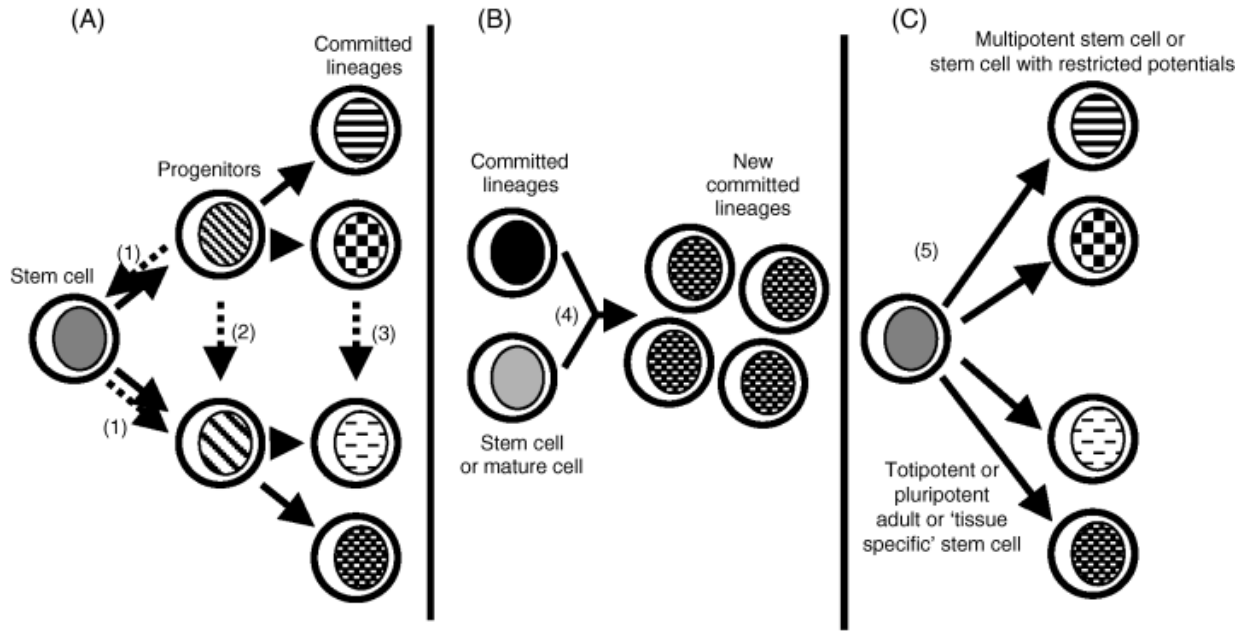


Figure 8. Pathways proposed for stem cell plastity. The pathways to new cell identities may be explained by the following processes: (1) dedifferentiation of mature or lineage-restricted cell to a more immature progenitor or precursor, followed by differentiation to another lineage. (2) Transdetermination, whereby stem cell potential is redirected giving rise to cell types of a different stem/precursor cell. (3) Transdifferentiation, the mechanism via which a differentiated cell can gain the phenotype of another differentiated cell. (4) Cell fusion and (5) a totipotent or pluripotent stem cell gives rise to a multipotent stem cell or a stem cell with restricted potential (Martin-Rendon and Watt, 2003).

1.1.4.1 Stem Cell Plasticity within haemopoietic tissues

Stem cells that generate the haemopoietic system are developmentally mesodermal in origin and, until recently it was commonly accepted in biology, that once committed to haemopoietic lineage should be unable to switch between lineages. However, recent studies have provided evidence that stem cells in haemopoietic tissues have the potential to generate cell types other than those of the tissues in which they reside and this potential of stem cells has been extensively reviewed (Orkin and Zon 2002) . A few selected examples of stem cell plasticity or potentiality demonstrated in human studies are described in the table 4. This non-hierarchical reversible phenotype of stem cells in haemopoietic tissues is an advantage that can be exploited extensively in regenerative medicine.

Mesoderm-Endoderm Conversions

Since one of the goals of this research was the generation of hepatocytes from haemopoietic stem cells (cord blood derived stem cells), an appropriate example of germ layer transition would be mesoderm-endoderm conversions (Table 4).

In the liver, hepatocytes and biliary epithelium are endodermal in origin. The capability of haematopoietic stem cells of mesodermal origin to gain the phenotype of endodermal cell lineages has been reported both *in vitro* and *in vivo* in both rodent and human studies (Petersen, Bowen et al. 1999; Lagasse, Connors et al. 2000; Theise, Badve et al. 2000; Theise, Nimmakayalu et al. 2000; Schwartz, Reyes et al. 2002; Kakinuma, Tanaka et al. 2003; Lee, Kuo et al. 2004; Kang, Zang et al. 2005; McGuckin, Forraz et al. 2005).

Table 4. Examples of adult stem cell plasticity.

Stem cell origin	Lineage 'plasticity'	Somatic lineage transition?	Reference
Marrow	Neural cells	Mesoderm-ectoderm	(Mezey 2003)
Marrow	Liver hepatocytes	Mesoderm-endoderm	(Alison, Poulsom et al. 2000) (Theise, Nimmakayalu et al. 2000)
Marrow	Pancreatic endocrine cells	Mesoderm-endoderm	(Ianus, Holz et al. 2003)
Marrow	Glomerular mesangial cells	Mesoderm-endoderm	(Masuya, Drake et al. 2003)
Marrow	Epithelial and liver hepatocytes	Mesoderm-ectoderm	(Kleeberger, Rothamel et al. 2002) (Korbling and Estrov 2003)
Marrow	Blood vessel endothelium and vascular cells	Mesoderm-mesoderm	(Strauer, Brehm et al. 2001) (Assmus, Schachinger et al. 2002) (Stamm, Westphal et al. 2003)

1.1.5 Stem cell niches

Cell fate is regulated by complex and complementary successions of external and intrinsic stimuli (Phillips, Ernst et al. 2000). The external stimuli activate complex signaling pathways which in turn module intrinsic regulatory pathways via production of transcription factors (Shivdasani and Orkin 1996; Tenen, Hromas et al. 1997; Zhang and Li 2008).

1.1.5.1 Microenvironment ('Niches')

Recent studies demonstrate that in addition to intrinsic control of cell survival, self-renewal and differentiation (lineage determination); the surrounding environment plays a crucial role in cell fate decisions. These privileged microenvironments are also referred to as 'niches' (Watt and Hogan 2000; Spradling, Drummond-Barbosa et al. 2001; Fuchs, Tumbar et al. 2004; Li and Xie 2005; Xie and Li 2007) (Figure 9). These niches may be viewed as specific anatomic locations that regulate how stem cells participate in tissue generation, maintenance and repair. In tissue engineering such niches can be modeled or re-created *in vitro* to culture tissues better or mimic a physiological environment. Such niches protect stem cells from depletion, while protecting the host from over-exuberant stem-cell proliferation and hence consequent pathologies, such as tumours (Scadden 2006). The theory of a niche as a specialized microenvironment was first proposed almost 30 years ago by Schofield in reference to mammalian haematology, although experimental evidence was first provided by invertebrate models (Schofield 1978).

The regulatory elements in these niches include heterologous cells, non-cellular and extracellular matrix (ECM) constituents (Scadden 2006). ECM constituents include: cytokines, cell adhesion molecules (CAM), and extracellular non-protein elements, such as Calcium and 'homing' molecules (which may themselves be modulated by cytokines) with their corresponding receptors. Interactions between the stem cells and their microenvironments are presumably a prerequisite for enabling the stem cells to lodge in specific regions and then to respond to the localized developmental stimuli (Hoffbrand AV 2001). The importance of this environmental control is considered greater for stem cells then for mature cells (Hoffbrand AV 2001). Establishment of ‘Niches’ is needed for organ regeneration. Disease conditions may be associated with destruction of or absence of the stem cell niche and this has to be restored, so that the organ can repair itself. Examples include: benign age related conditions like hair graying which is proposed to be associated with defective self-maintenance of melanocyte stem cells within their niche in the hair follicle (Nishimura, Granter et al. 2005); pathological age related degenerative disorders

such as Parkinson's disease and Alzheimer's disease (Carlson and Conboy 2007) and malignant diseases such as Leukemias (Lane, Scadden et al. 2009),

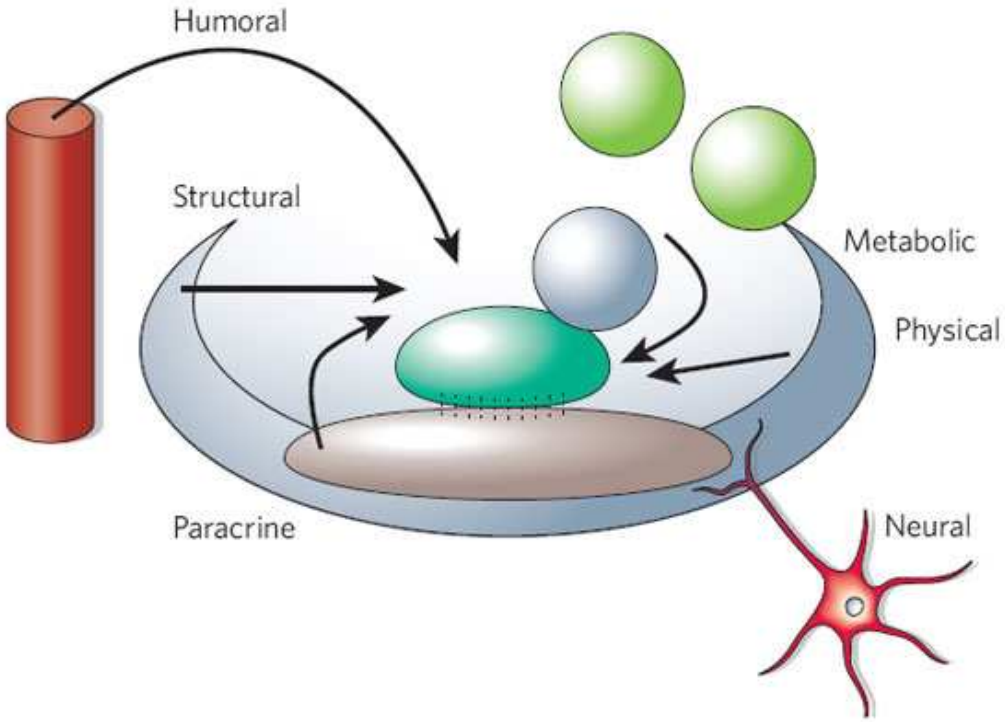


Figure 9. Cellular and non-cellular components of stem cell niche. The stem cell system is regulated by architectural space, physical interactions with neighboring cells, neural signals, paracrine (hormonal factors produced by neighboring cells) and endocrine (hormonal factors produced by distant glands and secreted into the circulation) signals from local or distant sources and metabolic products of tissue activity. Stem cell is depicted in dark green (Scadden, 2006).

Stem/progenitor cells utilized in this study were derived from the umbilical cord and umbilical cord blood. The following section is an introduction to the afterbirth and its various components.

1.2 Introduction to the Umbilical cord and cord blood

Afterbirth comprises: the placenta, umbilical cord and chorioamnion ('foetal membranes'). The structures are collectively known as the 'foetal adnexa'. The placenta and cord are extra-embryonic tissues derived from the trophoblast. The placenta connects the foetus to the uterine wall via the umbilical cord, and is the organ by means of which the nutritive, respiratory, and excretory functions of the foetus are carried on. It is composed of foetal and maternal portions (The Bartleby.com edition of Gray's *Anatomy of the Human Body*). The umbilical cord connects the foetus to the placenta. Umbilical cord blood (UCB) is the blood that is obtained from the umbilical vessels.

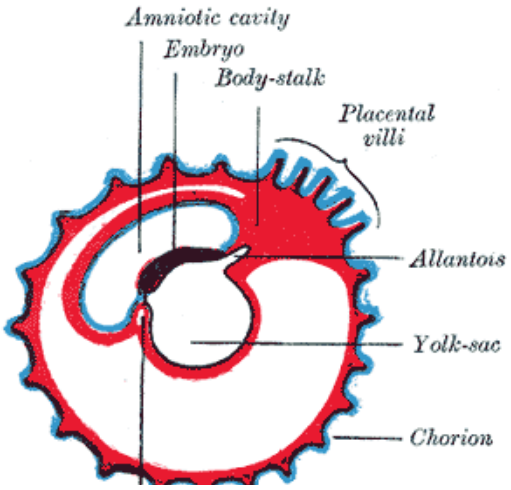
A. The Placental

The placenta is an intra-uterine organ formed by the fusion of extra-embryonic and maternal tissues. The word *placenta* comes from the Latin for *cake,* from Greek *plakóenta/plakoúnta*, accusative of *plakóeis/plakoús* - *πλακόεις*, *πλακούς*, "flat, slab-like", referring to its round, flat appearance in humans. Except from a small maternal contribution to the placenta, the foetal adnexa develop from the extra-embryonic tissues of conceptus. During the embryonic period, the precursors of the fetal adnexa are the developmental adnexa, which include the extra-embryonic membranes (amnion, chorion, umbilical vesicle, also known as the yolk sac or vitelline sac, and allantoic diverticulum) and the connecting (or body) stalk. Later during embryonic develop the connecting stalk becomes covered by amnion to form the umbilical cord. In humans, the placenta averages 22 cm (9 inch) in length and 2–2.5 cm (0.8–1 inch) in thickness (greatest thickness at the center and become thinner peripherally). It typically weighs approximately 500 grams (1 lb). It has a dark reddish-blue or maroon color. It connects to the fetus by an umbilical cord.

The placenta also illustrates metabolic and endocrine functions. It produces both protein as well as steroid protein hormones. Protein hormones including: human placental lactogen (hPL) also known as somatomammotropin, human chorionic gonadotropin (hCG), human chorionic thyrotropin (hCT) and human chorionic corticotrpin (hCACTH). Steroid hormones including: progesterone and oestrogen.

The placenta and the fetus might also be regarded as foreign allografts in the mother and hence must escape the maternal immune system. The placenta serves in several ways to avoid immune rejection; it avoids detection through secretion of Neurokinin B containing phosphocholine molecules. This mechanism is applied by parasitic nematodes to avoid detection by the immune system. There are small lymphocytic suppressor cells in the foetus that inhibit maternal cytotoxic T cells. This is done through inhibiting the response to interleukin 2 (Il-2) (Clark, Chaput et al. 1986).

A



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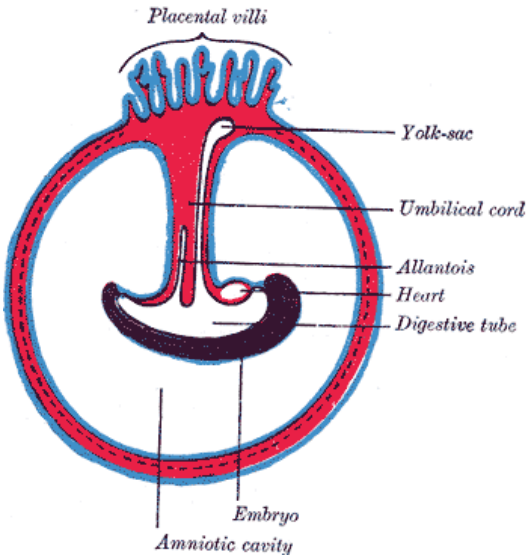

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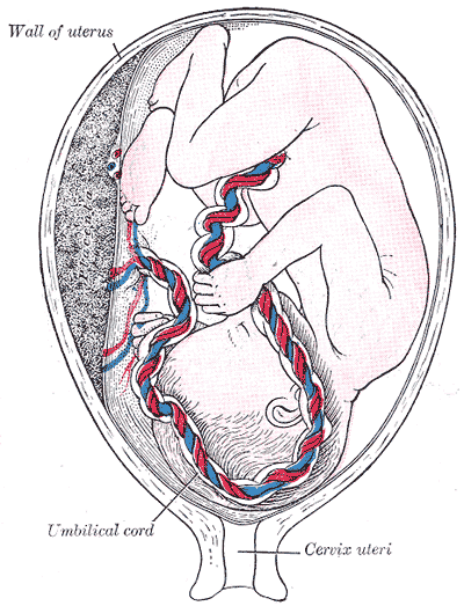


Figure 10. Development of the placenta and the umbilical cord. (A) Conceptus at approximately 3.5 weeks. All the fetal adnexa are visible at this stage. (B) A later stage in the development of the umbilical cord. The umbilical cord embryonically constituents the vitelline vessels, vitelline duct, right and left umbilical veins, and a pair of umbilical arteries. As development progresses the vitelline vessels and duct, together with the right umbilical vein, undergo atrophy and disappear; and thus the cord, at birth, contains a pair of umbilical arteries and one (the left) umbilical vein. (C) Foetus in utero, near term gestation. The diagram illustrates the structure of the umbilical cord near full term. Note the presence of one umbilical vein (blue vessel) and two umbilical arteries (red) wound around the vein. (The Bartleby.com edition of Gray's *Anatomy of the Human Body*)

B. The umbilical cord

The human umbilical cord (UC) is embryologically derived at day 26 of gestation. The umbilical cord at full term, as a rule, is about equal to the length of the foetus, *i.e.,* about 50 cm, but it may be greatly diminished or increased. The umbilical cord is essentially composed of amniotic epithelium covering mucoid mesenchymal connective tissue (Wharton's jelly). Sarugaser *et al* postulated that this connective tissue was derived from a mesenchymal precursor cell population located within the umbilical cord. They reported that these cells were most likely located in close vicinity to the vasculature, thus close to their source of oxygen and nutrients. They called these cells human umbilical cord perivascular (HUCPV) cells (Sarugaser, Lickorish et al. 2005).

The umbilical cord inserts into the chorionic plate of the placenta. It is composed of two arteries and a single umbilical vein. These vessels are developmentally derived from the allantoic vessels. At the junction of umbilical cord and placenta, the umbilical arteries branch radially to form chorionic arteries (Figure 11). The chorionic arteries further branch before they enter into the villi. In the villi, they form an extensive arteriocapillary venous system, bringing the fetal blood extremely close to the maternal blood; but no intermingling of fetal and maternal blood occurs. This is defined as the placental barrier and allows selective movement of endogenous and exogenous compounds across the placenta. This protects the developing foetus from the harmful effects of toxins, chemicals, cytokines, micro-organisms etc. The umbilical vessels are responsible for exchange of gases, nutrients and waste products between fetal and maternal circulations across the placental membranes. The umbilical arteries deliver deoxygenated blood to the capillaries of the placental villi where gaseous exchange takes place between maternal and fetal circulations. Oxygenated blood is returned to the fetus via the umbilical vein. Maternal blood in the intervellous spaces is supplied and drained by maternal vessels.

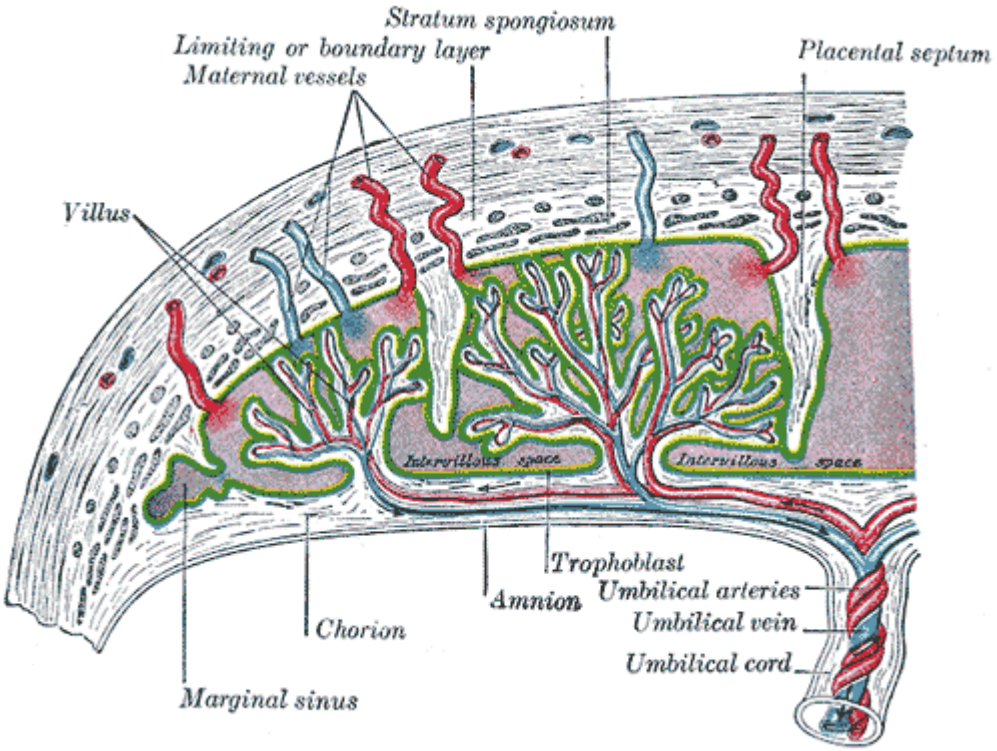


Figure 11. Cross section of the placenta illustrating branching of the umbilical arteries to form the chorionic arteries. These vessels branch further before they enter into the villi. In the villi the extensive arteriocapillary venous system allows communication between foetal and maternal circulation without direct mixing of blood. This is know as the placenta barrier. (The Bartleby.com edition of Gray's *Anatomy of the Human Body*)

1.2.1 The afterbirth as a stem /progenitor cell resource

Foetal stem cells, comprises a broad stem cell class and can be isolated from two distinct sources, the foetus proper and the extra-embryonic structures. The extra-embryonic tissues are a rich stem cell reservoir with many advantages over both embryonic and adult stem cell sources. The afterbirth is routinely discarded at parturition, thus allowing little ethical controversy over harvesting of the resident stem cell populations. In addition, the extracorporeal nature of these stem cell sources assist isolation, eliminating patient risk that may accompany adult stem cell isolation. Further more, the relatively large volume of extra-embryonic tissues and the ease of physical manipulation theoretically interprets as an increase in the number of stem cells isolated.

1.2.1.1 Umbilical cord blood

The first isolated foetal stem cells were haematopoietic, derived from human umbilical cord blood. Thus, cord blood represents the prototypic foetal stem cell source. UCB-derived stem cells enjoys an intermediate niche between ES and ADS with the added advantages of being ethically sound and associated with completely non-invasive methods for collection of the cord blood. Until the advent of cord blood banking in the mid 90s, placenta and umbilical cord were considered clinical by-products, but ever since cord blood has created hope as a new alternative in the management of various disease conditions for which there is no cure or the current treatment options are inefficient. Cord blood provides an easily available and rich source of haemopoietic and non-haemopoietic stem cells. Term and preterm umbilical cord blood contains an equal or significantly higher number of early and committed progenitor cells when compared with bone marrow and greatly surpasses that of adult peripheral blood (Mayani, Gutierrez-Rodriguez et al. 1998). A lot of work has been done in the area of exploring the potential of stem cells in cord blood. One of such exciting studies is the work done by Mcguckin *et al*, who investigated the multipotential capability of cord blood-derived stem cells (Forraz, Pettengell et al. 2002; McGuckin, Forraz et al. 2004). In 2005, McGuckin *et al* reported the world's first reproducible production of cord blood-derived stem cells expressing embryonic stem cell markers (*McGuckin et al, 2005*). Their work was supported by other reports concerning the existence of circulating embryonic stem cell-like cells during foetal development (*Jiang et al, 2002; Korbling and Estov, 2003*).

Another population of non-haematopoietic multipotent stem cells termed, mesenchymal stem cells (MSCs) has been identified in UCB (Erices, Conget et al. 2000; Mareschi, Biasin et al. 2001; Bieback, Kern et al. 2004; Kogler, Sensken et al. 2004; Markov, Kusumi et al. 2007). These multipotent cells (Kang, Zang et al. 2006) have unique immunoregulatory

features that suppress lymphocyte proliferation *in vitro* (Gerson 1999) and exhibit high self renewal potential (Bartholomew, Sturgeon et al. 2002). MSCs have potential to differentiate into tissues of all three germ layers: including bone, cartilage, fat, muscle, endothelial cells, neuronal, glial cells and liver (Bruder, Jaiswal et al. 1997; Kopen, Prockop et al. 1999; Pittenger, Mackay et al. 1999; Erices, Conget et al. 2000; Woodbury, Schwarz et al. 2000; Campagnoli, Roberts et al. 2001; Horwitz, Gordon et al. 2002; Krampera, Glennie et al. 2003; O'Donoghue, Choolani et al. 2003; Lee, Kuo et al. 2004; Hong, Gang et al. 2005; Li, Qu et al. 2005; Chien, Yen et al. 2006; Lange, Bruns et al. 2006; Ong, Dai et al. 2006; Snykers, Vanhaecke et al. 2006; Lu, Teng et al. 2007; Paunescu, Deak et al. 2007; Tamagawa, Oi et al. 2007; Yen, Chien et al. 2007; Lu, Dong et al. 2008).

Although, term UCB is reported to be a source of MSCs (Erices, Conget et al. 2000; Mareschi, Biasin et al. 2001; Bieback, Kern et al. 2004; Kogler, Sensken et al. 2004; Markov, Kusumi et al. 2007), this source of MSC is subjected to a lot of controversy (Gutierrez-Rodriguez, Reyes-Maldonado et al. 2000; Campagnoli, Roberts et al. 2001; Mareschi, Biasin et al. 2001; Wexler, Donaldson et al. 2003; Kogler, Sensken et al. 2004; Lee, Kuo et al. 2004; Yu, Xiao et al. 2004; Kern, Eichler et al. 2006). Kogler *et al*, reported that the amount of MSC in UCB was one MSC in every 20ml of UCB (Kogler, Sensken et al. 2004). Additionally, previous studies have demonstrated that only in 63% of UCB units could MSCs be isolated in culture with a frequency of MSC-like cells ranging from 0 to 2.3 clones per 1x 10^8 MNC cells (Bieback, Kern et al. 2004). This makes the isolation of UCB sourced MSCs very much dependent on the quality and volume of the cord blood, hence making cord blood not a very efficient resource for MSC compared to the umbilical cord.

1.2.1.2 Umbilical cord

The umbilical cord (UC) has recently been recognized as a rich source of stem/progenitor cells, namely Mesenchymal stem cells (Romanov, Svintsitskaya et al. 2003; Wang, Hung et al. 2004; Sarugaser, Lickorish et al. 2005) (Figure 13). Wharton's jelly, first described by Thomas Wharton in 1656 (Wharton 1996) is the matrix filling the umbilical cord and is a rich source of MSCs. Chacko and Reynolds (Chacko AW 1945) described these cells residing in Wharton's jelly as smooth muscles, but Takechi *et al* (Takechi, Kuwabara et al. 1993) refined the description to 'myofibroblats' after insitu labelling of vimetin, desmin, α-actin, and myosin, which was confirmed by Kadner *et al* (Kadner, Hoerstrup et al. 2002). These cells are present in relatively high numbers, with an average of 400,000 cells isolated per umbilical cord (Karahuseyinoglu, Cinar et al. 2007). This is significantly greater that the yield from adult bone marrow. These cells grow adherent to plastic and express specific

pattern of cell surface determinants (CD105, CD90, CD73, and CD44). This cell fraction is distinct from the haematopoietic and pluripotent stem cells present in cord blood and do not express blood-cell determinants (CD34, CD45). These Mesenchymal cells can be expanded very efficiently *in vitro* and have been propagated in culture for more than 80 population doublings (Marcus and Woodbury 2008).

In vivo studies involving the transplantation of UC MSCs are limited, but encouraging. These cells have been transplanted into animal models of disease (severe muscle damage, ischemic rodent brain and hemiparkinsonian) and have demonstrated very promising results (Conconi, Burra et al. 2006; Weiss, Medicetty et al. 2006; Ding, Shyu et al. 2007)

1.2.1.3 Amniotic fluid

Of all the extra-embryonic sources, amniotic fluid is the only one where harvest occurs prior to parturition. This trait may prove increasingly important as in utero cell-based therapies progress (Muench 2005). Amniotic fluid contains a heterogeneous population of cells displaying a range of morphologies. Most of these cells are epithelial in nature and have a limited capacity to proliferate in nature. These cells are predominantly derived from the fetal skin, urogenital, respiratory and digestive tract. Additional cells derived from the inner surface of the amniotic membrane add to the mix. The cellular composition of amniotic fluid changes with gestation, coinciding with the maturation of the foetus (Torricelli, Brizzi et al. 1993). Stem cells with the characteristics of HSCs within amniotic fluid were first isolated and described in 1993 by Toricelli *et al* (Torricelli, Brizzi et al. 1993). However, non-haematopoietic Mesenchymal stem cell population within amniotic fluid was only recently reported (In 't Anker, Scherjon et al. 2003). A subset of these cells have reportedly tested positive for Oct4 expression highlighting their possible pluripotent nature (Prusa, Marton et al. 2003). It has been reported that these MSCs express both mesodermal and ectodermal gene products. This characteristic is consistent with the emerging concept that stem cell populations exist in a multi-differentiated state. The proliferation capacity of these cells meets or exceeds that described for adult human MSCs. Additionally, these cells have demonstrated the ability to differentiate into various tissues derived from all three germ layers (In 't Anker, Scherjon et al. 2003; Bossolasco, Montemurro et al. 2006; Tsai, Hwang et al. 2006; coppi, Georg Bartsch et al. 2007). Amniotic fluid cells have been transplanted into animal models of disease but have produced contradictory results (Sartore, Lenzi et al. 2005; Chiavegato, Bollini et al. 2007). More work is demanded to resolve these apparently contradictory outcomes and to pronounce amniotic fluid MSCs as a possible alternative for cell transplantation therapies.

1.2.1.4 Placenta

This large fetomaternal organ is an attractive tissue for the isolation of stem/progenitor cells. The sheer volume of a full term placenta, weighing more than 500g, almost 15 times the weight of the average umbilical cord makes it a rich source of multipotent cells. MSCs have been isolated from dissociated placental tissue based on plastic adherence. These cells express the pluripotency markers Oct4 and Rex1. These cells exist in a multi-differentiated state- simultaneously expressing ectodermal, mesodermal and endodermal genes. These cell have demonstrated tremendous *ex vivo* proliferation capacity and the ability to differentiate into tissue representing all three germ layers (Fukuchi, Nakajima et al. 2004; Yen, Huang et al. 2005; Chien, Yen et al. 2006). Placental-derived MSCs have also demonstrated the ability to support the growth of exogenous HSCs derived from cord blood in culture (Zhang, Li et al. 2004).

Placental-derived MSCS have been utilized in *ex vivo* engineering of complex biological structures such as artificial heart valves (Schmidt, Mol et al. 2006). Additionally, these cells have also been exploited in *in vivo* studies on animal models and demonstrated interesting results (Kogler, Sensken et al. 2004).

1.2.1.5 Amniotic membrane

The amniotic membrane or the amnion is one of the first recognizable tissues derived from the epiblast. This tissue is an avascular structure generated early in development from multipotent cells residing in two distinct areas of the developing blastocyst. During subsequent development, the cells of the amnion are not exposed to the same signals responsible for the gradual fate restriction of cells within the embryo proper. It is thus postulated that the stem cell populations sequestered within the amnion might retain the potency of the epiblast cells from which they are derived. Several multipotent cells have been isolated from the amnion, including MSCs (Marcus and Woodbury 2008). Amniotic membrane has been extensively used as a biological dressing to treat chemical and thermal burns. The clinical success of amniotic membrane transplantation is greatly due to its immunoprivileged characteristics. If this trait is indeed shared by its constituent stem cell populations, they may prove very useful for allogeneic transplantation strategies (Akle, Adinolfi et al. 1981; Adinolfi, Akle et al. 1982). In addition, these cells secrete a number of immunosuppressive factors that target the innate and adaptive immune systems, which may support longterm survival of grafts following transplantation (Kamiya, Wang et al. 2005; Li, Niederkorn et al. 2005).

Some of the isolated stem cell populations were reported to be positive for pluripotency markers and demonstrated the capacity to differentiate into tissue derived from all three germ layers (Miki, Lehmann et al. 2005).

Amnion-derived stem/progenitor cells have been transplanted in animal models of brain diseases and have illustrated promising results (Kakishita, Elwan et al. 2000; Kosuga, Sasaki et al. 2001; Sankar and Muthusamy 2003)

1.2.1.6 Umbilical vessels

MSCs have also been identified in umbilical vessels (Butler, Nolan et al.; Ishige, Nagamura-Inoue et al. 2009).

Stem/progenitor cells derived from cord blood have been extensively studieds and have demonstrated clinical utility. More recently the umbilical cord has gained a lot of attention over other extra-embryonic tissues as an alternative stem cell source for cell transplantation approaches.

It may be safely concluded that, cord and cord blood-derived stem/progenitor cells offer multiple advantages over truly adult stem cells and over embryonic stem cells. With a global population of 6 billion people and a global birth rate in access of 130 million per year, umbilical cord and cord blood possibly provide the most readily accessible and ethically sound alternative source of stem cells (McGuckin, Forraz et al. 2005). Although UCB contains fewer primitive haemopoietic precursor cells than earlier trimester fetal bone marrow, it still provides a source of stem cells at a younger stage than mobilized peripheral blood (MPB) and bone marrow (Tocci, Roberts et al. 2003).

Other differences between these stem cell sources include UCB and MPB (Mobilized Peripheral Blood) haemopoietic precursors are slowly cycling, whereas those in foetal and adult bone marrow have a higher proportion in G_2, M and S phases. In terms of ontogeny, UCB cells are at the intermediate point between embryonic and adult life and have longer telomeres and high proliferation potential. In addition, UCB has an increased capacity for self-renewal (Emerson 1996). UCB units also have a reduced risk of viral contaminations (cytomegalovirus or Epstein Barr virus) (Chang and Seto 1979; Stagno, Pass et al. 1986; Bertolini, Lazzari et al. 1995).

Haematological transplantation using UCB further demonstrated a lower incidence of graft-versus host disease for allogeneic graft (Gluckman and Rocha 2006; Rubinstein 2006). UCB induces better tolerance for human leukocyte antigen (HLA) mismatches when compared to bone marrow, probably due to the immaturity of the immune cells or controlled by UCB dendritic cells and/or natural killer cells (Cohen and Nagler 2004; Liu, Law et al. 2004). They may be the only source of allogeneic HSCs (Hematopoeitic stem cells) available to patients with rare HLA types and hence to ethnic minorities, to siblings suffering from diagnosed haematological disorders and for urgent unrelated donor transplants.

The past 30-40 years has seen great successes in HSC therapies. These provide curative therapies for patients with haematological and non-haematological disorders. Currently more than 85 diseases are either treatable with or ameliorated with the use of umbilical cord blood.

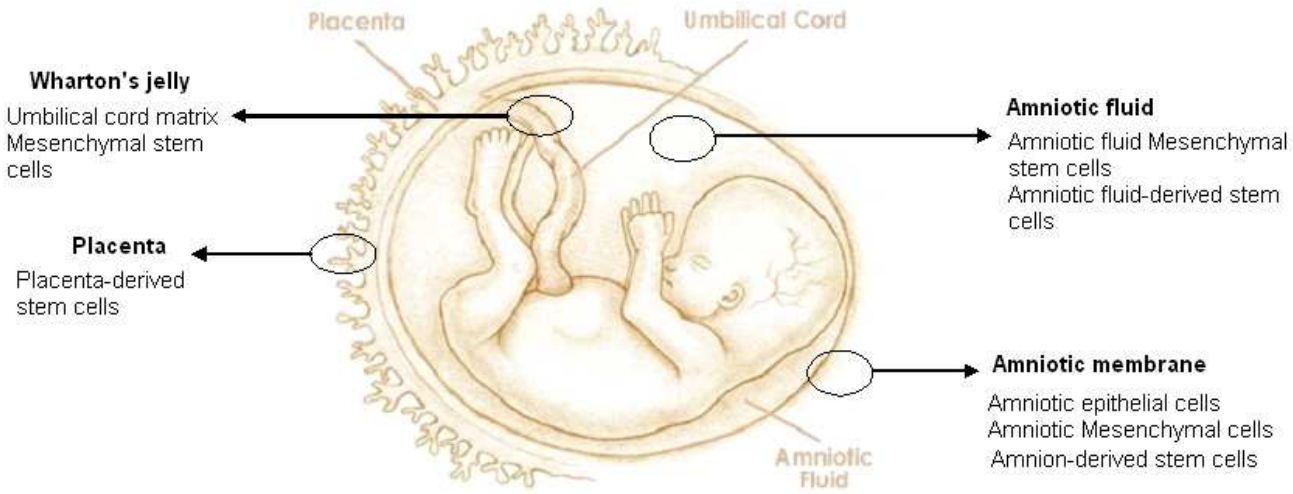


Figure 12. Extra-embryonic stem cell sources. The diagram illustrates that stem/progenitor cells have been isolated from all extra-embryonic tissues including: the amniotic membrane, amniotic fluid, Wharton's jelly and placenta. Adapted from (Marcus and Woodbury 2008)

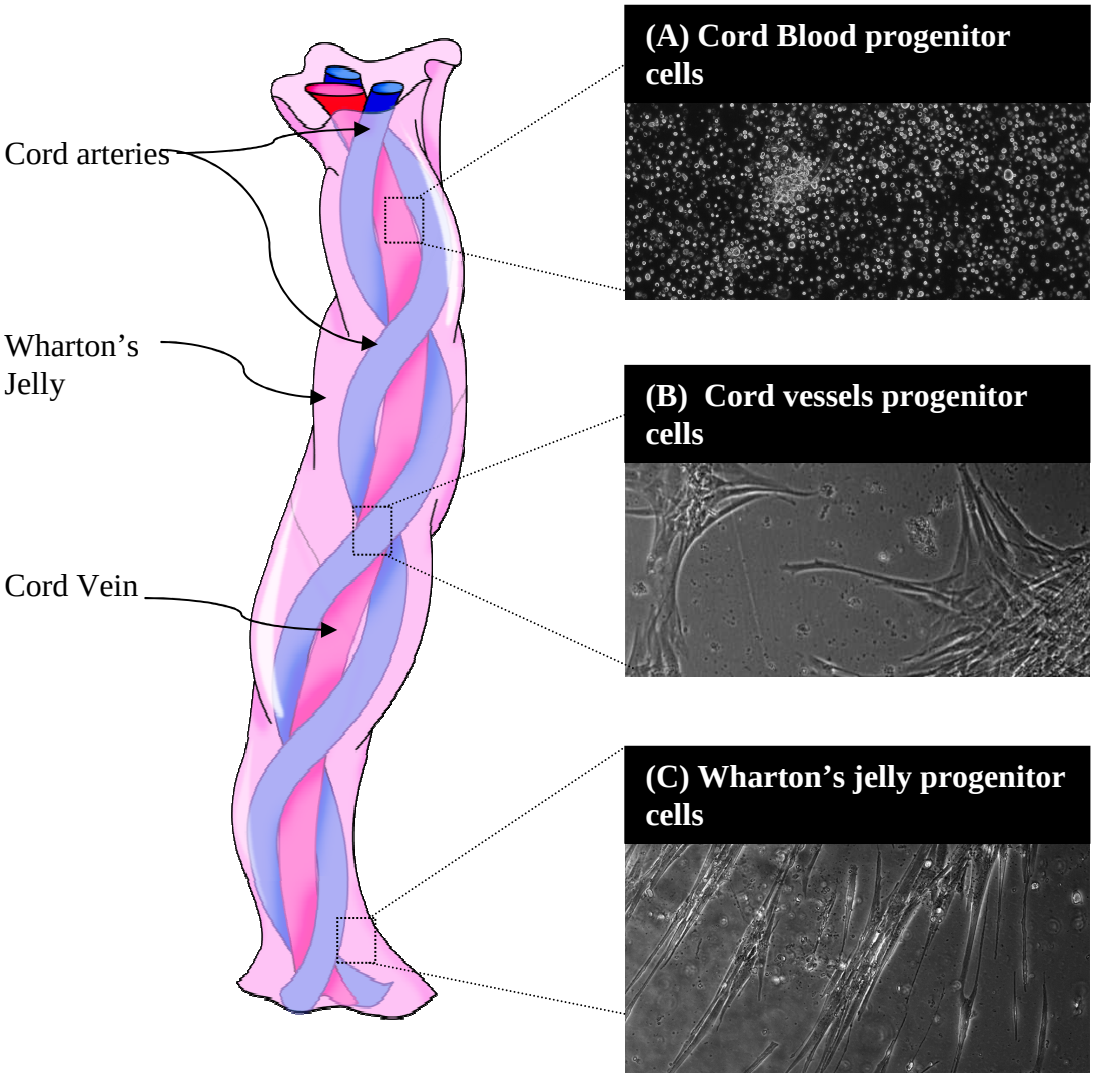


Figure 13. Stem/progenitor cells derived from the umbilical cord. (A) Populations of cord blood cells, these cells have a tendency to form aggregates upon culture *in vitro*. (B) Cord vessel progenitor cells with star-like phenotype. This phenotype is lost after the first passage subsequent to which the cells take on a standard MSC phenotype as shown in (C).

1.2.2 Clinical application of cord blood

The present day is witness to more than 2400 stem cell based therapies in early human trials around the world, based on the Clinical Trials Database (www.clinicaltrials.gov). The beginning of stem cell research dates back to 1866, when Ernst Neumann first postulated the bone marrow as a blood forming organ with a common stem cell for all haematopoietic cells. Further studies followed to increase the understanding of early mammalian development and more recently substantial work has been done in the area of stem cell biomedical research to help improve our understanding of how stem cells influence the organism to maintain its homeostasis.

The clinical application of stem cell transplantation in the management of disease has made tremendous development in the past 3 decades. 20 years ago, it was one disease treatable with cord blood; 10 years ago about 10-15 and now over 85 diseases are treatable with cord blood.

The first clinical use of cord blood was in 1930s, where is was safely used as a substitute for peripheral blood for performing transfusions (Halbrecht 1939). HLA-matching was not available then and no adverse effects were noted; hence the feasibility of cord blood administration to a non pre-conditioned host was suggested.

The first traceable umbilical cord blood transplant was performed in 1970 in a 16-year boy with acute lymphoblastic leukemia (Ende M 1972). This procedure is less reported in literature. The boy received cord blood units from 8 different unrelated donors, untested for HLA compatibility. Only one unit engrafted, but the patient remained in remission with maintenance chemotherapy until his last follow-up appointment at 9 months. The results of this transplant fuelled research into umbilical cord blood as a stem cell resource and in 1980s it was reported that umbilical cord blood contained haematopoietic stem cells that may be suitable for transplantation.

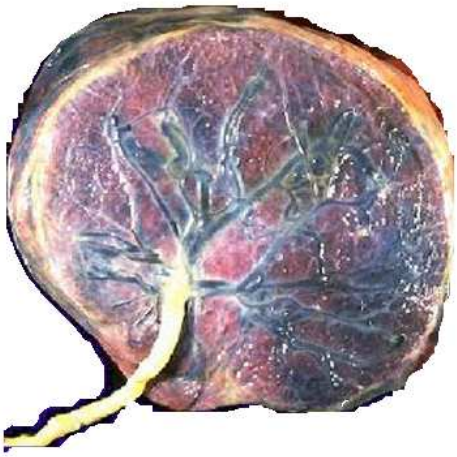
In 1988, the first reported cord blood transplantation was executed to cure Fanconi's anemia in a 5-year boy, using blood from his HLA-identical baby sister's umbilical cord, in Paris, France (Gluckman, Broxmeyer et al. 1989). This report lead to the establishment of cord blood banks worldwide for the collection and cryopreservation of cord blood for allogeneic haematopoietic stem cell transplants.

Since the first reported cord blood transplantation 20 years ago, the number of umbilical cord blood transplantations (UCBT) from siblings and unrelated donors has increased dramatically. In 2006, it was estimated that more than 5000 patients had undergone UCBT

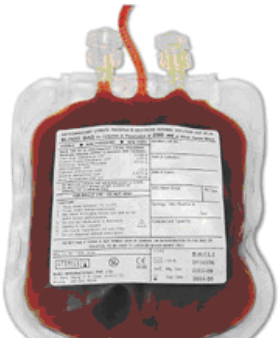
from unrelated donors (Rocha and Gluckman 2006). By 2009, 20,000 UCBT were performed in children and in adults (Gluckman and Rocha 2009).

In 2006, the inventory of Netcord, the cooperative network of large experienced UCB banks, had over 100,000 cryopreserved UCB units ready for clinical use and a reasonably accurate worldwide estimate would be 250,000 UCB units (Rocha and Gluckman 2006). By 2009 that number had increased to 400,000 in more than 50 banks worldwide (www.bmdw.org) (Gluckman and Rocha 2009). A survey of the International Bone Marrow Transplant Registry (CIBMTR) estimates that after 1998, 20% of stem cell transplants performed in young patients (<20 years) were cord blood transplants. In Japan, approximately 50% of haematopoietic stem cell transplants from unrelated donors were performed with cord blood cells. This development is due to the organization of international registries for outcome data collection named Eurocord (www.eurocord.org) and CIBMTR (www.cibmtr.org) and of cord blood banks network named Netcord (www.netcord.org) and NMDP (www.nmdp.org) (Gluckman and Rocha 2009).

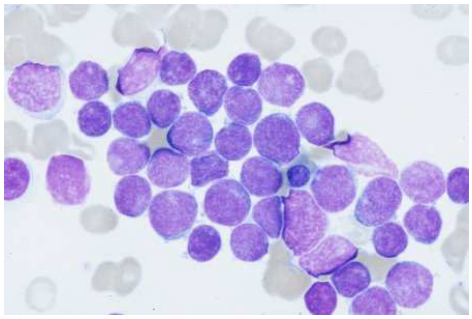
Figure 14. History of cord blood clinical applications.



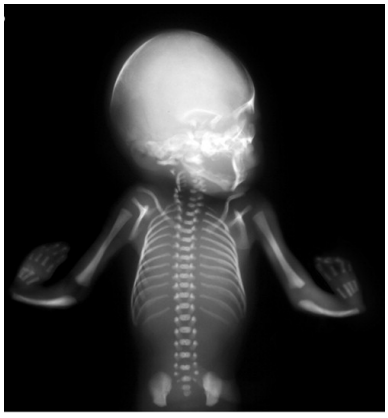
Afterbirth (placenta and umbilical cord)



1930 Cord blood as a substitute for peripheral blood for transfusion (Halbrecht 1939)



1970 first ever UCBT for treatment of acute lymphoblastic leukaemia (Ende M 1972)



1988 First reported UCBT for treatment of Fanconi's anemia (Gluckman, Broxmeyer et al. 1989)

Today, more than 20,000 UCBTs performed in children and in adults (Gluckman and Rocha 2009)

UCB stem/progenitor cells display several advantages over other ADS sources (BM being the most commonly used resource). These benefits include:

- Logistical and clinical advantage: prompt availability of transplant; patients receive UCB transplants in a median of 25-36 days earlier than those receiving BM) (Barker, Krepski et al. 2002).

- Less frequent and less severe acute graft-versus-host disease (GVHD) than BM which contains a higher number of activated T cells.

- Less stringent criteria for HLA donor-recipient selection; tolerance of 1-2 HLA mismatches out of 6. This allows extension of the donor pool.

- Lower incidence of infections by latent viruses.

- Lack of risk to the donor.

- Higher frequency of rare haplotypes compared to bone marrow registries, thus allowing easier targeting of ethnic minorities.

-Umbilical cord blood transplantation criteria iclude:

Two factors that are the key determining factors for engraftment are the number of nucleated cells (NC) and $CD34^+$ cells infused and the number of HLA differences (Gluckman, Rocha et al. 2004; Kamani, Spellman et al. 2008). The latest recommendations are: to choose UCB units with ≤ 2HLA disparities and >2.5 x 10^7 NC/kg or ≥2x 10^5 $CD34^+$ cells/kg. In non-malignant diseases where the risk of rejection is higher the dose should be increased and units with <3.5 x 10^7 NC/kg and 2 or more HLA incompatibility avoided. If a single UCB unit proves insufficient than, two units with a combined total dose of ≥3x 10^7 NC/kg and if possible not more than 1 HLA difference between the two units and the patient should be used (Gluckman and Rocha 2009).

Cord blood application was initially limited to the treatment of paediatric haematological malignancies due to the low cell yield from cord blood units of modest volume. Discovery of the need for less strict HLA donor-recipient selection permitted cord blood to be applied in adult patients lacking BM donors (Sanz, Saavedra et al. 2001; Schonberger, Niehues et al. 2004; Cornetta, Laughlin et al. 2005; Lekakis, Giralt et al. 2006). Additionally, outside the area of oncology, the clinical use of cord blood has expanded into various areas of disease management, such as : reconstitution of a defective immune system, correction of congenital haematological abnormalities and inducing angiogenesis. Table 5 illustrates some of the cord blood clinical studies concentrating on non-malignant diseases. The examples provided here are by no means exhaustive, but merely demonstrate the remarkable advances that have been made in the area of cord blood stem cell engineering and re-enforce the fact that stem cells hold tremendous promise for the future of regenerative medicine and cellular therapy.

Chronic diseases such as diabetes mellitus, stroke, ischemic heart disease, haemoglobinopathies and chronic liver cirrhosis are a social repercussion and have a huge impact on the health care system. They are a global priority of health and demand definite attention. The statistical incidence and prevalence of some of these chronic disease conditions is shocking. For example, in 2006 the International Diabetes Federation indicated that 6% of adults in the world (246 million people worldwide) suffer from diabetes. The highest prevalence was found in Eastern Mediterranean and Middle East region where 9.2% of adults are affected (BMJ 2006; 333:1191 (9 December), doi:10.1136/bmj.39055.608507.DB). It was predicted that the worldwide prevalence of diabetes will increase to 7.1% of adults, totaling 380 million people, by 2025. The Eastern Mediterranean and Middle East region will continue to have the highest overall prevalence, with 10.4% of the adult population having diabetes by 2025. Despite its long history the only curative treatment available for diabetes today, especially type 1 diabetes, is pancreatic beta cell transplantation.

Another common killer is liver cirrhosis. The WHO calculated that 20 million people worldwide have cirrhosis of the liver and/or liver cancer, arising predominantly among the estimated 500 million people (nearly 10% of the world population) who suffer from hepatitis B (HBV) and hepatitis C (HCV) viral infections. Each year an estimated 2 million people die of liver disease (www.hepalfe.com/images/chronic liver disease) (Rozga 2006).

In the present day the only management available for such chronic diseases is supportive or palliative therapy ultimately demanding cell transplantation for cure in some cases; beta cell

transplantation in the case of diabetes, bone marrow transplantation for thalassemia and hepatocyte transplantation for liver cirrhosis. However, the scarcity of available donors and the complications associated with the operative procedure justifies the requirement for alternative treatment options for these illnesses. Cord blood stem cells could prove to have potential in developing cellular therapy for treatment of these and other similar disease conditions

Table 5. Non-malignant disorders treated with cord blood.

Disorder	Number Treated	Outcome	Reference
Hurler's disease	20	17 out of 20 children alive a median of 905 days after transplantation with complete donor chimerism and normal peripheral blood α- L-iduronidase activity	(Staba, Escolar et al. 2004)
Duchenne Muscular Dystrophy	1	On day 42 post transplantation, obvious improvement in walking, turning the body over and standing up was noted	(Zhang, Chen et al. 2005; Zhang, Feng et al. 2005)
Malignant infantile osteoporosis	1	Normalization of spine bone mineral density was noted	(Jaing, Hou et al. 2006)
Buerger's disease	4	Ischemic rest pain suddenly disappeared. Digital capillaries were increased in number and size	(Kim, Han et al. 2006)
Spinal cord injury	1	Within 41 days post transplantation, improvement in sensory perception and movement in the SPI patient's hips and thighs was noted. Regeneration of the spinal cord at the injured site	(Kang, Kim et al. 2005)
Krabbe's disease	25	Progressive central myelination and continued gains in developmental skills, and most had age-appropriate cognitive function and receptive language skills in patient subset	(Escolar, Poe et al. 2005)
Omenn Syndrome	1	T cell reconstitution	(Tomizawa, Aoki et al. 2005)

Disorder	Number Treated	Outcome	Reference
Non-healing wounds	2	Accelerated healing	(Valbonesi, Giannini et al. 2002)
Refactory anaemia	3	All patients were alive and free of disease at between 17 and 59 months after UCBT	(Ooi, Iseki et al. 2005)
Diamond-Blackfan anaemia	1	Successful seroconversion to vaccines (Diphtheria, pertussis, tetanus, rubella, measles and BCG) administered 22-34 months post transplant	(Azuma, Hirayama et al. 2001)
Severe chronic active Epstein Barr virus	1	Complete remission without circulating EBV-DNA continued for 15 months post transplant	(Ishimura, Ohga et al. 2005)
Behcet's disease	1	23 months post UCBT, the patient was doing well and no signs or symptoms were noted	(Tomonari, Tojo et al. 2004)
Mucopolysaccharidosis type IIB (Hunter's symdrome)	1	2 years post UCBT ~ 55% normal plasma iduronase sulfarase activity was restored and abnormal urinary excretion of glycosaminoglycans was nearly completed resolved	(Mullen, Thompson et al. 2000)
Wiskott-Aldrich syndrome			(Slatter, Bhattacharya et al. 2006)
Chronic Granulomatous Disease			(Bhattacharya, Slatter et al. 2003)

Adapted from (Riordan, Chan et al. 2007)

1.2.3 Cord blood banking

Scientific data generated from research into the pluripotent nature of UCB-derived stem cells greatly highlights the need and importance of cord blood banking.

Banking is the process whereby UCB stem cells are saved for potential medical uses in the future. The procedure includes the process of cord blood collection from the umbilical cord which can be performed in utero (during third stage of labour, prior to delivery of the placenta) or ex utero (after the umbilical cord has been clamped and completely detached from the baby). Both procedures can yield similar amounts of cord blood (on average between 70-120ml per unit). It is encouraged that unless there is a very strong medical indication otherwise, that only ex utero collection is warranted. The procedure poses no harm in any way to the mother or the baby. It is a completely non-invasive process. The blood collected is then subjected to plasma depletion and removal of red blood cell compartment and is tested to determine whether it meets the eligibility standards. The processed cord blood unit (buffy cells) which contains, in very low quantity, the stem cells is then cryopreserved by freezing in liquid nitrogen at -196° C with a cryopreservative mixture. This enables cellular aging and damage to be halted.

Thus far, two main types of cord blood banks exist:

(i) Public cord blood banks storing anonymized units that can be released to any patients with a partial or total HLA match;

(ii) Private 'family' banks which against a fee, store units for exclusive use by the family paying for the service.

Public banks have come into existence in many countries since the early 1990's. These banks usually meet strict quality control criteria and national/international accreditation. The rationale behind public banking is that they offer the advantage of having the sample already stored in a bank, and if there is a match the unit can be shipped to the hospital immediately. On the other hand, bone marrow banks are subject to complexity of HLA and immunological matching and other logistical complications that hinder the timely localisation of a potential bone marrow donor.

However, when compared to a world registry exceeding 11 million bone marrow donors, only 700,000 cord blood units are stored in approximately 50 cord blood banks in about 20 countries.

Private cord blood banks charge a fee to store umbilical cord blood samples for about 20-25 years. It has been estimated that approximately 700,000 units are stored in private banks worldwide, including 500,000 in the USA and just over 135,000 in Europe. An issue associated with private banking is that not enough private cord blood banks are complying

with regulations and accreditations for processing quality control. So there is a rigorous need for strict regulatory frameworks to better prevent unreliable service. Additionally, some cord blood banks are struggling financially so it is of critical importance to find the most effective and economical processing and storage methods (McGuckin and Forraz 2008; Christina Basford 2010). Many factors affect the success of processing and the yield of stem cells generated (McGuckin, Basford et al. 2007), so it remains an important priority to find the best possible methods of cord blood separation, that will allow even cord blood units of small volumes to prove clinically viable.

More recently, in addition to cord blood, cryopreservation of the umbilical cord has also been made available to the public in some countries. An example of such a facility is the Tissue Regeneration Therapeutics (TRT) in Toronto, in collaboration with Create Cord Blood Bank (CCBB) that was launched in 2006. The focus of this tissue bank lays in cell therapy applications of MSCs for regenerative therapy in cell transplantation.

One of the chief goals of this study was the *in vitro* differentiation of cord and cord blood derived stem/progenitor cells toward hepatobilary lineage. The aim was to try to produce an *in vitro* model for application in biomedical research and extracorporeal liver assist devices and eventually to be applied in cell therapy.

The following sections provide a concise introduction to liver structure and development.

1.3 Introduction to the liver- a unique organ

The liver is a intricate organ composed of cells, such as hepatocytes, hepatocyte precursor cells (Scadden 2006) , stellate cells, Kupffer cells, epithelial cells, sinusoidal epithelial cells, biliary epithelial cells, and fibroblasts (Volker 2007). The liver is involved in over 150 different vital functions including: metabolism, detoxification, and maintenance of homeostasis in the body. The liver also possesses endocrine functions, production of many serum proteins into the blood, blood clotting factors and an exocrine function, secreting large amounts of bile into the digestive tract (Lemaigre and Zaret 2004; Volker 2007). Correct liver functions are fundamental to human health and loss of these can be severely compromising.

1.3.1 Gross anatomy and physiology of the liver

The Liver (Hepar) is the largest gland in the body. It is situated in the upper and right part of the abdominal cavity, occupying almost the whole of the right hypochondrium, the greater part of the epigastrium and not uncommonly extending into the left hypochondrium as far as the mammary line. The anatomical details of the liver illustrated below were taken from the online version of Gray's Anotomy of the Human Body (Bartleby.com)

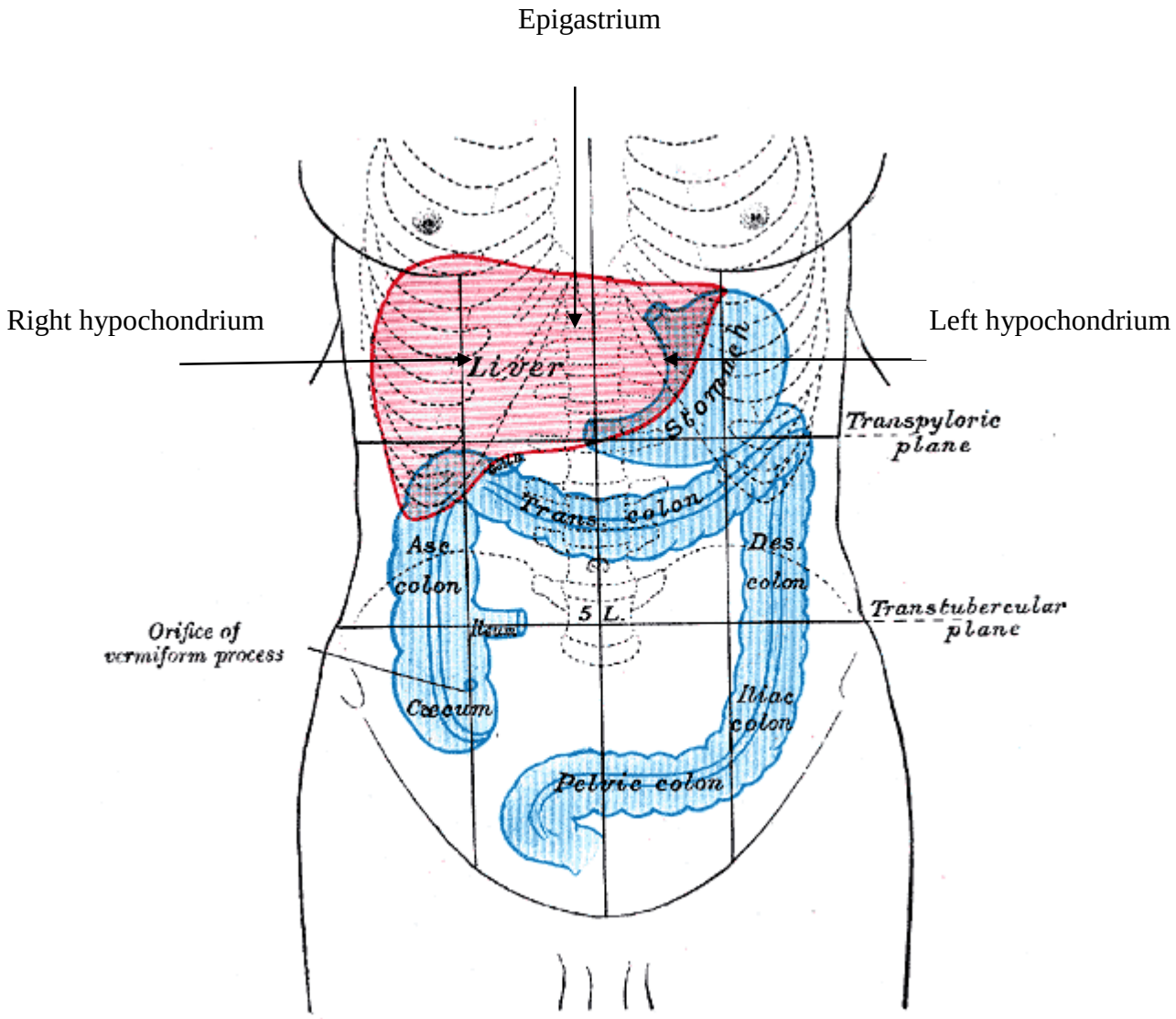


Figure 15. Surface anatomy of the liver. View from front of abdomen, showing surface markings of the liver, stomach and great intestines. Bartleby.com; Anatomy of the Human Body (Henry Gray- 1821-1865)

Weight- In adult males the liver weighs from 1.4 to 1.6 kg and in adult females from 1.2 to 1.4 kg. It is relatively much larger in the fetus than in the adult, constituting, in the former, about one-eighteenth, and in the latter about one thirty-sixth of the entire body weight.

Shape- it presents the appearance of a wedge, the base of which is directed to the right and the thin edge towards the left (Figure 15).

Measurements- the greatest transverse measurement of the liver is from 20 to 22.5cm. Vertically, near its lateral right surface, it measures 15-17.5cm.

Consistency- soft solid and friable

Color- dark reddish brown

Specific gravity- 1.05

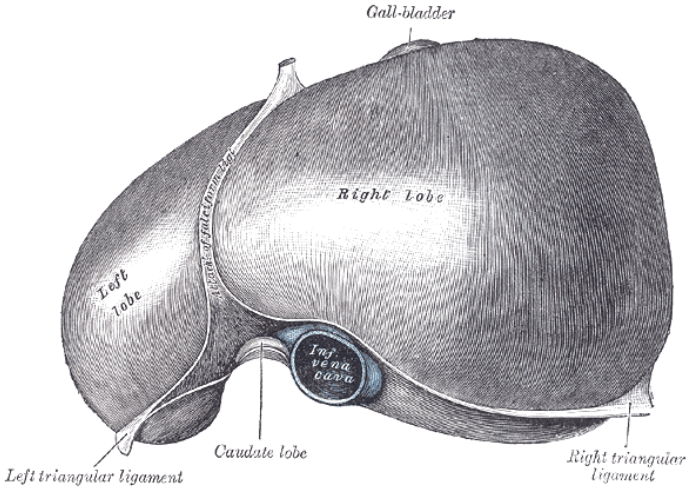
Structure of the liver- the substance of the liver is composed of lobules, held together by an extensively fine areolar tissue, in which ramify the portal vein, hepatic ducts, hepatic artery, hepatic veins, lymphatics and nerves. The entire structure is invested by a serous and a fibrous coat.

Structural unit of the liver- the lobules (lobuli hepatis) constitutes the chief mass of the hepatic structure. Each lobule consists of a mass of hepatic cells, arranged in irregular radiating columns between which run the blood channels (sinusoids) and minute bile capillaries (Figure 17). Each lobule measures approximately 1 to 2.5 mm in diameter.

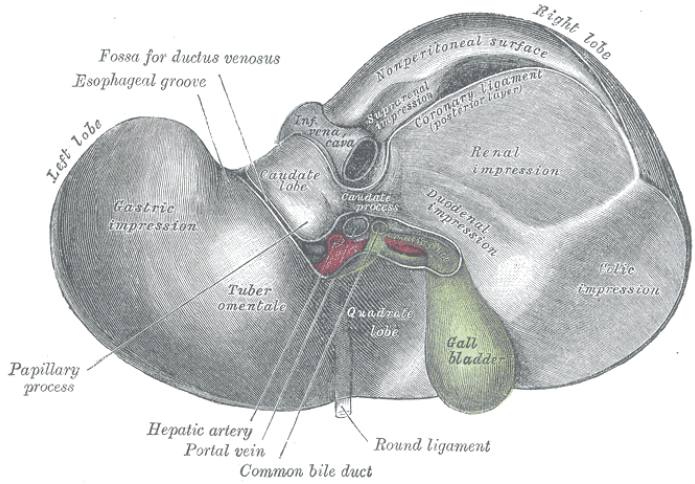
Functional unit of the liver- the hepatic acinus is the functional unit of the liver. The acinus is oriented around the afferent vascular supply of the liver lobules and hence represents a unit that is of more relevance to liver function (Figure 18).

Hepatocyte- or liver cells make up about 80% of the liver mass and are the functional cells. Hepatocytes are polyhedral in form and measure from 12-25 μ in diameter. These cells contain one or two distinct nuclei. The nucleus exhibits an intranuclear network and one or two refractile nucleoli. The cells are usually granular, some of which are protoplasmic, while others contain glycogen, fat or an iron compound.

(a) Superior surface of the liver



(b) Inferior surface of the liver



(c) Inferior and posterior surface of the liver

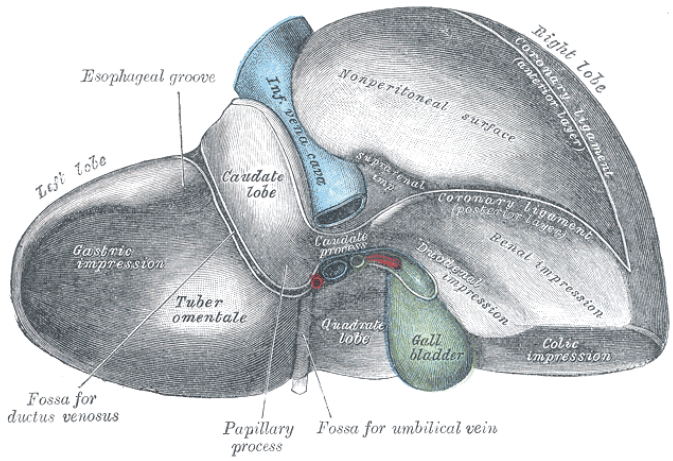


Figure 16. Surfaces of the liver. (a) Superior surface (*facies superior)* is attached to the diaphragm and the anterior abdominal wall by the falciform ligament. The line of attachment of the falciform ligament divides the liver into two parts, the right and left lobes. (b) Inferior surface of the liver (*facies inferior; visceral surface*). (c) Inferior anf posterior surface of the liver

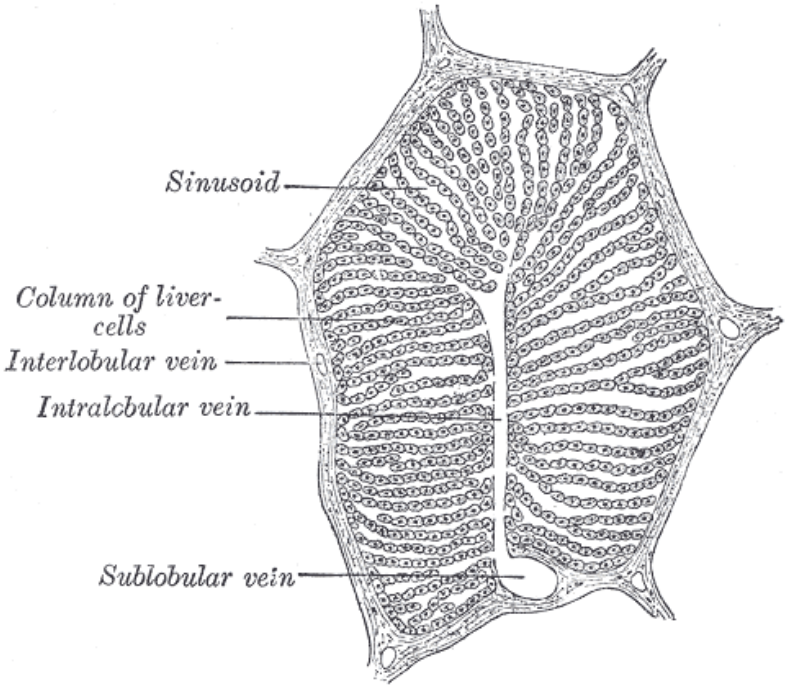


Figure 17. Structure of a single liver lobule. Image from porcine liver (x60)

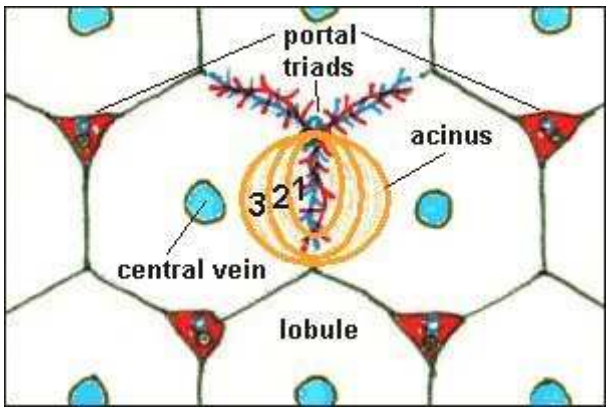


Figure 18. Liver acinus of porcine liver. The acinus is the functional unit of the liver and is oriented around the afferent vascular supply of the liver lobule.

1.3.2 Liver Development

The liver is derived from the endoderm, one of the three germ layers during gastrulation. The endoderm defines the primitive gut and gives rise to the epithelial compartment of the gastrointestinal tract. The endoderm also gives rise to other organs including the pancreas and the thyroid gland (Figure 19A) (Lemaigre 2009). Fate mapping studies in mice where endodermal cells were labeled with a dye showed that liver progenitor cells originate from three endodermal domains: two domains are paired and located laterally, and the third domain is found along the ventral midline (Figure 19B) (Tremblay and Zaret 2005; Zaret 2008).

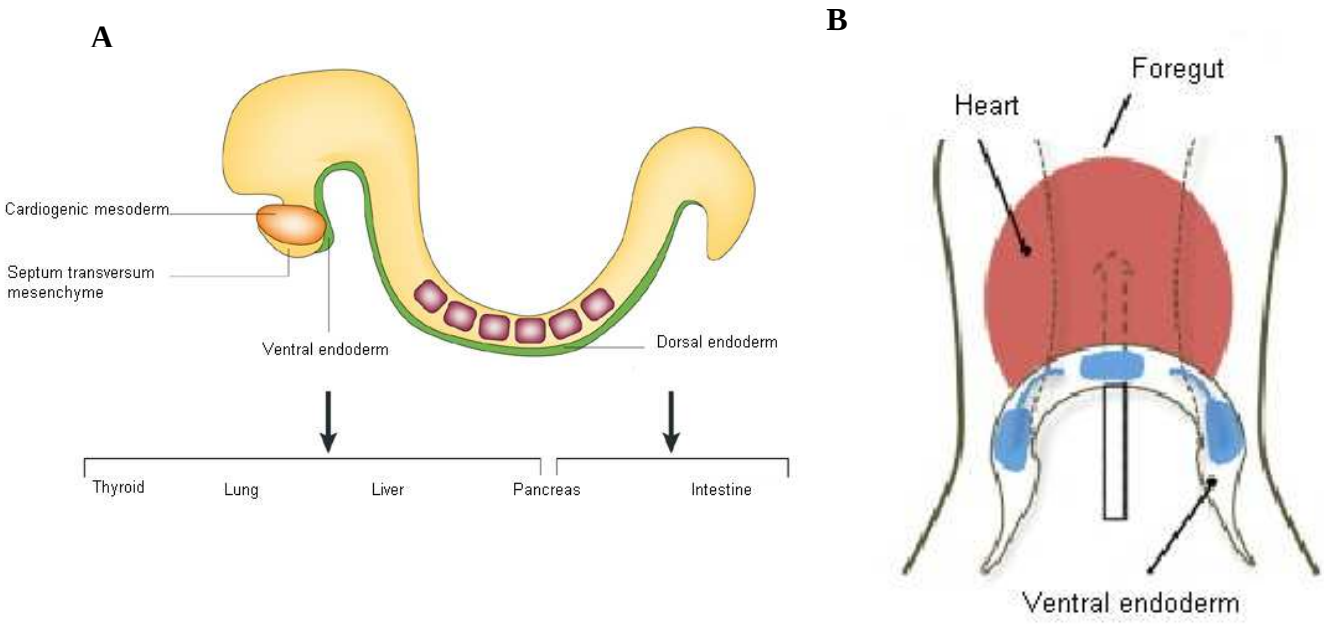


Figure 19. Formation and patterning of the endoderm associated with development of the liver. (A) The figure depicts a parasagittal section of a mouse embryo at the six-somite stage (~ 8.25 days gestation), at the time when endodermal patterning begins (Zaret 2002). (B) A schematic representation of a mouse embryo (4 to 10 somite stage) with a ventral view on the anterior intestinal portal showing the blue areas that correspond to the hepatic progenitor domains in the ventral endoderm. (Lemaigre 2009)

Both the endocrine as well as the exocrine parenchyma of the liver is derived from the endoderm. The ultimate fate of endodermal cells is determined by various factors that influence the differentiation potential of these cells (Figure 20).

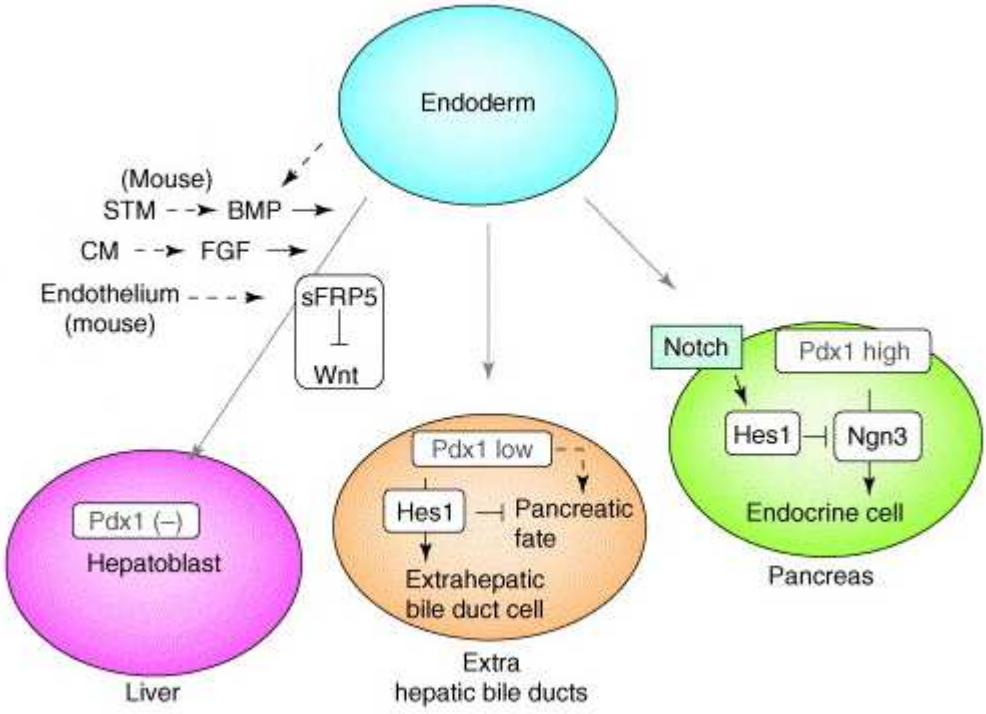


Figure 20. Factors influencing differentiation of the endoderm into liver, extra-hepatic bile ducts and pancreas. Liver induction signals in mammalian liver are illustrated. STM (Septum transversum),CM (Cardiac mesoderm), BMP (Bone morphogenenic protein, FGF (Fibroblast growth factor, Pdx1 (Pancreatic and duodenal homeobox 1), Hes1 (Haematopoietically expressed homeodomain) and Ngn3 (Neurogenin 3).

The liver arises in the form of a diverticulum from the ventral surface of that portion of the gastrointestinal tract which later forms the descending part of the duodenum. The diverticulum grows upward and forward into the septum transversum and gives off two solid buds of cells which represent the right and the left lobes of the liver. The liver buds grow into columns or cylinders, termed hepatic cylinders, which branch and anastomose to form a close meshwork. The vitelline and umbilical vessels are invaded by this network, which disrupts these vessels into a series of capillary-like vessels termed sinusoids. These sinusoids ramify in the meshes of the cellular network and ultimately form the venous capillaries of the liver.

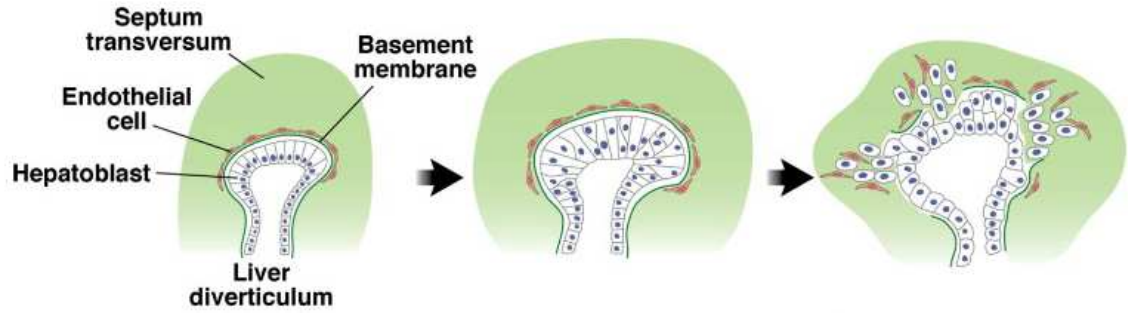


Figure 21. Budding of the liver out of the endoderm. The diagram schematizes the changes in morphology of the liver when it buds out of the endoderm.

By the continued growth and ramification of the hepatic cylinders the mass of the liver is gradually formed. The original diverticulum from the duodenum forms the common bile duct, and from this the cystic duct and the gall bladder arise as solid outgrowths which later acquire a lumen.

As liver development progresses and the liver undergoes enlargement, it together with the ventral mesogastrium of the forgut are gradually differentiated from the septum transversum. The liver projects downward into the abdominal cavity from the under surface of the septum transversum.

About the third month of embryonic development, the liver almost fills the abdominal cavity, and its left lobe is nearly as large as the right. From this period the relative development of the liver is less active, more especially that of the left lobe. The left eventually undergoes a degree of degeneration and becomes smaller that the right.

1.3.2.1 Developmental stages and molecular mechanisms that regulate liver development

Development of the hepatic parenchyma takes place in distinct developmental stages, although intertwining and overlap between the stages is highly likely (Duncan 2003). The molecular mechanisms involved in the different stages of liver development are detailed below:

A. Competency and specification

Competency depicts the innate ability of a cell type to follow a given developmental pathway provided it is exposed to the appropriate inductive cues (Gilbert 2003) (pg 142-144).

Tissue interactions define the precise location and timing of hepatogenesis during embryonic development. Large changes in the patterns of gene expression accompany the differentiation of the multipotent ventral endodermal cells towards a hepatic phenotype. In rodent embryos the onset of liver ontogeny is at embryonic day (E) 8.5.

Nicole Le Douarin demonstrated using the chick model, that the ventral endoderm had to be in close contact with precardiac splanchnic mesoderm and the septum transversum mesenchyme for it to follow a hepatic fate (Douarin 1975). This discovery was later validated by other reports (Fukuda 1979; Fukuda-Taira 1981). It was later noted that fibroblast growth factors (FGF1-, -2 and 8) produced by cardiac mesoderm and bone morphogenic proteins (BMPs) produced by the septum transversum act in concert to trigger the onset of hepatogenesis and make the ventral endoderm competent to interpret and respond to signals that induce the hepatic development program (Jung, Zheng et al. 1999; Rossi, Dunn et al. 2001).

Zaret *et al* reported the transcription factors HNF3 (FoxA) and Gata4 could modulate competency intracellularly (Gualdi, Bossard et al. 1996; Zaret 1998; Chaya, Hayamizu et al. 2001). These factors are switched on after induction by the surrounding tissues. It has been shown that both transcription factors resulted in opening of compact chromatin (Cirillo, Lin et al. 2002) and hence promote gene transcription. Two members of the FOXA family, FoxA1 (HNF3α) and FoxA2 (HNF3β), are expressed throughout the definitive endoderm preceding the potentiators that specify the hepatic fate within the ventral endoderm (Ang, Wierda et al. 1993; Monaghan, Kaestner et al. 1993; Sasaki and Hogan 1993). Although Gata-4 has been shown to bind to Albumin enhancer, it has yet to be demonstrated that Gata-4 is expressed in the endoderm during the onset of hepatogenesis as well.

Once the ventral endoderm had been rendered hepatic competent, the mechanisms by which the cells of the endoderm actually follow and adopt a hepatic fate are a sequence of intracellular responses to these signaling events. This leads to specification of the committed ventral endoderm and simultaneous inhibition of pancreas formation in the proximal endoderm. It is worth noting that pancreatic programme is the default for this domain of the endoderm.

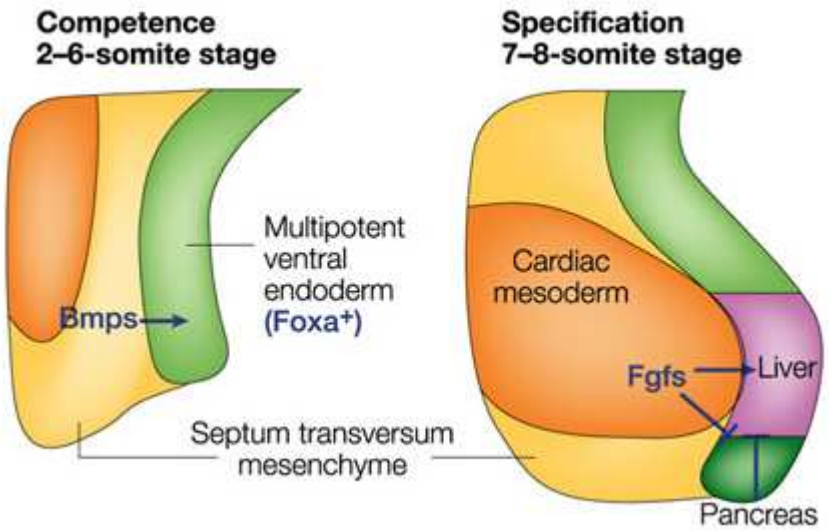


Figure 22. Competency and specification of the ventral endoderm. The septum transversum produces BMP (Bone morphogenic proteins) which in association with FGFs (Fibroblast growth factors) trigger the onset of hepatogenesis and make the ventral endoderm competent to interpret and respond to signals that induce the hepatic development program. This hepatic induction in the proximal endoderm is coupled with inhibition of pancreatic development from the endoderm. Ventral endoderm cells sufficiently distal to the cardiogenic mesoderm escape the latter inhibitory effect and initiate the pancreatic gene programme ((Zaret 2002).

B. Liver bud formation

After hepatic endoderm has been specified, it undergoes transitions that are elicited by signals that specify the endoderm. At approximately rodent E9.0-9.5, the hepatic endoderm begins to extend towards the midgut and the cells become columnar in shape. Various transcription factors promote the earliest steps of liver-bud emergence and morphogenesis: Hex (Haematopoietically expressed homeodomain) and Prox1 (prospero-related homeobox) genes. The septum transversum expresses various factors that regulate the synthesis of paracrine morphogens needed for hepatoblast growth. These include: Hlx (H2.0-like homeodomain protein), HGF (Hepatocyte growth factor), Smad2 and Smad3 (transcriptional mediators of TGF-β signalling pathway (Zaret 2002). Endothelail cells, which eventually form the vascular supply of the liver, have been shown to be a pivotal growth stimulus to the hepatic bud enabling the hepatoblasts to proliferate and migrate into the surrounding septum transversum (Matsumoto, Yoshitomi et al. 2001). At this stage the hepatic epithelial cells are referred to as bipotent hepatoblasts and express Gata4, HNF4α, HNF6, AFP and albumin and biliary cytokeratins (Ck17, Ck19) (Kinoshita and Miyajima 2002; Lemaigre and Zaret 2004; Zhao and Duncan 2005; Shafritz, Oertel et al. 2006).

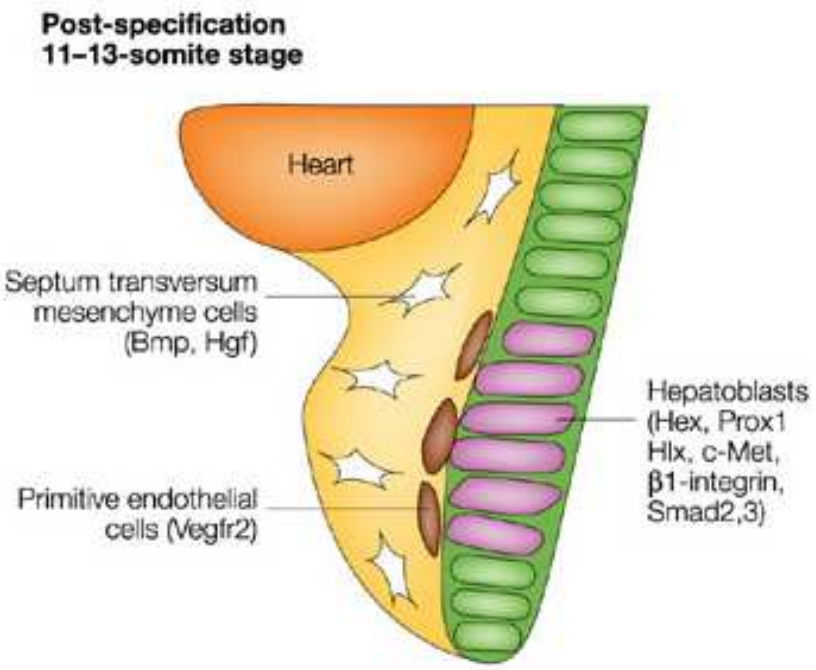


Figure 23. Post specification and liver bud formation. After the hepatic endoderm has been specified it begins to extend towards the midgut. At the same time hepatic endoderm cells become columnar in shape. Cells such as STM (Septum transversum mesenchyme) cells, primitive endothelial cells, signalling molecules (such as BMP, HGF and VEGFR2) and transcription factors (such as Hex, Prox1, Hlx and c-Met) are essential to promote morphogenesis of the liver bud itself. BMP (bone morphogenic protein), c-Met (HGF receptor), HFG (hepatocyte growth factor), VEGFR2 (Vascular endothelail growth factor receptor 2) (Zaret 2002).

C. Differentiation and functional maturation

At rodent E11-12, the liver primarily becomes a haematopoietic organ. HSCs originating from extra-hepatic organs colonize the liver bud and emit growth signals for further development of the liver. Consequent, hepatoblasts continue to proliferate and commitment to hepatocyte and/or biliary epithelial lineages is initiated (Snykers, De Kock et al. 2009). Expression data of marker genes are consistent with hepatocytes and bile duct cells (cholangiocytes) being derived from hepatoblast (Shiojiri 1984; Germain, Blouin et al. 1988). Hepatocyte nuclear factor 6 (HNF6) promotes hepatocyte over biliary cell formation in early development but positively controls later differentiation of biliary epithelia and the morphogenesis of the bile duct and the gall bladder. HNF4 directly promotes hepatocyte gene expression and the formation of hepatocyte epithelial morphology (Zaret 2002). Differentiation along cholangiocyte lineage is promoted by Notch signaling pathways (Lemaigre and Zaret 2004; Zhao and Duncan 2005; Clotman and Lemaigre 2006). Mesenchymal cells or non-parenchymal liver cells excrete HGF which antagonizes differentiation along cholangiocytic lineage and supports the growth and differentiation of foetal hepatocytes. The hormone insulin synergistically promotes this effect. Subsequently, OSM mostly produced by HSCs, and glucocorticoids induce partial hepatic maturation and suppression of embryonic haematopoiesis. OSM fails to induce differentiated liver phenotype on its own, implying the importance of glucocorticoids as trigger for hepatic maturation (Snykers, De Kock et al. 2009). At this point, although the cells continue to proliferate, most of them are unipotent and irreversibly committed to either the hepatocytic or cholangiocytic lineage. Complete functional hepatic maturation ultimately takes place after birth aided by HGF, produced by surrounding non-parenchymal liver cells (sinusoidal, stellate and endothelial cells (Lemaigre and Zaret 2004; Shafritz, Oertel et al. 2006; Snykers, De Kock et al. 2009).

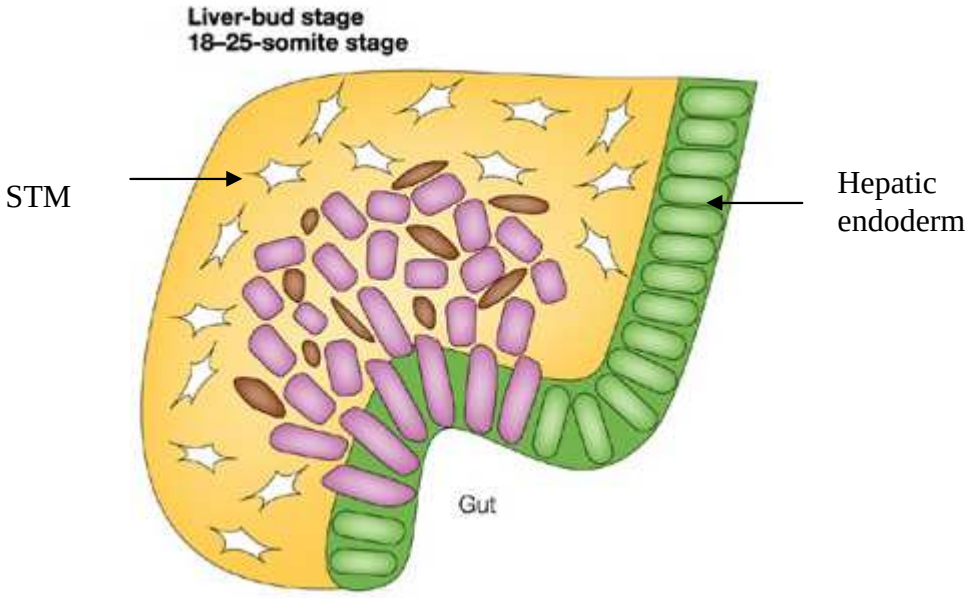


Figure 24. Final differentiation and liver bud formation. Liver-bud morphogenesis is marked by remodeling of the extracellular matrix around the hepatoblasts and of E-cadherin-based connections between the cells, and proliferation and migration into the surrounding STM (septum transversum mesenchyme) (beige). The outgrowth of the liver bud is promoted by surrounding primitive endothelial cells (or angioblasts). These endothelial cells eventually coalesce around spaces in the loose STM and create vesicles that fuse to form blood vessels (not shown). Haematopoietic cells then invade the growing liver and the organ becomes distinct from the gut epithelium (Zaret 2002).

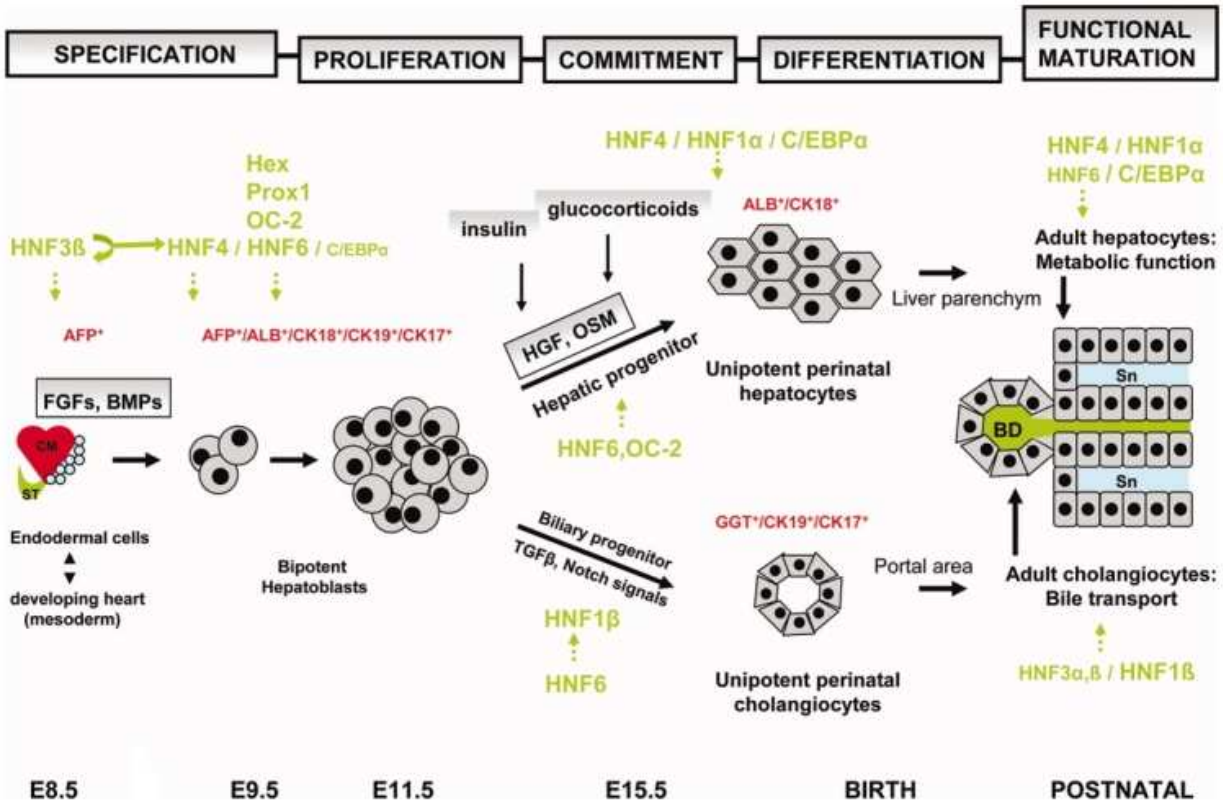


Figure 25. Schematic representation of foetal liver development. The establishment of fully functional liver tissue is accomplished only after postnatal stages and follows a sequential array of tightly regulated intra- and extracellular signaling pathways. To distinguish the level of expression and/or regulating role among diverse liver-enriched transcriptional factors, different letter sizes have been used in this diagram. ALB (albumin), AFP (α-fetoprotein), BMP (bone morphogenic proteins), FGF (fibroblast growth factors), GGT (γ-glutamyltransferase), HGF (Hepatocyte growth factor), HNF (Hepatocyte nuclear factor), OC-2 (Onecut transcription factor), ST (Septum transversum), TGF (Transforming growth factor). (Snykers, De Kock et al. 2009)

1.3.3 Hierarchy of Transcription Factors that regulate liver-specific gene expression during liver development

The figure below summarizes the chief transcription factors involved in hepatogenesis. These transcription factors belong to various familes: HNF1α and HNF1β belong to the variant homeodomain-containing proteins; HNF3α (FoxA1) belongs to winged helix protein family; HNF4α is a member of nuclear hormone receptor family and HNF6 is from the onecut homeodomain protein family of transcription factors.

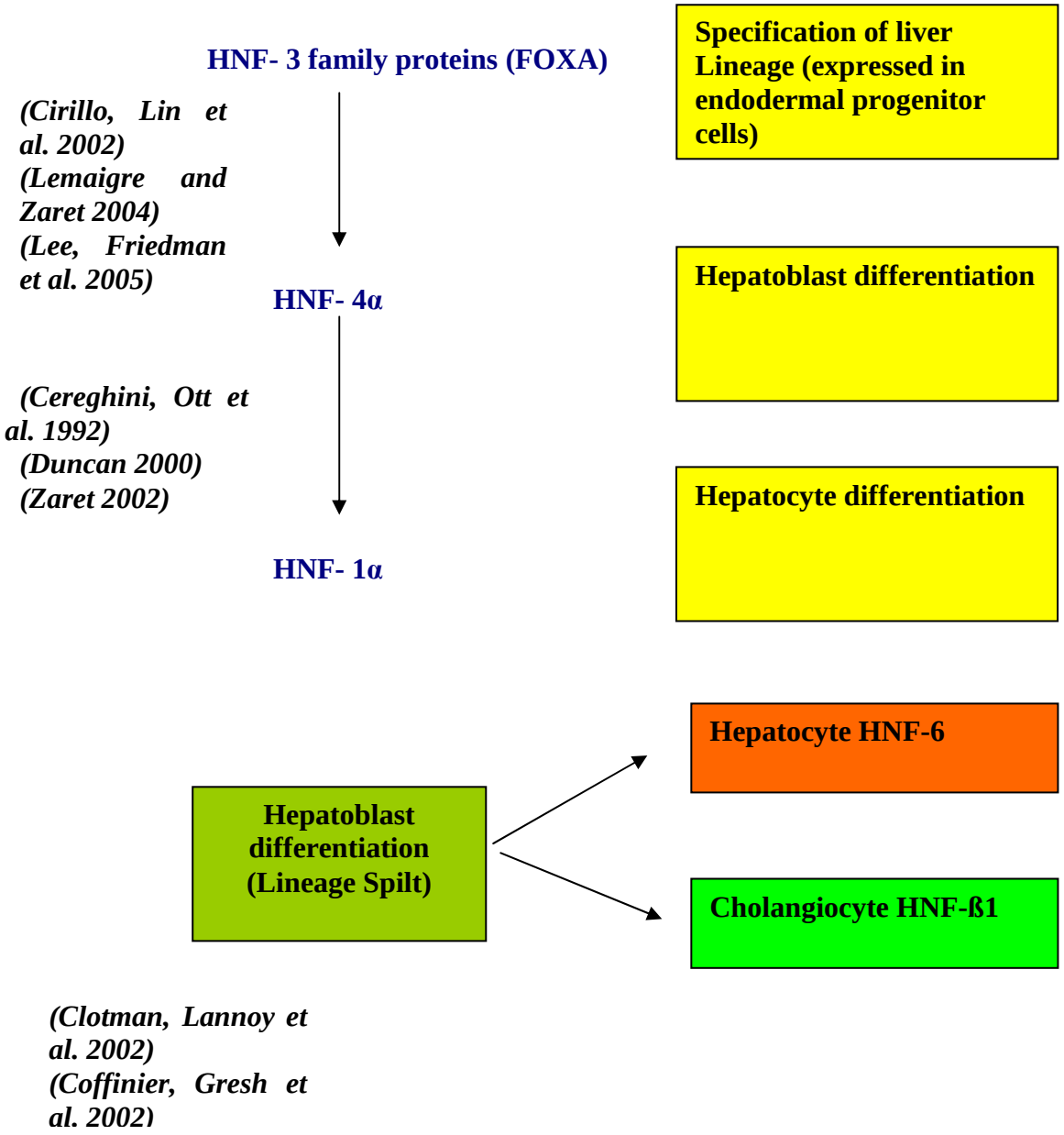


Figure 26. Transcriptional regulation of liver-specific gene expression during hepatogenesis.

1.3.4 Liver disease- global health burden

Liver disease is highly prevalent worldwide. The World Health Organization estimated in 2006, that approximately 20 million people worldwide suffered from cirrhosis of the liver and/or mitotic lesions of the liver, the majority arising from the 500 million persons (approximately 10% of the world population) who are victims of hepatitis B (HBV) or hepatitis C (HCV) viral infections (Rozga 2006). Hepatic failure alone, accounts for 1-2 million deaths per annum, and is nominated the 5th leading cause of death around the globe. Decompensated liver function and ultimate failure has many culprits including: excessive alcohol consumption, aggressive forms of fatty liver disease, fibrosis, inflammatory liver conditions and unregulated ingestion of common over-the-counter medication, such as acetaminophen (Tynelol) (Rozga 2006). Within the UK, acetaminophen over dosage contributes to 48% of poisoning admissions to hospitals and is involved in an estimated 100-200 death per year (Hawkins, Edwards et al. 2007).

1.3.4.1 Mechanism of liver damage and ultimate fate

The liver is an exceptional organ; in an event of parenchymal cell loss, the mammalian liver can cite at least three apparently distinct cell lineages to contribute to regeneration and repair after damage (Figure 27). First line of defence is provided by hepatoblasts/hepatocytes which are themselves believed to be functional stem cells of the liver (Shafritz and Dabeva 2002; Fiegel, Park et al. 2003). More severe liver damage calls upon activation of a potential stem cell compartment located within the intrahepatic biliary tree, giving rise to cords of bipotential cells that ultimately differentiate to hepatic or biliary epithelial cells. These hepatic stem/progenitor cells are referred to by different names including ‘oval cells’ and ‘small hepatocyte-like progenitors’ (SHPCs) in rodents (Gordon, Coleman et al. 2000; Roskams, Theise et al. 2004) and ‘intermediate cells’ in humans (Roskams, Theise et al. 2004; Roskams 2006). This cell population has been shown to have ‘bipotential characteristics’ expressing morphological and immunophenotypic features typical of both hepatic and biliary epithelial cells. Wilson and Leduc, 1958, were the 1st to describe activation of this ‘reserve cell compartment’, which in humans is described as ‘ductular activation’ or ‘progenitor cell activation’ (Leduc 1958). A third population of stem cells with hepatic potential resides outside the liver in the bone marrow. These haematopoietic stem cells may contribute to the low renewal rate of hepatocytes, but in the face of severe and/or extensive damage can contribute significantly to regeneration under very strong positive selective pressure (Petersen, Bowen et al. 1999; Alison, Poulsom et al.

2000; Theise, Badve et al. 2000; Theise, Nimmakayalu et al. 2000; Kleeberger, Rothamel et al. 2002)

Under conditions where the insult to the liver is extensive and/or chronic, the liver is crippled by hepatocyte senescence due to telomere shortening that is associated with ongoing proliferation during the prolonged chronic history of disease. During incidents where the intrinsic proliferative and clonogenic capacity of inherent hepatocytes/hepatoblasts is affected, extrinsic stem cells with hepatogenic potential are chemo-attracted towards to the site of liver injury, finding a niche and attempt to salvage the liver. It should be noted, however, that in the diseased liver, there may be lack of the major growth stimulus and/or absence of substantial growth advantage for haematopoietic stem cells to correct and repair the damaged tissue. Another important factor is the intrinsic genetic regulation of stem cells (Geiger, Rennebeck et al. 2005). In addition, there are many other regulatory systems involved, which dictate the replacement of damaged, aged or diseased tissue with new cells. Stem cells secrete factors which act through a paracrine system and play a role in regulating tissue regeneration. Systemic factors are also important in modulating signaling pathways critical to activate tissue specific stem cells (Conboy, Conboy et al. 2005). Liver cells (hepatocytes, cholangiocytes, and progenitor cells), mesenchymal cells (Kupffer cells, endothelial cells, hepatic stellate cells) and the liver stroma collectively form the liver 'stem cell niche' that regulates stem cell proliferation, maintenance and cell fate decisions. The importance of such cellular and non-cellular interactions was demonstrated by Kon *et al*, who elegantly illustrated *in vitro* differentiation of small rat liver progenitor cells into hepatocytes upon co-culture with non-parenchymal supporting cells (Kon, Ooe et al. 2006). Additionally, the fact that hepatocytes, which have a great growth potential *in vivo*, when isolated and put in culture pose a huge challenge to maintain alive and differentiated also further re-enforces the importance of such niches (Scharschmidt, Waggoner et al. 1975) . Even if stem cells did tend to retain much of their intrinsic proliferative potential when old, age-related changes in the systemic environment and niche in which stem cells reside preclude full activation of these cells for productive tissue regeneration

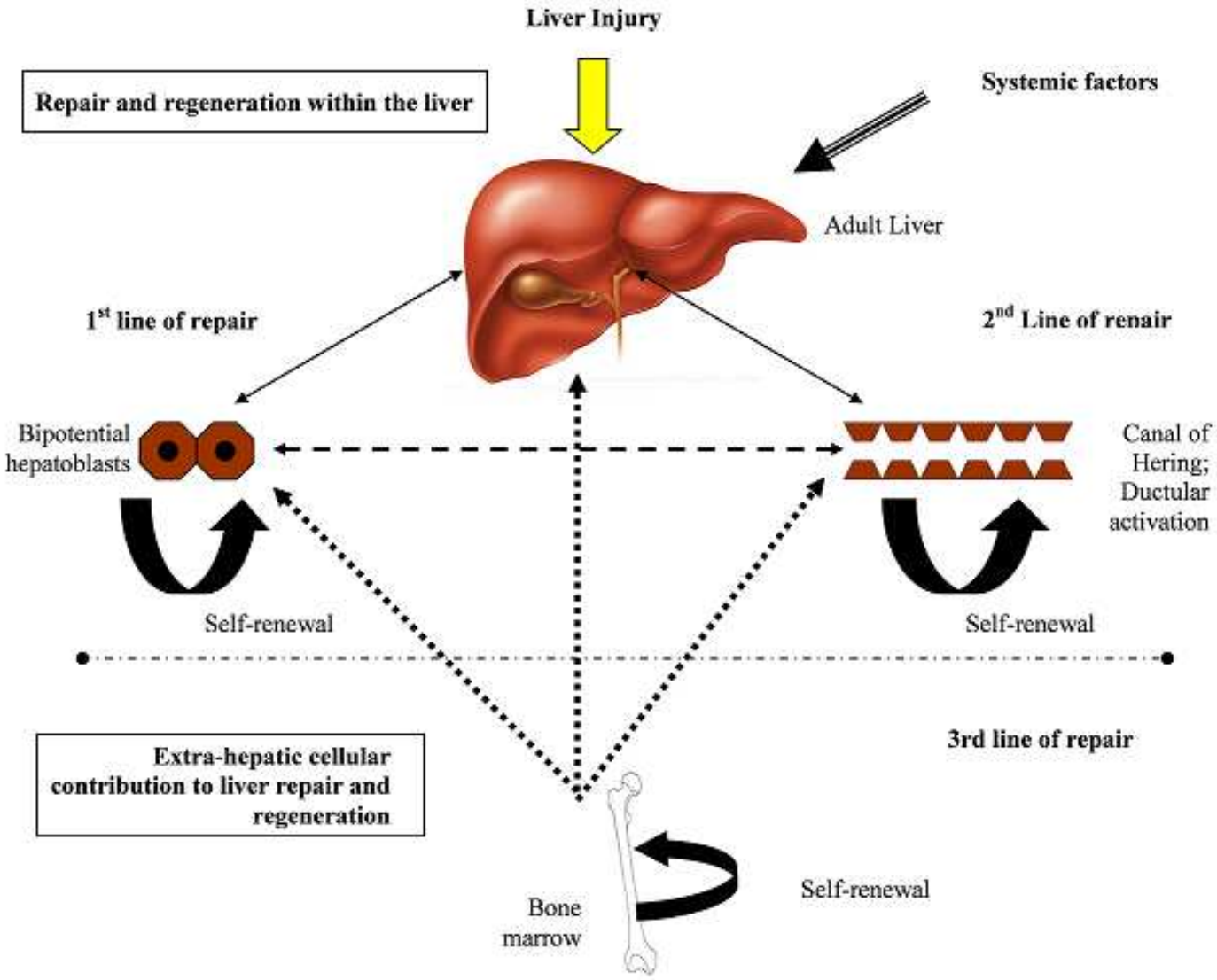


Figure 27. Three different cell lineages that contribute to liver regeneration and repair after injury. First line of defense in the face of injury is provided by bipotential hepatoblast which are capable of differentiating into hepatocytes and cholangiocytes. These are believed to be functional stem cells of the liver and are capable of self-renewal after loss. When hepatocyte renewal is compromised, or the liver damage is more severe, this calls upon activation of bipotential stem/progenitor cells in the canal of Hering, located in the intra-hepatic biliary tree that take over the burden of regeneration. Under conditions where the insult to the liver is extensive and/or chronic or the intrinsic proliferative capacity of the liver is compromised, extrinsic stem cells from the bone marrow harbor to the site of injury and provide a third line of repair and regeneration. It should be noted, however, that systemic factors also play a pivotal role in modulating signaling pathways critical to tissue regeneration.

1.3.5 Current mode of management of liver disease- restrictions and limitations

Despite the worldwide prevalence and significant morbidity and mortality associated with severe liver disease, medical advancement so far allows successful treatment of compromised liver function through resection and transplantation surgeries only (Belle, Porayko et al. 1997; Fong, Sun et al. 1999). This is an invasive procedure and is limited by the availability of donor organs. Less that 30% of patients on a waiting list receive a transplant (Ascher, Lake et al. 1993; Lee 1993). Non-treatable and/or incurable liver conditions impending transplantation, are subject to supportive and palliative modes of management. These comprise non-biological and biological liver support systems. These supportive modalities have been introduced to standard therapy since 1950s, to bridge patients awaiting organ transplant.

1.3.5.1 Non-biological or artificial liver support systems

Non-biological systems primarily provide detoxification/purification by removing toxins of hepatic failure. The repertoires of known substances that accumulate in the blood in hepatic failure aggravate injury to the liver. These substances inhibit the natural ability of the liver parenchyma to regenerate and simultaneously suppress the ability of residual hepatocytes to perform organ-specific functions (sick cell syndrome). Additionally, these substances cause neurological abnormalities and failure of the immune system and other organs (kidney and lung). These substances include not only small molecular weight toxins (ammonia, phenols, false neurotransmitters, free bile acids, etc.) but also mediators of inflammation (cytokines, chemokines, anaphylatoxins, etc.). Vasoactive substances, endototoxins, cell growth inhibitors (e.g TGFβ1) and other known and currently unknown harmful substances are also included (Rozga 2006). Some of the latest advancements in blood purification technologies include: MARS ® (Molecular adsorbent recycling system), SPAD (Single-pass albumin dialysis) and SEPET (Selective plasma filtration), but their utility is limited by their inability to provide missing liver functions.

1.3.5.2 Bio-artificial livers (BAL) - hepatocyte-based extracorporeal devices

Bio-artificial or hepatocyte-based devices represent advancement in the management of hepatic disorders. These cellular-dependent (animal or human cells) systems are able to provide whole liver functions, including detoxification, biosynthesis and biotransformation. However, it is worth noting that in addition to the risk of xenosis in the animal cell-based

systems (mainly of porcine origin) additional well-conducted studies are warranted to better demonstrate safety and proof of concept of these devices (Rozga 2006).

Thus far, development of an effective liver assist technology has proven challenging because of the complexity of liver functions that must be replaced, as well as the heterogeneity of the patient population.

Alternatively cellular therapies including hepatocyte transplantation can be used either to replace or increase the number of functional hepatocytes. The current source of hepatocytes is from discarded livers not suitable for whole organ transplantation, yet again limiting its accessibility. These cells are also used to establish primary cultures. Such primary cultures of hepatocytes have been hindered by their short lifespan and the rapid loss of hepatic function under *in vitro* conditions. There is still a great need for new sources of stem/progenitor cells with an ability to differentiate into functional liver cells. Isolation of an expandable population of adult human pluripotent stem cells will be an attractive alternative for current therapies. Before that, however, we need to better understand the cellular and molecular (genetic and epigenetic) mechanisms responsible for liver cell differentiation. Therefore, adequate *in vitro* platforms and animal models of liver disease are of great importance.

1.3.6 Cellular therapy of liver disease- Regenerative medicine, an alternative approach in healthcare

Liver transplantation is currently the only successful treatment for acute hepatic failure or end stage liver disease. However, as noted earlier a major limitation to its use is serious donor shortage. For this reason, considerable attention has recently been focused on identifying alternatives modes of management for fatal liver disorders with regenerative medicine occupying the limelight. The following section elaborates briefly on the different cell sources that have been exploited in regenerative therapy of liver diseases.

A. Hepatocyte transplantation

Hepatocyte transplantation was believed to be a potential solution to this problem. Several reports demonstrated in animal models, rescue from lethal hepatic failure post primary hepatocyte transplantation. This was achieved through efficacious donor chimerism (Makowka, Rotstein et al. 1980; Rajvanshi, Kerr et al. 1996; Rajvanshi, Kerr et al. 1996; Gagandeep, Rajvanshi et al. 2000). Two clinical trials have previously demonstrated the effectiveness of hepatocyte transplantation for the treatment of liver-based congenital metabolic disorders. Fox *et al*, transplanted allogeneic hepatocytes into the liver of a patient with Crigler-Najjar syndrome type I (Fox, Chowdhury et al. 1998). The patient survived for 11 months with partially corrected hyperbilirubinemia. Additionally, Dhawan *et al*, reported transplantation of cryopreserved hepatocytes into patients with inherited factor VII deficiency resulting in gradual reduction in their requirement for recombinant factor VIIa until, after 8 to 10 weeks, requiring only 20% of their original dose (Dhawan, Mitry et al. 2004). It is still questionable, however, whether hepatocyte transplantation can contribute to the rescue of liver-based metabolic disorders over long-term. Furthermore, *ex vivo* expansion of mature hepatocytes is not feasible because long-term cultivation of hepatocytes is associated with hypofunction of hepatocyte metabolism. It would, therefore, be greatly valuable if functional hepatocytes could be generated from stem cells, which could be propagated *in vitro*.

B. Foetal liver progenitor cells

Research looking into the role of stem cell application in liver disease has, unfortunately, been subject to various confines (table 6). Liver progenitor cells have been identified in embryonic and foetal livers and have been successfully differentiated into hepatocyte or bile epithelial cells (Rogler 1997; Lazaro, Rhim et al. 1998; Kubota and Reid 2000). Such studies have been restricted by the low yield of cells and the inadequate *in vitro* expansion after isolation (Czyz, Wiese et al. 2003). Sandhu *et al* and Oertel *et al*, both demonstrated in rat models that foetal hepatic stem/progenitor cells exhibited potency for reconstitution of

adult liver but only under a particular set of conditions (Sandhu, Petkov et al. 2001; Oertel, Menthena et al. 2008). Thus, it still remains unclear whether adult hepatic stem cells or progenitor cells, can reconstitute recipient livers not subjected to genetic modification. To answer this question, therapeutic transplantation of hepatic stem cells must be carried out using minimal pre-treatment and without genetic modification of the recipients.

To overcome the problem of low cell yield, attempts were made by several groups to immortalize hepatocytes using viruses (Cai, Ito et al. 2000; Allan JE 2002) or the enzyme telomerase (Wege, Le et al. 2003). These approaches allowed expansion of hepatocytes but were compromised by phenotypic changes and karyotypic abnormalities over prolonged culture durations (Delgado, Parouchev et al. 2005).

C. Embryonic stem cells

The introduction of embryonic stem cells (ESC) provided another potential source of human hepatocytes. Mouse embryonic stem cells (mESCs) have been shown to be able to differentiate down the hepatocyte lineage which can integrate into liver tissue and produce albumin (Chinzei, Tanaka et al. 2002; Yamamoto, Quinn et al. 2003; Teratani, Quinn et al. 2005; Cai and Grabel 2007). Similar work has been demonstrated in human embryonic stem cells (hESCs) as well (Baharvand, Hashemi et al. 2006; Hay, Zhao et al. 2007). Although ESCs have brilliant proliferation and differentiation properties they cannot be used in the clinic because of their genetic instability and a high risk of tumour formation. ESCs are also limited by the ethical issues that surround the use of human embryos associated with the fact that their retrieval requires destruction of an embryo. The scientific realism surrounding ESCs is that the production of large quantities of homogenous cells/tissue for clinical application are difficult and there could be potential complications associated with the animal feeder layers on which human ES cells tend to rely on in addition to the risk of teratomas. Hence it is unlikely that embryonic stem cell (ESC)-derived treatments will be available for clinical use anytime soon.

D. Induced pluripotent stem cells

Recently developed techniques by Takahashi and Yamanaka for pluripotency induced in adult fibroblast gives an interesting alternative for ESC (Takahashi and Yamanaka 2006) . These induced pluripotent stem cells can be generated without ethical concerns, but their genome instability and low efficiency of cell production raises the same concerns as for ESCs when considering their clinical use.

E. Adult stem cells

Adult stem cells (predominantly bone marrow stem cells) have been extensively investigated as a potential source of liver stem cells and as a means to regenerate the

diseased liver. This attention was fuelled by the identification of a population of liver stem cells in rodent (oval cells) that expressed HSC markers (e.g. Thy-15). And, hence it was postulated that these cells most probably originated from the bone marrow (Petersen, Bowen et al. 1999). Subsequently, BM transplants in animal models of liver disease were carried out and analysis suggested that BM could contribute to a mature hepatocyte population (Alison, Poulsom et al. 2000; Theise, Badve et al. 2000). These exciting results lead to further investigation of this resourceful stem cell population but the results obtained were contradictory with little evidence for a significant repopulation of the liver parenchyma by BM-derived cells (Kanazawa and Verma 2003; Menthena, Deb et al. 2004; Thorgeirsson and Grisham 2006; Vig, Russo et al. 2006). Additionally, proponents suggested that any rescue resulting from BM transplantation was the result of cell fusion of the BM-derived cells with the diseased hepatocytes, resulting in a form of cellular gene therapy. Furthermore, it was suggested that the unique selection pressure seen in these animal models triggered the fused cells to divide to repopulate the liver (Willenbring, Bailey et al. 2004). It is worth noting that a selection pressure of this magnitude is unlikely to be available for most clinical applications.

Mesenchymal stem cells (MSCs) derived from various tissues including bone, fat and dental tissue have also been explored as an alternative source of donor cells. Animal studies have previously demonstrated the ability of human adipose tissue MSC-derived hepatocytes to restore some liver function in nude mice with acute liver failure (Banas, Teratani et al. 2009). Additionally, van Poll *et al*, showed that systemic infusion of soluble factors secreted from MSCs provided a survival benefit and prevented the release of liver injury biomarkers (van Poll, Parekkadan et al. 2008). Thus it may be argued that the transplanted MSCs did not play a crucial role in reconstitution of the recipient liver, but instead, the soluble factors that they secreted were liver protective. Additionally, contradictory results have demonstrated that MSCs do not differentiate in to hepatocytes in rat livers (Popp, Slowik et al. 2007). Such apparently contradictory results have been commonly seen in this field and probably reflect a combination of factors, including the use of varying cell derivation and differentiation protocols, and the fact that the models of liver injury are often differing and rarely repeated. Although, more *in vivo* research is definitely needed to address these issues, MSCs do appear to ameliorate liver injury in some way or the other. Moreover, it may be that both the exact derivation and pre-conditioning of the starting cells and the host liver environment will determine the resulting phenotype of the transplanted cells.

The clinical studies reported for BM therapy for liver disease involve infusion of the enriched haematopoietic cell fraction ($CD133^+$, $CD34^+$ or monocytes) (am Esch, Knoefel et

al. 2005; Terai, Ishikawa et al. 2006; Lyra, Soares et al. 2007). Interestingly, the majority of *in vitro* and *in vivo* experimental work suggests that the MSC population in BM are inducible into hepatocyte-like phenotype, and there is very few data suggestive that HSCs have a similar potential. The logic, however, for using the haematopoietic cell fraction in cirrhotic liver conditions may be due to the modulating and remodelling effect of the monocyte-macrophage population on scar tissue (Duffield, Forbes et al. 2005). As pointed out, the results achieved from ADS research as an alternative cell source for the management of liver disease has been exciting but, has also simultaneously highlighted many grey areas that need further investigationS.

A novel stem cell source that has been explored more recently is the umbilical cord and cord blood. This stem/progenitor cell reserve provides a very abundant and valuable resource that still remains to be exploited therapeutically in the management of liver injury and disease.

As far as cell therapy of end-stage liver disease is concerned, it may be safe to conclude that stem/progenitor cells that can be efficiently differentiated into a hepatocyte phenotype can be used for *ex vivo* purposes, such as liver support devices, toxicology studies and drug testing. For transplantation procedures, however, it is essential to ensure that the cells infused are pre-programmed to either differentiate into or remain as hepatocytes *in vivo* post transplantation, and research thus far is some way short of this mark. Additionally, it is worth considering what the best treatment approach would be. Would it prove more fruitful to transplant hepatocyte-like cells in the extremely harsh environment of a cirrhotic liver, where endogenous professional hepatocytes find it hard to survive or to modify the cellular and extracellular milieu, to allow the endogenous hepatocytes and progenitor cells to regenerate the liver? The latter may well be a more realistic medium-term goal for cell therapy.

Table 6. Limitations of various stem/progenitor cells with potential for management of liver disease.

Embryonic/foetal liver progenitor cells	Immortalized hepatocytes Viruses/enzyme (telomerase**)**	Embryonic stem cells	Induced pluripotent stem cells	Adult stem cells Bone marrow/peripheral blood
a) Low yield of cells b) Inadequate *in vitro* expansion (Campard, Lysy et al. 2008)	a) Compromised by phenotypic changes and karyptype abnormality over prolonged culture durations. (Airey, Almeida-Porada et al. 2004)	a) Problems of genetic instability b) High risk if teratoma c) Ethical issues d) Difficulties to produce large quantities of homogenous cells/tissue e) Complications associated with feeder layers (Campard, Lysy et al. 2008,35)	a) Genetic instability b) Low efficiency of cell production (Airey, Almeida-Porada et al.)	a) Invasive collection procedures b) Differentiation potential widely studied but proponents question the very existence of the process claiming that cell fusion is responsible for the phenomena. (Alexander, Roberts et al. 1996)

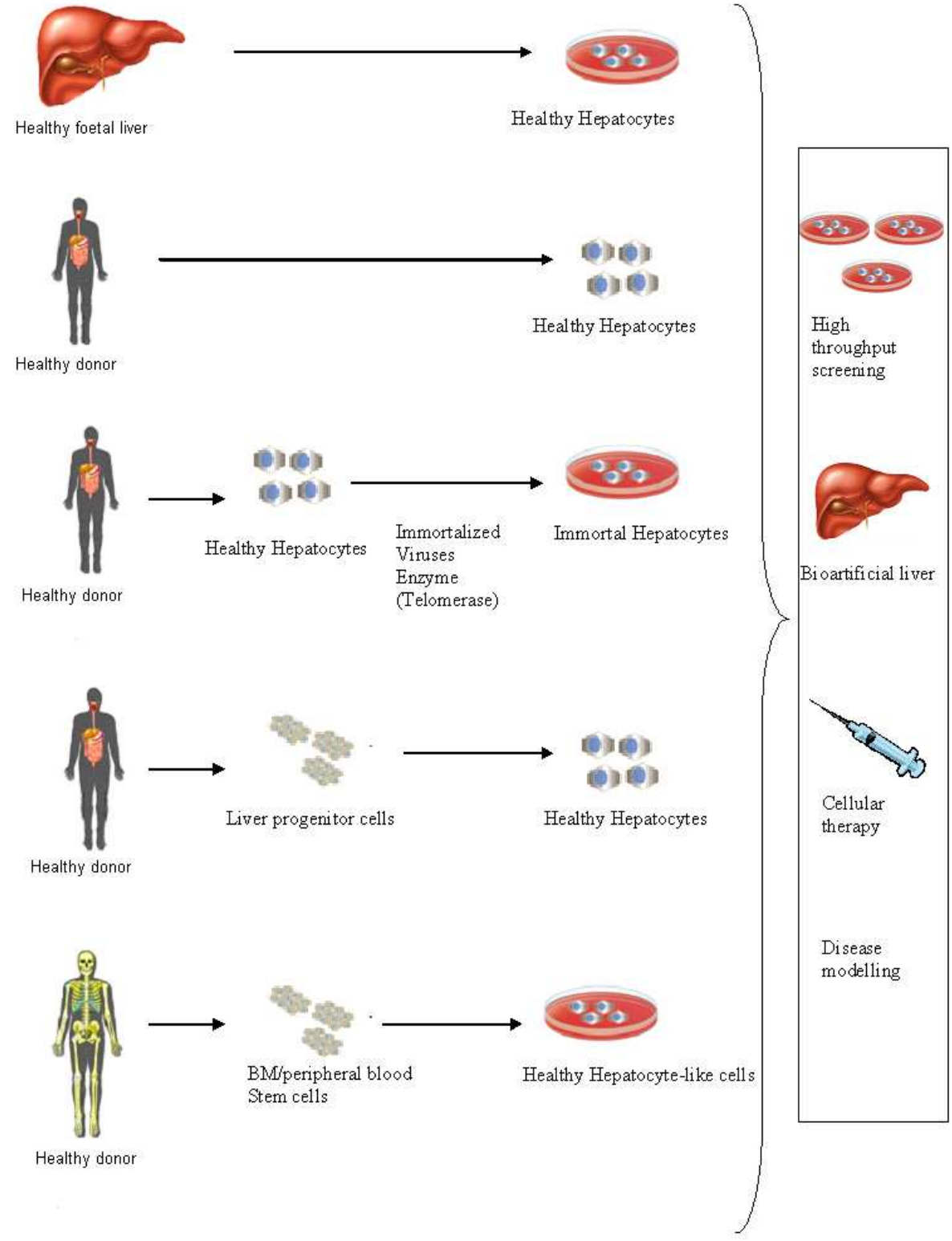


Figure 28a. Schematic illustration of hepatocyte sources for cellular therapies for *in vitro* modelling of liver disease. *Ex vivo* adult hepatocytes can be isolated from healthy donor livers, yet these cells are scarce due to donor shortage and upon isolation have a limited proliferation capacity and lose functionality. Alternatively, adult and foetal liver progenitor cells can be extracted from healthy donors and *in vitro* directed hepatocyte differentiation induced. Modified from (Dalgetty, Medine et al. 2009)

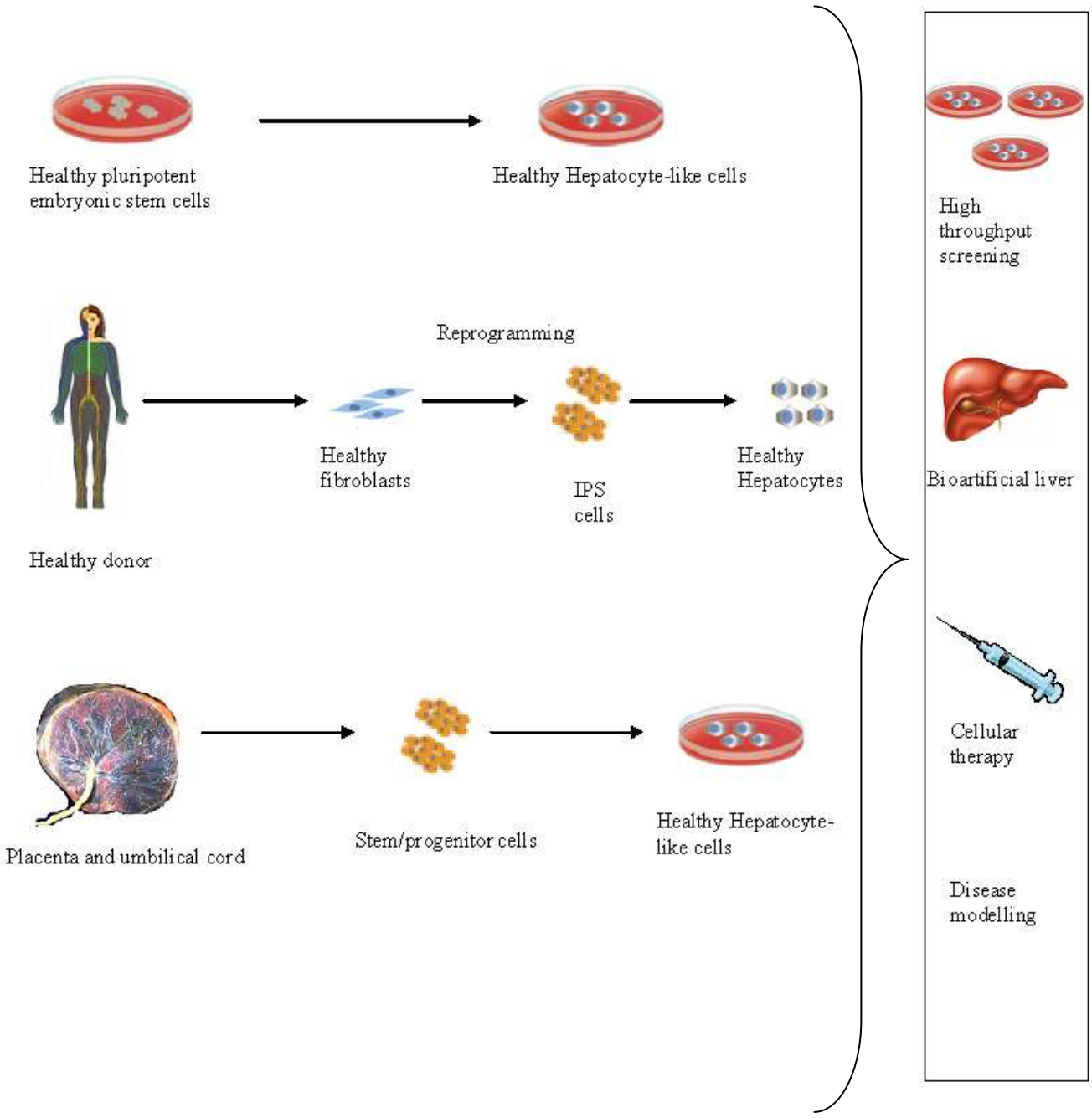


Figure 28b. Schematic illustration of hepatocyte sources for cellular therapies for *in vitro* modelling of liver disease. Human embryonic stem cells derived from healthy blastocyst undergo expansion and differentiation into hepatocyte-like cells by employing extracellular signals to mimic human liver development. Human induced pluripotent stem (IPS) cells can be generated by reprogramming healthy somatic fibroblast cells from healthy donors followed by induction of hepatocyte-like cell differentiation. Both the above sources of hepatocytes are associated with complications in terms of clinical applicability. A much safer and more resourceful alternative are the stem/progenitor cells extracted from the umbilical cord and cord blood. This primitive cell source provides an ethically sound and unlimited supply of stem/progenitor cells that can be exploited in *in vitro* hepatocyte differentiation systems and hence provides an ideal model to study liver development and simultaneously offers a valuable tool for cellular therapy of liver diseases.

1.3.7 Hepatic differentiation properties of adult stem cells

Work done on adult stem cells is in close context of ageing, cell repair strategies and haematopoiesis. Previous studies have shown the possibility of turning bone marrow (BM) and umbilical cord blood (UCB) stem cells into hepatic-like cells *in vivo* and *in vitro* (Di Campli, Piscaglia et al. 2004; Lee, Kuo et al. 2004; Aurich, Koenig et al. 2005; Hong, Gang et al. 2005; McGuckin, Forraz et al. 2005; Nonome, Li et al. 2005; Baharvand, Hashemi et al. 2006; Kang, Zang et al. 2006; Tang, Zhang et al. 2006; Zhan, Wang et al. 2006; Hay, Zhao et al. 2007). The ability of bone marrow to contribute to hepatocytes was first demonstrated by Petersen *et al*., who showed that bone marrow cells transplanted into lethally irradiated mice engrafted in the recipient's liver and differentiated into liver stem cells (called oval cells in mice) or mature hepatocytes (Petersen, Bowen et al. 1999). Soon after, Alison *et al* and Theise *et al* also illustrated in rodents and in humans that hepato-biliary cells could be derived from bone marrow (Alison, Poulsom et al. 2000; Theise, Badve et al. 2000; Theise, Nimmakayalu et al. 2000). With Y-chromosome staining and liver specific markers they detected bone marrow-derived hepatocytes in livers of irradiated mice and humans after gender-mismatched bone marrow transplantation. Gordon *et al*, reported that human CD34+ cells mobilized in peripheral blood by administration of G-CSF (Granulocyte- Colony Stimulating Factor) followed by leukapheresis and re-infusion of these cells in patients with liver insufficiency, resulted in improved liver function in patients (Gordon, Levicar et al. 2006). These interesting results, however, were not reproducible by several groups including Wagers *et al*, Kanazawa *et al* and Cantz *et al* (Wagers, Sherwood et al. 2002; Kanazawa and Verma 2003; Cantz, Sharma et al. 2004). Even when results were replicable by groups, as Lagasse *et al* (Lagasse, Connors et al. 2000), they were noted not to be the result of direct differentiation but rather occurring due to fusion of haematopoietic stem cells with recipient hepatocytes in the animal models (Vassilopoulos, Wang et al. 2003; Wang, Willenbring et al. 2003).

Although the exact mechanism for the effect on liver function is not clear and little is known as to which bio-molecular and biochemical pathways regulate such differentiation, such data may reflect activation of genes corresponding to a hepatocyte differentiation program upon exposure to the injured liver environment.

Although proponents see ADS as an attractive alternative to the use of embryonic stem cells in regenerative medicine, opponents have questioned the very existence of the process claiming that cell fusion is probably responsible for the phenomenon. Several critics have challenged the concept of stem cell plasticity. Issues have included the inability to reproduce data, and the suggestion that some apparently reprogrammed ADS could be engrafted cells

fusing with cells in their new location. This opinion is based on experiments exploring the outcome of co-culturing BM with highly volatile embryonic stem cells, but not ADS, and noting occasional tetraploid cells from the fusion of the two cell types (Alison, Poulsom et al. 2003; Preston, Alison et al. 2003). A better understanding of the mechanisms of lineage specific differentiation and plasticity of pluripotent stem cells would provide critical clues for the use of stem cells in regenerative medicine. For this purpose the scientific community needs to develop reliable *in vitro* and animal models that will allow for better understanding of how to efficiently differentiate ADS and UCB stem cells into functional hepatic tissue. A new approach of liver tissue-engineered constructs in 3-dimensional environment *in vitro* gives a unique tool for preclinical and toxicological studying of ADS and UCB for their clinical applications in a future. Hence, as previously elaborated earlier it may be safely concluded that, umbilical cord and cord blood-derived stem cells offer multiple advantages over truly adult stem cells and over embryonic stem cells for liver differentiation.

1.3.8 Can Cord and Cord Blood Stem Cells Regenerate Liver Tissue?

Taking into consideration the superiority of umbilical cord blood stem cells over bone marrow derived cells; several groups explored the potential of human umbilical cord blood to generate hepatocyte and biliary epithelial cells. These experiments were performed using mononucleated cells from cord blood for *in vivo* transplantation in animal models. *In vivo* studies were first performed in sheep by Almeida-Poroda *et al* and then in rodents by Newsome *et al*, Wang *et al* and Ishikawa *et al*, by transplanting cells into sub-lethally irradiated NOD-SCID mice (Ishikawa, Drake et al. 2003; Newsome, Johannessen et al. 2003; Wang, Ge et al. 2003; Almeida-Porada, Porada et al. 2004). Although these recent publications highlight the differentiation potential of human UCB cells and were able to produce hepatocytes, further characterization of these differentiated cells was demanded. In 2005, Sharma and colleagues, for the first time characterized these human UCB-derived hepatocyte-like cells after *in vivo* experiments in mouse animal models, and demonstrated the ability of these cells to express human albumin and human hepatocyte-specific antigen, Hep Par1 (Sharma, Cantz et al. 2005). They compared BM cells with human UCB mononucleated cells in parallel controlled studies and showed that cord blood was superior to BM in its differentiation potential. It could have been argued that because neonatal UCB stem cells are different from BM stem cells and may have a reservoir of preformed hepatic progenitors, which may not be present in the BM preparation, they demonstrated better results (Mayani and Lansdorp 1998; Kubota, Storms et al. 2002). However, to avoid such questions, Sharma *et al* performed the experiments using both adult and neonatal BM cells for transplantation but were unable to illustrate an increase in frequency of BM-derived hepatocytes in the model livers of neonatal BM transplantation (Sharma, Cantz et al. 2005).

Until 2005, all the research done on human cord blood was performed using cord blood-derived mononucleated cells and the studies were executed in animal models. Mononucleated cells, constitutes the entire white blood cell/leukocyte compartment of blood, of which stem cells are a component. Although, UCB demonstrates a better tolerance for Human leukocyte Antigen (HLA) mismatch compared to ADS, perhaps due to the immaturity of its immune cells, the production of a purified population of stem cells from cord blood for transplantation would further reduce any risk of immune-related graft rejection. Mcguckin *et al* reported the world's first production of haematopoietic cells, expressing embryonic stem cell markers, from cord blood. These cells were termed Cord Blood-derived Embryonic-like stem cells (CBEs) and were produced by exposing UCB to an immuno-magentic cell separation technology that allows sequential removal of nucleated granulocytes, haematopoietic myeloid/lymphoid progenitors and erythrocytes, leaving

behind a purified population of very immune naive stem/progenitor cells (McGuckin, Forraz et al. 2005). Subsequently other reports concerning the existence of circulating embryonic stem cell-like cells during foetal development were generated (Jiang, Vaessen et al. 2002; Korbling and Estrov 2003). McGuckin *et al* further demonstrated the differentiation potential of these cells and showed that these cells were capable of generating endodermal tissues, including hepatocyte-like cells in 2-dimensional and 3-dimensional culture systems, expressing hepatocyte-specific markers (McGuckin, Forraz et al. 2005) as well as pancreatic-like cells testing positive for insulin and C-peptide (Denner, Bodenburg et al. 2007). This was achieved by thoroughly studying classic liver biology and development and creating an artificial culture system that closely resembles the natural micro-environment that is needed for normal development of the liver (Figure 29). Similar protocols have been reported in human embryonic stem cell-derived hepatic cells but not in cord blood studies (Baharvand, Hashemi et al. 2006). A few other research groups have also been able to successfully differentiate cord MSCs into hepato-bilary cells (Hong, Gang et al. 2005; Kang, Zang et al. 2005). Campard and colleagues are amongst the few research groups that demonstrated differentiation of cord matrix (Wharton's jelly) MSCs into hepatocyte-like cells (Campard, Lysy et al. 2008).

It is worth noting, that since the isolation MSCs from cord blood is inefficient, extraction of haematopoietic stem/progenitor cells may prove more resourceful when using cord blood, as irrespective of the size of the cord blood units these cells can be isolated from nearly every cord blood specimen processed. The umbilical cord, on the other hand, is a brilliant resource for MSCs. However, one of the limitations of MSCs is their strict dependence on selected lots of foetal bovine serum that has limited clinical applicability of *ex vivo* expanded MSCs. This drawback, however, may be overcome by restoring to low serum or serum-free culture systems as demonstrated by Reinisch *et al*, who for the first time demonstrated propagation of both BM and UCB MSCs in bovine serum-free systems using human platelet lysate-conditioned medium (Reinisch, Bartmann et al. 2007). Both these aspects: (i) generation of artificial liver tissue *in vitro* and (ii) use of defined culture media for stem cells differentiation, are crucial for establishing a reliable *in vitro* platform for studying cellular and molecular mechanisms of stem cell differentiation towards liver.

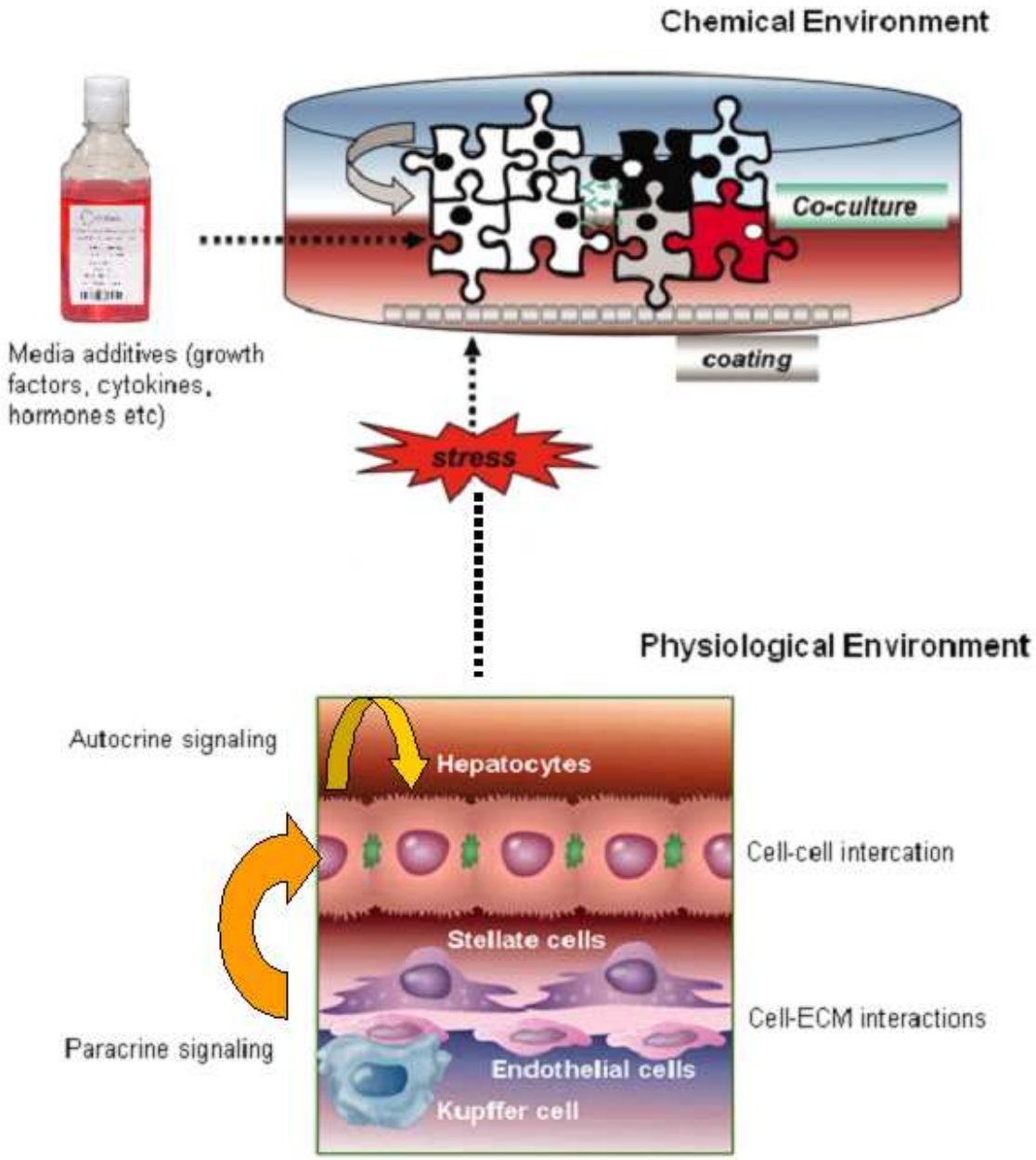


Figure 29. Hepatocyte microenvironment *in vitro* and *in vivo*. Artificial culture systems are created by closely studying physiological liver biology and replicating it *in vitro*. Modified from (Snykers, De Kock et al. 2009)

1.3.9 Liver tissue-engineering

It is worth noting that most *in vitro* hepatic tissue models available for research and development use 2-dimensional culture systems. These systems fail to represent the physical cell-cell interactions of a 3-dimensional human liver and also do not always produce scientific data that can be fully translated to physiological interpretation. The quest for an efficient cellular technology that can be exploited for the management of end-stage liver diseases has paved the way for 3-dimensional tissue engineering. Such technology employs synthetic biodegradable porous scaffolds and rotational cell culture systems, also referred to as bioreactors, which allow cells to grow and differentiate in a 3-dimensional environment facilitating cell-cell interaction. Groups like Baharvand *et al* have utilized scaffolds to effect 3-D hepatic differentiation of human embryonic stem cells (Baharvand, Hashemi et al. 2006) and Mcguckin *et al* have demonstrated similar results from umbilical cord blood by employing the microgravity bioreactors (McGuckin, Forraz et al. 2005).

1.3.10 Clinical trials

After several reports in animal models , Theise *et al* was the first to confirm the ability of bone marrow to generate hepatocytes and cholangioctyes in humans by evaluating archival autopsy and biopsy liver specimens obtained from gender mismatched therapeutic BM transplantations and from orthotopic liver transplantations (Theise, Nimmakayalu et al. 2000). Approximately 11 BM stem cell transplantation clinical trials for treatment of liver diseases in humans have been published thereafter (Houlihan and Newsome 2008). These include work done by Mohamadnejad and colleagues in 2007, which carried out autologous bone marrow enriched $CD34^+$ haematopoietic stem cell transplantation into hepatic arteries of four decompensated liver cirrhosis patients. Although a mild degree of improvement of liver function was noted in two out of four patients, one died due to radiocontrast nepropathy and hepatorenal failiure. Thus they concluded that intra-hepatic artery is not a safe mode of stem cell transplantation but could not preclude the use of $CD34^+$ cells via other routes of administration (Mohamadnejad, Namiri et al. 2007). Conversely, clinical trials performed on similar patients by Lyra *et al (Lyra, Soares et al. 2007; Lyra, Soares et al. 2007)* and Gordon *et al* (Gordon, Levicar et al. 2006), using mononucleated cell-enriched BM cells, reported intra-hepatic artery route of infusion safe and feasible. Mohamadnejad *et al* further reported on the safety and feasibility of autologous bone marrow mesenchymal stem cell transplantation in patients with liver decompensation due to cirrhosis. This time, however, the stem cells were administered via a peripheral vein in four patients. No post-operative side effects were noted and all patients showed evidence of

improvements of liver function and a better quality of life (Mohamadnejad, Alimoghaddam et al. 2007). The efficiency of intravenous infusion was confirmed by studies performed in mice by Kou *et al* (Kuo, Hung et al. 2008). Terai *et al* and Gordon *et al*, reported transplantation of autologous BM stem cells and human $CD34^+$ stem/progenitor cell populations mobilized into the blood by granulocyte colony-stimulating factor respectively, and both noted improvement of liver functions in patients post transplantation (Gordon, Levicar et al. 2006; Terai, Ishikawa et al. 2006). It seems clear that trials of BM stem cells treatment in patients with liver disease are still at a preliminary stage and a better understanding of the physiology and mechanism of action of BM stem cell in animal models of liver disease is needed. The results of these clinical trials are, however, very exciting and open the roads for exploring the potential of umbilical cord and cord blood stem cells for cellular therapy of liver disease. In addition to intra-hepatic delivery of stem cells for treatment of liver disease, tissue engineering offers novel opportunities for the generation of extracorporeal liver devices. Such technology would allow temporary replacement of liver function buying time till a suitable organ is available for transplantation.

Thus far the research done into exploring the potential of the umbilical cord and cord blood for regenerative management of end-stage disease is commendable. Although the outcome of the various studies highlights extra-embryonic tissues as an indispensable reserve with immense potential for liver therapy, limitations such as lack of consensus in immunophenotype of liver progenitor cells, uncertainty of the physiological role of reported candidate stem/progenitor cells, long-term efficacy and safety challenge the use of these cells in humans. Current molecular techniques of stem cell identification are confounded by cell fusion, horizontal gene transfer, incomplete differentiation and chimera formation. It is exciting to note that stem cell transplantation and phase 1 trials of bone marrow transplantation in humans for liver diseases (Mohamadnejad, Alimoghaddam et al. 2007; Mohamadnejad, Namiri et al. 2007) are underway but require more robust verification. More research is definitely demanded to help identity the best source of stem/progenitor cells that can transferred from the bench to the bedside for the management of patients with severe or life-threatening liver disorders.

Goals and scope of this study

The scope and ambition of this study was to harness the multi-potential property of umbilical cord and cord blood stem/progenitor cells to engineer human 2-dimensional and 3-dimensional functioning hepatic tissue models. These models are to be used as a platform to better understand the biology of liver development, to be exploited in bio-artificial liver assist technology, used in biotechnology and pharmaceutical applications (drug toxicology studies) and finally to be applied in regenerative medicine for advancement in cell therapy for liver disease.

Stages of the project included:

- Umbilical cord and umbilical cord blood collection only after written informed consents.
- Selection of cord blood stem/progenitor cell populations.
- Isolation of umbilical cord Mesenchymal stem cells.
- Optimization of expansion protocol facilitating commitment of cord blood stem cells to meso-endodermal lineages.
- Directed 2-D umbilical cord and UCB stem cell *in vitro* differentiation into hepatocytes and cholangiocytes (biliary epithelium) driven by growth factors, amino acids, minerals, extracellular matrices and cell culture media combinations.
- Hepatic cell characterization by morphology and phenotyping.
- Umbilical cord and cord blood derived differentiated stem/progenitor cells were compared with other liver cell populations at different stages of development including:
    - **HepG2** (liver cancer cell line)
    - **Human foetal liver cells**
    - **Human adult liver cells**
- Progression from 2-D cell culture to 3-D tissue engineering. Integration of cells, bioscaffolds/biomaterials, bio-chemical and physical cues to engineering 3-D hepatic tissue constructs, using 3D bioreactor technology.
- Functional characterization: assessment of some key functions of the engineered hepatic tissue constructs.

2 Materials and Methods

2.1 Consent and collection of umbilical cord and cord blood

2.1.1 Informed consent

Written informed consents were obtained from all participants (Appendix A). Uniform standards for collection of cord blood units were observed without alterations of safe obstetric practice.

2.1.2 Cord blood collection

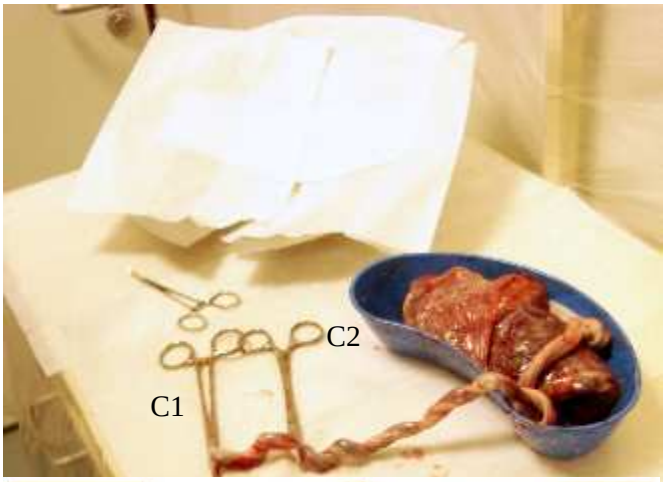
Umbilical cord blood (UCB) specimens were collected from full-term third stage of labour deliveries after elective caesarean sections. All specimens were collected according to the Royal Victoria Infirmary (RVI) and Newcastle university ethical regulations. The protocols followed, were reviewed by the National Health Service local ethics committee. Cord blood units were collected at the maternity unit in the Royal Victoria Infirmary, Newcastle upon Tyne.

Blood samples were collected in 250ml blood collection bags (Baxter PL146-CPDA-1-35ml; Baxter, Derrfeild, IL, USA) containing 35ml anticoagulant (Citrate Phosphate Dextrose-A, CPD-A).

Cord blood collection was only instigated after completion of the third stage of labour with no interference with the timing of cord clamping and disconnection of the placenta and umbilical cord from the newborn.

The umbilical cords were clamped by the performing surgeon at three positions along the length of the cord (Figure 30). Upon receipt of the cord, blood gases were collected between clamps one and two. These samples are sent off for analysis to determine the level of gases in the neonate. Subsequently cord blood collection for research was commenced. Blood collections were performed under strict sterile conditions. Cord blood was collected between clamps two and three (Figure 30). Blood was collected only from the umbilical vessels and not from the placenta. Placental manipulation and milking of the cord were not performed during the collection procedure. Gravity and natural movement was used to facilitate drainage of the umbilical vessels.

A



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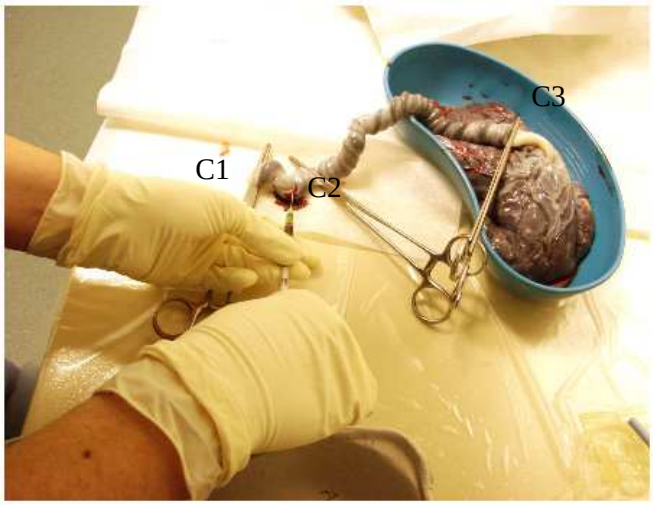


Figure 30. Clamping of the umbilical cord after the third stage of labour. (A and B) Blood gases were collected between clamp 1 (C1) and clamp 2 (C2) and (B) cord blood was subsequently collected between clamp 1 (C2) and clamp 3 (C3).

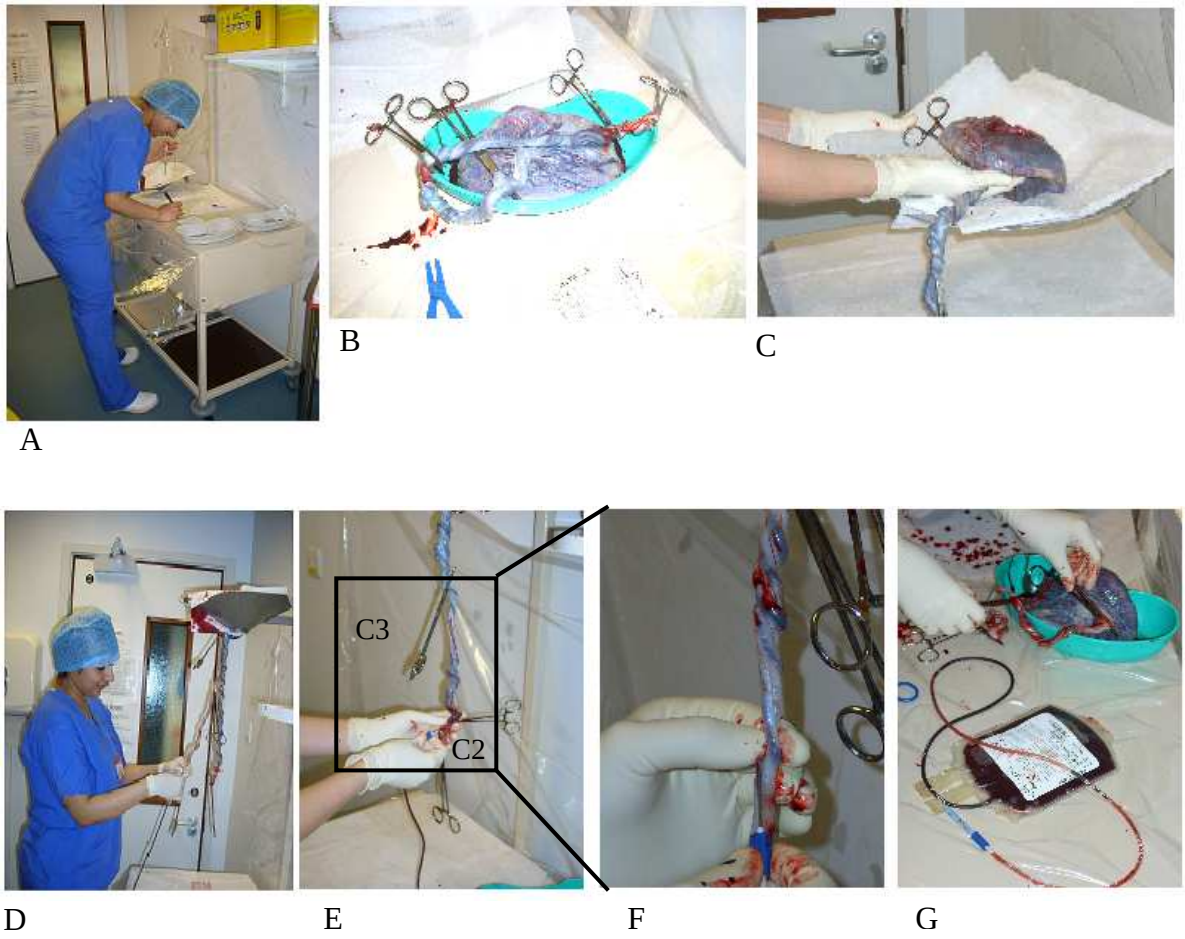


Figure 31. Collection of umbilical cord blood after the third stage of albour.

(A) Apparatus used for cord blood collection. (B) Clamping of umbilical cord by the obstetric surgeons. (C and D) Placenta and cord transferred to funnel-shaped instrument and suspended to allow gravity to facilitate cord blood collection. (E and F) Cord blood collection between clamp 2 (C2) and clamp 3 (C3). (G) 250ml blood collection bag (Baxter) containing anticoagulated cord blood.

2.1.3 Cord collection

Once all the blood from the umbilical cord vessels was drained, the first two clamps were removed from the cord and 10cm of the cord was cut from the distal end using a sterile scalpel. The piece of cord was then transferred into Hanks' Balanced Salt Solution (x1) (HBSS) (Invitrogen; 1600 Faraday Ave, Calsbad, CA) in a 50ml falcon tube until processing was undertaken.

2.1.4 Criteria for transport and storage of samples prior to processing

Transport of cord and cord blood units from remote collection sites to the processing and storage facility is a quality concern due to time and temperature sensitivity of the samples. Both parameters play an important role in the potential decline in cell viability. The samples utilized for this project were all transported at room temperature and processed within 24 hours of collection. Wada *et al* noted a 1 per cent drop in cell viabilities for every 4 hours increase in transit time (Wada, Bradford et al. 2004). And, Hubel *et al* reported that cord blood units processes within 24 hours after collection exhibited very little loss of cell viability (Hubel, Carlquist et al. 2004).

2.1.5 Tissue Culture standards

To evade bacterial and fungal contamination all tissue culture including processing of umbilical cord and cord blood samples as well as preparation of reagents, was performed in a specialised tissue culture facility with class II vertical laminar flow hoods, with or without fitted dissection microscopes (Zeiss), observing sterile methodology throughout. These facilities were sterilised by fumigation biannually. Additionally, all reagents, equipment, materials and hoods were sprayed with 70% ethanol. All waste liquids and cells were treated with Virkon^{TM} (Medisave, VK734))before disposal. All Culture media was filtered with a 0.2µm acrodisc syringe filter (PALL Life Sciences, 0.2µm supor membrane, PN 4612, PALL corporation, Ann Arbor, USA) and was used or disposed of within four weeks of preparation. Cells were routinely examined with a Zeiss Axiovert 200M fluorescence microscope to assess cell viability and state of culture. To minimize cell stress from cold temperatures or acidic pH changes, all media was warmed to 37°C, and CO_2 equilibrated by placing in an water bath for 30 minutes prior to usage. All wash buffers utilized were made fresh to avoid potential contamination between experiments.

2.2 Isolation of mononuclear cell fraction from cord blood

Recent successful clinical reports have implied the use of UCB in the management of several disease conditions. In addition, UCB may alleviate some of the problems associated with adult stem cell transplantation. Cord blood banks could minimize the problems connected with finding suitable stem cell donors and incidence of graft rejection would be diminished. Several different methods of cord blood separation are available today and in this study we have analysed the most recent and frequently applied techniques in an attempt to indentify the most efficient method for separation and cryopreservation.

2.2.1 Density gradient method of cord blood separation

Density gradient method of cell separation exploits the differences in densities to separate blood into its various components.

Thorsby and Bratile (1970) slightly modified the technique pioneered by Bøyum for isolation of Lymphocytes from blood using sodium diatrizoate (9.1%) and polysaccharide (5.7%). This technique was pioneered by Bøyum in 1968. Other authors, Harris and Ukayiofo (1969), Ting and Morris (1971) have emphasized the reliability of this technique for separation of lymphocyte preparation from anti-coagulated blood stored at room temperature for up to 6 hours.

Ficoll-Paque PREMIUM (GE Healthcare Bio-Science AB; Cat no. 17-5442-02, Sweden) is a ready made, sterile solution for the isolation of pure lymphocyte suspensions. This density gradient media is manufactured according to GMP compliant measures. This media is composed of polysucrose and sodium diatrizoate. It illustrates the following physical characteristics: density 1.077 +_ 0.001 g/ml, osmolality 290 +/- 15 mOsm.

Ficoll causes aggregation of RBC, thereby increasing their sedimentation rate. The sedimentation of leucocytes is only slightly affected and can be collected from the upper part of the tube when the erythrocytes have settled.

In this study anti-coagulated blood samples were transferred from blood bags to 50ml skirted funnel tubes and diluted in equal volumes of Dulbeco's Phosphate buffered salt solution (PBS) (PAA laboratories GmbH, Cat no. H15-002, Strass 1, Pasching, Austria). Four volumes of diluted blood was then overlaid onto one volume of Ficoll (GE Healthcare Bio-Sciences AB, Cat. No. 17-5442-02, Sweden) prior to centrifugation (Jouan CR422. St-herblain, France) (400g (1350rpm), 30 minutes, 4°C, Acceleration 1, Deceleration 0). Centrifugation aids in accelerating the density gradient separation. The buffy layer of white blood cells (WBCs) is at the interface between the plasma and Ficoll (Figure 32). Ficoll

preserves the functional and morphological integrity of the cells. Additionally, this method does not penetrate biological membranes (http://www4.gelifesciences.com, 2007).

The mononucleated cell (MNC) layer was collected with a sterile Pasteur pipette, washed once in PBS and pelleted (500g, 10mins) before re-suspension in PBS. Cell aliquots were taken for viability/enumeration using Trypan blue (0.25% in PBS, Sigma- Aldrich, and Poole, UK).

It has, however, been suggested that in order for Ficoll to work at its optimal potential it should be used in conjunction with a hydroxyethyl starch separation. This is because the modified two-step approach gives a superior WBC/MNC recovery (median 94.2 ±2.44% Vs. 90.2±5.8%) (Schwinger, Benesch et al. 1999). Lymphoprep offers a similar approach to Ficoll, but it uses a mixture of metrizote and polysaccharide (http://www.axis-shield.com, 2007).

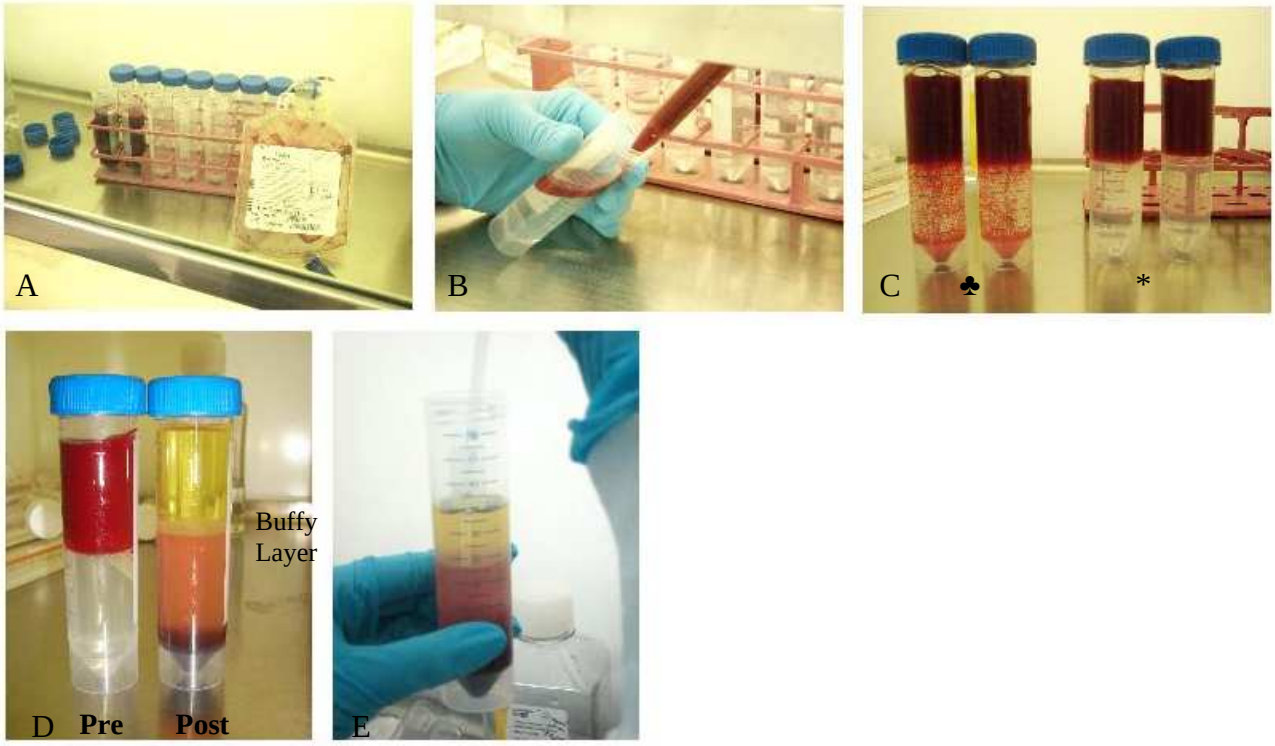


Figure 32. Density gradient method for cord blood cell separation. (A) Umbilical cord blood samples transferred from blood bags to 50ml skirted falcon tubes and diluted in equal volumes of (x1) Earle's basic salt solution (EBBS). (B) Four volumes of diluted blood was then overlaid onto one volume of Ficoll. (C) Samples immediately after overlaying on Ficoll (*) and post 10 minutes standing at rtp (♣). Note density dependent RBC sedimentation to the bottom of the falcon tubes. (D) Pre and post centrifugation of the blood samples. Centrifugation aids in accelerating the density gradient cell separation. Note the buffy layer (WBC rich) at the interface between the plasma and Ficoll. (E) Post centrifugation the buffy layer (WBC rich) was collected with a sterile pasteur pipette and washed once with (x1) PBS and pelleted.

2.2.2 Hetastarch- Hydroxyethy Starch method for cord blood separation

The umbilical cord blood units were transferred to 150 ml transfer bags (Baxter, R4R2001) then HES (Baxter 1001999959, 6 % Hetastarch in 0.9 % Sodium Chloride) was added to the UCB at a concentration of 20 % blood volume. The bag was then centrifuged at 125 x g for 10 mins, with the brake off (Jouan CR422. St-herblain, France). This stops disruption of the RBC pellet. Using a plasma expresser (Fenwal BM-1. Lake Zurich IL, USA), the supernatant containing the desired nucleated cells was expressed off into a second transfer bag (Figure). The second bag was then centrifuged at 400 to 500 x g for 10 mins (Jouan CR422, St-herblain, France). Again using plasma expresser, the supernatant was removed into a third transfer bag and this time discarded, leaving the pellet of nucleated cells in the second bag. These nucleated cells were then resuspended in Human Serum Albumin (HSA) (Bio Products Laboratory PL08801/006. Elstree, UK).

2.2.3 PrepaCyte-CB method for cord blood separation

PrepaCyte-CB is the first product originated from BioE's patented PrepaCyte technology platform. PrepaCyte is manufactured under U.S. FDA cGMP regulations. PrepaCyte-CB's negative selection process leaves desired cells unmodified. Preliminary results have shown PrepaCyte-CB improves TNC (total nucleated cell) and CD34+ cell recoveries when compared to Hetastarch-based methods (Basford, Forraz et al. 2009). In addition, PrepaCyte-CB removes more than 98 percent of red blood cells from the final product.

In this study Cord blood samples were thoroughly mixed on a blood collection mixer (Genesis CM-735; Hackensack, NJ, USA) and then transferred to the PrepaCyte-CB kit as illustrated in figure 33. The cord blood collection bag was connected with the PrepaCyte-CB system with a connecting tube thus allowing blood to drain into the PrepaCyte system. For optimal recovery of cells, a portion of the reagent-cord blood mixture was drained back into the collection bag, mixed and the contents were transferred back into the PrepaCyte system. Tubing between the cord blood bag and the PrepaCyte kit was the heat-sealed and the blood collection bag discarded. The PrepaCyte kit containing the reagent-blood mixture was the mixed on a rocker for 3-5 minutes, 15-20 rocks/min. After mixing, the PrepaCyte was suspended on a plasma expressor (Fenwal BM-1) for 30 minutes at room temperature to allow unwanted cells to aggregate and sediment to the bottom of the processing bag by causing an antibody dependent agglutination of red cells. The TNC-rich Supernatant containing the entire white cell compartment of cord blood was transferred to the next bag for centrifugation at 400-500 g for 10 minutes, with low break to avoid disruption of the pellet (Jouan CR422). After centrifugation, TNC and stem cell fraction was pelted thus

allowing unwanted second supernatant (predominantly plasma compartment of blood) to drain back through the system into the first processing bag already containing the unwanted agglutinated red blood cell compartment of the cord blood sample. The TNC and stem cell fraction was then either transferred into the cryopreservant bag or used in the laboratory for tissue culture purposes. The entire processing protocol took approximately 60 minutes to complete.

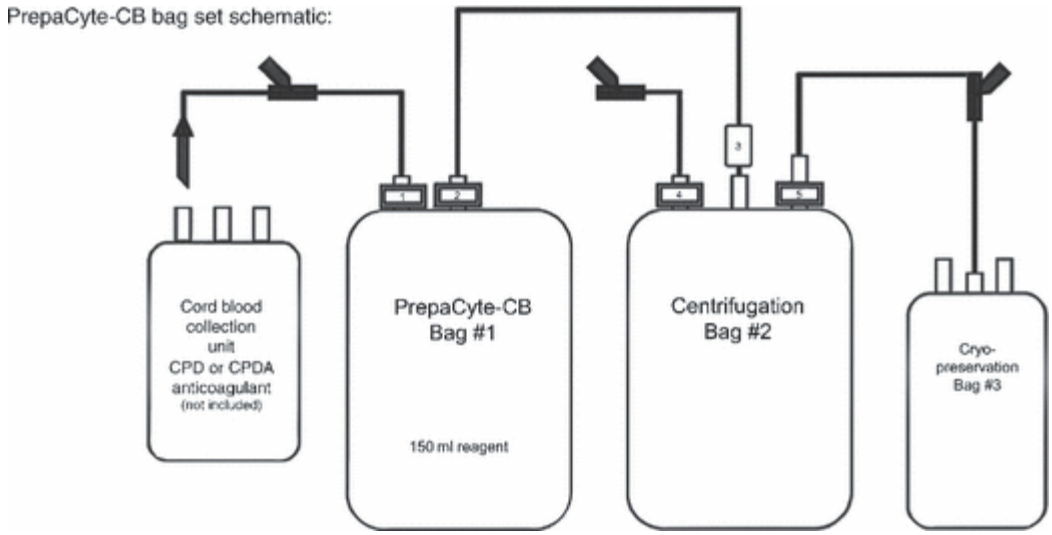


Figure 33. Schematic diagram of the PrepaCyte-CB bag set. Once the UCB unit is added to the bag set, it flows sequentially through as the process progresses until the desired nucleated cells reach the cryopreservation bag. (Basford, Forraz et al. 2009)

2.2.4 Plasma depletion method of cord blood separation

Plasma depletion is a non-red blood cell-depleted cord blood processing technique. It allows substantial plasma depletion from the UCB unit but does not remove red blood cells (RBCs). In this study, blood collection bags were connected to sterile 300ml Baxter transfer bags (transfer bag 1). Cord blood was allowed to flow through. The tubings were heat sealed and the collection bag removed. The pre-processing sampling was then performed in a biological safety cabinet. The blood units were thoroughly mixed prior to removing samples for analysis. A weighing scale was employed to balance the bags prior to centrifugation for 10 minutes at 2400rpm (temp 15-26°C). The bags were then carefully removed from the centrifuge and placed on a plasma extractor. The transfer bag 1 was then connected with a second sterile 150ml transfer bag (transfer bag 2). This bag was placed on an electric weighing scale and the scale was set to zero. The tubing of transfer bag 2 was clamped with a haemostat. Once the integrity of both bags was checked, the handle of the extractor was gently released to close the plate and the haemostat on the tubing was slowly released. The volume of the plasma expressed into transfer bag 2 was calculated by the weight of the bag. The tubing between the bags was re-clamped before any RBCs entered the tubing. The handle of the extractor was then released. Any air remaining in the blood bag after removal of the plasma was extracted with a syringe. Transfer bag 2 containing the expressed plasma was then heat sealed and removed. The content of transfer bag 1 was then thoroughly mixed and a sample was removed for further analysis. A cryobag was then attached to transfer bag 1 containing the plasma depleted UCB unit. The UCB was transferred into the cryobag using sterile technique. The extra tubing and transfer bag 1 were heat sealed and removed. The cryobag was the stored in an upright position at 2-8°C for a minimum of 30 minutes before being transferred to the control rate freezer in cold aluminium cassettes.

2.2.5 Sepax- fully automated sterile system for cord blood processing

This was a new fully automated, functionally closed and therefore sterile system, which is controlled by computer software (Figure 34). It permits isolation of the haematopoietic stem cell (HSC) rich buffy-coat of an UCB unit to a final volume of 10 to 90 ml. Each unit is separated with a single use kit, which is inserted into the machine.

The UCB unit was introduced into the machine where it filled the central chamber. Whilst filling, the system simultaneously sediments the UCB unit. The sample was then centrifuged at speeds of up to 1900 x g and the blood components were separated concentrically. Then, using optical sensors and motorised stopcocks the blood components

were directed to individual blood bags and extracted from the UCB unit. (http://wwwbiosafe.ch 2007). This method was recently validated by the Besançon Cord Blood Bank in France. They found that TNC recoveries were higher with the Sepax system than with Hetastarch (HES) separation methods (80.3 ± 7.7% versus 76.8 ± 9.1%) and that CD34+ cell recovery was also higher with Sepax (86 ± 11.6% versus 81.5 ± 12.5%) (Lapierre, Pellegrini et al. 2007).

A

B

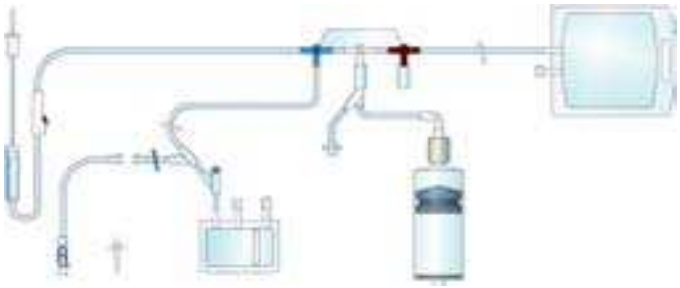


Figure 34. Sepax- fully automated sterile system for cord blood separation. This is a versatile cell separation technology which consists of the compact Sepax main processing unit (A) and single-use kits (B) allowing the controlled separation of cellular products in a fully automated and closed environment- no other equipment is required. (http://www.sinarvin.ir/index_files/sepax_kits.htm).

2.3 Umbilical Cord processing

The piece of umbilical cord previously collected was immersed in HBSS (Gibco, cat. no. 14170120) until further processing was initiated. Two separate methods of cord processing were investigated and the manual dissection of the cord proved to be an easier and more efficient technique.

2.3.1 Isolation of stem/progenitor cells from umbilical cord Wharton's jelly

2.3.1.1 Enzymatic digestion of the cord

The piece of cord (approximately 10cm in length) was transferred into a petri dish half filled with HBSS. Sterile forceps and a scalpel were used for the cord dissection. The cord was cut along its length with a scalpel and the vessels were removed using the forceps. The cord and its matrix (including the Wharton's jelly) were cut into small 4-6mm pieces and transferred into a 50ml falcon tube filled with HBSS. The tube was centrifuged at 250g for 5 minutes. The supernatant was discarded and the cord pieces were washed in serum-free DMEM (Invitrogen, Cat. no. 11039-021) and centrifuged at 250 x g for 5 minutes. The cord pieces were then transferred into a Petri dish and incubated with Collagenase (2mg/ml) overnight in an incubator at 37°C. The cord pieces were then washed with serum-free DMEM and incubated with 2.5% Trypsin at 37°C for 30 minutes with regular agitation. Fetal bovine serum (FBS) was introduced to inhibit the enzymatic reaction. The cord pieces were washed in serum-free DMEM and then cultured in DMEM-F12 10% FBS.

2.3.1.2 Manual dissection of the cord

The piece of cord was dissected in a similar method to the enzymatic digestion protocol. The 1-3mm cord pieces were then immediate transferred into culture medium (DMEM-F12 10% FBS, 1% penicillin-streptomycin (Invitrogen, Cat. no. 15140-122), 1% fungizone (Invitrogen, Cat. no. 15290-026) in 6 well plates.

In both methods, medium was not changed until 5 days after isolation. This allowed adequate time for the cord stem/progenitor cells to migrate out and adhere to the culture plates.

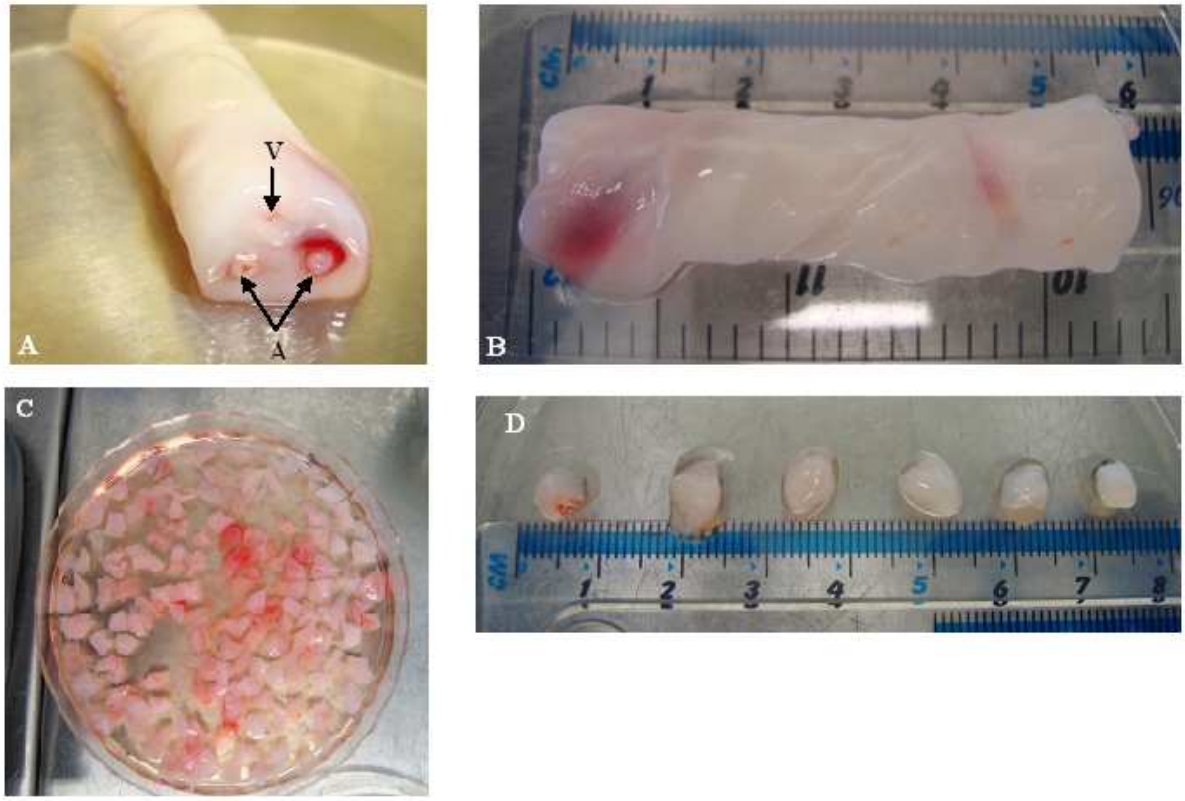


Figure 35. Processing of umbilical cord specimens. (A) Transverse section of umbilical cord showing two umbilical arteries (A) and an umbilical vein (V). (B) Measurement of umbilical cord specimen prior to processing. (C) Umbilical cord pieces post dissection in HBSS. (D) The umbilical cord specimens were dissected resulting in 4-6mm long pieces which were then transferred into culture medium for Mesenchymal stem cell isolation.

2.4 Sample analysis post cord blood and umbilical cord processing

2.4.1 Enumeration of cell samples

2.4.1.1 Haemocytometer

Cells were enumerated and assessed for viability by diluting cells with Trypan blue solution (0.25% in PBS). Cells were then counted with a Neubauer standard haemocytometer. The Trypan blue solution allows discrimination between dead cells (staining blue due to impaired membrane integrity) and viable cells (remaining translucent) to be made.

Formulation:

Cell number

Cells in central and four corner squares counted. This represents cells * 10^5 / ml

% viability

Viable cells/ total cell count * 100

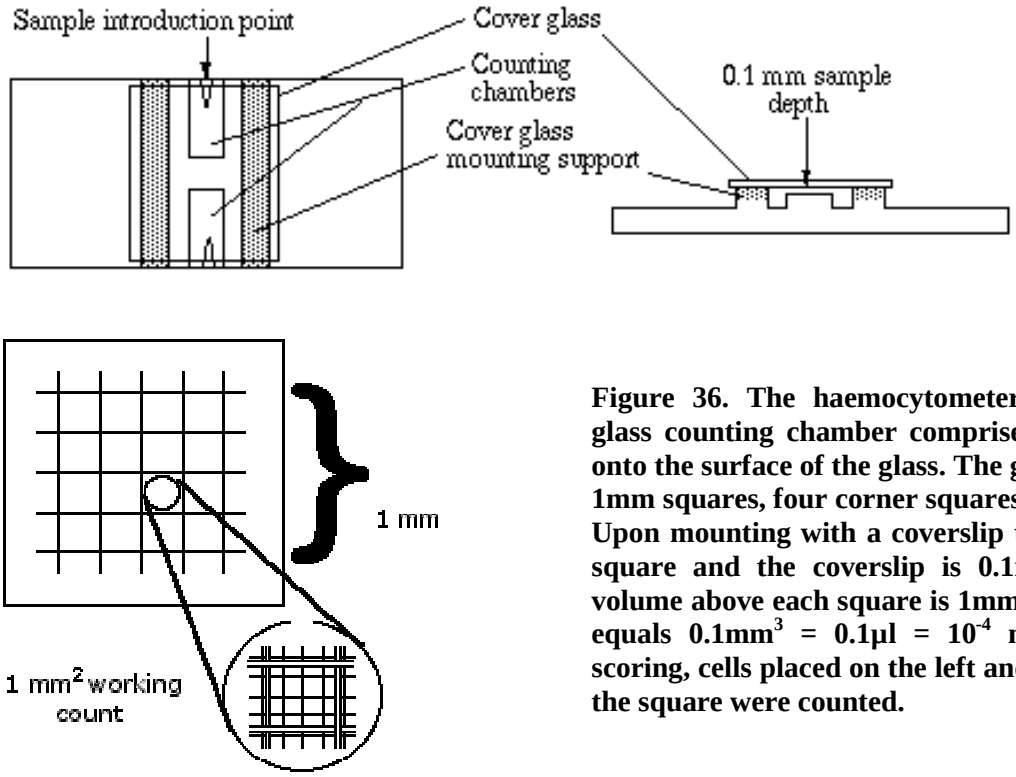


Figure 36. The haemocytometer. The haemocytometer glass counting chamber comprises grids that are etched onto the surface of the glass. The grids consist of five large 1mm squares, four corner squares and one middle square. Upon mounting with a coverslip the height between each square and the coverslip is 0.1mm. Consequently, the volume above each square is 1mm x 1mm x 0.1mm, which equals $0.1mm^3$ = 0.1µl = 10^{-4} ml. To avoid repetitive scoring, cells placed on the left and top borders and inside the square were counted.

2.4.1.2 Differential cell count

Differential cell counts on the UCB units was done using a COULTER® A^C.T diff 2^{TM} Analyzer (Beckman Coulter, Inc. Fullerton, CA 92835). This is a compact system that features low-volume automated one step sample processing and haematological analysis. The analyzer provides a complete blood count (CBC) together with a comprehensive differential count of the cord blood samples. A sample of 1ml was applied to the machine. The WBC count and differential determined the number of WBCs and the percentage of each type of WBC in each unit of blood.

2.4.2 Colony forming unit (CFU)

Mononuclear cells from cord blood were counted using the Beckman Coulter analyzer for the pre- and post-processed samples. $1x10^6$ cells from each sample was added to Iscoves's Modified Dulbecco's Medium (IMDM) (Gibco/Invitrogen 21980-032, Daisley, UK) + 20% FCS + supplements to make a final volume of 0.5ml. This was then added to 3.5ml aliquot of methyl cellulose culture medium (Stem cell Technologies 04534, USA) to make a total volume of 4ml. Gentle mixing with a 20ul pipette was performed to avoid frothing. Three small gridded petri dishes (Nunc brand, VWR 734-2114, Westchester, PA, USA) were labeled and placed in a large petri dish (Figure 37). 1ml of the cell mix was transferred gently into two of the small petri dishes using a pipette. One of the small petri dishes was filled with a volume of sterile distilled water (not more that 2/3 full). The dishes were incubated at 37°C (95% O_2 and 5% CO_2) for 14 days. The number of Granulocyte-Macrophage colonies and other lineages present were assessed using an inverted light microscope. If the growth was plentiful the number of colonies in only 20 squares was noted. This was achieved by counting the number of colonies in every eight square of the grid until 20 squares had been counted. This figure was multiplied by 11.25 to give the total number of cells in the whole plate. If the colony growth was poor then all the colonies in all the squares were counted. The colony numbers of the three test plates were averaged. And the number of colonies per 10^5 cells was calculated.

E.g. if cells were plated at 1.0 $x10^6$ per plate and resulted in 85 colonies per plate then:

Colonies per 10^5 cells = 85 ÷ 10

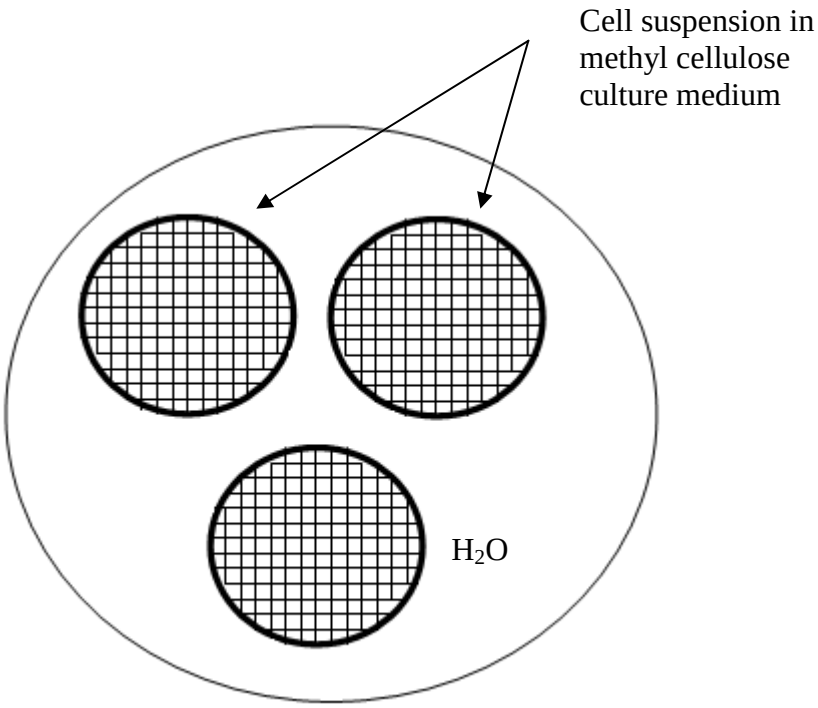


Figure 37. Colony forming unit assay. Three small gridded petri dishes were placed in a larger petri dish. One was filled with sterile water and the other two with 1ml of cell suspension in methyl cellulose culture medium. The dishes were incubated at 37◦C (95% O_2 and 5% CO_2) for 14 days.

2.4.3 Flowcytometric analysis

Cellular characterisation (extra- and intra-cellular) and antigen expression profiles of the test samples was investigated by flowcytometry. For the purpose of this project flow cytometric analysis was carried out using a Becton Dickinson FACS calibre flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Sample Preparation

Samples were prepared as follows:

Cord blood analysis: samples were prepared in 9 tubes. 100µl of blood (200µl in tube 6 as the population examined in this tube was very small) and 50µl of respective antibody mixture were added to each of the nine tubes. Tubes were then incubated at room temperature, in the dark, for 20 minutes. After incubation, the cells were lysed and washed using the BD FACS Lyse/Wash Assistant. The samples were then ready to be analysed on the flow cytometer.

Table 7. Antibody cocktails in each FACS tube used to characterize cord blood derived stem/progenitor cells

Tube Number	Tricolour (antibody cocktails)-Cluster of Determinants (CD)
1	45/14/7ADD
2	3/4/45
3	3/8/45
4	3/56/45
5	3/19/45
6	34/7ADD
7	25/4/45
8	Lin1/11c/ HLA-DR
9	Lin1/123/ HLA-DR

Each antibody is detailed in the table 8

Table 8. Details of antibodies used to characterize cord blood-derived stem/progenitor cells

Antibody (CD)	Immunoglobulin Subtype	Fluorochrome	Source
3	IgG1	FITC	555332; BD Pharmingen, Franklin Lakes, NJ, USA
4	IgG1	PE	347327; BD Pharmingen, Franklin Lakes, NJ, USA
8	IgG1	PE	S340055; Caltag/Invitrogen, Paisley, UK
11c	IgG1	PE	555392; BD Pharmingen, Franklin Lakes, NJ, USA
14	IgG1	PE	MHCD 1404-4; Caltag/Invitrogen, Paisley, UK
19	IgG1	PE	555413; BD Pharmingen, Franklin Lakes, NJ, USA
25	IgG2b	FITC	558689; BD Pharmingen Franklin Lakes, NJ, USA
34	IgG1	PE	345802; BD Pharmingen Franklin Lakes, NJ, USA
45	IgG1	FITC	555482; BD Pharmingen, Franklin Lakes, NJ, USA
56	IgG1	PE	345802; BD Pharmingen, Franklin Lakes, NJ, USA
123	IgG1	PE	340545; BD Pharmingen Franklin Lakes, NJ, USA
7AAD	IgG1		559925; BD Pharmingen Franklin Lakes, NJ, USA
HLA-DR	IgG2	PE-Cy5	555813; BD Pharmingen Franklin Lakes, NJ, USA
Lin 1	IgG1/2b	FITC	340546; BD Pharmingen Franklin, Lakes, NJ, USA

Mesenchymal stem cell analysis: samples were prepared as follows: 1-2 x10^6 cells in PBS were incubated with 5µl of each antibody (table 9) in the respective tubes. The tubes were incubated and the subsequently washed as for the cord blood samples before analysis on the LSRII.

Table 9. Antibody cocktails used to characterize umbilical cord derived Mesenchymal stem cells.

Tube Number	Multi-Colour (antibody cocktails)- Cluster of Determinants (CD)
1	29/34/44/45/73/105/166
2	44/45/90/106/133

Each antibody is detailed in the table 10.

Table 10. Details of antibodies used to characterize umbilical cord-derived Mesenchymal stem cells.

Antibody (CD)	Immunoglobulin Subtype	Fluorochrome	Source
14	IgG2a	Pacific-Blue	558121; BD Pharmingen Franklin, Lakes, NJ, USA
34	IgG1	PE-Cy7	348791; BD Pharmingen Franklin, Lakes, NJ, USA
29	IgG1	Alexa Fluor 700 (A700)	303020; BioLegend
44	IgG2b	Pacific Blue	103020; BioLegend
45	IgG1	APC-Cy7	557833; BD Pharmingen Franklin, Lakes, NJ, USA
CD 73	IgG1	PE	550257; BD Pharmingen Franklin, Lakes, NJ, USA
90	IgG1	FITC	555595; BD Pharmingen Franklin, Lakes, NJ, USA
133/1	IgG1	APC	130-090-826; Miltenyl Biotech GmbH
105	IgG1	Alexa Fluor 647 (A647)	323212; BioLegend
106	IgG1	PE/Cy5	305808; BioLegend
166	IgG1	FITC	ab33403; AbCam

2.5 Selection of a homogenous stem/progenitor cell population from mononuclear cell fraction

The antigen profile of haematopoietic stem/progenitor cells (HSPC) is utilized by most investigators to either positively or negatively select HSPC from heterogeneous blood samples. Positive selection relies on monoclonal antibodies (conjugated to various compounds) designed specifically against markers expressed on primitive HSPC. CD34 and CD133 antigens are amongst these antigenic markers, which are commonly aimed for the selection of HSPC away from developing and differentiated cells. Inversely, negative selection (also known as 'depletion') exploits monoclonal antibodies or chemicals with specific affinity for mature and differentiated cell markers, eliminating the differentiated haematopoietic lineages from a restricted group of lineage negative (LinNeg) HSPC population (Civin, Strauss et al. 1990; Miltenyi, Muller et al. 1990; Engelhardt, Lubbert et al. 2002).

2.5.1 Separation of non-haematopoietic lineage negative cell population (CBE) from mononuclear cells applying in-house LinNeg protocol

A negative immuno-magnetic selection strategy was employed for the depletion of desired MNC from differentiating and mature haematopoietic cells. This technique allows a primitive and more homogenous source of haemopoietic and possibly non-haemopoietic stem/progenitor cells to be obtained. This immuno-magnetic technique separates cells according to the presence or absence of cell surface molecules recognized by a monoclonal antibody coupled to magnetic beads either primarily or via a secondary antibody (Figure 38).

MNC obtained after Ficoll treatment of whole cord blood samples were incubated for 20 minutes at 4°C with human gammaglobulins (2% in PBS). This allows blocking of non-specific Fc receptors. Cells were then incubated for 30 minutes at 4°C with the following mouse monoclonal anti-human antibodies (all IgG isotypes): (i) anti-CD45 (MEM 28, Autogen Bioclear), (ii) anti-glycophorin-A (JC159, Dako, UK), (iii) anti-CD33 (WM53, Autogen Bioclear, UK) and (iv) anti-CD7 (WM31, Autogen Bioclear). Centrifugational washes in dextrose-albumin were then applied (400g, 10 minutes, 4°C).

Cells were subsequently secondarily labelled with Dynabeads human IgG4 monoclonal anti-pan mouse IgG (Dynal, Invitrogen,) for 30 minutes. Cell fraction was then applied to a Dynal Magnetic Particle Concentrator. Application to the magnetic field causes retention of the positive cell fraction (magnetic bead labelled fraction) whilst eluting the negative fraction. The negative cell fraction, termed 'Lineage Negative' cells (LinNeg) were collected

with a sterile Pasteur pipette. After centrifugation (400g, 10 minutes, 4°C), LinNeg cells were counted with a haemocytometer and assessed for viability. The LinNeg protocol although valuable for selection of a very small non-haematopoietic progenitor cell population, proved to be very expensive, time consuming and labour intensive. Thus, commercial kits from StemSep were resorted to for the enrichment of progenitor cell population from cord blood units post Ficoll treatment.

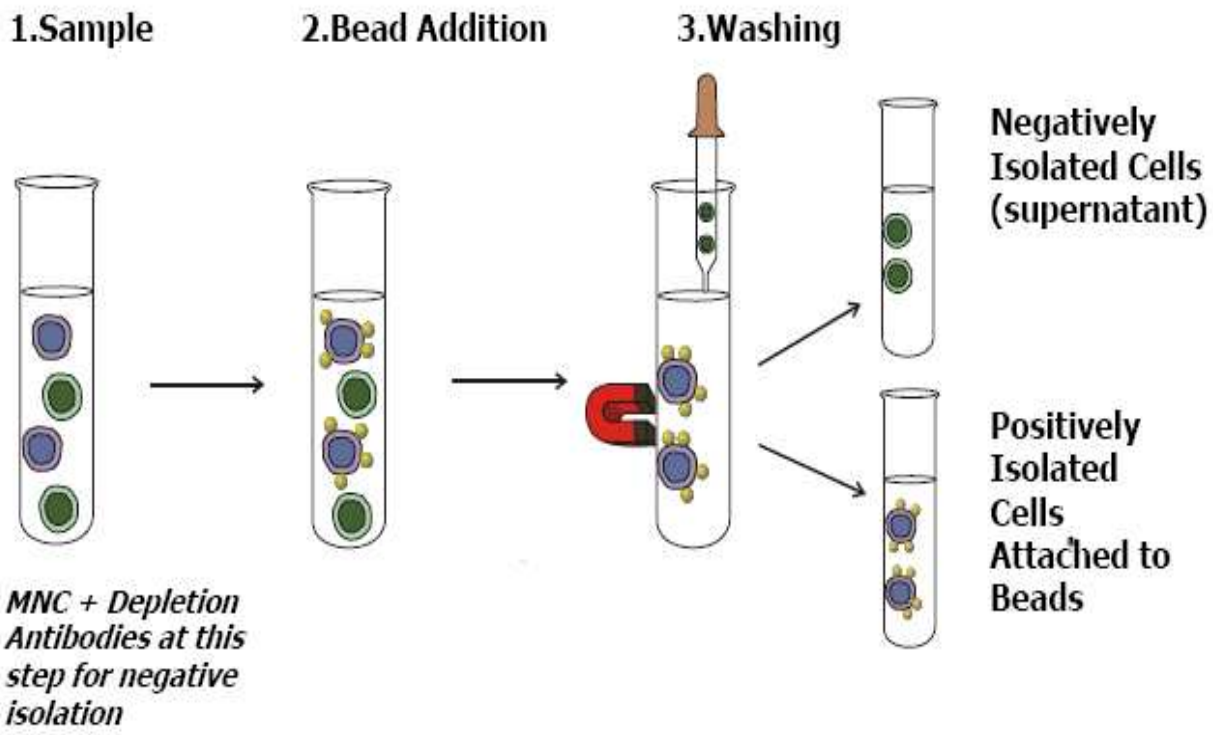


Figure 38. Negative immuno-magnetic selection strategy. The figure illustrates a negative immuno-magnetic selection strategy employed for the isolation of a primitive and more homogenous source of stem/progenitor cells from differentiated and mature haematopoietic cells. Cells are separated according to the presence or absence of cell surface molecules recognized by monoclonal antibodies coupled to magnetic beads. The monoclonal antibodies utilized in this study include: anti-glycophorin-A, anti-CD45, anti- CD33 and anti-CD7. Cells are subsequently secondarily labelled with Dynabeads human IgG4 monoclonal anti-pan mouse IgG and cell fraction applied to the magnetic field causing retention of the positive cell fraction whilst eluting the negative fraction. The negative fraction ' Lineage Negative' constitutes the desires cell fraction.
http://www.invitrogen.com/downloads/IVGN_SC_Capabilities_Jan_2006.pdf

2.5.2 Haematopoietic stem/progenitor cell extraction using commercial kits- Human Primitive Haematopoietic Progenitor Cell enrichment Kit (StemSep)

Mononuclear cells (MNC) obtained post Ficoll processing were pelleted and re-suspended in separation medium (PBS+2% FBS) (07905; StemCell Technologies) within the acceptable range 2-8 x 10^7 cells/ml, as recommended by the kit (StemSep; 14057 (14067), StemCell Technologies). StemSep Enrichment Coctail containing mouse derived monoclonal bispecific tetrameric antibody complexes (IgG1) against cell surface antigens on human haematopoietic cells and dextran (CD2, CD3, CD14, CD16, CD19, CD24, CD36, CD38, CD45RA, CD56, CD66b, glycophorin A) (14057C; StemCell Technologies) was added to the cell suspension at concentration of 100µl/ml of cells. The cells were mixed well by pipetting and incubated at 4°C for 30 minutes (Figure 40). Magnetic colloid (colloidal suspension of magnetic dextran iron particles in saline pH 7.0-7.5) (10051; StemCell Technologies) was added to the cell-antibody suspension at a concentration of 60µl/ml of cells, mixed well and incubated at 4°C for 30 minutes. During the incubation period the cooled MACS separation columns were prepared. These columns have been developed for the gentle depletion of magnetic microbead labelled cells. They come in different sizes depending on the number of magnetically labelled cells (LD columns, capacity 1-5 x10^8 cells, 130-0742-901; LS columns, capacity 10^8 cells, 130-042-401; MS columns, capacity 10^7 cells, 130-042-201). The columns contain an optimized matrix and when placed in a permanent magnet allow a high-gradient magnetic field to be generated. This permits the microbeads, which are extremely small, superparamagnetic particles to be retained from the cell suspension. The columns were prepared by washing them from top down with 2ml of priming medium (PBS). This allowed the hydrophilic coating of the columns to be rinsed out and thus avoided rapid filling of the columns with cell suspension. The magnetically labeled cell suspension was mixed properly by pipettimg several times and applied to the column. The cell suspension runs through the column matrix at a rate of approximately 0.2-0.25ml/min and the effluent was collected. The column was then washed with PBS twice with 2ml of separation medium (PBS+2% FBS) (07905; StemCell Technologies) and the total effluent was collected as the depletion fraction. The depletion fraction was then characterized by flow cytometric analysis using antibody cocktails mentioned in section 2.4.3, to observe whether the kit allowed enrichment of the desired stem/progenitor cell population.

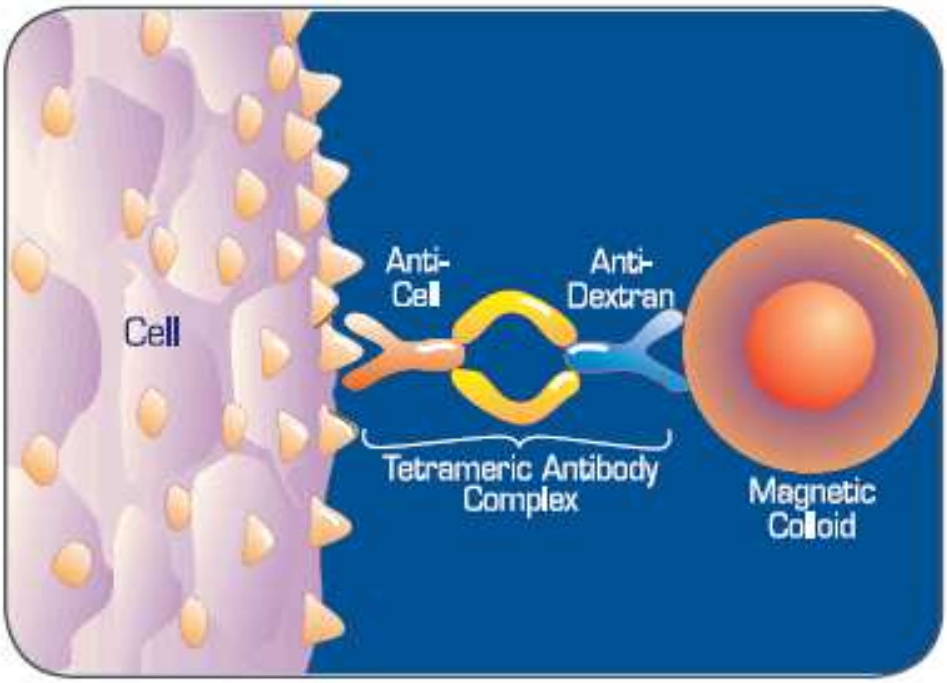


Figure 39. Schematic diagram of StemSep demonstrating tetrameric antibody complexes (TAC) magnetic labeling of human cells. StemSep® Human Hematopoietic Progenitor Cell Enrichment Kit

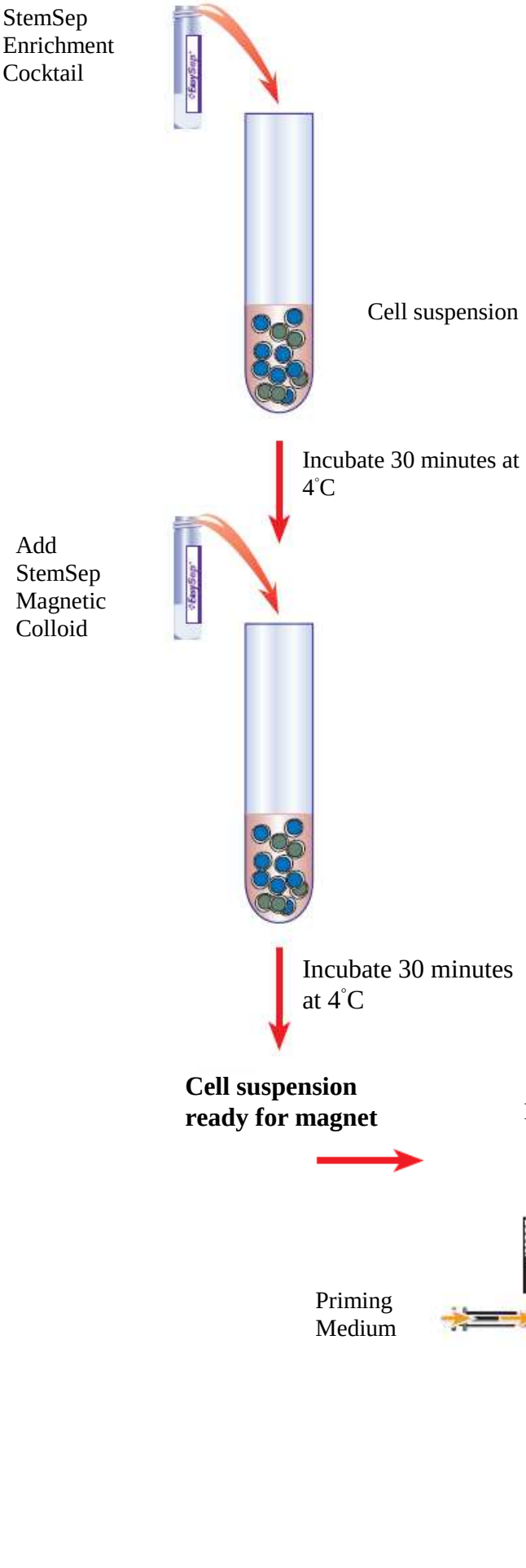


Figure 40. StemSep human progenitor cell enrichment kit. The schematic diagram illustrates the steps involved in extraction and isolation of haematopoietic stem/progenitor cells using the commercial StemSep kit. http://www.stemcell.com/en/Products/All-Products/StemSep-Human-Primitive-Hematopoietic-Progenitor-Cell-Enrichment-Kit.aspx

2.6 Expansion and commitment of isolated stem/progenitor cell populations

One of the biggest clinical disadvantages of stem/progenitor cells derived from cord blood is that there are not adequate cells in a single cord blood unit to meet the needs of an adult patient. Consequently, several groups have made efforts at attempting to expand cord blood stem cells. A part of this study was also dedicated to this task.

MSCs from umbilical cord, however, are blessed with a high proliferation potential and thus can be expanded efficiently *ex vivo*.

2.6.1 Expansion of Mesenchymal stem/progenitor cells from umbilical cord

Post isolation of MSCs from umbilical cord these cells were cultured in Mesenchymal stem cell growth medium (MSCGM) 10% fetal bovine serum (FBS) (Invitrogen, 16000-036). This medium was formulated by adding MSCGM SingleQuots (Lonza, Cat. no. PT-4105, Walkersville, MD, USA) to Mesenchymal stem cell basal medium (MSCBM) (Lonza, Cat. no. PT-3238, Walkersville, MD, USA) to make MSCGM. The media was supplemented with Penicillin-streptomycin (Invitrogen, Cat. no. 15140-122) and Fungizone (Invitrogen, Cat. no. 15290-026) both at a concentration of 1:100 to evade bacterial and fungal contamination. The first medium change was carried out 4 days after initial plating of cells to allow the cells adequate time to adhere to the plate forming a monolayer. The cells were expanded in MSCGM for two weeks and then transferred to DMEM-F12 (Gibco, Cat. no. 11039-021) 10% FBS for further propagation and passaging.

2.6.2 Expansion and commitment of Haematopoietic and non-haematopoietic stem/progenitor cells

The yield of selected stem/progenitor cell population isolated from cord blood MNCs is usually very low and depends of the volume and quality of the cord blood unit. In addition the tolerance of these cells to the artificial *in vitro* culture conditions and hence the resulting cell viability is low. A part of this project was focused to trying to devise a system whereby these cells could be simultaneously expanded and committed along the desired endodermal cell lineage. The cytokines used in this effect are listed in the table 11.

Table 11. Expansion and endoderm commitment medium for cord blood stem/progenitor cells

Reagents	Morphogen Supplier and Code	Final volume
DMEM-F12 (1:1)	Invitrogen, Gibco 11039-021	22832.5µl
5% FBS (Stock 100%)	Invitrogen 16000-036	1250µl
ITS liquid media supplement (100x)	Sigma 13146	250µl
Heparin Sodium Salt (2.5µg/ml)	Sigma-Aldrich H4784	125µl (500µg/ml stock)
Collagen IV- from human placenta, acid soluble (1µl/ml)	Sigma-Aldrich C5533	25µl
Penicillin-streptomycin (1:100)	Invitrogen 15140-122	250µl
Fungizone (1:100)	Invitrogen 15290-026	250µl
Recombinant Stem Cell Factor (rh SCF) 20ng/ml	ImmunoTools 11343407	5µl (100µg/ml stock)
Recombinant Hepatocyte Growth Factor (rh HGF) 20ng/ml	ImmunoTools 11343415	5µl (100µg/ml stock)
Recombinant human Fibroblast Growth Factor- acidic (rh FGF-acidic) 20ng/ml	ImmunoTools 11343555	5µl (100µg/ml stock)
Recombinant thrombopoietin (rh TPO) 10ng/ml	ImmunoTools 11133345	2.5µl (100µg/ml stock)
Final Volume		25000µl

2.7 Passaging of cells

When cells reach confluency, they must be passaged or subcultured. Failure to subculture confluent cells results in a reduced mitotic index and eventually ends in cell death. Passaging of cells starts with detachment of attached cell monolayers from the surface of primary culture by either: enzymatic detachment (Trypsinization or Accutase) or by mechanical means (cell scraping) (Greiner bio-one GmbH, Cat. no. 541 070, Germany).

Trypsinization was performed using Trypsin-EDTA (0.25% Trypsin, 0.02% EDTA; 59428C Sigma-Aldrich) for 5-7minutes at 37 °C in the incubator. Once the cells had detached FBS (26140 Gibco, lot.366182; Grand Island, N.Y. U.S.A) was added to inactivate the enzyme reaction and then the Trypsin-EDTA solution with the cell suspension was transferred into a centrifuge tube. The flask was rinsed out with a pipette-full (3-5ml) of PBS (x1) and that was transferred into the same centrifuge tube (Jouan CR422). This minimized cell loss. The cell suspension was then centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded leaving behind 200-500µl supernatant to re-suspend the cells in.

Alternatively, Accutase (in Dulbecco's PBS containing 0.5mM EDTA; L11-007, PAA Laboratories GmbH; Haidmannweg) was used. Accutase is a mixture of proteolytic and collagenolytic enzymes from invertebrate species. Its non-mammalian source makes Accutase guaranteed free of BSE, Parvovirus and other viruses that may be found in Trypsin. It is a ready to use solution for detachment of cells. Accutase was used in the same manner as Trypsin EDTA solutions were used to detach adherent cells but did not require foetal bovine albumin for enzyme inactivation. Accutase allowed a more gentle enzymatic detachment meaning that the cells could be incubated with Accutase for up to 45 minutes.

Mechanical detachment was performed using cell scrappers (Greiner bio-one GmbH. Cat. no. 541 070, Germany). This can be applied as the sole method of cell passaging or in combination with enzymatic detachment.

2.8 Growth curve

To evaluate the growth characteristics of the isolated cell populations growth curves were carried out. The rate of growth of each cell population was calculated by counting the total number of cells in duplicate wells every day for 4 weeks. The results were plotted on a log-linear scale.

2.9 Cytogenetic analysis of expanded cells- analysis of genetic stability

2.9.1 Cell preparation

The protocol for cytogenetic analysis of cells was provided by Dr Simon Zwolinski, Cytogenetics department, Institute of Human Genetics, Centre for Life.

Cells for cytogenetic analysis were cultured as monolayers in flasks. 200µl of 1/5 Colcemid (Demecolcine) was introduced into the culture medium. Colcemid is related to colchicine but is less toxic. It depolymerises microtubules thus limiting microtubule formation (inactivates spindle fibre formation). This leads to cell cycle arrest in metaphase.

After the 3 hours incubation period, the supernatant in the flasks (medium containing colcemid) was transferred into a labelled centrifuge tube. The cells are then trypisinized Trypsin-EDTA (0.25% Trypsin, 0.02% EDTA; 59428C Sigma-Aldrich). The cell suspension was then centrifuged at 500 x g for 5 minutes. The supernatant was discarded leaving behind 200-500µl supernatant to re-suspend the cells in. 100µl of 0.075M KCL (5.59g of KCL in 1L of dd water) was then added to the cell suspension. The cells were given a gentle mix with a pipette and the tube was topped up with 0.075M KCL. The cell suspension was then placed in a 37°C water bath for 5 minutes and then centrifuged at 500 x g for 5 minutes. The KCL was carefully decanted without disrupting the pellet. A few drops of fixative (3:1 Methanol: Acetic acid) was then added to resuspend the cells. The centrifuge tube was topped up with fixative and then centrifuged at 500 x g for 5minutes. The fixative was pipetted off gently. A few drops of fresh fixative were added to the cells and the cells were then stored at -20°C until slide preparation was undertaken.

2.9.2 Slide preparation

The slide preparation was courtesy of Dr Simon Zwolinski, Cytogenetics department, Institute of Human Genetics, Centre for Life. Once the slides were prepared they were stained using a G banding protocol. The staining was kindly performed by Simon Zwolinski.

2.9.3 Slide Analysis

The microscope used to analyse the slides was Nikon Eclipse E400. The capture software was CytoVision version 3.93 (supplied by Applied Imaging 2007). Slide analysis was done courtesy of Dr Simon Zwolinski.

2.10 Cryopreservation of cells

2.10.1 Cryopreservation of umbilical cord blood

Umbilical cord blood unprocessed and post processing units, were cooled to 4°C before being transferred to a cryostore bag (CS250n, Quest Biomedical, Solihull, UK). Dimethyl sulphoxide (DMSO) (Origen 210002, Origen, Austin, TX, USA) mixed with Dextran 40 (Baxter Healthcare B5043) was used as the cryopreservant for the cord blood samples. The cryopreservant was also cooled to 4°C, and then was introduced to the cells at a concentration of 10%. The samples were cryopreserved in a control rate freezer (Planar Kryo 560-16, Sunbury-on-Thames, UK) using the following protocol: **Start temperature = 4°C, step I = hold at 4°C for 10 minutes, step II = -2°C/min to -5°C, step III = -1°C/min to -40°C and step IV= -5°C/min to -100°C**. Samples were then transferred to the gaseous phase of a liquid nitrogen Dewar vessel. Cord blood units were cryopreserved for a minimum of 14 days before thawing and further analysis was carried out.

2.10.2 Cryopreservation of Cord Mesenchymal stem cells (MSCs)

Dimethyl sulphoxide (DMSO) (Origen 210002, Origen, Austin, TX, USA) mixed with foetal bovine serum (26140 Gibco, lot.366182; Grand Island, N.Y. U.S.A) were used as the cryopreservant for MSCs. The cryopreservant was cooled to 4°C and then introduced to the cells at a concentration of 10% in 1.8ml round bottom Nunc Cryo Tubes (363401; Nunc, ThermoFisher Scientific; Nunc GmbH & Co. KG; Langenselbold, Germany). The cells were frozen at a concentration of 1 x 10^6 cells/ml of cryopreservant.

2.11 Thawing protocol

2.11.1 Thawing of umbilical cord blood

The thaw protocol applied in this project was derived from Rubinstein's method (Rubinstein, Dobrila et al. 1995). The cord blood samples, both unprocessed and processed units, in cryostore bags (CS250n, Quest Biomedical, Solihull, UK) were removed from liquid nitrogen Dewar vessels and transferred to a water bath at 37°C. The blood units were then gently manipulated in the water to allow the sample to defrost slowly. Once completely thawed, the cryostore bag was connected to two separate transfer bags (Baxter PL1813) using aseptic technique. The first bag (Bag 1) was empty and the second bag (bag 2) contained 100ml Dextran 40 (Baxter Healthcare B5043)/ Albumin (Bio Products Laboratory PL08801/006) solution, in a 1:1 ratio. Half of the Dextran/Albumin solution in transfer bag 2 was then transferred to the thawed unit in the cryostore bag and mixed thoroughly. This mixed solution was then transferred to the first transfer bag (bag 1). The remainder of the Dextran/Albumin solution was then transferred to the cryostore bag to rinse out any residual thawed blood sample and this was then also transferred into transfer bag 1. Transfer bag 1 was then heat sealed and detached from the cryostore bag and transfer bag 2, which were disposed of. The sample in transfer bag 1 was then centrifuged (Jouan CR422) at 500 x g for 10 minutes. This transfer bag was then connected to a third transfer bag (bag 3) which was used for drainage of the supernatant post centrifugation with the help of a plasma expressor (Fenwal BM-1). The pellet in bag 1 was the resuspended in Dextran/Abumin to its original post processing volume.

2.11.2 Thawing of Mesenchymal stem cells

Mesenchymal stem cells in Nunc Cryo Tubes (363401; Nunc, ThermoFisher Scientific; Nunc GmbH & Co. KG; Langenselbold, Germany) were thawed in a 37°C water bath similar to the umbilical cord blood samples. Once completely thawed the samples were transferred to a centrifuge tube containing 10-15mls of PBS and centrifuged at 500 x g for 5 minutes. The supernatant was then decanted and the cell pellet was re-suspended in 1ml of culture media (DMEM-F12) or PBS.

2.12 Liver specific differentiation culture system *in vitro*

2.12.1 Culture conditions

In vitro differentiation was performed in 2-D and 3-D culture systems. 2-D differentiation experiments were performed in plastic culture flasks (T25cm^2 and T75cm^2, canted neck, ventilated culture flasks; Iwaki, Scitech DIV, Asahi Techno glass, Japan), 6-well, 24-well and 96-well plates (Techno Plastic Products, Switzerland). Cells were also seeded on Slide Flasks (Nunc, Rochester, NY, USA) and extra-cellular matrix (ECM) coated 30mm cover slips (Thermanox ® plastic coverslips, Nunc, Rochester, NY, USA) positioned in 24-well plates for ease of subsequent immunocytochemistry analysis.

The ECM proteins utilized for coating:

1. Fibronectin (Human) (Invitrogen, Cat. No. 33016015)
2. Gelatin (Tissue culture grade, BioReagent, Type B, sterile) (G1393, Sigma-Aldrich, Aryshire, UK)

After 1 week of *ex vivo* expansion UCB-derived stem/progenitor cells were stimulated with a range of differentaitive hepatic and biliary cues for 1week. The morphogens and growth factors exploited to drive stem/progenitor cells towards hepatic differentiation were added to medium. Cells were differentiated both in EGF supplemented and EGF depleted media.

Table 12. Differentiation medium

Reagents	Morphogen Supplier and Code	Working Concentration
DMEM-F12 (1:1)	Invitrogen, Gibco 11039-021	47167.5µl
B27 supplement (x50) (1:50)	Gibco-Invitrogen 17504-044	1000µl
N-2 Supplement (x100) (1:100)	Gibco-Invitrogen 17502-048	500µl
Collagen IV- from human placenta, acid soluble (1µl/ml)	Sigma-Aldrich C5533	50µl
Heparin Sodium Salt (2.5µg/ml)	Sigma-Aldrich H4784	250µl (500µg/ml stock)
Penicillin/streptomycin (1:100)	Invitrogen 15140-122	500µl
Fungisone (1:100)	Invitrogen 15290-026	500µl
Recombinant Hepatocyte Growth Factor (rh HGF) (20ng/ml)	ImmunoTools 11343415	10µl (100µg/ml stock)
Recombinant human Fibroblast Growth Factor-acidic (rh FGF-acidic) 20ng/ml ♣	ImmunoTools 11343555	10µl (100µg/ml stock)
Recombinant Epidermal Growth Factor (rh EGF) 20ng/ml	ImmunoTools 11343407	10µl (100µg/ml stock)
Recombinant Stem Cell Factor (rh SCF) 5ng/ml	Immuno Tools 11343325	2.5µl (100µg/ml stock)
Final volume		50000µl

♣ In EGF depleted culture system EGF cytokine was omitted from the culture medium. The cells were then transferred to maturation medium for a further 1week. Cells were checked and photographed at different stages of differentiation and maturation.

Table 13. Maturation medium

Reagents	Morphogen supplier and Code	Final volume
DMEM-F12 (1:1)	Invitrogen, Gibco 11039-021	46067.5µl
B27 supplement (x50) (1:50)	Gibco-Invitrogen 17504-044	1000µl
N-2 Supplement (x100) (1:100)	Gibco-Invitrogen 17502-048	500µl
Collagen IV- from human placenta, acid soluble (1µl/ml)	Sigma-Aldrich C5533	50µl
Heparin Sodium Salt (2.5µg/ml)	Sigma-Aldrich H4784	250µl (500µg/ml stock)
Penicillin/streptomycin (1:100)	Invitrogen 15140-122	500µl
Fungisone (1:100)	Invitrogen 15290-026	500µl
Dexamethasone (10-6M) 1µmol/L	Sigma-Aldrich D4902	1000µl
Recombinant, human Oncostatin M (OSM) 20ng/ml	Sigma-Aldrich O 9635	100µl (10µg/ml stock)
Recombinant Hepatocyte Growth Factor (rh HGF) 20ng/ml	Immuno Tools 11343415	10µl (100µg/ml stock)
Recombinant human Fibroblast Growth Factor-acidic (rh FGF-acidic) 20ng/ml	ImmunoTools 11343555	10µl (100µg/ml stock)
Recombinant Epidermal Growth Factor (rh EGF) 20ng/ml	Immuno Tools 11343407	10µl (100µg/ml stock)
Recombinant Stem Cell Factor (rh SCF) 5ng/ml	Immuno Tools 11343325	2.5µl (100µg/ml stock)
Total volume		50000µl (50ml)

2.12.2 Seeding of cells onto plates

Sterile tissue culture flasks and plates were seeded with the following cell concentrations:

Table 14. Seeding density of cells onto tissue culture flasks and plates

Plate Format	**Surface Area (cm^2)**	**Volume/well (µl)**	**Cell Density (cells/ml)**	
T75cm^2 ($75cm^2$/canted Neck, slim-type; 3123-075, Iwaki, Scitech DIV, Asahi Techno glass, Japan)	$75cm^2$	10000µl	**MNC** 10x10^6/ml	**MSC** 0.1x10^6/ml
T25 cm^2 ($25cm^2$/canted Neck, slim-type; 3103-025, Iwaki, Scitech DIV, Asahi Techno glass, Japan	$25cm^2$	5000µl	**MNC** 5x10^6/ml	**MSC** 0.02x10^6/ml
6-well plate (Zellkultur Test Platte 6; 92006, Techno Plastic Products, Switzerland)	8.962 cm^2	2000µl	**MNC** 0.5x10^6/ml	**MSC** 1500-5000/ml
24-well plate (Zellkultur Test Platte 6; 92024, Techno Plastic Products, Switzerland)	$1.862cm^2$	500-1000µl	**MNC** 0.5x10^6/ml	**MSC** 1000/ml
96-well plate (round bottom, Zellkultur Test Platte 96; 92096, Techno Plastic Products , Switzerland)	$0.36cm^2$	100-200µl	**MNC** 50000-80000/ml ♣ used predominantly for culturing of LinNeg or StemSep selected cells were cell yield is very low	
Slide Flask Nunc, Rochester, NY, USA	9 cm^2	2500-5000 µl	**MNC** 0.5x10^6/ml	**MSC** 1500-5000/ml

2.12.3 Three-Dimensional (3D) culture system

2.12.3.1 Scaffolds

Scaffolds are three-dimensional structures that play a pivotal role in *ex vivo* tissue engineering. Synthetic scaffolds are typically engineered to demonstrate a surface chemistry and microstructure adapted to facilitate cellular attachment, proliferation and differentiation. They also allow simultaneous inter-cellular communication and excretion of extracellular matrix components needed for the tissue formation. Biodegradable scaffolds are favored for tissue regeneration as they disintegrate without undesirable by-products and leave behind the cells they were meant to deliver.

2.12.3.2 Types of scaffolds

Scaffolds used in this study were:

- **OPLA (Open-Cell Polylactic Acid)** (3D OPLA Scaffold, 354614, Becton and Dickinson Biosciences, France) - synthesized from D, D-L, L Polylactic acid. These scaffolds are white/neutral in color and sponge/non-compressible in texture. They measure approximately 5mm x 3mm x 0.039cm^3 with a hydration capacity of 30µl and an average pore size of 100-200µm and weight 5.2-34mg. These scaffolds demonstrate facetted architecture, which is effective for culturing high density cell suspensions.

- **Macroporous hydrogels** (Protista): these cryogels were white/neutral in color and sponge/compressible in texture. They measured 12.5mm x 7.1mm with a pore size of approximately 10-120 microns. These scaffolds were sliced with a cryostat and then sterilized before use. The hydrogels applied in this study were:

- **Fibrinogen-gelatin**- composed of Fibronectin from human plasma, gelatin and glutaraldehyde

- **Laminin-Dextran**- composed of Laminin, dextran-LLA-HEMA (L-lactide-hydroxyethyl methacrylate)

These cryogels were provided non sterile and were sterilized by incubating them in 70% ethanol for at least 24 hours and then rinsed with (x1) PBS to remove ethanol prior to their application in tissue culture (as recommended by the supplier).

2.12.3.3 Seeding of cells on scaffolds

Scaffolds were gently placed into a small volume of culture medium in a culture dish. Scaffolds equilibrated with the medium were then transferred into the wells of a 24-well plate (1 scaffold per well) and gently dried with sterile cotton pads. Cells suspended in

culture medium were then directly injected with a 100µl pipette into the centre of the top of each scaffold at a density of 10^5per ml. Partially dehydrated scaffolds re-swell upon addition of the cell suspension. Scaffolds equilibrated with the cell suspension were left in dry wells for 1 hour in the incubator at 37°C and 5% CO_2. . This ensured cell adherence to the porous walls of the scaffolds. After 1 hour incubation the wells with scaffolds were filled with growth medium (2ml per well) and the plates were returned to the incubator.

2.12.3.4 Rotatory cell culture system (RCCS) or Bioreactor

The original concept of the rotatory cell culture system (RCCS) was conceived as a means of studying the effects of microgravity at the cellular level. The goal was to gain insight into the molecular mechanisms of the effects of microgravity on the astronauts, and to formulate counter measures. This technology was developed at NASA's Johnson Space Centre in 1996 (Navran 2004). The original purpose of the RCCS was to maintain cell cultures on the space shuttle before and during lift off. But ever since the usefulness of this technology became apparent in ground-based experiments, the RCCS has been exploited as a microgravity simulator in cell culture. Thus, allowing cells to be cultured in a 3 dimensional environment with minimal shear stress on the cells. The RCCS was designed with several features intended to minimize fluid shear (Hammond and Hammond 2001).

- **Biomechanics**

In practice, the rotational speed of the bioreactor vessel is set so that the sedimentation rate of a cell in the cell culture medium is minimized (Figure 41). The medium properties (viscosity and density) are not varied.

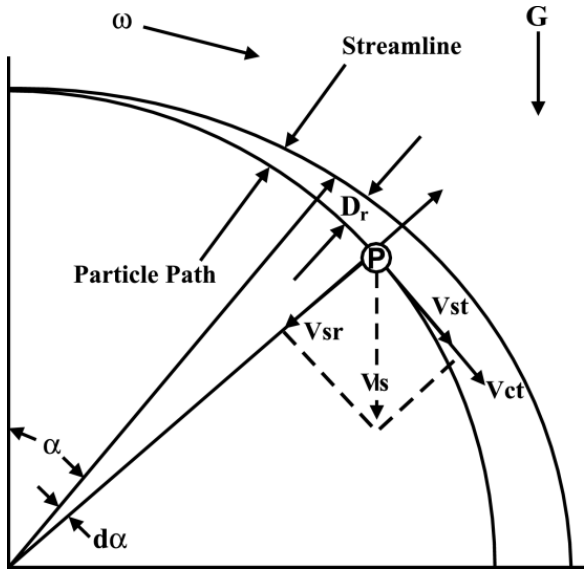


Figure 41. Vector velocity diagram. The diagram illustrates the forces acting on a particle (P) rotating in a fluid. Gravity-induced sedimentation Vs is resolved into radial Vsr and tangential Vst components. Centrifugal force results in an outwardly directed particle velocity, Vcr. The Coriolis motion Vct is tangential to the particle (Navran 2004).

Mass transport of nutrients and gaseous exchange is accomplished by a combination of bulk flow and diffusion, excluding the harmful influences of fluid shear stress on the cells.

- **Limitations of the RCCS**

The drawback of the RCCS is that as particle (cell) size increases, the rotational speed must also increase to keep the particles in suspension. Centrifugal force is then increased, causing particle collision on the vessel wall. The augmented rotation also causes an increase in shear stress. These two factors generate cell damage and limit the size of cell aggregates to less than 1cm in diameter (Navran 2004).

2.13 Phenotypic analysis of differentiated cells

2.13.1 Light microscope – Differential interference contrast (DIC) imaging

All DIC images were taken with Zeiss Axiovert 200M fluorescence/live cell imaging microscope (Carl Zeiss AG International, Germany). Software Axiovision version 4.5. The following objectives were used:

Plan Neofluar 5x/ 0,15

Plan Apochromat 10x /0,32 Ph1

LD Achroplan 20x/ 0,40 Korr Ph2

LD Achroplan 40x/ 0,60 Korr Ph2

LD Achroplan 63x/ 0,75 Korr Ph2

2.13.2 Immunocytochemistry (ICC)/Immunohistochemistry (IHC)

Immunocytochemistry and Immunohistochemistry were used for *in situ* visualization of cellular proteins. During the course of this project cellular proteins were labeled with fluorescent markers and visualized using confocal laser scanning microscopy.

Immunocytochemistry protocol

To allow precise appreciation of cellular morphology by microscopy, cells were grown on gelatin coated glass cover slips in 24 well plates. This manoeuvre permitted passaging and the subsequent need to resort to cytospin slides to be excluded, both of which may be associated with loss of original cellular morphology. Use of cover slips also had the added advantage of minimizing the total volume of antibody usage.

Adherent cells were washed with PBS once and not for more than 5 minutes. Cells were then fixed with a formalin free fixative, Accustain (A5472; Sigma-Aldrich Biotechnology LP and Sigma-Aldrich) for 20 minutes at room temperature for fixation of intra- and extracellular markers. The cells were then washed three times with PBS, 10 minutes each time. Cells could then be stored in PBS at 4°C for up to 72 hours before proceeding with the protocol. The cells were then permeabilized with 1% Triton X-100 (Sigma, Cat. no. T8787) (5ml of 100% Triton X in 495ml of (x1) PBS) for 15 minutes. This step was only needed for internal markers and was not necessary for the detection of surface markers. The cells were then blocked with blocking buffer (5% BSA (Albumin Solution from Bovine Serum, cell culture tested, 30% in DPBS, A9576, Sigma), 5% Goat serum (10% Non-Immune Goat

Serum, 50-0627, Zymed Laboratories, Invitrogen, Carlsbad, CA 92008, USA) in 0.1% Triton-X) for 1 hour. For surface markers the same blocking solution was used but without the addition of Triton X. The cells were then incubated with the primary antibody overnight at 4°C. The primary antibody working concentrations are listed in table (Table 15). Dual staining was performed by pre-mixing and adding the relevant primary antibodies together. Control immuno-labeling was carried out in all cases by excluding primary antibodies.

After the overnight incubation with primary antibody the cells were washed three times with PBS (x1), 10 minutes each time. The cells were then incubated with the appropriate secondary antibody for 60 minutes at room temperature. Post incubation the cells were washed three times with PBS (x1), 10 minutes each time. Nuclear DNA was counterstained by incubating cells with 500nM 4', 6-diamidino-2-phenylindole dilactate (DAPI) (D9564; Sigma-Aldrich) in PBS (x1) for 5 minutes. Cells were subsequently washed once in PBS(x1). Post-staining, cells cultured on cover slips were mounted on glass slides using anti-fade reagent (Gel Mount Aqueous Medium, G0918, Sigma-Aldrich) and they were analyzed using Confocal laser scanning microscopy.

Immunohistochemistry protocol

Paraffin embedded foetal and adult liver tissue sections were used as positive controls for immunocytochemistry data generated from cord and cord blood differentiated progenitor cells.

Paraffin embedded foetal liver tissue sections were provided courtesy of Steve Lisgo, Human Development Biology Resource (HDBR) department, a MRC-Wellcome Trust funded resource, run at two sites: the Institute of Human Genetics, Newcastle University and Institute of Child Health, University College London (http://www.hdbr.org/).

Paraffin embedded adult liver tissue sections were provided courtesy of Dr Mathew Wright, School of Biomedical Sciences, Newcastle University.

Tissue sections were de-waxed by immersing them in Histo-clear (Histological clearing agent) (HS-200; National Diagnostics) twice, 10 minutes each time. The sectioned were then gradually rehydrated by immersing them in ethanol (VWR International, EC no. 200-578-6, Leicester, UK) in descending order: 100%, 70% and 50%, 2 minutes each, followed by equilibration in Tris Buffered Saline (TBS) (100ml 1M Tris-HCL pH 7.5 (125g Trisma base, 600ml double deionised (dd) water, ph to 7.5 with concentrated HCL, made up to 1 litre), 30ml 5M NaCl, 870ml dd water) for 5 minutes. After tissue rehydration antigen retrieval was carried out to enhance staining intensity of antibodies. Pre-treatment with antigen retrieval reagents, in this case citrate buffer allowed breakage of the protein cross-links formed by formalin fixation and thereby uncovered hidden antigenic sites. The tissue sections on slides were positioned in a slide rack in a large beaker and covered with citrate buffer pH 6.0 (3.84g citric acid-Na3 salt, 2L dd water, pH to 6.0 with HCL). The beaker was covered with cling film which was pierced for venting. The slides were then heated in the microwave for 5 minutes and then allowed to cool by placing them under gently running tap water for 3 minutes. The tissue sections were then permeabilized in TBS-TX (1L TBS and 5ml Triton X-100) for 15 minutes and subsequently placed in blocking solution (10% FCS in TBS-TX) in a humid chamber for 1 hour at room temperature. The sections were then covered with primary antibody solutions (approximately 200µl per slide) and covered with parafilm. This allowed the antibody solution to cover the entire surface area of the slide and minimized evaporation. The sections were incubated with primary antibodies overnight at 4°C. Consequently the slides were washed in TBS-TX, three times, 10 minutes each time and then the secondary antibody was introduced. The secondary antibody was added in similar manner as the primary antibody but incubated in a humid chamber for 1 hour at room temperature. The slides were then washed in TBS-TX, three times, 10 minutes each time and then visualised with confocal laser scanning microscopy.

Tissue imaging

The cells and tissue sections were visualized with a confocal laser scanning microscope (CLSM) (Leica TCS SP2 UV, Leica Microsystems, Heidelberg, GMBH, Germany). The software used was LCS 2.61. The lens used was the HCX Plan Apo NA 1.32.

Imaging and software operation was performed by Dr Trevor Booth (Bio-Imaging Manager, Institute of Cellular Medicine, Newcastle University).

Table 15. List of antibodies utilized for immunocytochemistry/immunohistochemistry

Antibody	Immunoglobulin Subtype	Supplier and Code	Specificity of 1° Antibody	Source of 1° antibody	Working conc. of 1° Antibody	2° Antibody/ Supplier/ Code/ Working conc.
Anti-Human albumin	IgG	Sigma-Aldrich A 0433	Reacts with human albumin	Rabbit IgG fraction of antiserum	1:16	Alexa 488/Goat anti-rabbit IgG A11008 FITC/1:500
Anti-Human albumin	IgG	Sigma-Aldrich A 7544	Reacts with human albumin	Goat delipidized whole antiserum	1:16	Alexa 594/ Rabbit anti-goat IgG A11012 TxRed/1:500
Monoclonal anti-human α-fetoprotein	IgG2a	Sigma-Aldrich A8452	Recognizes α-fetoprotein present in human. No cross reaction with human serum albumin	Mouse ascites fluid	1:500	Alexa 594/ Goat anti-mouse IgG2a A21135 TxRed/1:500
Polyclonal anti- Ck 7	IgG	Abcam Ab31014	Reacts with human Ck7	Rabbit	1:100	Alexa 488/Goat anti-rabbit IgG A11008 FITC/1:500
Monoclonal anti-Ck 18	IgG1	Sigma-Aldrich C 1399	Reacts with Ck18 in human-derived epithelial cells	Mouse ascites fluid	1:100	Alexa 647/Goat anti-mouse IgG1 A21240 Cy5/ 1:500
Monoclonal Anti-Ck 8	IgG1	Abcam Ab9023	Reacts with human and mouse	Mouse	1:100	Alexa 647/Goat anti-mouse IgG1 A21240 Cy5/ 1:500
Monoclonal anti-Ck19	IgG2a	Sigma-Aldrich C 6930	Reacts with human Ck19	Mouse cell culture supernatant	1:100	Cy™5/ Goat anti-mouse IgG2a 115-175-206 Cy5/1:500
Monoclonal anti-Vimentin	IgM	Sigma-Aldrich V2258		Mouse Ascites fluid	1:200	Alexa 594/Goat anti-mouse IgM A21044 TxRed/1:500

Antibody	Immunoglobulin Subtype	Supplier and Code	Specificity of 1° Antibody	Source of 1° antibody	Working conc. of 1° Antibody	2° Antibody/ Supplier/ Code/ Working conc.
Polyclonal Anti-Gata 4	IgG	SantaCruz Biotech Sc-9053	Reacts with mouse, rat and human Gata-4	Rabbit	1:100	Alexa 594/ Goat anti-rabbit IgG A11012 FITC/1:500
Polyclonal anti- CPS1	IgG	Abcam ab3682	Reacts with mouse, rat and human CPS1	Rabbit Anti-serum	1:5000	Alexa 594/ Goat anti-rabbit IgG A11012 FITC/1:500
Monoclonal anti-HGFR	IgG	Abcam ab51067	Recats with human HGFR	Rabbit	1:100	Donkey anti-goat IgG NorthernLights™ NL493/ NL003 Light green /1:500
Polyclonal Anti-LDLR	IgG	Abcam Ab30532	Reacts with Mouse, Rat and human LDLR	Rabbit	1:100	Alexa 594/ Rabbit anti-goat IgG A11012 TxRed/1:500
Polyclonal Anti-Oct4	IgG	Abcam ab27985	Reacts with Mouse, Rat and human	Goat	1:500	Goat anti-mouse IgG2b Red™-X 115-295-207 Rhodamine/1:500
Monoclonal Anti-Sox2	IgG2a	R&D MAB2018		Mouse	1:50	Cy™5/ Goat anti-mouse IgG2a 115-175-206 Cy5/1:500
Polyclonal Anti-Nanog	IgG	R&D AF1997		Goat	1:20	Donkey anti-goat IgG NorthernLights™ NL493/ NL003 Light green /1:500
Polyclonal Anti-GFAP	IgG	Abcam ab7779		Rabbit	1:1000	Alexa 594/ Goat anti-rabbit IgG A11012 TxRed/1:500
Monoclonal Anti-Nestin	IgG1	R&D MAB1259		Mouse	1:100	Alexa 488/ Goat anti-mouse IgG1 A21121 FITC/1:500

All antibody working concentrations were made in PBS

2.14 Protein Analysis- SDS-PAGE and Western Blotting

Western blot or alternatively protein immunoblot is an analytic procedure used to detect specific proteins. It depends on the ability to resolve the individual proteins (macromolecules) in a size-dependent manner. The method was pioneered by George stark (Renart, Reiser et al. 1979) and refined by Towbin (Towbin, Staehelin et al. 1979) and named by Burnette (Burnette 1981). Western blot is a sequential protocol that was executed as follows:

2.14.1 Tissue preparation

A combination of mechanical and biochemical techniques are employed to denature protein samples. The target proteins investigated in this project were predominantly located in the cytoplasm and mitochondria so RIPA (RadioImmuno Precipitation Assay) buffer was used for lysate preparation.

RIPA buffer contains the ionic detergent sodium deoxycholate as an active constituent. As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. These events are slowed down by keeping the samples on ice (4°C) throughout the lysis procedure and appropriate inhibitors were added fresh to the lysis buffer. In this case 100µl of Phenylmethylsulfonyl Fluoride (PMSF) (protease inhibitor) and one protease inhibitor cocktail tablet (Roche Diagnostics, GmbH, Cat. no. 11 836 170 001, Mannheim, Germany) were added to 10ml of RIPA buffer. 100µl of lysis buffer was used for lysis of $1x10^6$ cells. The cell-lysis buffer mixture was incubated on ice for 30 minutes with gentle regular mixing with a pipette. The lysed sample was then centrifuged at 12000 rpm for 20 minutes at 4°C. The supernatant was then aspirated and transferred to a new tube and the pellet discarded. The samples were then stored on ice before proceeding to the next stage.

2.14.2 Protein quantification

Bradford (Coomassie Blue G-250 dye-binding) assay was used for measuring total protein concentration. The method applied was adapted from Bradford M.M., 1976 (Bradford 1976).

The method is based on the proportional binding of the dye to proteins. It is a colorimetric assay; as the protein concentration increases, the colour of the test sample becomes darker. Coomassie absorbs at 595nm. The protein concentration of the test samples were determined by comparison to that of a series of protein standards know to reproducibly exhibit a linear absorbance profile in this assay. Bovine serum albumin (BSA) was used as a

standard protein in this project. Cell lysates and standards 0-1mg/ml (table 16) were plated in duplicates (10µl per well) in a 96-well clear flat bottom ELISA plate (Techno Plastic Products, Cat. no. 92096, Switzerland) leaving column 1 blank. Dilution of the protein samples (1:10) was required for the resulting absorbance to fall within the linear range of the assay. 200µl of diluted Bradford reagent (Bio-Rad Protein Assay; 500-0006, bio-Rad Laboratories GmbH, HeidemannstraBe 164, 80938 Mὺnchen) (diluted with water 4:1) was introduced to each well and allowed to stand for 5 minutes at room temperature. The Multiskan was switched on for at least 10 minutes prior to reading the plate and the optimal density of the samples was read at 595nm. The results generated were used to construct a standard curve to which the unknown values obtained could be compared to determine their concentration. The curve and the data from Bradford were used to determine the unknown protein concentration. Once the protein concentration in each sample was determined the samples were frozen at -20°C or -80°C for later use.

Table 16. Standards created from Bovine Serum albumin (BSA) stock 1mg/ml

Volume of Stock BSA	Volume of (Double distilled) water	Final concentration of Standard
0µl	30µl	0.0 mg/ml
1.5µl	28.5µl	0.05 mg/ml
3µl	27µl	0.1mg/ml
7.5µl	22.5µl	0.25 mg/ml
15µl	15µl	0.5 mg/ml
22.5µl	7.5µl	0.75 mg/ml
30µl	0µl	1 mg/ml

2.14.3 Denaturation and reduction of the protein

The fraction of the protein recognized by antibodies is the epitope and this domain may reside within the 3D conformation of the protein. To enable antibodies to access this portion it is necessary to unfold the protein structure, i.e. denature it. This procedure was carried out by using a loading buffer with the ionic detergent sodium Dodecyl sulphate (SDS), and the mixture was boiled at 95-100°C for 5 minutes. The volume of loading buffer added was equal to the volume of the cell lysate.

Table 17. Reducing loading buffer (LBR)

Reagent	Volume	Supplier
(Double distilled) Water	4ml	
0.5M Tris pH 6.8 (60.5g Trizma base in 1L dd water, pH to 6.8 with conc. HCL)	1ml	Trizma base Sigma-Aldrich T1503
Glycerol	0.8ml	Sigma-Aldrich G8773
10% Sodium Dodecyl Sulfate (SDS) (10g SDS in 100ml dd water)	1.6ml	Sigma-Aldrich L3771
0.05% Bromophenol Blue (BPB) (5mg of BPB in 10ml dd water)	0.6ml	Sigma-Aldrich B0126
1M DTT (DL-Dithiothreitol)	80µl	Sigma-Aldrich D9779

Samples were mixed by vortexing (Vortex-Genie, Jencons- PLS, Scientific Industries INC, Model G-560E, Serial no. 2-109523) both before and after heating for good resolution. SDS denatures proteins by wrapping around the polypeptide backbone and hence confers a negative charge to the polypeptide in proportion to its length. The denatured polypeptides thus lose their complex secondary and tertiary structures and become rods of negative charge clouds with equal charge densities per unit length. Reduction of disulphide bridges by β-mercaptoethanol or dithiothreitol (DTT) is necessary before proteins can adopt the random-coil configuration necessary for separation by size. In this case DTT was used at a concentration of 150µl of 1M DTT per 1ml of LBR. In denaturing SDS-PAGE separations, therefore, migration is determined not by intrinsic charge of the polypeptide, but by molecular weight.

To enable visualization of the migration of proteins, an anionic dye molecule (bromophenol blue) was included in the loading buffer. Since the dye molecule is small, it migrates faster than any of the components in the mixture to be separated and hence provides a migration front to monitor the separation progress. Glycerol was also included in the loading buffer to increase the viscosity of the sample to be loaded and hence allowed the sample to be maintained at the bottom of the well, restricting overflow and uneven gel loading.

2.14.4 Gel electrophoresis

The proteins in the samples were separated using one dimensional polyacrylamide gel electrophoresis. SDS-PAGE (SDS Polyacrylamide Gel Electrophoresis) maintains polypeptides in a denatured state as they run along the gel. Polyacrylamide gels are formed from the polymerization of two compounds, acrylamide and N, N-methylene-bis-acrylamide

(Bis-acrylamide). Bis is a cross-linking agent for the gels. Introduction of ammonium persulphate along with N, N, N', N'-Tetramethylethyl-enediamine (TEMED) (Sigma, T9281) initiates polymerization of the gel. The gels are neutral, hydrophilic, three-dimensional networks of long hydrocarbons cross linked with methylene groups. The separation of molecules within a gel is determined by the relative size of the pores formed within the gel. The resolution and pore size is determined by the total amount of acrylamide present (%T) and the amount of bis cross-linker (%C). As the total amount of acrylamide increases, the pore size decreases.

In this project a 4% stacking gel was used, which allowed the proteins to form a nice tight band at the interface between the stacking gel and the separating gel. A 9% separating gel was utilized for the runs in this study as the proteins investigated rangd in molecular weight between 40 and 200 KDalton in size.

Table 18. Polyacrylamide gels

Reagents	**Stacking Gel (4%)**	**Reagents**	**Separating Gel (9%)**	**Supplier**
(**Double distilled) Water**	6.3ml	(**Double distilled) Water**	5.1ml	
0.5M Tris pH 6.8 (60.5g Trizma base in 1L dd water, pH to 6.8 with conc. HCL)	2.5ml	**1.5M Tris pH 8.8** 181.65g Trizma base in 1L dd water, pH to 8.8 with conc. HCL)	2.5ml	Trizma base Sigma-Aldrich T1503
10% SDS	100µl	**10% SDS**	100µl	Sigma-Aldrich L3771
Acrylamide/Bis- acrylamide 49:1 ratio	1ml	**Acrylamide/Bis-acrylamide 49:1 ratio**	2.25ml	Sigma-Aldrich AO924
Ammonium persulfate (APS)	100µl	**Ammonium persulfate (APS)**	50µl	Sigma-AldrichA3678
N, N, N', N'-Tetramethylethylenediamine (TEMED)	10µl	**N, N, N', N'-Tetramethylethylenediamine (TEMED)**	5µl	Sigma-Aldrich T9281

The wells of the gel were gently flushed with running buffer with an 19G fine needle syringe (Syringe, BD 1ml, Terumo, Cat. no 0197, Leuvrn, Belgium) (19G microlance 3, BD, Cat. no. 2013-08,Ireland). This was performed to ensure even filling of the wells. Protein samples were loaded into the wells in the gel. 10µg of protein was loaded into each well. The first well was reserved for the protein ladder (5µl) (PageRuler Plus Prestained Protein Ladder, SM1811, Fermentas Life Sciences, Sheriff House, UK). The tank was the filled with running buffer (table 19) and voltage applied to the gel. The gels were run at a voltage of 120 Volts for 20 minutes (until the protein ladder appeared to separate) and then

at a constant voltage of 110 volts for 40 minutes (until the bromophenol blue had migrated to the bottom of the gel ensuring that the proteins have separated into various bands in each well.

Table 19. Running buffer

Reagents	X10 Running Buffer
(Double distilled) Water	1L
Trizma Base	30g
Glycine	144g
SDS	10g

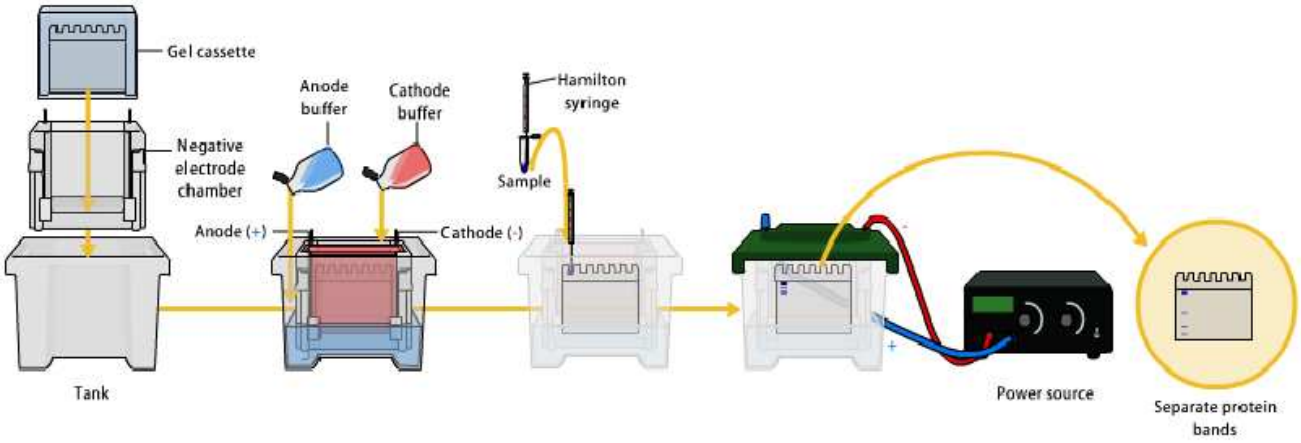


Figure 42. SDS-polyacrylamide gel electrophoresis. SDS-PAGE maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure and thus allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size. The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration the better the resolution of higher molecular weight proteins. Samples are loaded into wells in the gel. One lane is usually reserved for a ladder. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement separate into bands within each lane (http://en.wikipedia.org/wiki/Western_blot**).**

2.14.5 Transfer of protein bands from gel onto PVDF membrane

To make the proteins accessible to antibody detection they are moved from within the gel onto a membrane made of polyvinylidene difluoride (PVDF). Transfer was done in wet conditions during this project. PVDF membranes (Amersham Hybond-P, RPN303F, Lot. NH0031, GE Healthcare, Amersham Place, Little Chalfont, Buckinghamshire) were pre-treated by soaking the membrane in methanol for 1-2 minutes. This was followed by a quick soak in water and then incubation in ice cold transfer buffer for 5 minutes. The wet membrane was then placed on top of the gel, and a stack of transfer buffer soaked filter papers placed on top of that. This stack was sandwiched between two buffer soaked sponge pads and transferred to a tank filled with transfer buffer. The electro-transfer was performed in a cold room at a constant voltage of 110 volts for 2 hours.

Table 20. Transfer buffer

Reagents	(x1) Transfer Buffer
(Double distilled) Water	2L
Trisma Base	6.06g
Glycine	28.8g
Methanol	400ml

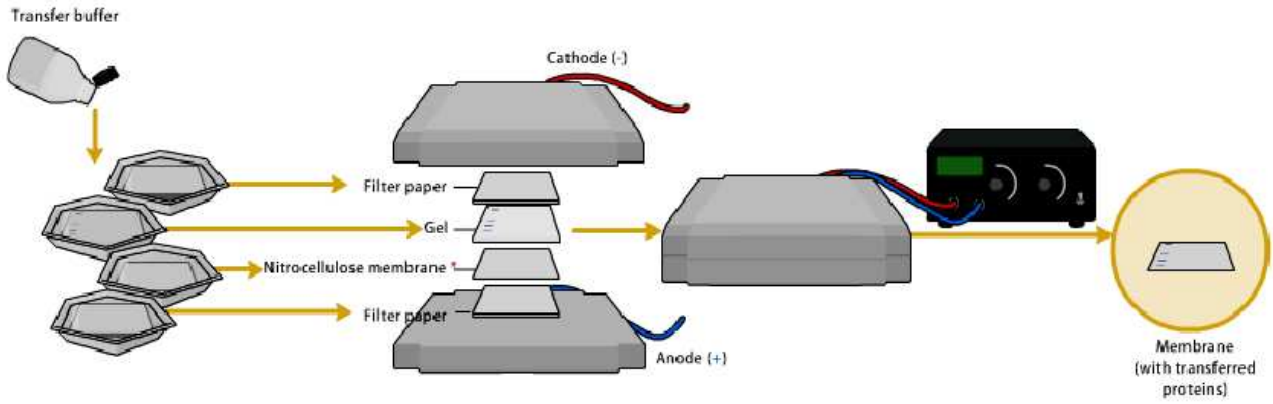


Figure 43. Transfer of proteins from gel onto membrane. Electroblotting was used for protein transfer. This technique uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel (http://en.wikipedia.org/wiki/Western_blot**).**

2.14.6 Visualization of all separated proteins after migration

Ponceau S dye (P7170-IL, Sigma-Aldrich) was used to visualize the uniformity and overall effectiveness of protein transfer from the gel to the membrane. The membranes were incubated with Ponceau S dye (1:10 dilution with water) for 3-5 minutes at room temperature on an agitator. The protein bands, if transferred effectively, appear as well-defined reddish-pink bands. The membranes were then washed extensively in (x1) Tris Buffered Saline-Tween (TBS-Tween) until the dye was washed off.

Table 21. (x20) TBS

Reagents	(x20) Tris Buffered Saline Solution (TBS)
NaCL	234g
Trizma base	48.4g
(Double distilled) Water	1L

Table 22. (x1) TBS

Reagents	(x1) Tris Buffered Saline Solution (TBS) 1Litre	Supplier
(x20) TBS	50ml	
Tween-20	1ml	Sigma-Aldrich 27,434-8
(Double distilled) Water	949ml	

2.14.7 Blocking of the PVDF membranes

The membranes with the transferred protein bands were then blocked in blocking buffer for 1 hour at room temperature on an agitator. Blocking the membranes prevented non-specific binding of the primary and/or secondary antibodies to the membrane.

Table 23. Blocking buffer

Reagents	Blocking buffer	Supplier
(x20) Tris buffered Saline (TBS)	2.5ml	
Tween-20	0.6ml	Sigma-Aldrich 27,434-8
Low-fat milk (Marvel)	2.5g	Dried skimmed milk, R. No. 92962, Marvel, Dublin, Ireland

2.14.8 Antibody staining

The membranes were incubated with the primary antibody (diluted in incubation buffer) (table 25) overnight at 4°C on an agitator to enable adequate homogenous covering of the membrane and prevent uneven binding.

Table 24. Incubation buffer

Reagents	Incubation Buffer
(x1) Tris Buffered Saline (TBS)-TX	50mls
Low-fat milk (Marvel)	150mg

Post overnight incubation with primary antibody the membranes were washed in (x1) TBS-TX, three times, and 10 minutes each time. The washes were done at room temperature on an agitator. This step ensured that all excess unbound antibodies were washed off.
The membranes were then incubated with the appropriate secondary antibody (diluted in incubation buffer) for 1 hour at room temperature on an agitator.
The membranes were then washed in TBS-TX, three times, and 10 minutes each time, to get rid of any unbound secondary antibody.

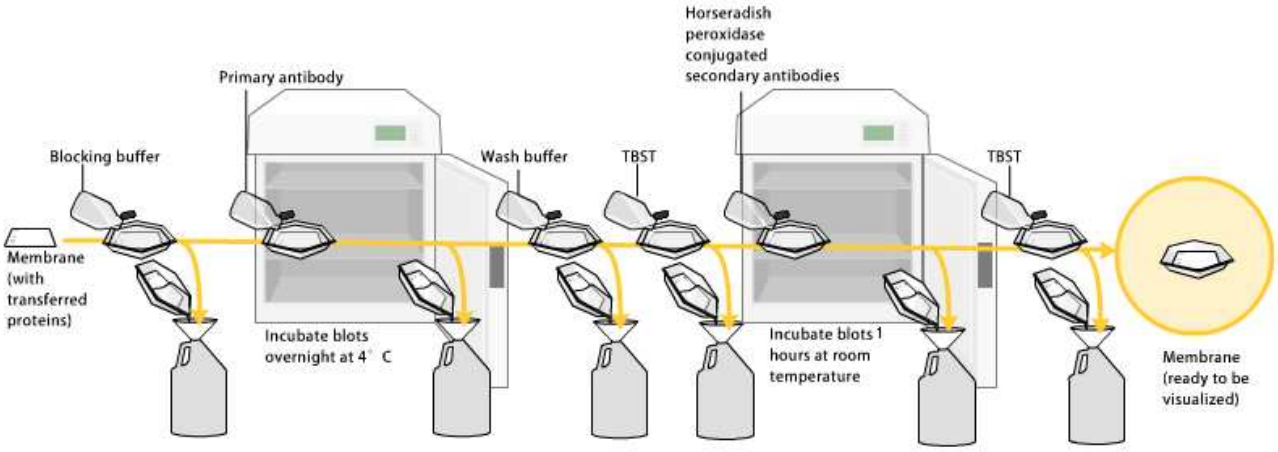


Figure 44. Blocking and antibody staining of membranes with transferred proteins. During the detection process of proteins, the membranes were "probed" for the protein of interest with antibodies which were linked to reporter enzymes, which when exposed to an appropriate substrate drives a colourimeic reaction and produces a colour. This was carried out in a two step process where the proteins were first probed with primary antibodies overnight at 4°C followed by washing and subsequent exposure to secondary antibodies which were conjugated to a reporter enzyme, in this case horseradish peroxidase (http://en.wikipedia.org/wiki/Western_blot**).**

Table 25. List of antibodies used for western blotting

Antibody	Immunoglobulin Subtype	Supplier and Code	Specificity of 1° Antibody	Source of 1° antibody	Working conc. of 1° Antibody	2° Antibody/ Supplier/ Code/ Working conc.
Anti-Human albumin	IgG	Sigma-Aldrich A 7544	Reacts with human albumin	Goat	1:1000	Donkey anti-goat IgG-HRP conjugated/ 1:7000 Sc-2020, Lot. C1207, Santa Cruz Biotechnology
Monoclonal anti-human α-fetoprotein	IgG2a	Sigma-Aldrich A8452	Recognizes α-fetoprotein present in human.	Mouse	1:500	Anti-Mouse IgG F $(ab^1)_2$ HRP conjugated/1:7000 SAB-100, Stressgen Corp Bioreagents
Polyclonal anti- Ck 7	IgG	Abcam Ab31014	Reacts with human Ck7	Rabbit	1:2000	Anti-rabbit IgG (whole molecule) HRP conjugated/1:7000 A-0545 Sigma-Aldrich
Monoclonal Anti-Ck 8	IgG1	Abcam Ab9023	Reacts with human and mouse	Mouse	1:500	Anti-Mouse IgG F $(ab^1)_2$ HRP conjugated/1:7000 SAB-100, Stressgen Corp Bioreagents
Monoclonal anti-Ck19	IgG2a	Sigma-Aldrich C 6930	Reacts with human Ck19	Mouse	1:50	Anti-Mouse IgG F $(ab^1)_2$ HRP conjugated/1:7000 SAB-100, Stressgen Corp Bioreagents
Polyclonal anti- CPS1	IgG	Abcam ab3682	Reacts with mouse, rat and human CPS1	Rabbit	1:1500	Anti-rabbit IgG (whole molecule) HRP conjugated/1:7000 A-0545 Sigma-Aldrich
Monoclonal anti-HGFR	IgG	Abcam Ab51067	Recats with human HGFR	Rabbit	1:1000	Anti-rabbit IgG (whole molecule) HRP conjugated/1:7000 A-0545 Sigma-Aldrich

Antibody	Immunoglobulin Subtype	Supplier and Code	Specificity of 1° Antibody	Source of 1° antibody	Working conc. of 1° Antibody	2° Antibody/ Supplier/ Code/ Working conc.
Polyclonal Anti-CYP3A4	IgG	Courtesy of Dr Mattew Wright ♣		Rabbit	1:3000	Anti-rabbit IgG (whole molecule) HRP conjugated/1:7000 A-0545 Sigma-Aldrich
Polyclonal Anti-CYP2A	IgG	Courtesy of Dr Mattew Wright ♣		Rabbit	1:4000	Anti-rabbit IgG (whole molecule) HRP conjugated/1:7000 A-0545 Sigma-Aldrich
Polyclonal Anti-CYP2E	IgG	Courtesy of Dr Mattew Wright ♣		Rabbit	1:5000	Anti-rabbit IgG (whole molecule) HRP conjugated/1:7000 A-0545 Sigma-Aldrich
Monoclonal Anti-β Actin	IgG	Abcam Ab8226		Mouse	1:1000	Anti-Mouse IgG F $(ab^1)_2$ HRP conjugated/1:7000 SAB-100, Stressgen Corp Bioreagents

♣ Institute of Cellular Medicine, Newcastle University

2.14.9 Detection

ECL Plus western blotting detection system (GE Healthcare, Cat. no. RPN2132, Buckinghamshire HP79NA, UK) was used for visualisation of the protein band. The ECL detection kit uses an acridan-based substrate that releases a high level of sustained light output. In addition, ECL kit generates an intense long-lasting chemifluorescent signal at 440nm that can be detected on a fluorescence imager with appropriate filters.

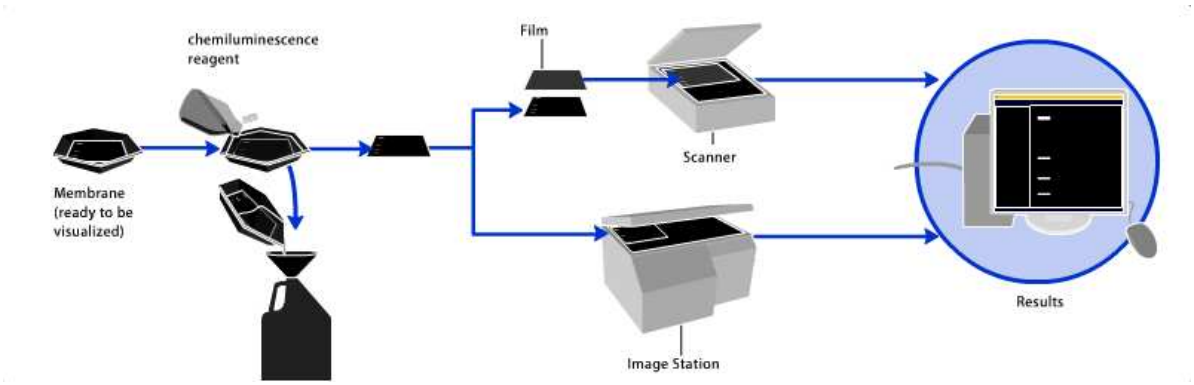


Figure 45. Chemiluminnescent detection of proteins. This detection technique depends on incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light emitted was then detected on a fluorescence imager which captured a digital image of the western blot. The substrate used in this project was ECL Plus western blotting detection reagent which is acridin-based. (http://en.wikipedia.org/wiki/Western_blot**)**

2.15 Molecular analysis

An RNase-free work environment was used for all molecular methodology. Class II vertical laminar flow hoods were used and all work surfaces and materials were wiped with RNase Away solution (Invitrogen, Cat. no. 10328-011, CA, San Diego).

2.15.1 RNA isolation

RNA isolation from cell pellets was performed with Rneasy Mini and Micro kits supplied by Qiagen. The Micro kit (74034, Qiagen GmbH, D40724 Hilden) was applied when the cell numbers were $5x10^5$ and below. RNA isolation from cell numbers greater that $5x10^5$ were done with the Mini kit (74104, Qiagen). The kits were used according to the manufacturer's instructions. The protocols for both kits are available at the following websites: Mini Kit (http://www1.qiagen.com/HB/RNeasy/MiniKit_EN) and Micro kit (http://www1.qiagen.com/Products/RNeasyMicrokit.aspx#Tabs=t2). In both cases, an additional step of in-column DNase digestion was carried out. 10µl DNAse 1 stock (1500 Kunitz units dissolved in 550µl of RNase-free water) (79254, Qiagen) was diluted in 70µl RDD buffer (provided in the Dnase kit) and mixed gently. The DNase 1 mix was then introduced directly to the RNeasy MinElute spin column membrane very gently, and placed on the bench top for 15 minutes at room temperature (RNeasy Micro Handbook; Page 21; http://www1.qiagen.com/Products/RnaStabilizationPurification/RNeasySystem/RNeasyMicroKit.aspx#Tabs=t2). Generally, DNase digestion is not required for RNA purified with the Qiagen kits. The silica-membrane technology of the columns provides efficient removal of the majority of the DNA without DNase treatment. However, complete DNA removal was desired as the RNA was used for real-time PCR applications which can be quite sensitive to even minute amounts of DNA contamination. (http://www1.qiagen.com/Products/Accessories/RNase-FreeDNaseSet.aspx#Tabs=t2).

2.15.2 RNA quantification- Nanodrop

To evaluate the purity and the integrity of the mRNA produced the samples were tested on a nanodrop. An A_{260}/A_{280} ratio of 1.9-2.1 was considered as pure RNA.

2.15.3 cDNA preparation

First-strand cDNA was generated using SuperScript VILO cDNA Synthesis Kit (11754-050; Invitrogen, Carlsbad, CA 92008, USA). This kit comprises: the 10X SuperScript Enzyme Mix (SuperScript III RT, RNaseOut Recombinant Ribonuclease Inhibitor and a proprietary helper protein) and the 5X VILO Reaction Mix (random primers, MgCl2, and dNTPs in a buffer formulation). SuperScript III RT is an engineered version of M-MLV RT with

reduced RNase H activity and increased thermal stability. The kit was used as per suggested by the manufacturer (http://tools.invitrogen.com/content/sfs/manuals/vilo_cdna_synthesis_man.pdf).

The Thermocycler (Master/Gradient, Eppendorf AG, 22331, Hamburg, Germany) was used to run the first-strand cDNA synthesis reactions. The SuperScript VILO cDNA Synthesis Kit components were mixed in a tube on ice and then the RNA was included (up to 2.5µg). The Thermocycler programme used was as follows: incubation at 25°C for 10 minutes, 42°C for 60 minutes, and then the reaction terminated at 85°C for 5 minutes. The resulting cDNA was then either used directly or stored at -20°C until further application. The purity of the single-strand DNA produced was tested with a nandrop. The ratio of absorbance values at 260nm and 280nm gives an estimate of DNA. An A_{260}/A_{280} ratio of 1.8-2.0 was considered as pure cDNA.

2.15.4 Primers

A list of the primers used during the course of this study is elaborated in the table 26. All primers were self designed using the following web provided programmes:

- **NCBI (National Centre for Biotechnology Information)** - the nucleotide database. During primer designing, particular attention was focused on trying to ensure, where possible, that the mRNA sequence selected included a segment of two exons. This allowed application of introns (genomic DNA) during polymerase chain reaction to be avoided.

http://www.ncbi.nlm.nih.gov/nuccore

- **Primer BLAST (Basic Local Alignment Search Tool)** - the selected mRNA sequence was then fed into the Primer BLAST software and primers were designed, ranging in length between 150-250 bp.

http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome

- **Nucleotide BLAST**- once primers were designed they were checked for any similarities with other biological sequences by using the Nucleotide BLAST database. This step helped avoid non-specific and unwanted products from being amplified during the PCR.

http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome

Table 26. List of primers applied in this study

Oligo Name	Sequence (5'-3')	Product size (bp)	Annealing Tm°	Melting Tm°
AFP-F	AGCTTGGTGGTGGATGAAAC	248	60°	80°
AFP-R	CCCTCTTCAGCAAAGCAGAC		60°	80°
Albumin-F	AAGCTGCCTGCCTGTTGCCA	156	65°	83°
Albumin-R	GCTCAGGCGAGCTACTGCCC		65°	83°
CLDN2-F	TCGAACCTCATTGTCAGCAG	154	60°	80°
CLDN2-R	ACGCTGAGGAAGTTCTCCAA		60°	80°
CYP1B1-F	AAGGTGCTTGGAGTTTACCTGGC	251	65°	75°
CYP1B1- R	CTCCGGTAGAAACACAATGAGGG		65°	75°
CYP2C9-F	TGCATGCAAGACAGGAGCCACAT	429	65°	81°
CYP2C9-R	CGGCACAGAGGCAAATCCATTGA		65°	81°
CYP2E1-F	CCCGAGACACCATTTTCAGAGGA	229	65°	81°
CYP2E1-R	AGAAACAACTCCATGCGAGCCAG		65°	81°
CYP 3A4 -F	CAAGACCCCTTTGTGGAAAA	175	65°	85°
CYP 3A4	CGAGGCGACTTTCTTTCATC		65°	85°
CYP7B1-F	GCCCATAGGACTAAACTACAGCC	256	65°	75°
CYP7B1-R	GGGCTTTGTGACTAAGGACAAAC		65°	75°

CYP11B1-F	AAACGCCATAGACTGGGTTGCTG	235	65°	81°
CYP11B1-R	AATGCAGGCAGTGCCCTCTTGAA		65°	81°
CYP20A1-F	CTCGTCCTTTATGCCCTTGGTGT	230	65°	81°
CYP20A1-R	ACCTGTCCCTCCACAGAAAGTAG		65°	81°
CYP27B1-F	CTTTGAGAGGAAGGGTGAAGCCT	256	65°	75°
CYP27B1-R	GGGGCAAACCCACTTAATAGTGG		65°	75°
GATA-4-F	GCAGCAGCGAGGAGATGCGT	194	65°	85°
GATA-4-R	GGGGAGAGCTTCAGGGCCGA		65°	85°
HGFR-F	CAGGCAGTGCAGCATGTAGT	201	60°	80°
HGFR-R	GATGATTCCCTCGGTCAGAA		60°	80°
HNF1B-F	TCTGCTCTCCCACGGCTCCC	148		
HNF1B-R	GCTGGTCACCATGGCGCTGT			
HNF3B-F	TGCCATGCACTCGGCTTCCAG	129	65°	85°
HNF3B-R	CCCAGGCCGGCGTTCATGTT		65°	85°
HNF4A-F	GCCACCCCTGAGACCCCACA	159	65°	85°
HNF4A-R	AGCCCCAGCGGCTTGCTAGA		65°	85°
LDLR-F	GCTTGTCTGTCACCTGCAAA	190	60°	80°
LDLR-R	AACTGCCGAGAGATGCACTT		60°	80°

NANOG-F	GATTTGTGGGCCTGAAGAAA	155	65°	82°
NANOG-R	AAGTGGGTTGTTTGCCTTTG		65°	82°
OCT-4A-F	CGTGAAGCTGGAGAAGGAGA	91	65°	82°
OCT-4A-R	CTTGGCAAATTGCTCGAGTT		65°	82°
PAX6-F	GAGGTCAGGCTTCGCTAATG	91	65°	86°
PAX6-R	TGGTGATGGCTCAAGTGTGT		65°	86°
RPL13A-F	CCTGGAGGAGAAGAGGAAAGAGA	126	65°	80°
RPL13A-R	TTGAGGACCTCTGTGTATTTGTCAA		65°	80°
SDHA-F	TGGGAACAAGAGGGCATCTG	84	65°	76°
SDHA-R	CCACCACTGCATCAAATTCATG		65°	76°
SOX-2-F	GGCAGCTACAGCATGATGCAGGACC	131	65°	86°
SOX-2-R	CTGGTCATGGAGTTGTACTGCAGG		65°	86°

2.15.5 Quantitative PCR (real-time)

Real-time PCR allowing detection and quantification of nucleic acid sequences was performed by the 7900HT Fast Real-Time PCR system (Applied Biosystems). SYBR Green JumpStart *Taq* ReadyMix (20 mM Tris-HCl, pH8.3, 100 mM KCl, 7 mM MgCl2, 0.4 mM each dNTP (dATP, dCTP, dGTP, TTP), stabilizers, 0.05 unit/ml *Taq* DNA Polymerase, JumpStart *Taq* antibody, and SYBR Green I) (S4438, Sigma-Aldrich) was used as a fluorescent DNA binding dye, that binds all double-stranded DNA and detection is monitored by measuring the increase in fluorescence throughout the cycle. SYBR green has an excitation and emission maxima of 494nm and 521nm, respectively. The JumpStart *Taq* antibody inactivates the DNA polymerase at room temperature. When the temperature is raised above 70°C in the first denaturation step of the cycling process, the complex dissociates and the polymerase becomes fully active. This helps prevent non-specific product amplification and hence leads to generation of more accurate Ct (Cycle threshold) values. The reactions were executed in 384-well clear optical reaction plates (ABI PRISM, Applied Biosystems, Cat. no. 4309849, USA). The PCR reaction mix for each primer per well was set up as demonstrated in the table below:

Table 27. PCR reaction mix for each primer

Reagents	**Volumes per well**
SYBR green (SYBR Green Jumpstart *Taq* ReadyMix, S4438, Sigma)	5µl
Forward Primer (10µM)	0.5 µl
Reverse Primer (10µM)	0.5 µl
RNase-Free Water	3 µl
cDNA (2.5-10ng)	1 µl
Total volume	10 µl

Each primer was run in triplicates for all the samples investigated and for each primer tested the following controls were applied:

- **Negative control**- RNase-free water

- **Positive controls**-

* Foetal liver tissue (7-12 weeks fetal development)

* Adult liver cDNA (Biobank cDNA kit, PrimerDEsign Ltd BioBank cDNA)- 1.92ng per reaction

* HepG2 (liver carcinoma cell line)

The plates were centrifuged at 4000 rpm for 5 minutes and then run in a 7900HT Fast Real-Time PCR Thermocycler. In each case 40 cycle amplification was performed and the cycling parameters were as follows:

Table 28. Real-Time PCR thermocycler parameters

Initial Denaturation	94°C	2 minutes
	40 Cycles	
Denaturation	94°C	15 seconds
Annealing, extension and read fluorescence	60°C or 65°C depending on the primer	1 min
Hold	4°C (to allow the products to be run on a gel subsequently)	

The data generated was visualized in a two-dimensional plot of log of relative fluorescence intensity (y-axis) vs. cycle number (x-axis). This is a kinetic PCR amplification curve and is used to determine the Ct (cycle threshold) values. Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceed the background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample and are used to determine the amount of template in each sample. Relative quantification of gene expression (i.e. the ratio between the amount of target DNA and a reference amplicon, usually a house-keeping gene, which illustrates invariant and constitutive expression) was

analyzed by inputting the Ct values (Excel format) into the Q-Base software. This software permits relative quantification of the genes being studied together with inter-run calibration and proper error propagation along the entire calculation track.

2.15.6 PCR Gel Electrophoresis

The PCR products (DNA) generated by the real-time thermocycler were analyzed by running them on an Agarose Gel. The movement of DNA molecules through the Agarose matrix with an electric field allows them to be separated based on their size.

A 1.5% Agarose gel (Seakem LE agarose, Cat. no. 50004, Lonza, Rockland, Me, USA) (1.5g Agarose in 100ml (x1) TAE) was used. 10 µl of GelRed (10,000x in H20, Cat. no. 41003, Biotium) was added to the gel to allow visualisation of the DNA bands. This nucleic acid stain was a safer alternative to ethidium bromide which is a mutagen. GelRed has almost identical spectra as ethidium bromide and is nonmutagenic, noncytotoxic and environmentally safe.

The PCR products were pre-mixed with Blue/Orange Loading dye 6X (0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0) (G1881, Promega). This dye is visible in natural light, co-sediments with DNA and allows tracking of migration during electrophoresis. 3µl of loading dye was mixed with the 10µl of the real-time PCR product and the total volume was then loaded into the Agarose gel with GelRed. The first well in the gel was reserved for a 50bp step ladder (G4521, Promega). This 50bp DNA Step Ladder consists of 16 DNA fragments ranging from 50bp to 800bp in exactly 50bp increments plus a 1,800bp "backbone" fragment. The gels were run for 1 hour and 45 minutes at 90 volts. After electrophoresis, the gels were visualized with a UV Transilluminator (GeneGenius, Bio Imaging System, Syngene, a division of Synoptics LTD). The gels were photographed with a CCD camera. Although the stained nucleic acid fluoresces reddish-orange, images are usually shown in black and white. The GeneTool analysis software, which comes with the system, was used for the analysis of gels.

Table 29. (x50) Tris-acetate buffer (1L)

Reagent	Volume/mass
Trisma base	242g
0.5M Na_2EDTA ph8.0	100ml
Glacial acetic acid	57.1ml
(Double Distilled) water	500ml

Table 30. (x1) Tris-acetate buffer (1L)

Reagent	Volume/mass
X50 TAE solution	20ml
(Double Distilled) water	980ml

2.16 Functional assessment of differentiated cells

2.16.1 Periodic acid Schiff staining

Periodic Acid Schiff (PAS) staining reagents are commonly used for the demonstration of lympocytes and mucopolysccharides. In this project, however, PAS protocol was used to examine glycogen deposits in differentiated umbilical cord and UCB-derived progenitor cells. A staining kit from Sigma: Periodic Acid Solution, 395-1 (Periodic acid 1g/dL) ; Schiff's Reagent, 395-2 (pararosaniline HCL, 1% sodium metasulfite 4%, in HCL 0.25 mol/L) ; Hematoxylin solution , Gill No.3, GHS-3 (certified hematoxylin 6g/L, sodium iodtae 0.6g/L, aluminium sulphate 52.8 g/L and stabilizer) was utilized and the staining procedure was adapted from Kang *et al* (Kang, Zang et al. 2005) and according to the manufacturer's instructions. It should be noted that the reagents involved in this procedure are corrosive and toxic and hence the staining protocol was performed with care.

When treated with periodic acid, glycols are oxidized to aldehydes. After reaction with Schiff's reagent a pararosaniline adduct is released that stains the glycol-containing cellular components bright magenta pink.

Cells used for this staining procedure were cultured on gelatin coated glass cover slips in 24-well plates. All stages of this procedure were carried out at room temperature on the bench top. Culture media was completely removed from the wells and the cells were gently rinsed in (x1) PBS once. The cells were then fixed by incubating them in 100% ethanol for 10 minutes with gentle agitation on a rocker. The cells were then rinsed in (x1) PBS and oxidization reaction was initiated by incubating them with 500µl of PAS reagent for 10 minutes on a rocker. Once again the cells were rinsed with (x1) PBS, twice, 5 minutes each time and 500µl Schiff's reagent was added to the cells for 15 minutes with gentle mixing on the rocker. Subsequently, the cells were rinsed with (x1) PBS, twice, 5 minutes each time. Haematoxylin was used to counter stain the nuclei. Cells were stained with Haematoxylin for 1 minute and then rinsed twice in (x1) PBS- one quick wash and then on a rocker for 5 minutes. The cells were then either directly visualized under a light microscope or the cover slips were mounted onto a glass slide and then visualized with a light microscope.

2.16.2 Indocyanine green dye uptake and release test

Indocyanine green (ICG) is an organic anion that is clinically used as a test to evaluate liver function since it is nontoxic and eliminated exclusively by hepatocytes (Branch 1982) (Berk PD 1986; Meijer DKF 1988). In this study Indocyanine green dye uptake and release by

cultured cells was used to identify functionally differentiated hepatocytes within the culture system. The protocol used was adapted from Wang *et al*, 2005 (Wang, Nan et al. 2005). Adherent cultured cells were washed with (x1) PBS and then incubated with ICG (Cardiogreen 21980, Standard Fluka) solution (1mg/ml) at 37°C for 20 minutes. It should be noted that ICG is not soluble in PBS and hence a (x10) concentrated solution was made in sterile water and then diluted to (x1) in culture media (DMEM-F12 plus supplements) (Yamada, Yoshikawa et al. 2002). Subsequently the cells were rinsed twice with (x1) PBS and replaced with culture media. Cellular uptake of ICG was then examined microscopically. The number of cells that had successfully taken up the green ICG dye was noted and the cells re-incubated at 37°C for a further 24hrs. The cells were regularly examined microscopically to note the time take for the cells to completely excrete the dye.

2.16.3 Cytochrome P450 Glo-Assay

A non-selective cytochrome (CY) P450 enzyme assay using a bioluminescent probe substrate that cross-reacts with multiple P450 enzymes was used to measure CYP activity in differentiated cells. The nonlytic P450-Glo assays measure the activities of CYP enzymes from native sources and allow the effects of analytes such as drugs to be tested. The P450-Glo substrates are CYP enzyme substrates that are derivatives of beetle luciferin {(4S)-4, 5-dihydro-2-6'-hydroxy-2'-benzothiazolyl)-4-thiazolecarboxylic acid}. The substrate used in this study was Luciferin-MultiCYP (Methyl 2-(6-methoxybenzo {d} thiazol-2-yl)-4, 5-dihydrothiazole-4-carboxylate) (P1731, Promega, 2800 Woods Hollow Road, Madison, USA). This chemically synthesized derivative of beetle luciferin is converted to a luciferin ester by several members of the CYP family with preference for CYP1A1, -1A2, -1B1 and -2D6. The luciferin ester produced in a CYP reaction can be detected with Luciferin Detection Reagent (LDR) that contains an esterase (V8931: Luciferin Detection Reagent (LDR), V859B; Reconstitution Buffer with esterase, V144B). The esterase converts the luciferin ester into luciferin and this is converted into light through the action of Ultra-Glo Luciferase. The luminescence produced is recorded with a luminometer. The LDR with esterase simultaneously stops the initial enzymatic reaction and initiates a stable glow-type luminescent signal with a half-life of greater that two hours. The amount of light produced is proportional to the amount of luciferin formed and, reflective of the CYP enzyme activity. The Luciferin-MultiCYP was used in accordance to the manufacturer's instructions (http://www.promega.com/tbs/tb325/tb325.pdf). It is important to note that the substrates and reaction products of the cell-based P450-Glo assays are cell permeable and agreeable with a nonlytic format. This property allowed multiplexing with cell viability assays so that

CYP P450 activity could be normalized to the number of viable cells in culture.

Umbilical cord and UCB cells used for this assay were cultured as monolayer in 24-well plates. Simultaneous CYP gene induction studies were performed by treating the cells with CYP enzyme inducers for 72 hours prior to treating the cells with the MultiCYP luminogenic substrate. The following inducers were used:

Table 31. Cytochrome P450 gene induction

Inducer	CYP Enzyme Upregulated	Pathway activated
Rifampicin (25μM)	CYP2C8 CYP2C9 CYP2C19 CYP3A4	Pregnane X Receptor (PXR) Constitutive Androstane Receptor (CAR)
Omeprazole (10μM)♣	CYP 1A1 CYP1A2 CYP1B1	Ary Hydrocarbon Receptor (AHR)

♣ Omeprazole (100mM) was provided courtesy of the company XT (XenoTech)

The inducers were added to the culture medium and incubated with the cells for 72 hours at 37°C. After induction the cells were washed with (x1) PBS (this was important as some compounds that induce CYP gene expression also inhibit the CYP enzyme activity that has been induced) and then replaced with fresh medium containing the MutliCYP substrate (0.1mM). To determine background luminescence luminogenic substrate was added in the medium to a set of empty wells (no cells). The cells were then incubated with the CYP substrate for 1 hour and 30 minutes at 37°C. Intracellular CYP enzymes convert the substrate to luciferin, which passes out of the cells into the medium. A small aliquot (50μl) of the culture medium from the test wells was removed, and the remaining cells were used to analyze cell viability using Trypan Blue and the haemocytometer. The 50μl aliquots of medium were transferred to a white-walled luminometer plate (Nunc, Dk-4000, Roskilde, Denmark). 50μl of LDR was added to the wells to initiate a luminescent reaction. The plates

were incubated at room temperature in the dark for 20 minutes. The luminescence was then read using a luminometer (Flouroskan, Ascent FL, Thermo LabSystems). The Flouroskan was used according to the manufacturers instruction with an integration time of 0.26 seconds per well. The net luminescence of each CYP assay was calculated by subtracting the luminescence of the minus-cell control wells. Fold changes in net signal from the inducer-treated samples as compared to the untreated control samples represented upregulation of CYP activity by the inducer.

Net luminescence of each sample= luminescence (RLU) of sample well- luminescence (RLU) of the minus-cell control well

Fold change after CYP induction= Luminescence (RLU) of induced sample- luminescence (RLU) of uninduced sample

RLU- Relative Light Units

Chapter 3

Selection and isolation of stem/progenitor cells from human umbilical cord blood

Evaluation of five different umbilical cord blood separation techniques

Immuno-magnetic methodology for stem/progenitor cell isolation by negative depletion

3 Introduction

Many different stem and progenitor cells have been postulated in cord blood with potential ranging from embryonic like to lineage-committed progenitor cells. Based on previous research (Mayani, Gutierrez-Rodriguez et al. 1998; Erices, Conget et al. 2000; Forraz, Pettengell et al. 2002; Bieback, Kern et al. 2004; Kogler, Sensken et al. 2004; McGuckin, Forraz et al. 2004; Markov, Kusumi et al. 2007) it was expected that a heterogeneous population of stem/progenitor cells could be isolated from cord blood. However, the category and quantity of primitive cells derived is governed by various peri-natal and post-natal parameters which include: gestational age, birth weight and volume of cord blood (Ballen, Wilson et al. 2001; McGuckin, Basford et al. 2007) as well as the external factors including the method of cord blood processing and the microenvironment in which cells grow.

3.1 Evaluation of five different umbilical cord blood separation techniques

3.1.1 Introduction

Since this research project endeavoured to develop clinically relevant protocols for liver differentiation, the first step was to evaluate and compare methods for optimal stem cell harvest from UCB.

Ever since the first reported successful reconstitution of the haematopoietic system of a child with Fanconi's anaemia using UCB in 1989 (Gluckman, Broxmeyer et al. 1989) UCB has been regarded as an established source of treatment for a range of disorders. Currently the most commonly used method of umbilical cord blood separation in many hospitals in the world, where small-to-medium amounts of samples are processed, is Hetastarch. However, each cord blood bank works to its own criteria for collection, processing and banking, and there is no standard operational procedure (SOP) that all cord blood banks abide to.

A major limitation when considering UCB for transplantation is total nucleated cell (TNC) yield recovered from a single cord blood unit of limited volume. This illustrates the importance of maximizing the efficiency of UCB processing. One of the objectives of this study was to evaluate the efficiency of some of the more commonly employed methods available for UCB processing and to find the best ways forward for hospitals to process cord blood prior to clinical application. The different separation systems are elaborated in materials and methods chapter (section 2.2). All the separation methods (except plasma depletion) have been developed to remove erythrocytes or red blood cells (RBCs) from

UCB, but permit recovery of non-erythroid subsets including leucocytes or white blood cells (WBCs) encompassing the stem/progenitor cell population and thrombocytes (platelets). Plasma depletion only removes the plasma compartment of whole cord blood.

3.1.2 Multi-parametric analysis of UCB samples pre- and post UCB processing and freezing

UCB samples pre- and post treatment with the different separation techniques were investigated and cell populations characterized by flowcytometry (materials and methods; section 2.4.3).

The post-processed samples were subsequently transferred to the gaseous phase of liquid nitrogen at -196°C and cryopreserved for a minimum of two weeks before thawing and subsequent characterization by flowcytometry.

3.1.3 Sample

A total of 65 UCB units were processed and 120 samples were collected to accomplish this number. For logistical reasons and for homogeneity of treatment, samples were collected only from Caesarean section births. Under no circumstance was the bith procedure adjusted or changed due to our study. Samples were from full-term healthy newborns. The data generated related to the following inclusion criteria: all UCB units had to fulfill a negative viral profile and infection status; UCB units were processed within 24hrs of collection and only if the volume of blood collected exceeded 40ml. The birth weight of the infants concerned with the study ranged from 2.41 to 4.42kg (average 3.52kg ± 0.38SD). Since the accepted cut-off volume for processing is 40ml (Armitage, Warwick et al. 1999; Van Haute, Lootens et al. 2005) the volumes of blood collected ranged from 62.5 to 138.9ml (average 88.36ml ± 21 SD). For each unit, analysis samples were taken pre and post processing, and post cryopreservation/thaw.

3.1.4 Statistical analysis

The results generated were statistically analysed using Student t Test where analysis of two groups of data was undertaken (2-tailed, paired or unpaired depending on the data analysed). A p value of <0.005 was considered as statistically significant. For three or more groups of univariate data, single-factor analysis of variation (ANOVA) was used to obtain p values. For three or more groups of bivariate data (with replicates), two-factor ANOVA with replication was used to obtain p values. Results with p values of less than 0.05 were considered statistically significant.

3.1.5 Red blood cell reduction efficiency

To evaluate RBC depletion and consequent volume reduction, erythrocyte counts and haemoglobin concentration (g/dl) in pre- and post- processing samples were used as the determining criteria. These values were provided by the Coulter cell count machine.

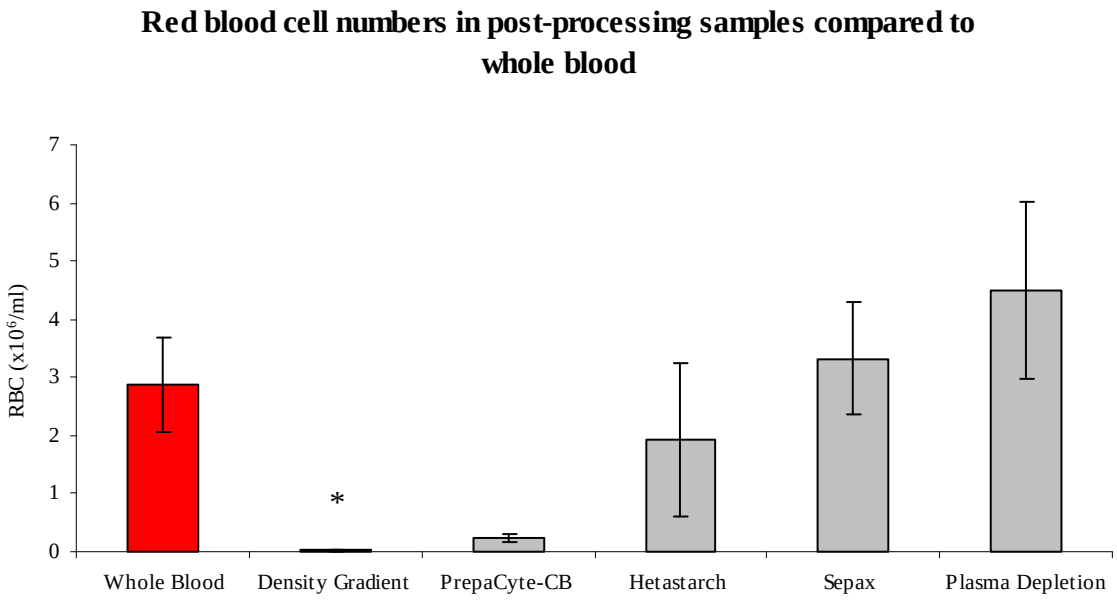
With regard to eliminating RBCs from the cord blood units, density-gradient separation systems (Ficoll-Plaque or Lymphoprep) proved to be the most efficient (Figure 45). Treatment with Ficoll resulted in x95.7 fold reduction in RBC number compared to whole blood (p=0.000964) and a corresponding x60.9 fold reduction in haemoglobin concentration (p=0.000904). Ficoll was the most efficient of the methods tested; superior to PrepaCyte-CB (p=7.36974E-06); Hetastarch (p=0.005384); Sepax (p=4.77539E-07) and plasma depletion (p=2.81517E-06). Plasma depletion, as expected, was ineffective in getting rid of RBCs (Figure 45).

From the results noted, it may be concluded that density gradient method of cord blood separation was the most efficient in RBC depletion. PrepaCyte-CB was second best.

Conclusion

Density gradient method of cord blood separation was the most efficient in RBC depletion. PrepaCyte-CB came in as second best.

(a) Red blood cell numbers



(b) Haemoglobin (g/dl) levels

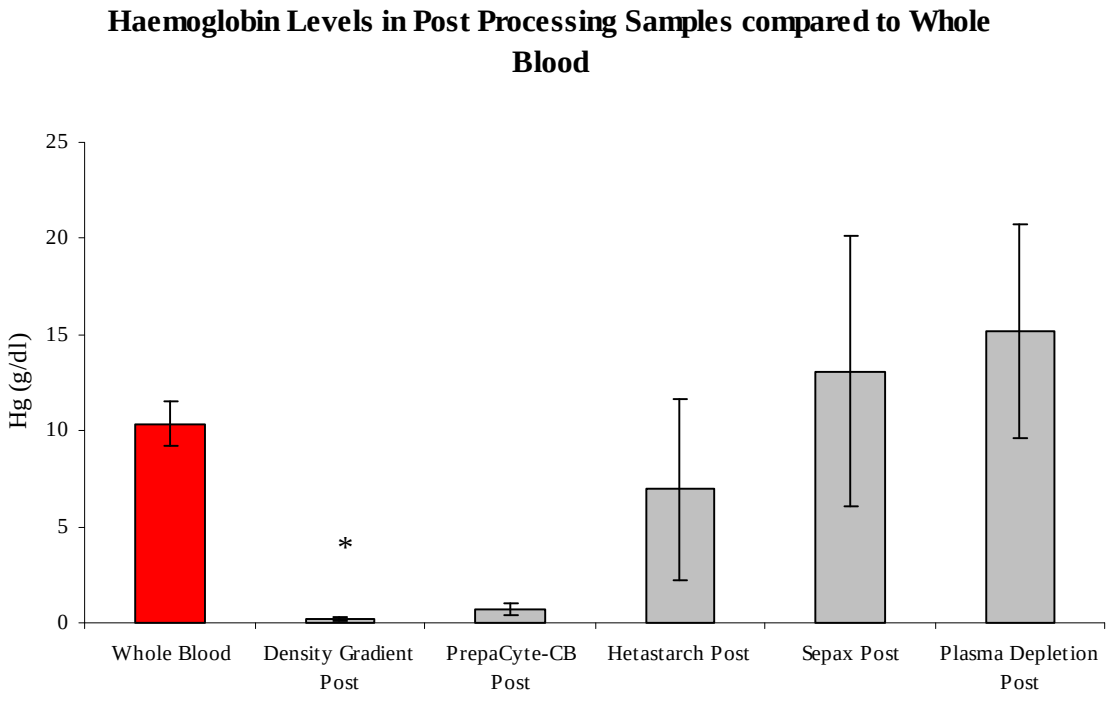


Figure 46. RBC and corresponding haemoglobin concentrations in cord blood samples post processing. (a) The graph illustrates changes in the number of RBCs after treatment with the different separation techniques. Density-gradient dependent processing of cord blood proved to be the most effective in depleting erythrocytes from the samples. (b) The graph illustrates corresponding Haemoglobin levels in cord blood samples post processing. As expected the alteration in Haemoglobin levels corresponded to the changes observed in the RBC numbers.

***** p**<0.001**

3.1.6 Total nucleated cell (TNC) fraction recovery

Total nucleated cell (TNC) fraction encompasses the entire non-erythroid compartment of blood excluding the platelets. TNC recovery was quantified using data from the differential cell counter (WBC count, RBC count and WBC differential count) combined with data derived from flow cytometric analysis, looking at $CD45^+$ cells (expressed by all leukocyte lineage committed cells) and their viability analysed by uptake of 7AAD.

Plasma depletion yielded the greatest TNC recovery post processing at 86.48% (±29.35 SD). TNC recovery was significantly more efficient for plasma depletion compared to Ficoll/Lymphoprep 19.13% (±10.88 SD) (p<0.005) and Hetastarch 44.94% (±20.06 SD) (p<0.005). PrepaCyte-CB 72.03% (±8.48 SD) and Sepax 83.48% (±18.58 SD) were only slightly less efficient than plasma depletion in terms of TNC recovery (both p>0.005) (Figure 47a)

Post freezing and subsequent thaw (compared to pre-processing cord blood units) the highest TNC recovery was illustrated by Sepax 48.83% (±28.87SD). This recovery was significantly superior to results obtained from Ficoll/Lymphoprep 8% (±10.51SD), PrepaCyte-CB 5.73% (±4.04 SD) and Hetastarch 9.73% (±5.06 SD) (all illustrating p<0.005). Although plasma depletion also yielded a lower TNC recovery post thaw compared to Sepax, the difference was not statistically significant (Figure 47a).

Corresponding to the previously elaborated post-thaw results, Sepax demonstrated the highest TNC recovery 58.48% (±30.74 SD) compared to post-processing cord blood samples. Although, the recovery values for the others methods were lower: Ficoll/Lymphoprep 42.84% (±32.79 SD), PrepaCyte-CB 7.96% (±6.11 SD), Hetastarch 21.66% (±6 SD) and plasma depletion 24.75% (±24.89 SD), the difference was only significant for PrepaCyte-CB (p<0.005) (Figure 47a).

In conclusion, Plasma depletion yielded the highest total TNC recovery post processing of cord blood units, followed closely by Sepax and PrepaCyte-CB. Density gradient proved to be the least effective. Post freezing and consequent thawing of the cord blood units, TNC recoveries where calculated with respect to both the unprocessed cord blood TNCs as well as to the post-processing TNCs. Although, the comparison to post processing TNC is interesting to note, what is of greater value is the recovery compared to the starting TNC. As a general rule, when comparing post thaw TNCs to post-processing TNCs, the recoveries calculated look much better as the post-processing TNCs are usually lower than

that of the unprocessed whole blood. In this respect, Sepax yielded the highest post thaw recovery compared to whole cord blood, followed by plasma depletion. PrepaCyte-CB was the least efficient. Density gradient, although, not very effective, generated results comparable to Hetastarch.

Thus, it may be inferred that for immediate clinical transplantation post cord blood processing, plasma depletion might be considered to be the best option. However, it should be noted that plasma depletion removes only the plasma fraction of whole blood leaving behind the entire cellular compartment, including platelets. Transfusion of platelet concentrate may not be desired in a transplantation setting. Based on this, Sepax or PrepaCyte-CB might prove to be better alternatives.

In situations, where processed cord blood units are to be cryopreserved for future use, Sepax might be the superlative choice for cord blood separation as it yielded the highest TNC recovery post freezing and subsequent thaw.

For purposes of research in the laboratory, density gradient might be a good option for cord blood processing. Although the TNC yield post processing was not brilliant, post thaw TNC recovery was comparable to Hetastarch. In addition, density gradient-dependent protocols for cord blood processing, have the advantage of being very time and cost effective.

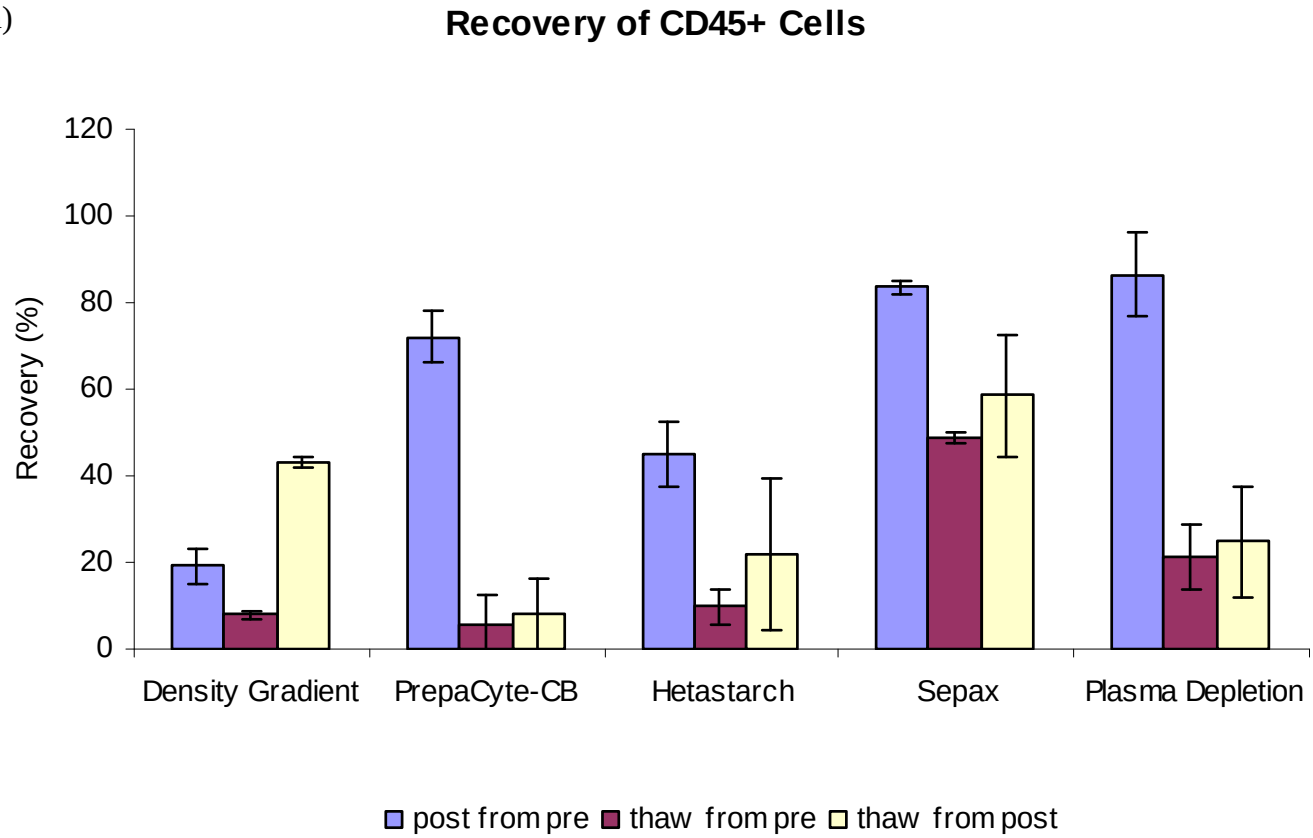
Conclusion

Immediate requirement for clinical transplantation entails PrepaCyte-CB or Sepax as the most appropriate option for cord blood processing.

Sepax is a better choice for cord blood processing if the units are aimed for cryopreservation.

For laboratory based research density gradient method of cord blood separation provides the most time and cost effective methodology.

(a)



(b)

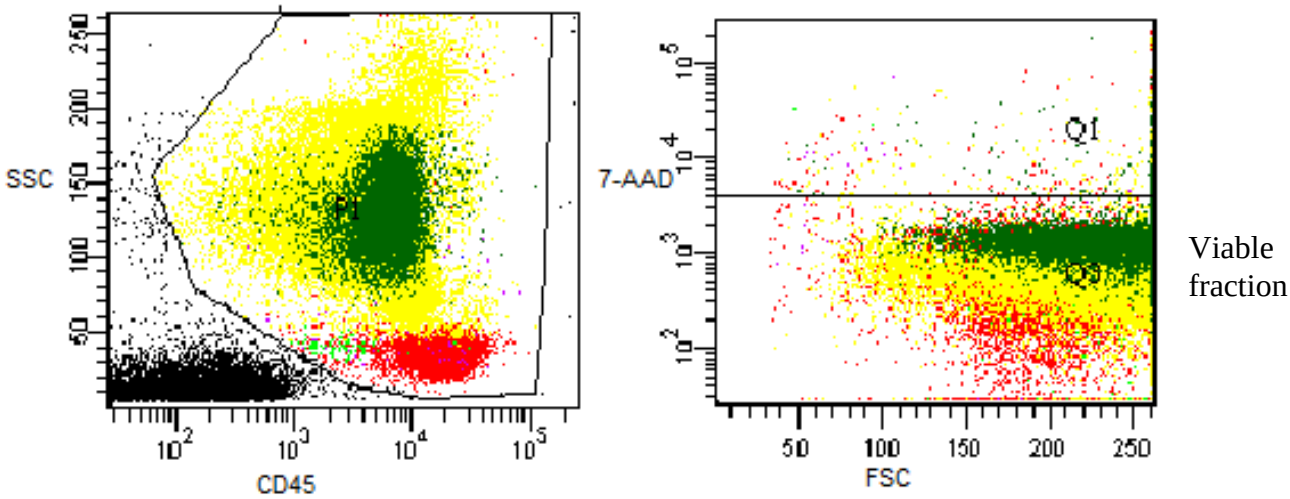


Figure 47. Recovery of nucleated cell fraction from cord blood post-processing and post-thaw for the different techniques investigated. (a) The graph demonstrates the percentage of TNC recovery during the different phases of cord blood processing. Post-processed samples compared with whole blood (post from pre), and post thaw. Post thaw analysis was done compared to pre-processed whole blood (thaw from pre) as well as compared to post-processed samples (thaw from post). (b) Dot plot FACS graphs illustrating TNC fraction (CD45) and percentage viability of this gated cell fraction (7AAD).

3.1.7 Haematopoietic Stem/progenitor cell population recovery

Haematopoietic stem/progenitor cells (HSC) are integrated in the nucleated cell compartment of blood. These cells may be categorized into three developmental stages based on expression of cell surface markers (McGuckin, Pearce et al. 2003; Christina Basford 2010).

- Early stage stem cells ($CD45^{+}low/CD34^{-}/CD133^{+}$)
- Mid stage stem cells ($CD45+low/CD34^{+}low/CD133^{+}$)
- Late stage stem cells ($CD45^{+}high/CD34^{+}/CD133^{-}$)

Recovery of CD34+ and $CD133^{+}$ cells was measured by using the nucleated cell count values from the differential cell counter combined with flow cytometric analysis, examining the $CD34^{+}$ and $CD133^{+}$ cells and their viabilities measured by uptake of 7AAD.

3.1.7.1 Early stage stem cells ($CD45^{+}/CD34^{-}/CD133^{+}$)

PrepaCyte-CB yielded the highest average recovery of 62.59% (±16.23 SD), followed closely by plasma depletion 60.1% (±28.5% SD). In comparison to the other methods: Hetastarch 48.4% (±26.25 SD), Sepax 48.5% (±15.66 SD) and Ficoll/Lymphoprep 13.66% (±9.36 SD) produced lower recoveries. PrepaCyte was significantly more efficient than Ficoll/Lymphoprep (p=8.87921E-07) but the difference in HSC recovery compared to the other methods was not statistically considerable (p>0.005) (Figure 48a).

Post thaw the highest average HSC recovery (compared to the original unprocessed samples) was noted in PrepaCyte 25.1% (±21.55 SD) and plasma depletion 25.5% (±10.2 SD). However, when compared to the other methods the difference was insignificant (p>0.005): Ficoll/Lymphoprep 8.16% (±6.67 SD), Hetastarch 20.4% (±19.6 SD) and Sepax 7.56% (±4.47 SD) (Figure 48a)

When post thaw counts were compared to the post-processed samples, the highest recovery was provided by Hetastarch 50.5% (±29.2 SD). The recoveries generated by the other methods were as follows: Ficoll/Lymphoprep 45.89% (±33.28 SD), PrepaCyte-CB 30.5% (± 25.39 SD), Sepax 23.23% (±20.23%) and plasma depletion 33.5% (±4.6 SD). The variation in the HSC recovery was not significant (p>0.005) (Figure 48a).

Conclusion

PrepaCyte-CB generated the highest recovery for early-stage HSCs post processing.

Density gradient proved to be the least effective in terms of early-stage HSCs recovery.

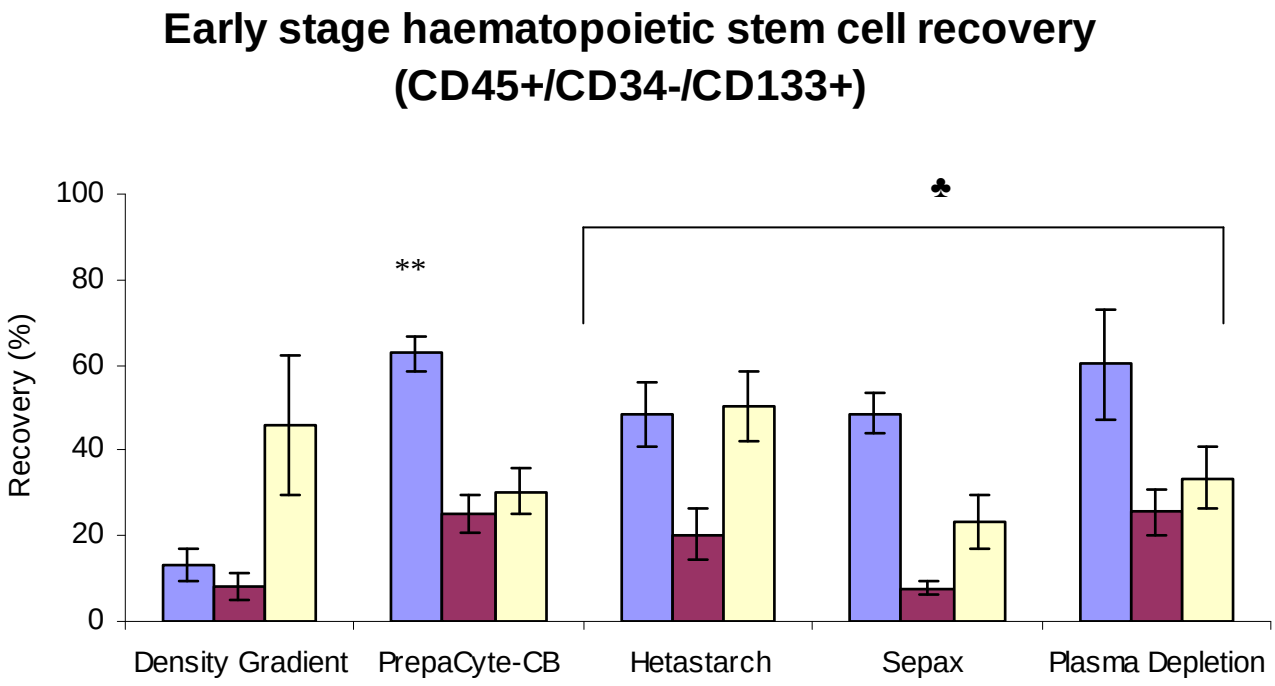
Post cryopreservation and consequent thaw, PrepaCyte-CB, once again, provided the highest recovery and density gradient, accompanied by Sepax were the least efficient.

Figure 48. Recovery of early haematopoietic stems cell population post-processing and thaw. (a) The graph demonstrates the percentage recovery of early stage haematopoietic stem cell (HSC) population post processing and post thaw. (b) Dot plot FACS graphs illustrating how early stage HSC fraction ($CD45^+/CD34^-/CD133^+$) were selected on FACS analysis. This cell population was gated within viable total nucleated cell (TNC) fraction ($CD45^+$). Percentage viability (7AAD negative) of each gated cell fraction is illustrated below each corresponding graph. (c) Quadrant FACS dot plot illustrating percentage of viable TNCs ($CD45^+$) positive for CD34 only (quadrant Q1-3), positive for CD133 only (quadrant Q13) and positive for both CD34 and CD133 (quadrant Q2-3). The cell population we were interested in was the cell population positive for CD133 and negative for CD34 (quadrant Q13). The cell Quadrant 3-3 is negative for both CD markers. Negative controls for each sample were the unstained samples (samples with secondary antibody alone) corresponding to the test sample for analysis.

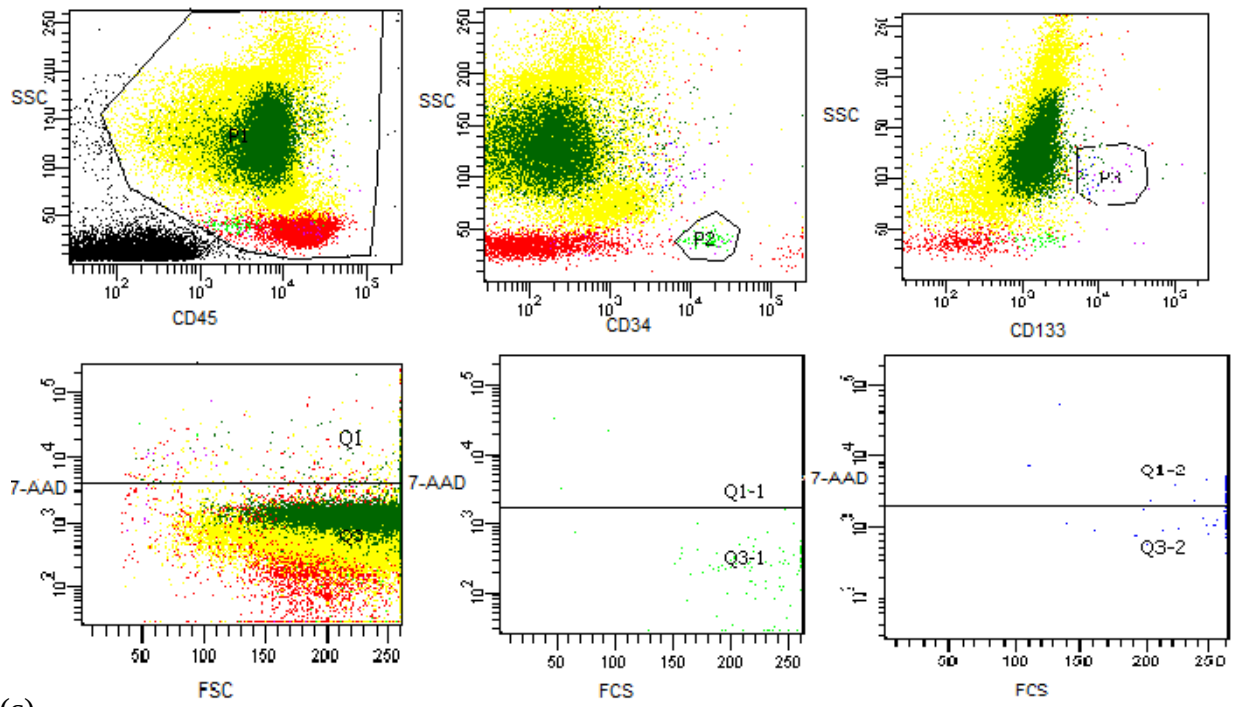
** $p<0.0000008$ (PrepaCyte was significantly more efficient than Density Gradient method)

♣ $p>0.005$ (HSC recovery by PrepaCyte was not significantly superior to the other methods of cord blood processing)

(a)


Early stage haematopoietic stem cell recovery
(CD45+/CD34-/CD133+)
Recovery (%)
100
80
60
40
20
0
**
♣
Density Gradient
PrepaCyte-CB
Hetastarch
Sepax
Plasma Depletion
post for Pre
thaw from pre
thaw from post

(b)


SSC
CD45
P2
CD34
P3
CD133
7-AAD
FSC
Q1
Q1-1
Q3-1
FCS
Q1-2
Q3-2
CD34
Q1-3
Q2-3
Q3
Q1 3
CD133

(c)

3.1.7.2 Mid stage haematopoietic stem cell ($CD45^+/CD34^+/CD133^+$) recovery

PrepaCyte yielded the highest recovery for mid stage HSCs 65.83% (±14.22 SD). Hetastarch and plasma depletion followed closely with recoveries of 64.21% (±17.89 SD) and 60.98% (±14.9 SD) respectively. Ficoll/Lymphoprep provided a recovery of 27.43% (±13.37 SD) and Sepax 54.3% (±26.79 SD). Although, both methods provided lower recoveries compared to PrepaCyte immediately post processing, the difference was not significant (p> 0.005).

Post thaw (average counts compared to pre-processing samples) the highest average mid stage HCS recovery was achieved by Sepax 67.99% (±134.35 SD). This was significantly more efficient than plasma depletion 21.78% (± 34.35) (p=0.005) and PrepaCyte 24.33% (±14.21 SD) (p=0.005). But, Hetastarch 26.7% (±22.69) and Ficoll/Lymphoprep 45.38% (±40.09) although demonstrating lower recoveries, the variance was not significant (p>0.005).

Post thaw average counts compared to post-processing counts showed Sepax to be the most efficient in terms of HSC recovery, 76.70% (±16.56 SD). This was significantly more efficient than plasma depletion, 26.39% (±15.56 SD) (p=0.005). Ficoll/Lymphoprep 46.85% (± 23.79 SD), PrepaCyte 37.94% (±28 SD) and Hetastarch 34.31% (±28.51 SD) demonstrated recoveries lower than Sepax, the difference did not prove to be statistically significant (p> 0.005).

Conclusion

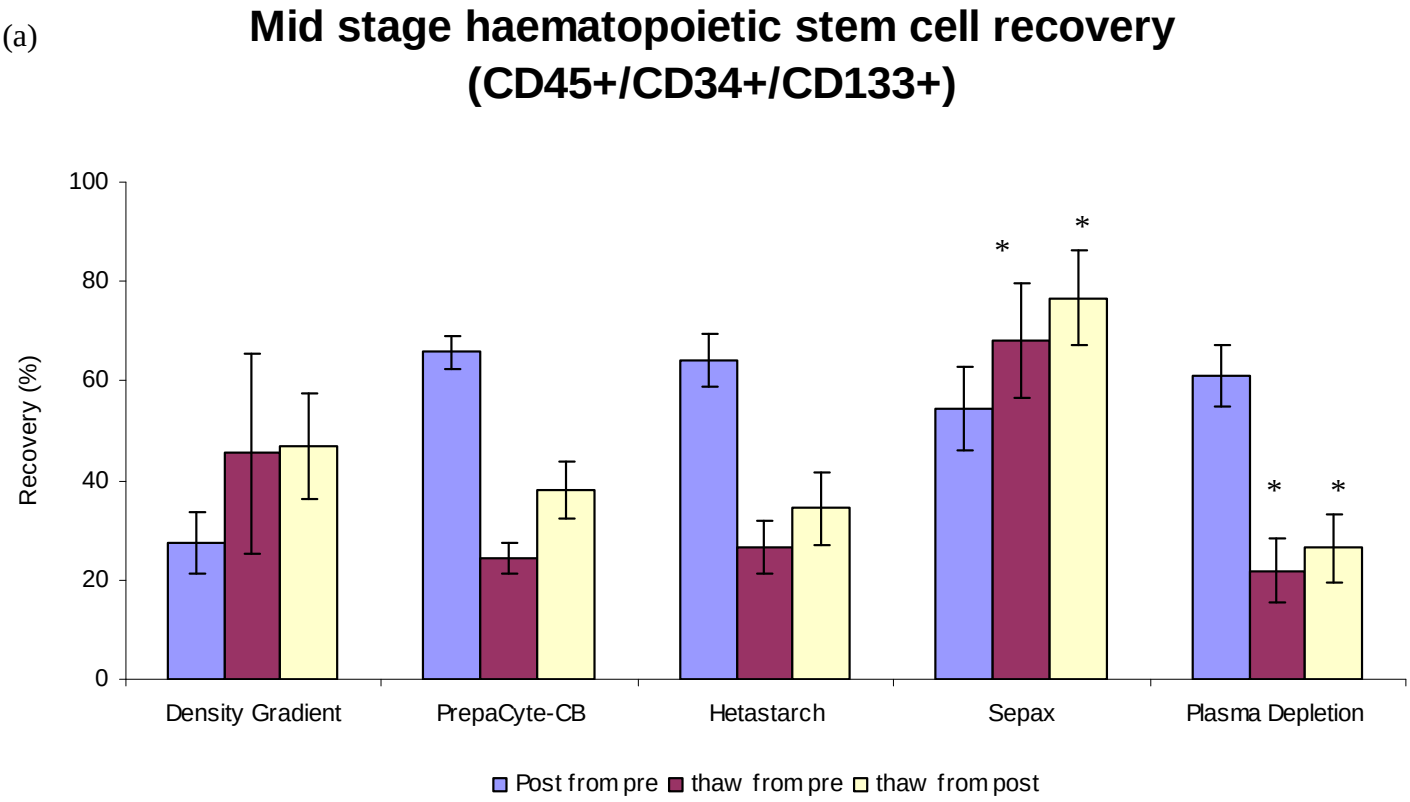
PrepaCyte-CB proved to the most efficient for mid-stage HSC recovery post processing. This result was followed closely by plasma depletion and Hetastarch. Density gradient was the least efficient.

Post-thaw, Sepax provided the highest recovery, followed by density gradient. Plasma depletion was the least effective.

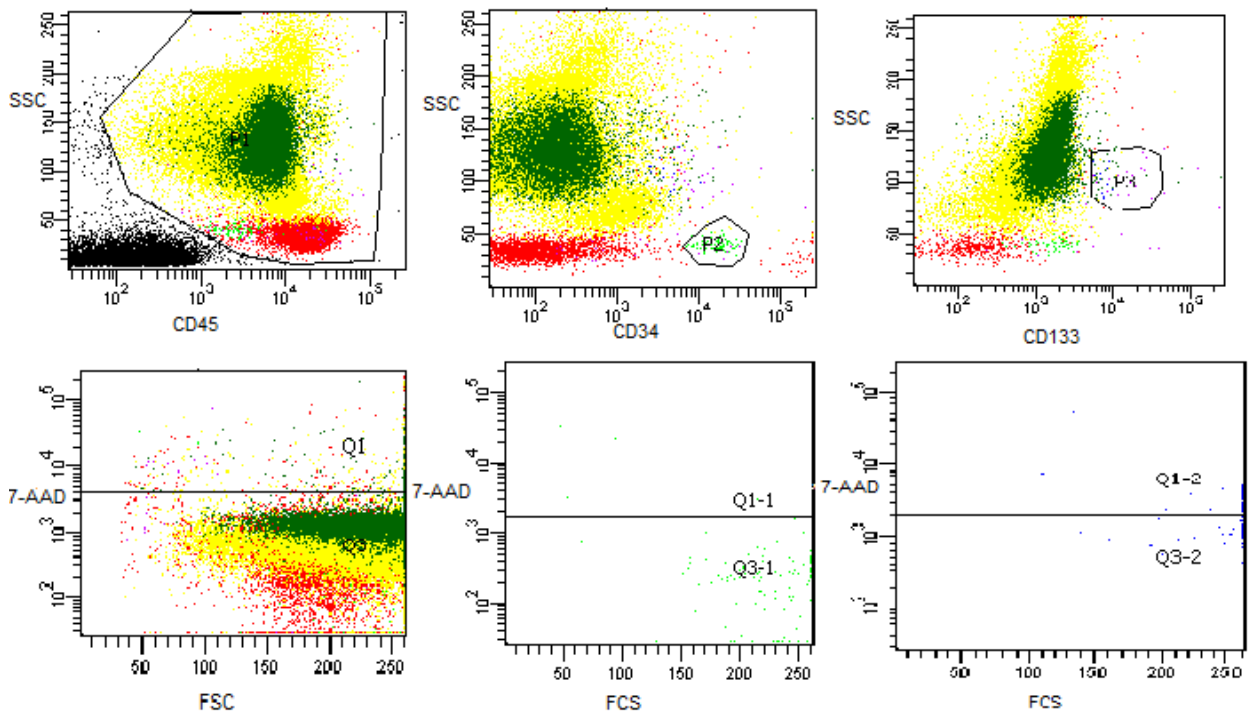
Figure 49. Recovery of mid stage haematopoietic stem cell population post-processing and thaw. (a) The graph demonstrates the percentage recovery of mid stage haematopoietic stem cell (HSC) population post processing and post thaw. (b) Dot plot FACS graphs illustrating mid stage HSC fraction ($CD45^+/CD34^+/CD133^+$). This cell population was gated within viable total nucleated cell (TNC) fraction ($CD45^+$). Percentage viability (7AAD negative) of each gated cell fraction is illustrated below each corresponding graph. (c) Quadrant FACS dot plot illustrating percentage of viable cells positive for CD34 only (quadrant Q1-3), positive for CD133 only (quadrant Q13) and positive for both CD34 and CD133 (quadrant Q2-3). Quadrant 3-3 is negative for both CD markers. Negative controls for each sample were the unstained samples (samples with secondary antibody alone) corresponding to the test sample for analysis.

*** $p<0.005$ (Sepax demonstrated the highest average HSC revovery post thaw and this was statistically sginificant when compared to plasma depeltion recoveries)**

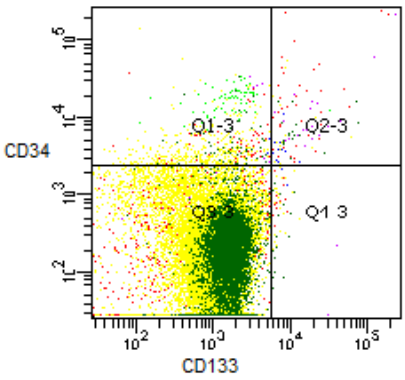
(a)


Mid stage haematopoietic stem cell recovery
(CD45+/CD34+/CD133+)
Recovery (%)
100
80
60
40
20
0
*
*
*
*
Density Gradient
PrepaCyte-CB
Hetastarch
Sepax
Plasma Depletion
Post from pre
thaw from pre
thaw from post

(b)


SSC
CD45
P1
SSC
CD34
P2
SSC
CD133
P3
7-AAD
FSC
Q1
7-AAD
FCS
Q1-1
Q3-1
7-AAD
FCS
Q1-2
Q3-2

(c)


CD34
CD133
Q1-3
Q2-3
Q1 3

3.1.7.3 Late stage haematopoietic stem cell (HSC) (CD45+/CD34+) recovery

PrepaCyte yielded the highest average recovery for late stage HSCs, 73.92% (±14.42 SD). This recovery was appreciably greater than the recoveries achieved by Ficoll/Lymphoprep 39.18% (±27.03 SD) (p=0.0003), Hetastarch 48% (±29.55 SD) (p=0.001) and plasma depletion 45.58% (±23.19 SD) (p=0.0006). Sepax, however, provided an average recovery which although slightly less then PrepaCyte, was still comparable in efficiency; 68.70% (±19.88 SD) (p=0.406).

Late stage HSC recoveries yielded post freeze-thaw (compared to the original unprocessed units) demonstrates highest average recoveries for Sepax 53.83% (±15.93 SD). The results observed were superior to recoveries obtained by PrepaCyte 30.59% (±17.01 SD) (p=0.001) and Hetastarch 24.93% (±24.55 SD) (p=0.004). The recoveries acquired by Ficoll/Lymphoprep 26.06% (±35.76 SD) and plasma depletion 25.21% (± 25.81 SD), while lower than PrepaCyte were not statistically considerable (p>0.005).

Post thaw recoveries compared to post processing samples illustrated highest recovery by Sepax 63.20% (±17.23 SD). The recoveries obtained by the other four techniques were less efficient, but the variance was not major (p>0.005): Ficoll/Lymphoprep 35.96% (±31.78 SD), Hetastarch 41.89% (±28.11 SD) and plasma depletion 36.20% (±36.97 SD). PrepaCyte, however, was significantly less efficient, providing an average recovery of 38.49% (±19.85 SD) (p=0.004).

Conclusion

PrepaCyte-CB proved to be the most efficient for the recovery of late-stage HSCs post processing, followed by Sepax. Density gradient was the least effective.

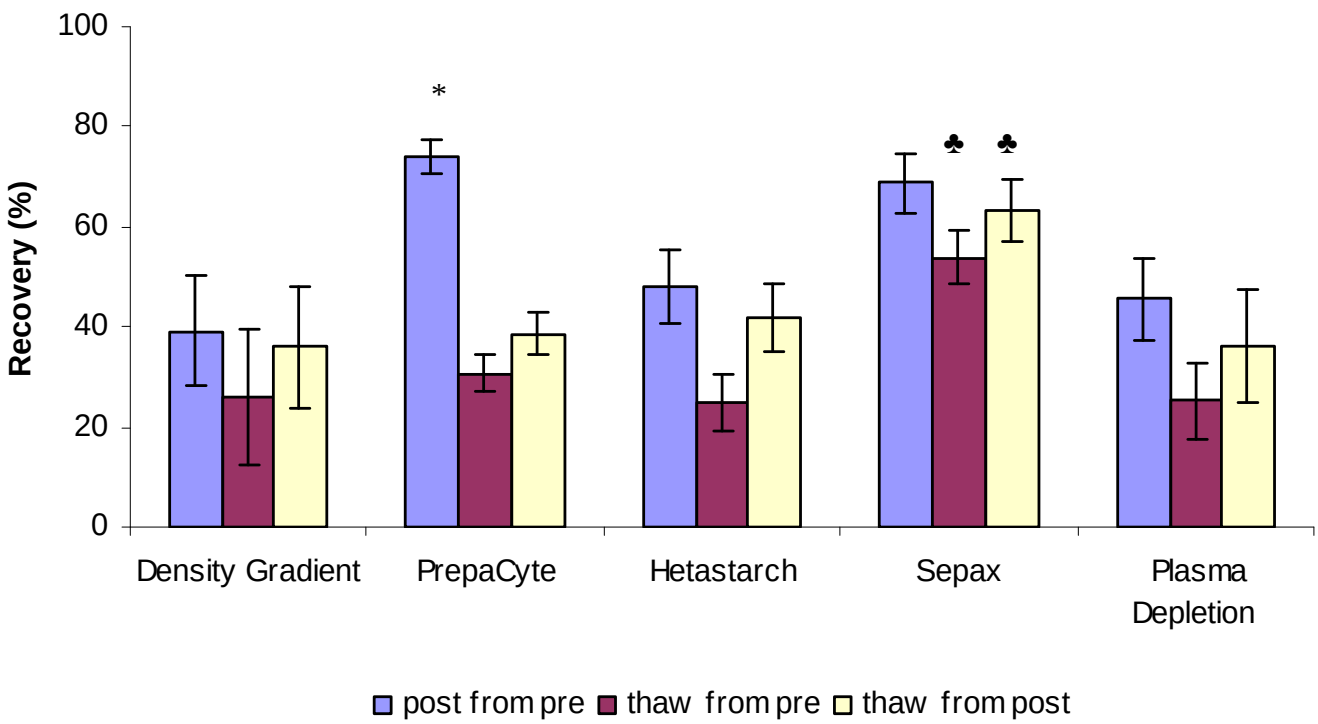
Post thaw, the highest recovery was noted in Sepax. Density gradient, although proving to be the least efficient, generated results that were comparable to the other three techniques.

Figure 50. Recovery of late stage haematopoietic stem cell population post-processing and thaw. (a) The graph demonstrates the percentage recovery of late stage haematopoietic stem cell (HSC) population post processing and post thaw. (b) Dot plot FACS graphs illustrating late stage HSC fraction ($CD45^+/CD34^+$). This cell population was gated within viable total nucleated cell (TNC) fraction ($CD45^+$). Percentage viability (7AAD negative) of each gated cell fraction is illustrated below each corresponding graph. Negative controls for each sample were the unstained samples (samples with secondary antibody alone) corresponding to the test sample for analysis.

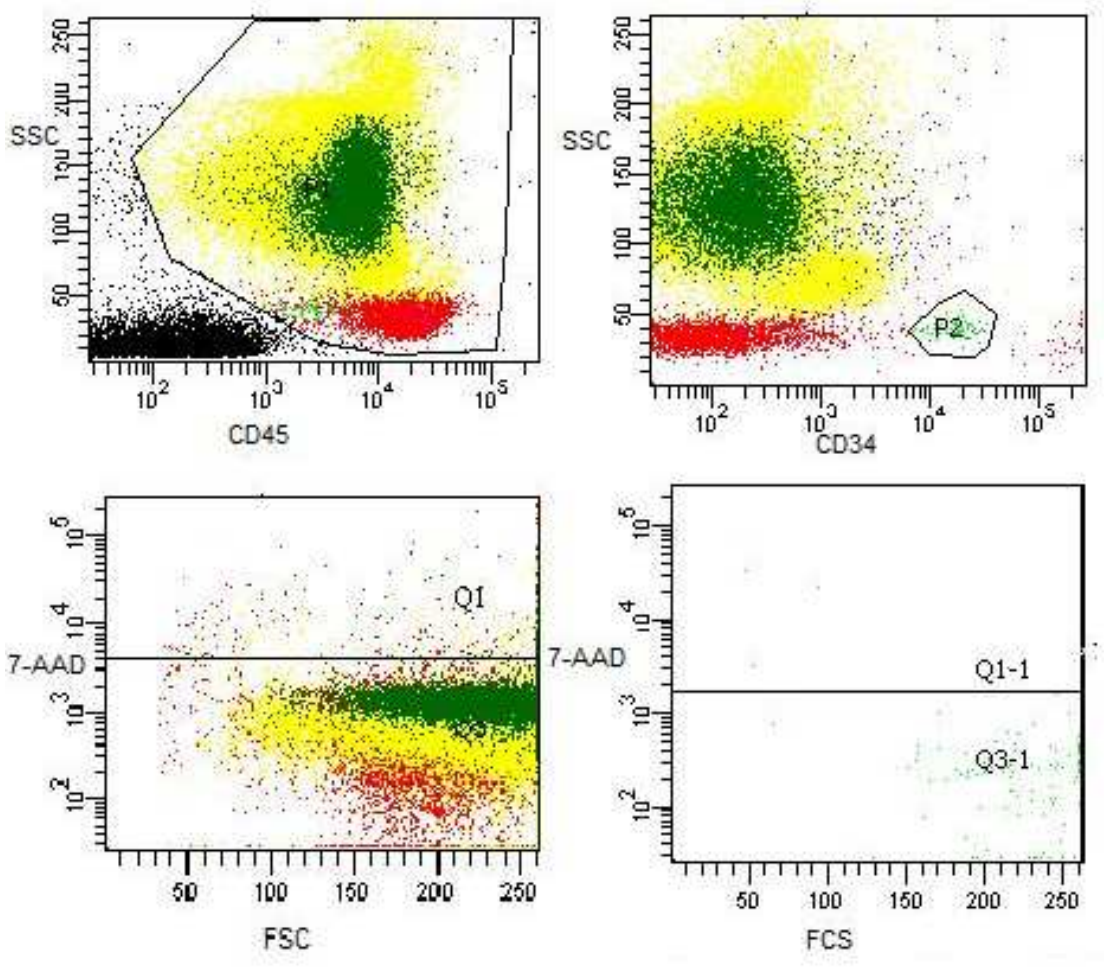
*** $p<0.005$ (PrepaCyte yielded highest HSC recovery post processing and this was significantly superior to Density Gradient, Hetastarch and plasma depletion)**

♣ $p<0.005$ (Sepax yieled highest HSC recovery post thaw and this was significantly superior to PrepaCyte and Hetastarch)

(a)


Late stage haematopoietic stem cell (CD45+/CD34+) recovery
Recovery (%)
100
80
60
40
20
0
*
♣
♣
Density Gradient
PrepaCyte
Hetastarch
Sepax
Plasma Depletion
post from pre
thaw from pre
thaw from post

(b)


SSC
CD45
SSC
P2
CD34
7-AAD
Q1
FSC
7-AAD
Q1-1
Q3-1
FCS

3.1.8 Non-haematopoietic stem cell (Lineage Negative cell fraction) recovery

It has been postulated that cord blood constitutes both haematopoietic as well as non-haematopoietic stem/progenitor cells. We investigated the non-haematopoietic cell fraction by using total nucleated cell counts provided by the differential cell counter and the data generated by flowcytometry. The Lineage negative population was characterized as the percentage of cells that tested negative for CD235a or Glycophorin-A (RBCs and reticulocytes), CD45 (T cells, B cells and monocytes), CD33 (myeloid-specific and some lymphoid cells) and CD7 (T cells) on flowcytometric analysis. Cell viability as assessed by 7AAD uptake was taken into consideration each time. The recovery of this primitive cell population was calculated for each processing technique both immediately post cord blood separation and post thawing of the cryopreserved processed samples.

Post-processing the highest recovery was noted in Sepax 77.31% (±45.79 SD). PrepaCyte and plasma depletion were the next best in line, with recoveries of 59.44 % (±7.38 SD) and 57.99% (±22.04 SD) respectively. Ficoll/Lymphoprep 10.02% (±5.36 SD) and Hetastarch 15.77% (±11.69 SD) were considerably less efficient in terms of LinNeg recovery (p=0.001 and p=0.003 respectively).

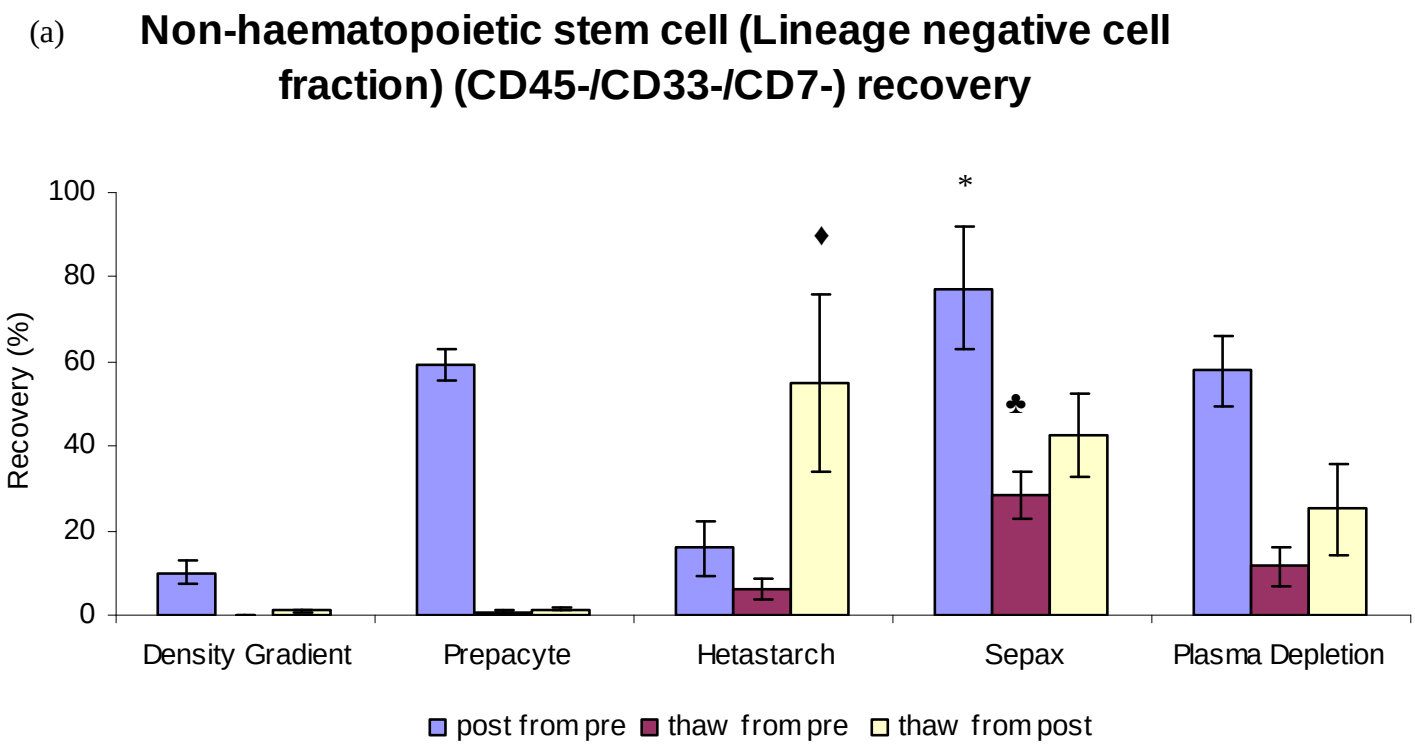
Post thaw (compared to unprocessed samples) the highest LinNeg recovery was also noted in Sepax 28.39% (±18.06 SD). This was a lot better that Ficoll/Lymphoprep 0.093% (±0.04 SD) (p=0.0008), PrepaCyte 0.79% (±0.46 SD) (p=0.0009) and Hetastarch 6.29% (±4.01 SD) (p=0.004). Plasma depletion illustrated a recovery of 11.46% (±12.48 SD) which, although lower that the recovery noted in Sepax separation, the difference was not statistically noteworthy.

Post thaw (compared to post-processing samples) the highest LinNeg recovery was demonstrated by Hetastarch 54.98% (±36.11 SD). The post thaw recoveries noted in all the other separation methods was less efficient; Ficoll/Lymphoprep 0.95% (±0.23 SD), PrepaCyte 1.29% (±0.66 SD), plasma depletion 25.02% (±29.3 SD) and Sepax 42.56% (±30.98 SD). The difference observed was statistically notable for density gradient (p=0.001) and PrepaCyte-CB (p=0.001).

Conclusion

Sepax was the most effective for recovery of lineage negative cells post processing, followed by PrepaCyte-CB. Density gradient yielded the least efficient recovery, but was comparable to Hetastarch.
Post thaw, Sepax once again proved to be the most efficient and density gradient was the least effective.

(a)



(b)

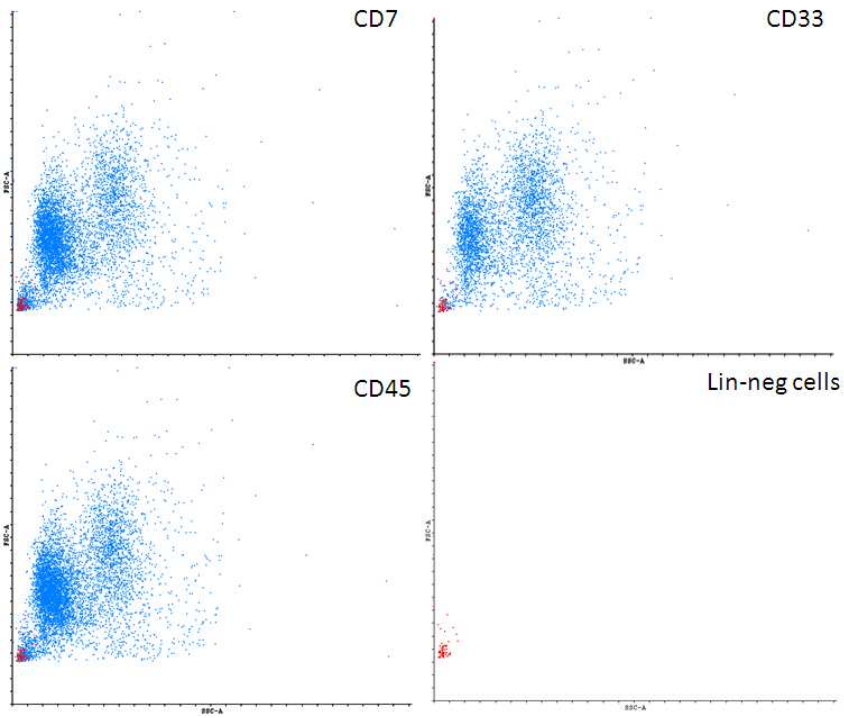


Figure 51. Recovery of lineage negative cell fraction post-processing and thaw. (b) Dot plot FACS graph illustrating Lineage negative cells (cell fraction negative for CD45, CD7 and CD33). Controls for each sample were the unstained samples (samples with secondary antibody alone) corresponding to the test sample for analysis.

* $p<0.005$ (Sepax demonstrated the highest Lineage negative fraction recovery post-processing. This was significantly superior to Density Gradient and Hetastarch methods of cord blood separation).

♣ $p<0.005$ (Sepax demonstrtaed the hightest post thaw yield compared to pre-processed cord blood.

♦ $p<0.005$ (Hetastarch demonstrated the highest LinNeg yield post thaw when compared with post-processed cord blood units).

3.1.9 Conclusion

Taken as a whole, the results generated in this study indicated that the best way forward for translational cellular medicine where cord blood transfusion or transplantation is demanded immediately is PrepaCyte-CB. PrepaCyte-CB showed very efficient TNC recovery post processing of fresh cord blood units. Additionally, this method proved very effective for RBC depletion and subsequent volume reduction. Recoveries of HSCs and lineage negative cellular fractions were also notably superior to that observed in the other techniques.

In situations where cord blood units are to be cryopreserved for possible future use then Sepax might be a better alternative for the processing of cord blood units. The highest TNC recovery post thaw was noted for Sepax. Similarly, the recoveries of both HSCs and non-haematopoietic stem cells (lineage negative stem cells) were very efficient. The only limitation of this processing technique is that it was not very effective in RBC depletion and volume reduction, which is a very important issue for cord blood banking.

In terms of *in vitro* research, density gradient method of cord blood processing is probably a good choice. This method was the most effective in RBC depletion. This is a matter of prime importance in cell culture. Remnant RBCs in culture eventually undergo lysis releasing various cytokines into the culture media. These cytokines provide signalling cues to stem/progenitor cells in culture ushering them towards undesired haematopoietic cell fates. Although, this technique proved to be the least effective in terms of TNC, HSC and lineage negative cell recovery post processing, the recoveries post thaw were acceptable and in some cases comparable to Sepax and PrepaCyte-CB. Furthermore, as noted earlier, density gradient was very time and cost effective to use.

3.2 Colony forming units

Clonogenic potential has been used as a measure for more mature haematopoietic progenitor cells. Each colony formed represents the progeny of a single pluripotent/multipotent stem cell. Hence, operationally, the number of colony forming units is a measure of the numbers of stem cells. The clonogenic potential of the cord blood samples, both pre and post-processing with the different separation techniques, were investigated. PrepaCyte generated the highest average CFU potential, 1327 CFU/10^6 cells post-processing. This was notable more efficient than the clonogenic potential noted in Ficoll/Lymphoprep 554.64 CFU/10^6 cells (p=0.00024), plasma depletion 859 CFU/10^6 cells (p=4.78E10-6). Average CFU counts in Sepax and Hetastarch treated units were only slightly inferior to PrepaCyte; Sepax 1110 CFU/10^6 cells and Hetastarch 1153 CFU/10^6 cells (both p values > 0.005).

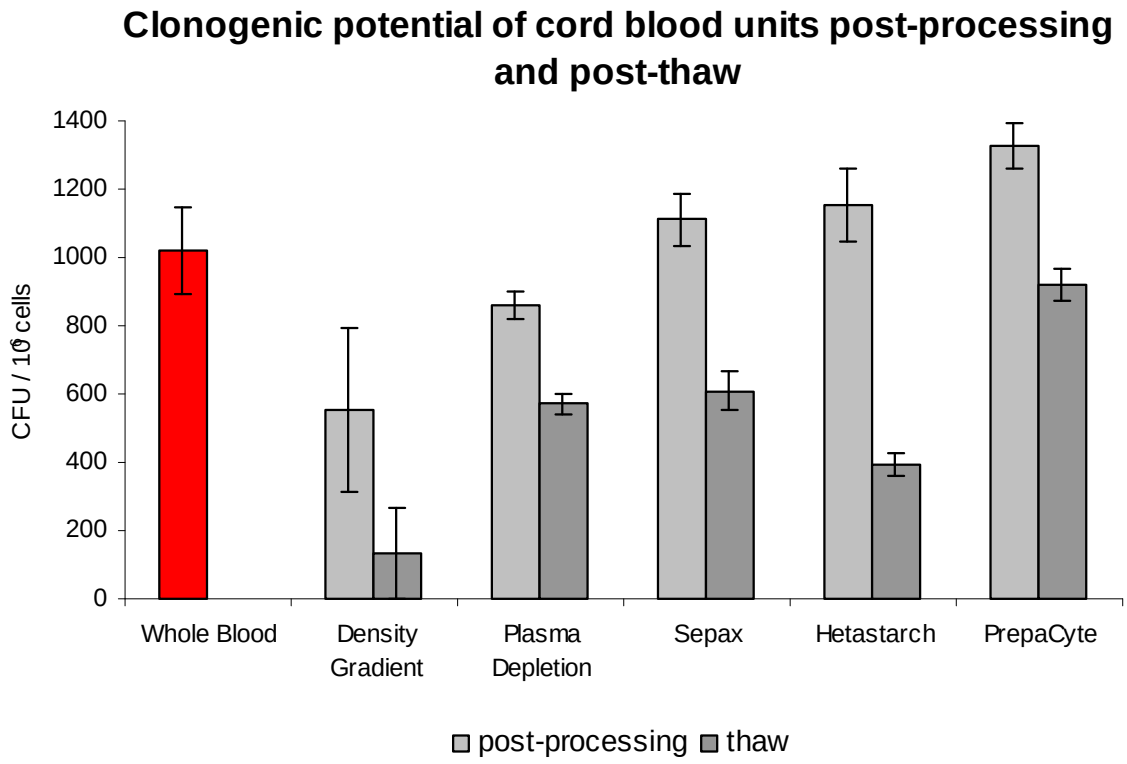
Post freeze-thaw procedure, CFU capacity was once again highest in PrepaCyte treated samples, 922.52 CFU/10^6 cells. This count was significantly better than the CFU units noted in the other techniques: Ficoll/Lymphoprep 133.33 CFU/10^6 cells (p=9.606E10-6), plasma depletion 540 CFU/10^6 cells (p=3.147E10-6), Sepax 608 CFU/10^6 cells (p=0.003) and Hetastarch 394.37 CFU/10^6 cells (p=9.29E10-11).

Conclusion

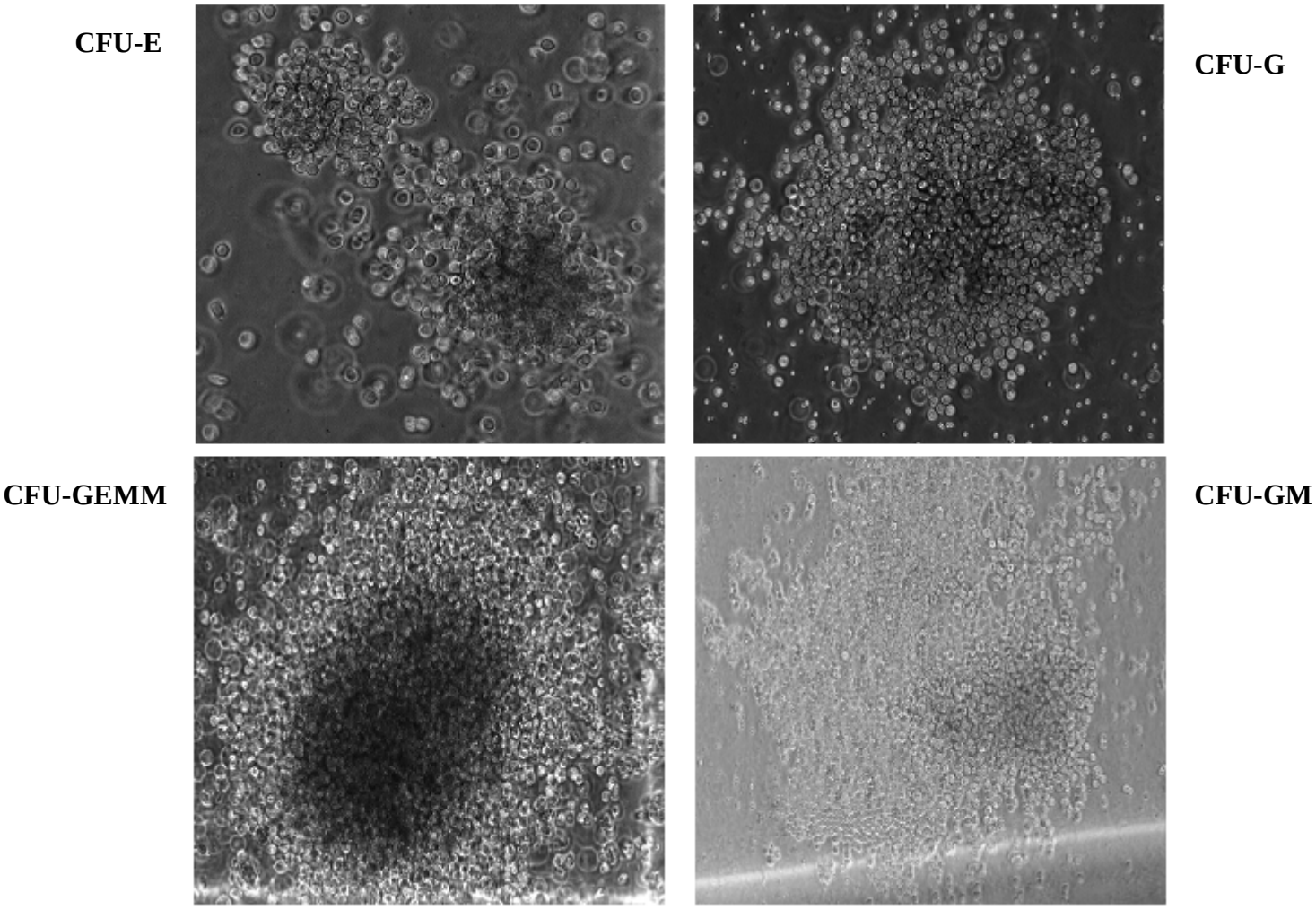
PrepaCyte-CB yielded the highest average CFUs, followed by Sepax and Hetastarch. Density gradient generated the lowest CFU count.

Figure 52. Clonogenic potential of cord blood samples post processing and post thaw. (a) A graphical presentation of the colony forming unit (CFU) potential of cord blood samples after processing and after subsequent thaw. (b) DIC images of CFUs. CFU-E (colony forming unit-erythroid): clonogenic progenitors that represent the more mature erythroid progenitors. They have less proliferative capacity. CFU- G (colony forming unit- granulocytes): clonogenic progenitors of granulocytes that give rise to a homogenous population of eosinophils, basophils or neutrophils. CFU-GM (colony forming unit- granulocyte, macrophage): progenitors that give rise to colonies containing a heterogeneous population of macrophages and granulocytes. CFU-GEMM (colony forming unit- granulocyte, erythrocyte, macrophage, and megakaryocyte): formed by the most primitive of progenitors in cord blood giving rise to erythroid, granulocyte, macrophage and megakaryocyte lineages. Images were taken at magnification of x20.

(a)


Clonogenic potential of cord blood units post-processing and post-thaw
CFU / 10^5 cells
1400
1200
1000
800
600
400
200
0
Whole Blood
Density Gradient
Plasma Depletion
Sepax
Hetastarch
PrepaCyte
post-processing
thaw

(b)


CFU-E
CFU-G
CFU-GEMM
CFU-GM

3.3 Analysis Discussion

During the analysis no one method came out consistently as best; so a league table was devised. Each method was rated for all the 7 analysis methods discussed above and was awarded 3 points for 1st place, 2 points for 2nd place and finally 1 point or 3rd place.

As mentioned in the previous section, depending on the primary use of UCB, different processing methods may be more applicable than others. Density gradient was the most efficient for RBC depletion; Plasma depletion and Sepax provided the highst TNC recoveries post processing and PrepaCyte-CB demonstrated the highest recovery for HSC populations. Based on the league table, however, PrepaCyte-CB was noted to be the best performer for many of the analysis methods. PrepaCyte-CB has the added advantage of being a closed system and hence is associated with a much reduced risk of contamination during processing. This is of particular importance to the developing world where access to a clean room is not economically feasible. Additionally, PrepaCyte does not require expensive laboratory equipment and this is a significant issue in less economically developed parts of the world where transplants are rarely peformed due to lack of necessary resources and facilities. It is worth noting that UCB transplants are currently unavailable to large sections of the second and third world countries. Thus, the choice of UCB processing should be carefully addressed with the view that UCB is readily available in a suitable form for patient treatment.

Table 32. Processing League Table

	Performance position and frequency			
Methods	**1st[a]**	**2nd[b]**	**3rd[c]**	**Points**
PrepaCyte-CB	4	2	1	17
Sepax	1	2	2	9
Plasma Depletion	1	1	2	7
Hetastarch	0	2	3	7
Density Gradient	1	0	0	3

[a]1st place worth 3 points, [b]2nd place worth 2 points and [c]3rd place worth 3 points
(7 analysis methods evaluated)

3.4 Isolation of stem/progenitor cells from cord blood by negative depletion

It has been demonstrated in the previous sections (section 3.1.7 and 3.1.8) that regardless of the technique utilized to process cord blood units, both haematopoietic and non-haematopoietic stem/progenitor cells were identified and characterized in cord blood samples.

Our subsequent aim was to investigate whether the desired cell populations could be selectively isolated and concentrated using the StemSep 'human primitive progenitor cell enrichment kit' and our in-house Lineage negative selection protocol.

The StemSep depletion kit is tailored to highly enrich $CD34^+$, $CD36^-$, $CD38^-$ and $CD45RA^-$ haematopoietic progenitors from fresh cord blood. Antibodies to 12 lineage-specific cell surface antigens are used to target mature, fully differentiated cells as well as lineage-committed progenitor cells for removal. The depletion cocktail includes antibodies to the following human cell surface markers: (CD2, CD3, CD14, CD16, CD19, CD24, CD36, CD38, CD45RA, CD56, CD66b, glycophorin A) (14057C; StemCell Technologies).

Cord blood buffy layer, post processing with Ficoll, was further treated with StemSep kit, which effectively rid the samples of any remnant RBCs, including reticulocytes (CD24, CD36 and glycophorin A); T cells (CD2, CD3, CD38, CD45RA, CD56); B cells (CD19, CD38, CD45RA); Natural killer cells (CD2, CD16, CD56); monocytes and macrophages (CD14, CD16, CD36, CD45RA); granulocytes (CD14, CD16, CD66b); lymphoid and myeloid precursors (CD24, CD56) and platelets (CD36). The negative selection post StemSep treatment contained cells of interest and was investigated for a number of stem cell markers by flowcytometric analysis: CD90, CD133, CD34, SSEA-4 (Stage-specific embryonic antigen 4). In each case, cell viability with 7-AAD was noted (Figure 53)

Likewise, buffy cells were treated with in-house Lineage negative selection protocol. This procedure employed glycophorin-A, which rid the samples of RBC remnants post Ficoll; CD45, which eliminated T, B cells and monocytes; CD33, which removed myeloid derivatives and some lymphoid cells and, CD7, which rid of T cells. Post procedure the negative cell selection was analysed for the stem cell markers noted above (Figure 53).

Both methods resulted in a significant further reduction in CD45 cell population post Ficoll: StepSep, an additional 8.7% reduction (p=0.0005) and in-house protocol, an additional 9.3% reduction (p=0.0003).

Post treatment with StemSep, $CD90^+$ cells (haematopoietic stem cell marker) illustrated a relative increase in cell concentration (p=8.17E-06). This was attributable to the selective removal of the undesired mature leukocytes and leukocyte precursors, leaving behind the cells of interest. Our in-house protocol also demonstrated a similar effect. The $CD90^+$ cell concentration, although lower than that noted in StemSep, was not notably inferior (p>0.005).

The CD34 recovery after StemSep treatment was slightly abridged compared to post Ficoll but the difference was not noteworthy (p>0.005). This small decrease in cell number was probably contributed to the elimination of late stage haematopoietic stem cells, which illustrate a strong expression of CD45; leaving behind early and mid-stage haematopoietic stem cells and lineage negative cells. Our in-house protocol, however, was unable to demonstrate a similar result. We noted a considerable decrease in the number of $CD34^+$ cell concentration (p=2.24E-05). This outcome was most likely due to the many washes incorporated in this protocol, which lead to cell loss. In addition, the sequential introduction of antibodies to the cells lead to prolongation of the protocol and hence contributed to decreased cell viability.

$CD133^+$ cells constitute a very small percentage of the cellular compartment of blood. This makes them a challenge to study due to low cell numbers, which undergoes further reduction during processing. StemSep failed to show a marked change in $CD133^+$ cell population compared to post Ficoll treated samples. Lineage Negative protocol, however, demonstrated a modest increase in $CD133^+$ concentration, which although small was statistically appreciable (p=0.0015).

We next examined the expression of SSEA-4, an embryonic stem cell marker. Both methods demonstrated a notable relative increase in the concentration of SSEA-4 positive cells post processing: StemSep (p=0.0006) and LinNeg (p=3.13E-07). Although, StemSep illustrated somewhat better results, the difference was not statistically significant (p>0.005).

So, it may be concluded that StemSep facilitated the selective isolation of mid-stage HSCs, as noted by efficient further reduction of $CD45^+$ cells (considerably eliminating late-stage HSCs that show strong CD45 expression) and a relative increase in the concentration of CD90 and CD34 positive cells. This kit, however, failed to demonstrate a notable increase in $CD133^+$ cell concentration, and thus did not prove very effective in the selection and concentration of early HSC populations. The relative increase in the concentration of SSEA-4 positive cells was impressive, demonstrating the ability of the kit to isolate and

concentrate stem/progenitor cells with ES cell attributes.

Our in-house protocol demonstrated better isolation for early HSCs (more efficient $CD133^+$ cell selection) compared to StemSep. Although CD90, CD34 and SSEA-4 positive cell recovery was less efficient then StemSep, the difference was not statistically significant.

Conclusion

Although our in-house protocol did prove effective in the selection and concentration of early HSC populations and non-haematopoietic stem cells, taking the overall results into view, it was decided that StemSep was a more efficient method.

StemSep protocol involved fewer steps, fewer washes and less manipulation of cells *in vitro*.

Lineage Negative protocol, on the other hand, was labour intensive. It was a very long protocol involving multiple steps, several washes and more cell manipulation. The prolonged *ex vivo* cell processing procedure involved, in addition to reducing cell viability also greatly increased the risk of bacterial and cellular contamination. It was for these reasons that it was decided to discontinue the lineage negative method and continue with StemSep kit for all further experiments.

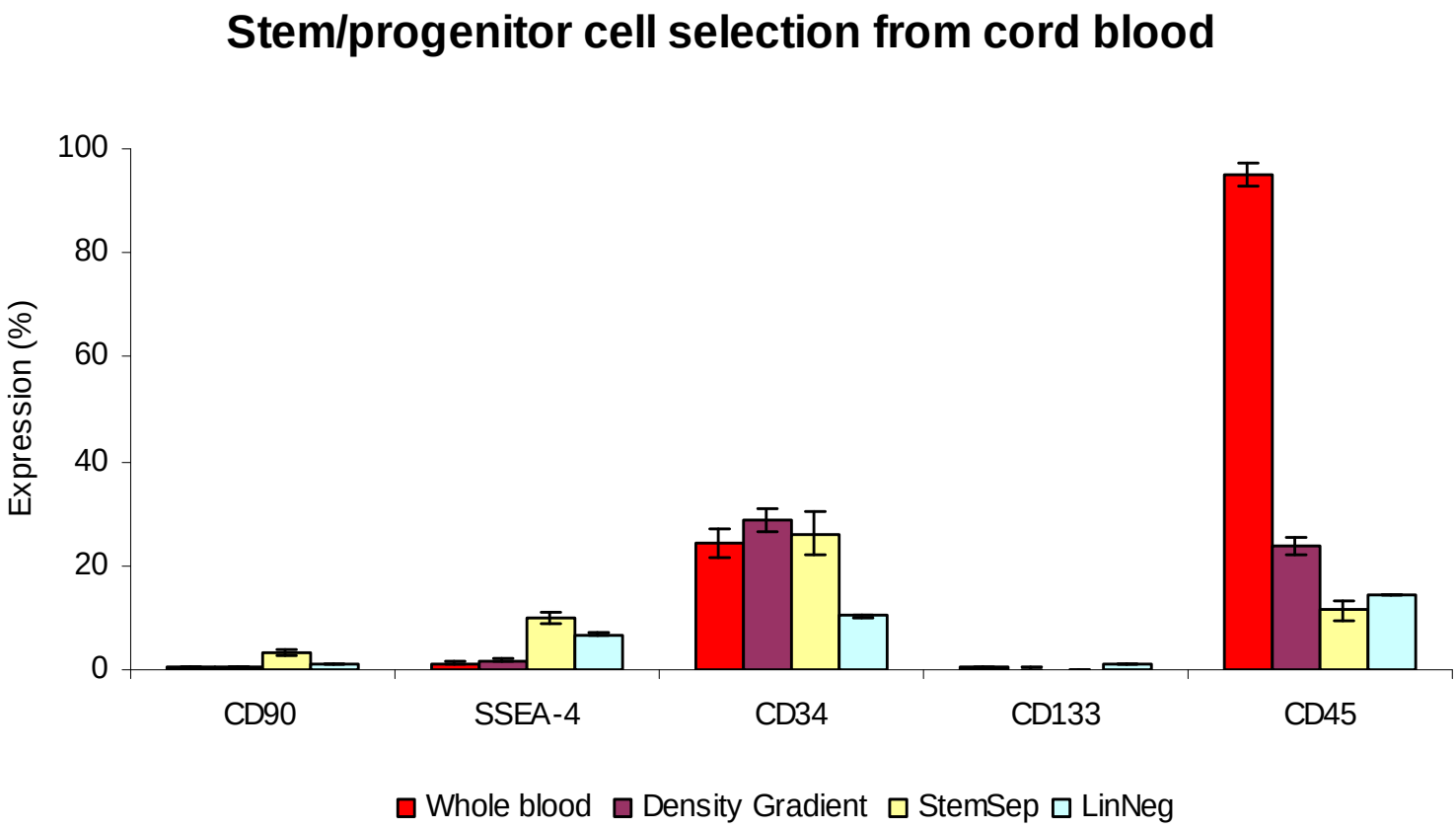


Figure 53. Percentage of expression of haematopoietic stem cell markers in cord blood post isolation using StemSep kit versus in-house Linegae negative protocol. The graph illustrates that StemSep facilitated the selective isolation of mid stage HSCs (greater $CD45^+$ cell removal decreasing late stage HSC population; these cells demonstrate strong CD45 expression). Simultaneous increase in SSEA-4 concentration illustrated the ability of the kit to isolate cells with embryonic stem cell attributes. In-house protocol proved to be more efficient for early stage HSC selection (more efficient $CD133^+$ cell selection) compared to StemSep. Although the resulting cellular expression of CD90, CD34 and SSEA-4 was less efficient than SetmSep the difference was not statistically significant (p>0.005). N=6

(b)

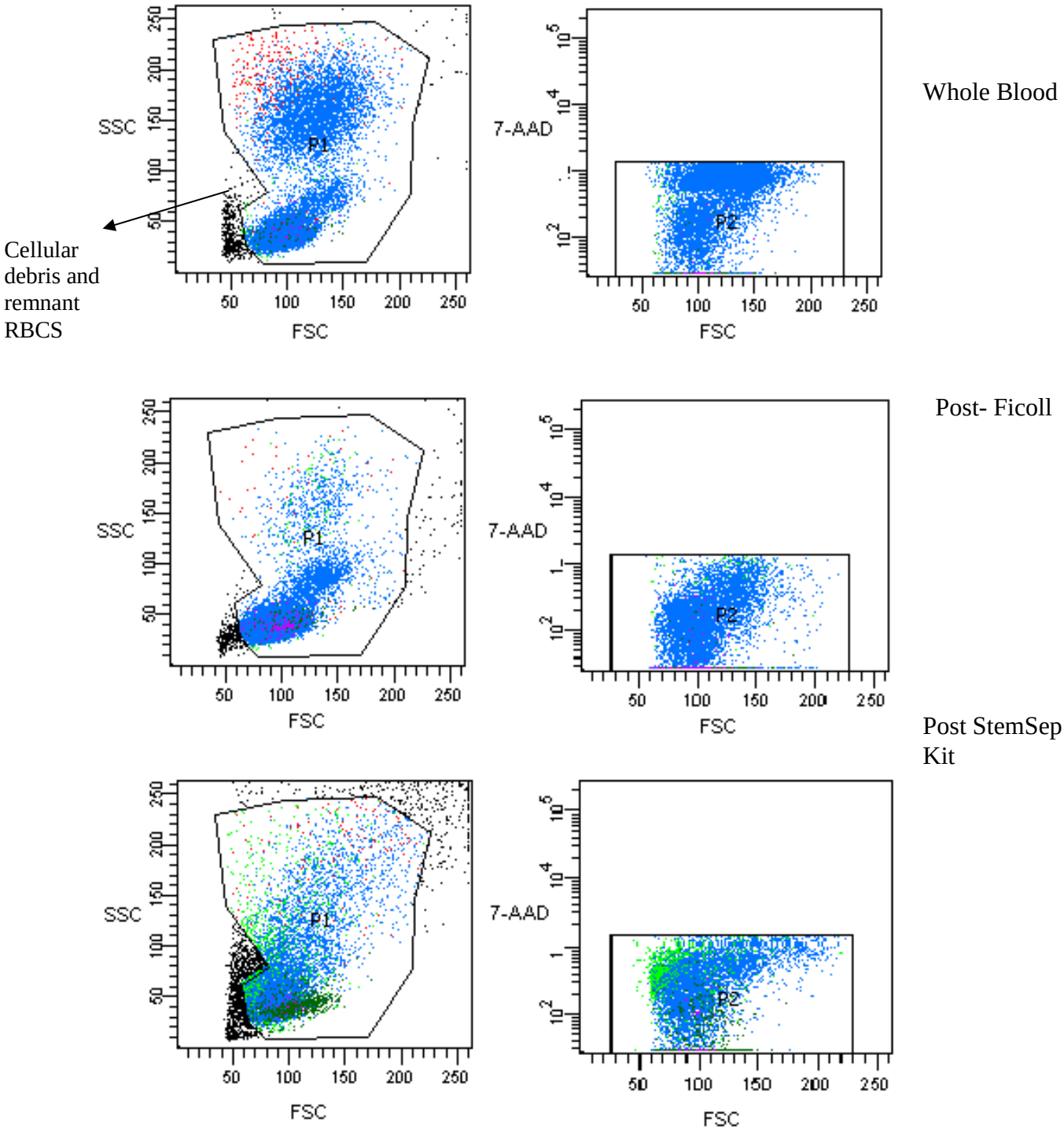


Figure 53b. Dot plot FACS graphs illustrating gated selection of HSC populations from white blood cell fraction isolated from whole blood. Note corresponding cellular viability (7-AAD negative cells) from the different stages of cord blood processing. Percentage of expression demonstrated is from the total cell population.

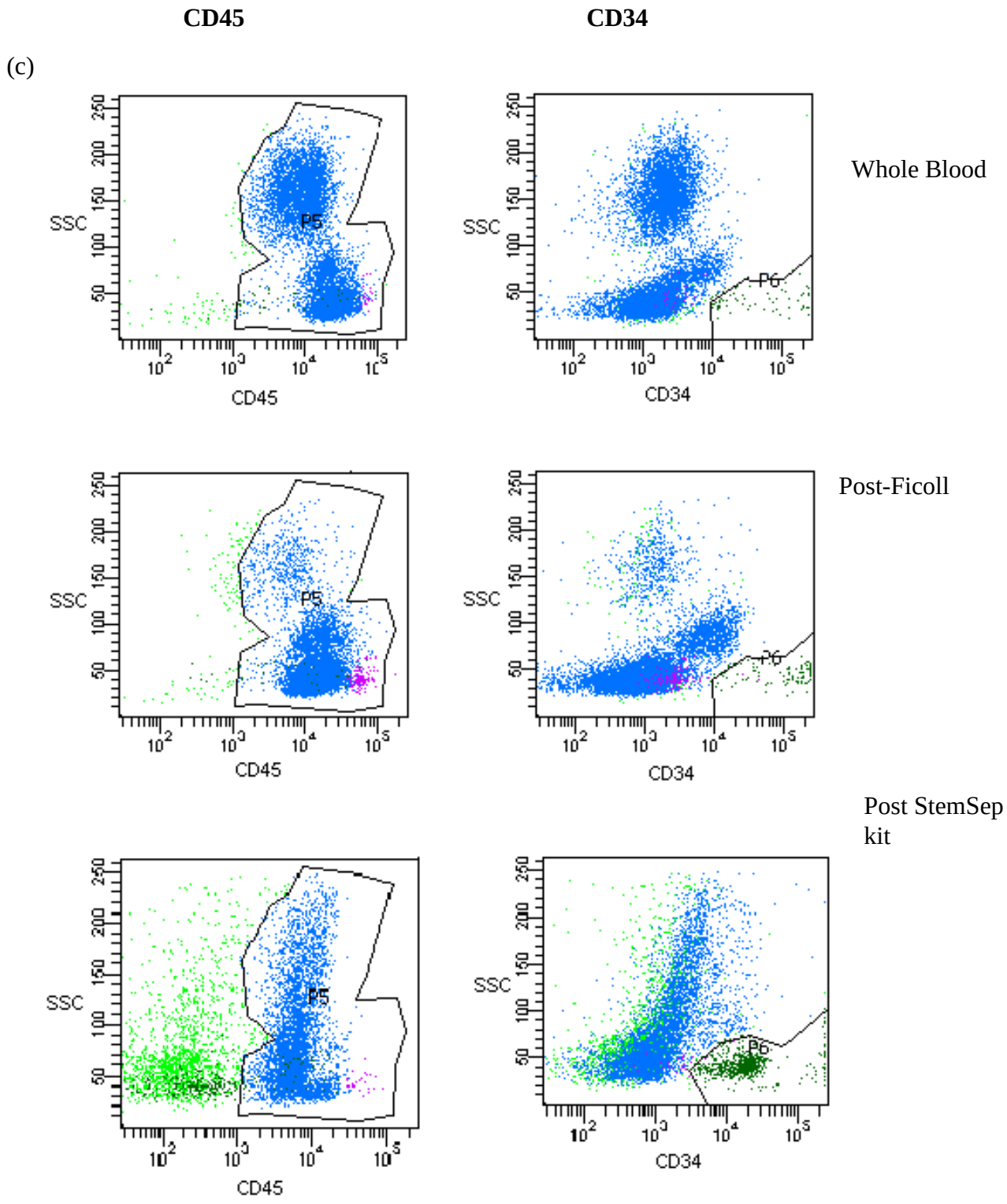


Figure 53c. Dot plot FACS graphs illustrating gated selection of $CD45^+$ cell population and within that population the concentration of $CD34^+$ cells. Percentage of expression demonstrated is from the total cell population.

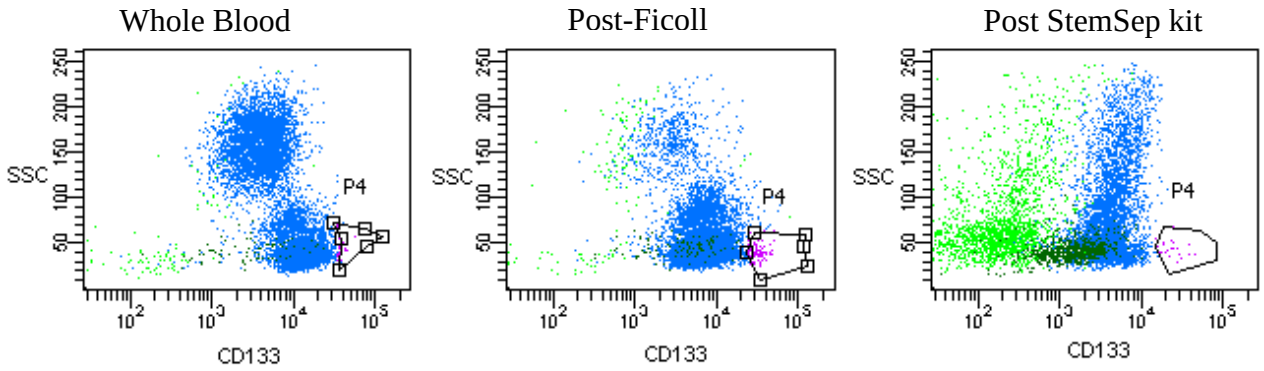
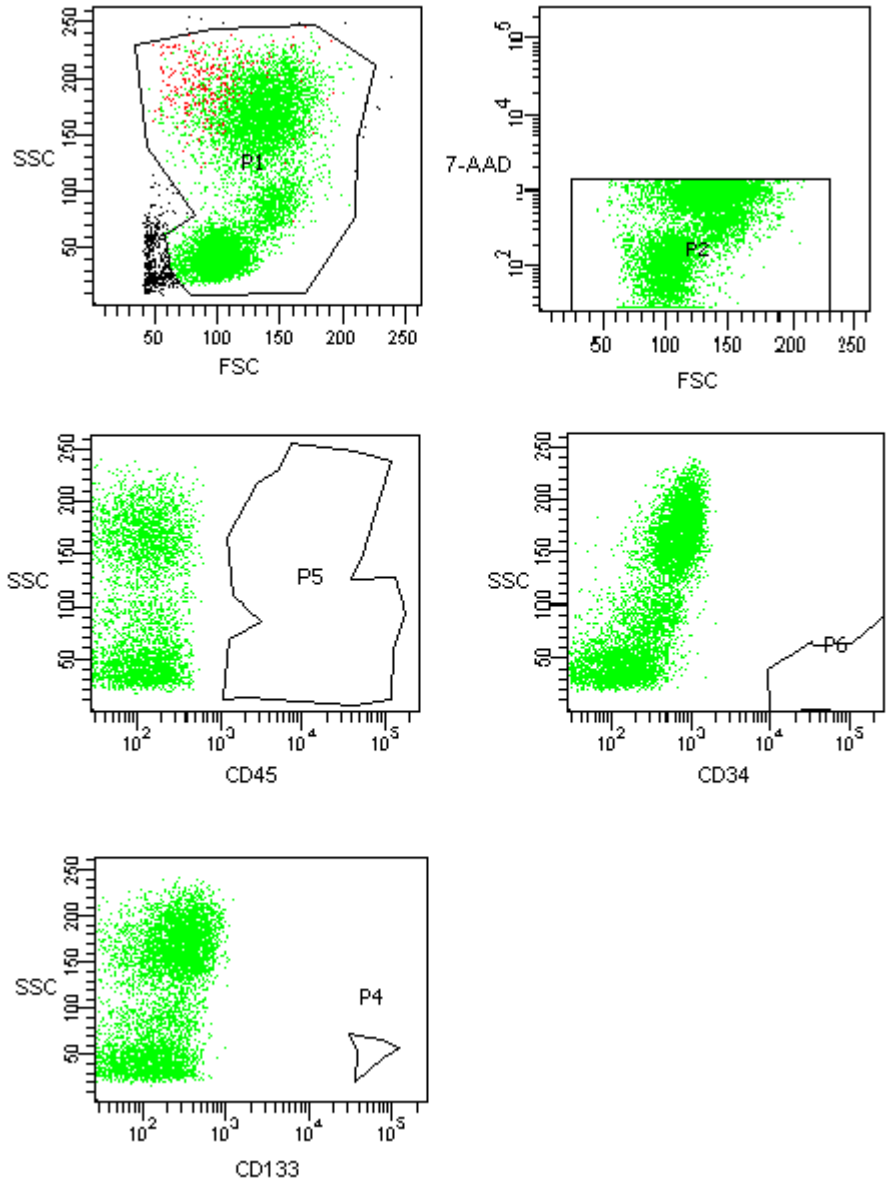


Figure 53d. Dot plot FACS graphs illustrating gated selection of CD133^{+} cellar population within CD45^{+} population.

(d) Negative control (Unstained samples)



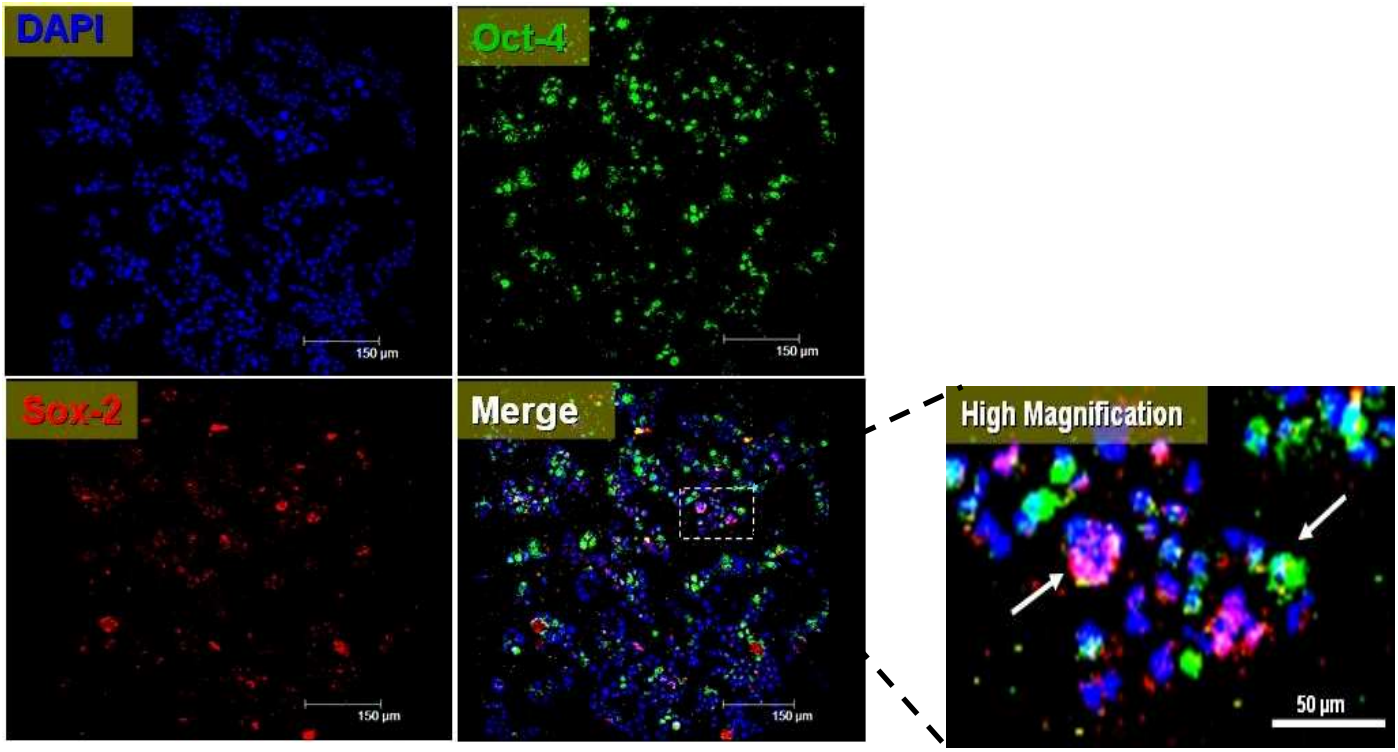
3.5 Characterisation of isolated cord blood stem/progenitor cells

3.5.1 Immunocytochemistry for pluripotency markers

The negative selection from the StemSep kit was further analysed for a number of embryonic stem cell proteins by immunocytochemistry: Oct4 (Pou5fl), Sox-2, Nanog. These transcription factors have been identified as crucial for the efficient maintenance of embryonic stem cell identify (Niwa, Miyazaki et al. 2000; Chambers, Silva et al. 2007; Masui, Nakatake et al. 2007). The examined cell population tested positive for all three pluripotency regulators, which co-localised to the chromatin (Figure 54). This close localisation of these three transcription factors in the chromatin has been previously reported in human ES cells (Boyer, Lee et al. 2005). It was reported for the first time, by McGuckin *et al*, that these markers were also expressed in cord blood-derived stem/progenitor cells (McGuckin, Forraz et al. 2006). These results were successfully reproduced in this study. Several other pluripotency transcription factors: Klf4 (Kruppel-like factor 4), Esrrb (Estrogen related receptor βeta) and Tcf4 (T cell factor 4) also share the global localisation to chromatin (Chen, Fang et al. 2008; Chen, Xu et al. 2008) but were not investigated in this project.

In addition, the cells were also tested for the presence of Ki-67 (MKI67), which is a cellular marker of proliferation (Scholzen and Gerdes 2000). Ki-67 protein is present during all active phases of the cell cycle (G_1, S, G_2 and mitosis), but is absent from resting cells (G_0). The cells tested positive for this proliferation marker, which too was limited to the nucleus.

(a)



(b)

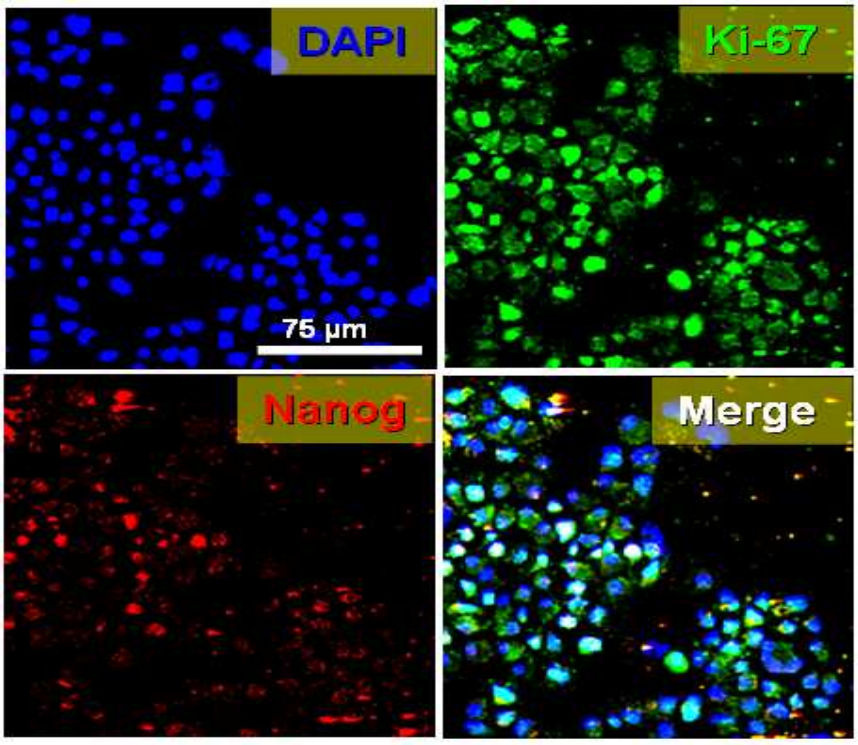


Figure 54. Expression of pluripotency markers in cord blood cells post StemSep isolation. The cells demonstrated positive expression of Oct4a, Sox-2 and Nanog. Cells were also positive for Ki-67, which is a proliferation marker. Cellular nuclei was stained blue with DAPI. Red=TxRed, Purple=Cy5 and Green= FITC. Images were taken at a magnification of x10 and x20. All positively stained images were acquired by setting negatives (secondary antibody only) as a control.

3.6 Expansion of cord blood-derived stem/progenitor cells- a significant challenge

One of the chief limitations with cord blood samples is the scarce stem/progenitor cell content that is attained from cord blood units of modest volume. *Ex vivo* expansion and maintenance of this cell population would have significant impact for stem cell enrichment of grafts deemed unsuitable for transplantation purposes owing to insufficient stem cell content. Several groups have made attempts at achieving stem/progenitor cell self-renewal *in vitro*. This includes attempts at optimization of culture systems incorporating growth factors and cytokines or by retroviral gene transfer as was done in bone marrow cells (Sorrentino 2004). The results observed, however, have been so far modest. In addition, the potential deleterious effect of insertional mutagenesis is a subject of great concern and is clinically very relevant.

What occurs in stem cell cultures *in vitro,* in the absence of any cytokines, is that that the cells divide symmetrically and give rise to two daughter cells committed to differentiation, a situation that eventually leads to depletion of the cultured stem cell pool in a matter of days. This happens because the critical interplay between intrinsic and extrinsic factors that exists *in vivo,* and is essential for the maintenance of stem cells has been disrupted. What is desired, however, is the creation of a culture system that will permit symmetrical division of the stem cells with one parent cell giving rise to two identical daughter stem cells, hence increasing stem cell numbers.

The ideal expansion system would include as few growth factors or cytokines as possible, should be short term and associated with minimal manipulation.

The expansion regime we formulated involved a limited number of mitogens and morphogens, hence obeying the concepts of 'minimal manipulation'. This was done with the perspective of transfer to clinical settings. The cocktail constituted: Dulbeco's Modified Eagle's Medium, 5 % fetal calf serum, Stem cell factor (SCF) (20ng/ml), acidic Fibroblast growth factor (aFGF) (20ng/ml) and Thrombopoietin (TPO) (10ng/ml). These cytokines were chosen based on the role that they play in regulation of stem cell niches. The doses selected were derived from literature review on previous research done in this area. SCF has been shown to play an important role in the regulation of HSCs in the stem cell niche in the bone marrow (Broudy 1997). SCF has been shown to contribute to the self renewal and maintenance of HSCs *in vivo* and increase the survival of HSCs *in vitro.* The expression of the receptor for SCF (c-kit) on HSCs at all stages of development stays constant (Kent, Copley et al. 2008). *In vivo,* the stromal cells that surround HSCs constitute part of the stem

cell niche, and release several ligands, including SCF. SCF has been shown to enhance adhesion to ECM proteins and stromal cells and thus may contribute to the maintenance of HSCs in the niche (Broudy 1997). It has previously been demonstrated that foetal HSCs are 6 times more sensitive to SCF than HSCs from adults (Bowie, McKnight et al. 2006; Kent, Copley et al. 2008). Hence, UCB- derived stem/progenitor cells, being at a more primitive developmental stage than adult stem cells were expected to show an effective response to SCF in the culture media.

Thrombopoietin (TPO), in addition to playing a pivotal in the development of megakaryocytes also plays a role in the development of HSCs. Kaushansky *et al* (Kaushansky 2002; Kaushansky and Drachman 2002) demonstrated that when used alone TPO does not influence stem cell kinetics, but when used in combination with other cytokines, for example SCF, it supports HSC survival and profoundly enhances stem cell proliferation. The importance of TPO and its receptor (c-Mpl) in haematopoiesis has been demonstrated in *in vivo* studies in mice by genetically eliminating c-Mpl receptors and demonstrating a much less potent stem cell repopulation capacity in the recipient murine bone marrow (Solar, Kerr et al. 1998). Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare human disorder that further demonstrates the role of TPO in haematopoiesis and stem cell biology (Ballmaier, Germeshausen et al. 2001). In this disorder faulty signalling response to TPO causes initial faulty megakaryocytes and platelets, and with time develops into aplastic anaemia due to failed haematopoiesis and deficiency of all haematopoietic progenitors.

Fibroblast growth factors have also been shown to play an important role in haematopoiesis. One of the proposed mechanisms of action of FGF is that it has the ability to interrupt BMP signalling which causes differentiation of human ES cells. Hence FGF in culture allows maintenance of undifferentiated proliferation of stem cells. This phenomenon has been elegantly demonstrated in human embryonic stem cells *(Xu, Peck et al. 2005).*

The formulated medium was further supplemented with ITS and heparin sulphate. ITS was applied as a maintenance factor and heparin sulphate functions as a modulators of FGF activity. Heparin sulphate binding protects FGF from denaturation and proteolytic degradation and provides a matrix-bound or cell-surface reservoir of this factor for the cells and is required for the activation of FGF high-affinity receptors (Quarto and Amalric 1994)**.**

In addition to exogenous cytokines, *in vitro* and *in vivo* studies have shown that stromal cells are also important in providing a microenvironment that enables primitive undifferentiated cells to indefinitely self-renew and inhibits differentiation and maturation. These cells also provide a rich environment of signals (cytokines, ECM proteins and

adhesion molecules) that control proliferation, survival and differentiation decisions. It is currently not known whether non-contact conditions are sufficient for *ex vivo* expansion or whether stromal binding is essential (Hofmeister, Zhang et al. 2007). A great deal of work has been done to investigate the importance of ECM components on the *ex vivo* expansion of cells. Previous studies have reported successful expansion of BM-derived MSCs on plates coated with various ECM: fibronectin, collagen and Matrigel (mixture of ECM components), and these cells were able to differentiate into hepatocyte-like cells (Schwartz, Reyes et al. 2002; Fiegel, Lioznov et al. 2003). It was suggested that the ECM could potentially modulate the local concentration of cytokines available and together with the stem cell microenvironment and cytokines regulate stem cell proliferation and differentiation fate. Conversely, Kang *et al* reported that that they were able to culture and expand UCB-derived MSCs in plastic culture flasks without treatment with ECM proteins and these cells were still able to differentiate into hepatocytes (Kang, Zang et al. 2006). Thus, there exists opposing views regarding the role of ECM proteins in *in vitro* stem/progenitor cell expansion.

The stem/progenitor cells in this study were cultured on ECM proteins: collagen IV or fibronectin coated plastic round bottom 96-well plates. StemSep kit yielded a very low cell count post treatment and for this reason culturing in round bottom 96-well plates allowed cells to aggregate at the bottom of the plate and permitted liberated inter-cellular communication that is required for cell fate decisions and maintenance. These cells grew as tight adherent clusters resembling the 'niche-like' structures described by Scadden and Sun *et al* (Scadden 2006; Sun, Jeong et al. 2007). This was observed as early as after 1 day post seeding and culture (Figure 55). Cell colonies were formed as clusters of small rounded cells, 4-5μ in size. These homogenous colonies, in due course, became surrounded by spindle-shaped cells and embodied a 'niche-like' architecture (Figure 55). This structure might be correlated to the hypothesis of Wilson and Trumpp (Wilson, Oser et al. 2007) ; that the quiescence stem cells are anchored in the centre of the niche, whereas self renewing stem cells are located close to the border separating the niche from the non-niche microenvironment, which would provide signals that determine cell fate. The surrounding cells play a key role in supporting the centrally located stem cells and provide signals that are key players in determining cell fate decisions. The perseverance of this 'niche' structure appears to be a key factor in survival of the stem cells as it was noted that loss of the supporting cells in the 'niche' structures resulted in ultimate loss of the stem cell colonies as well.

The cells were cultured *in vitro* for 25 days with media changes every other day. The cells were trypsinized and counted with a haemocytometer at regular intervals and the results are noted in Figure 56. The cells were doubled in number by day 4, but thereafter there was a constant decline in cell number till day 13 after which the cell numbers stayed constant at very low levels for approximately a week and then cell viability went down to zero. It is worth noting that, after the first week in culture the cells that did survive were the ones that existed in aggregates.

In conclusion, the applied expansion system resulted in a very modest increase in cell number and was incapable of maintaining the cells in culture for long term applications. Consequently, it was decided to limit the expansion period to duration of one week only. Since the cell viability decreased rapidly after day 10, commitment of cells towards endoderm lineage was undertaken simultaneous with the short-term expansion. This was done by the introduction of Hepatocyte growth factor (HGF) (20ng/ml) to the expansion media. Analysis with immunocytochemistry at the end of a one week treatment with the dual culture system illustrated positive expression for Gata-4 (endodermal marker), albumin (earliest hepatic marker) and Ck7 (hepatic stem cell marker) (Figure 57). The cells were very weakly positive for ectoderm markers Nestin and GFAP and were negative for Pax6, showing commitment towards endo-mesodermal lineage. This dual culture system permitted modest expansion and endoderm commitment of cord blood-derived stem/progenitor cells in a short time period.

Conclusion

The applied expansion system resulted in a very modest increase in cord blood stem/progenitor cell number and was incapable of maintaining the cells in culture for long term applications.

Commitment of stem/progenitor cells towards meso-endoderm lineage was undertaken simultaneous with short-term expansion.

The dual culture system permitted modest expansion and commitment of cord blood stem/progenitor cells to endoderm and liver lineage.

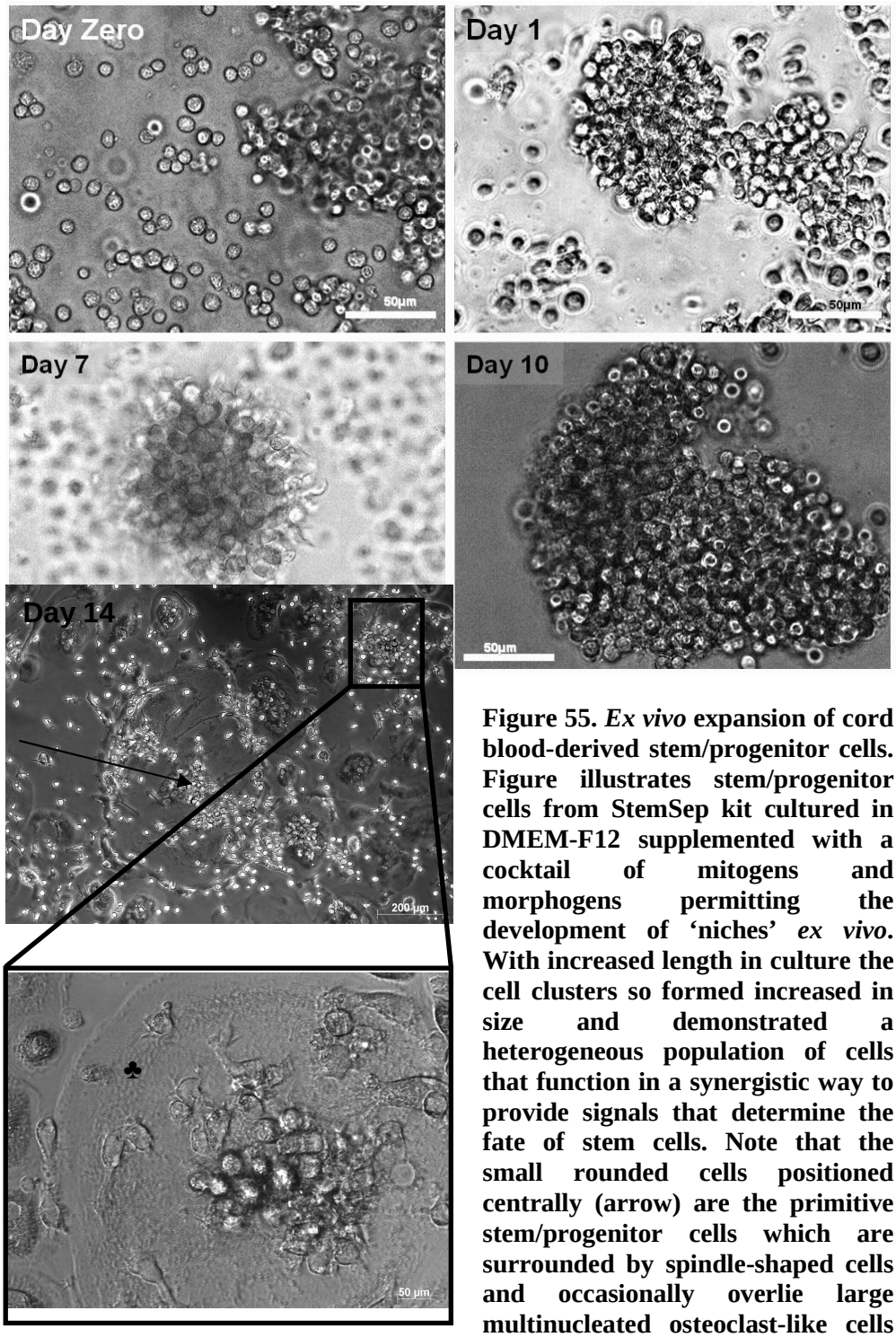
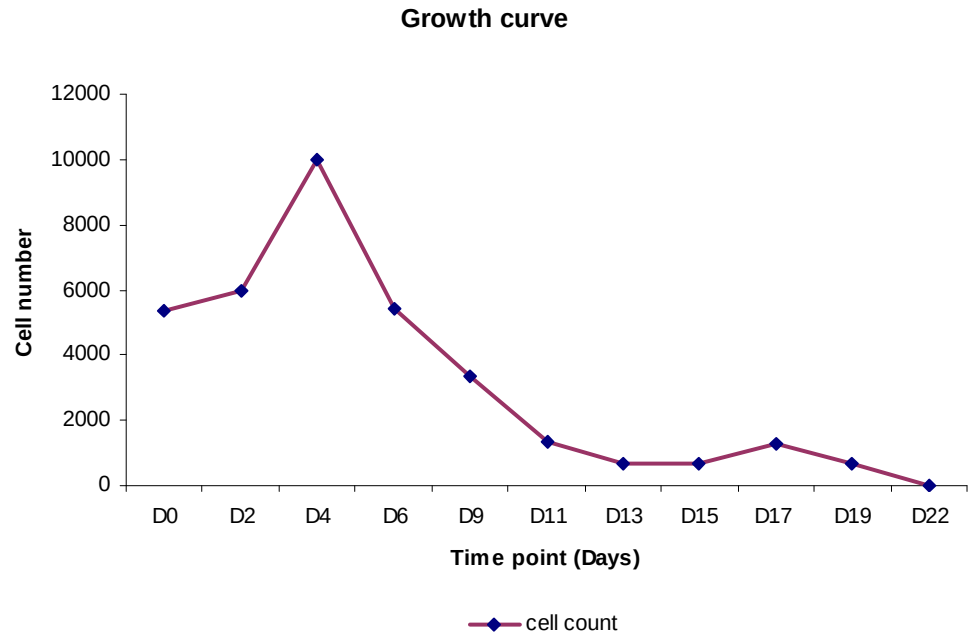


Figure 55. ***Ex vivo*** **expansion of cord blood-derived stem/progenitor cells. Figure illustrates stem/progenitor cells from StemSep kit cultured in DMEM-F12 supplemented with a cocktail of mitogens and morphogens permitting the development of 'niches'** ***ex vivo*****. With increased length in culture the cell clusters so formed increased in size and demonstrated a heterogeneous population of cells that function in a synergistic way to provide signals that determine the fate of stem cells. Note that the small rounded cells positioned centrally (arrow) are the primitive stem/progenitor cells which are surrounded by spindle-shaped cells and occasionally overlie large multinucleated osteoclast-like cells (♣). DIC images acquired at magnification 10, 20 and 40.**



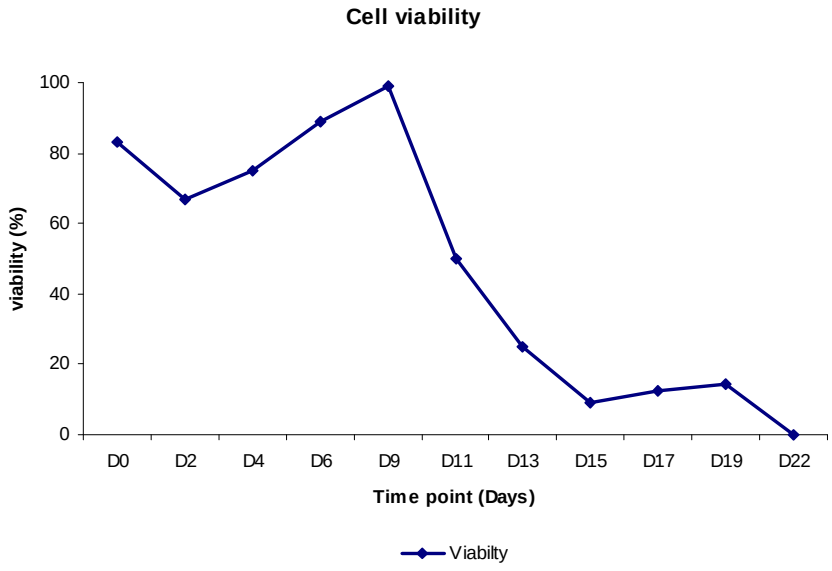
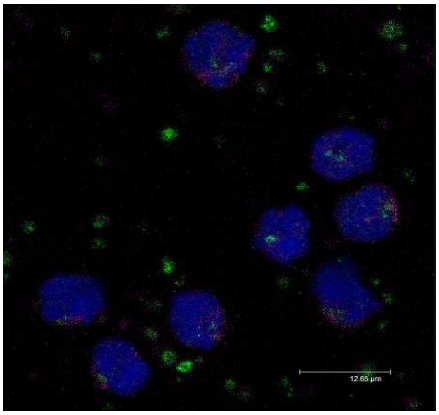
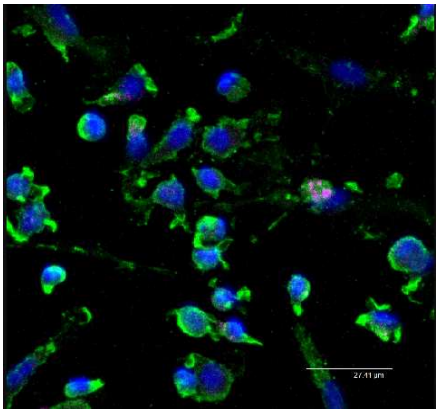


Figure 56. ***Ex vivo*** **proliferation assay for human cord blood-derived stem/progenitor cells. The average doubling time for these cells was four days after which the cellular viability deteriorated remarkably resulting in rapid fall in cell number as noted on the haemocytometer. (N=3)**

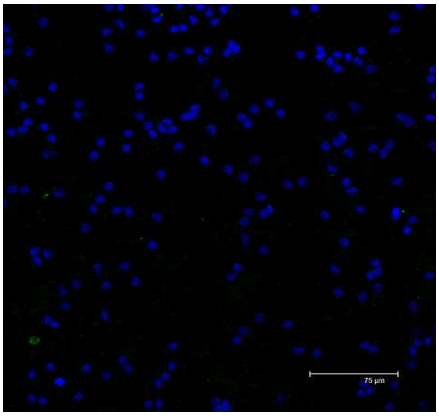
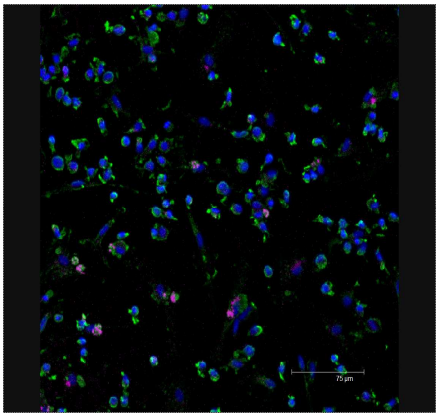
Figure 57. Simultaneous endoderm commitment of cord blood stem/progenitor cells during *in vitro* expansion. Post treatment with relevant mitogens, cells demonstrated positive expression of Gata-4 (endodermal marker) and were also positive for some liver related markers: albumin, AFP, Ck7 and Ck19. The cells were very weakly positive for Nestin and GFAP. Cellular nuclei were stained blue with DAPI. Red= TxRed, Purple=Cy5 and Green=FITC. Images were taken at a magnification of x10 and x20. All positively stained images were acquired by setting negatives (secondary antibody only) as a control. HPCs (Haematopoietic progenitor cells). N=4

Endoderm and hepatic commitment

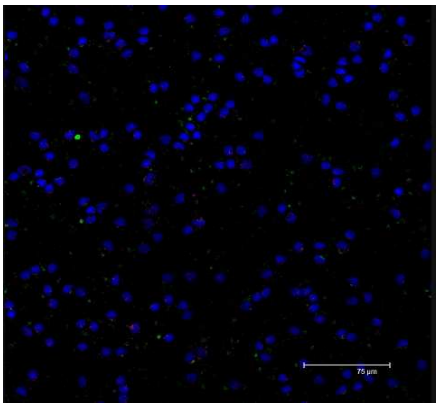
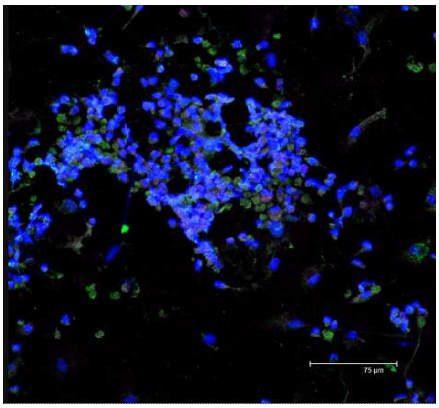
D0 HPCs **D7 HPCs**

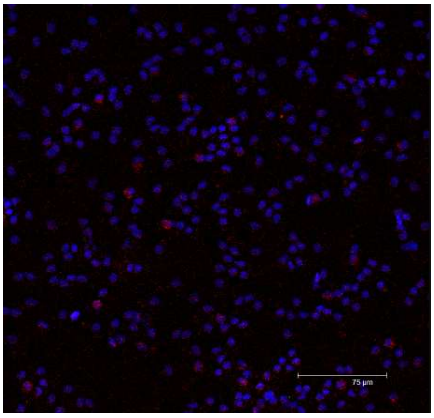
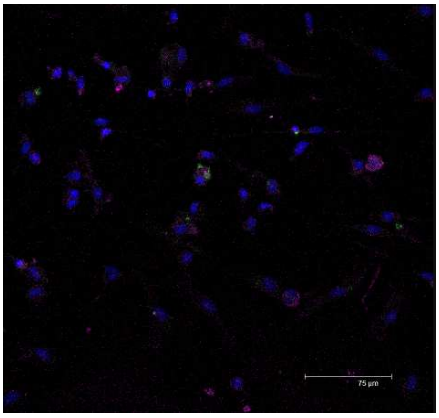
Gata4 Sox2

ALB AFP

CK7 CK19

Nestin GFAP
Pax6

3.7 Cytogenetic analysis of cord blood-derived stem/progenitor cells

One of the major concerns during *ex vivo* manipulation, culture and propagation of stem/progenitor cells is genetic instability and mutagenesis. Thus, karyotype analysis of cord blood stem/progenitor cells post isolation, propagation and lineage commitment was undertaken. The cells produced normal karyotype as illustrated in the figure 58.

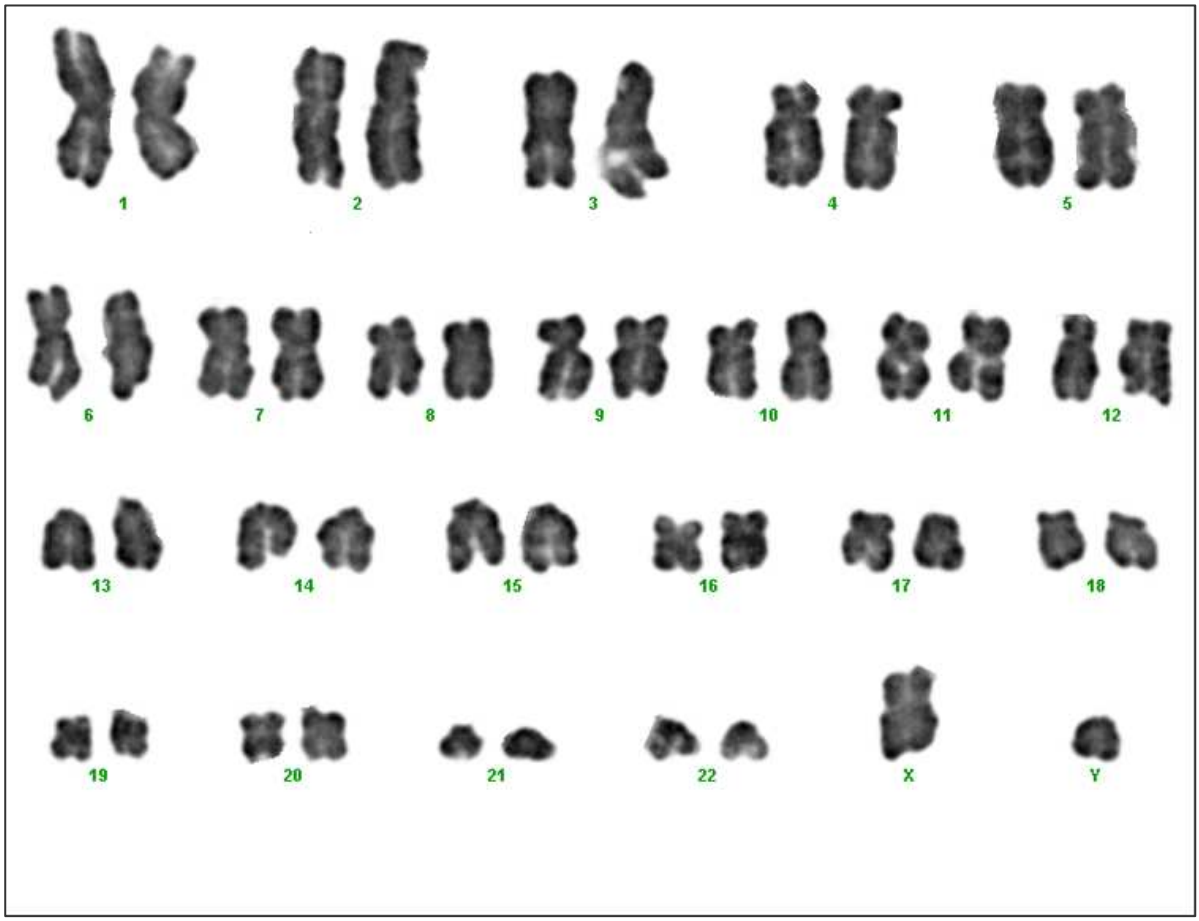


Figure 58. Karyotype of cord blood-derived stem/progenitor cells post short time *ex vivo* expansion. Post expansion cells demonstrated a normal karyotype. (N=2)

3.8 Multi-potential of selected stem/progenitor cells

The selected cord blood stem/progenitor population demonstrated multi-potential capacity. In addition to liver commitment (endoderm), other members of the group were able to differentiate the same stem/progenitor cell population into pancreatic cells (endoderm), neuroglial cells (ectoderm) and cardiac progenitors (mesoderm). Based on this result, it may be argued that these cells were pluripotent and capable of generating tissues of all three germ layers (Figure 59).

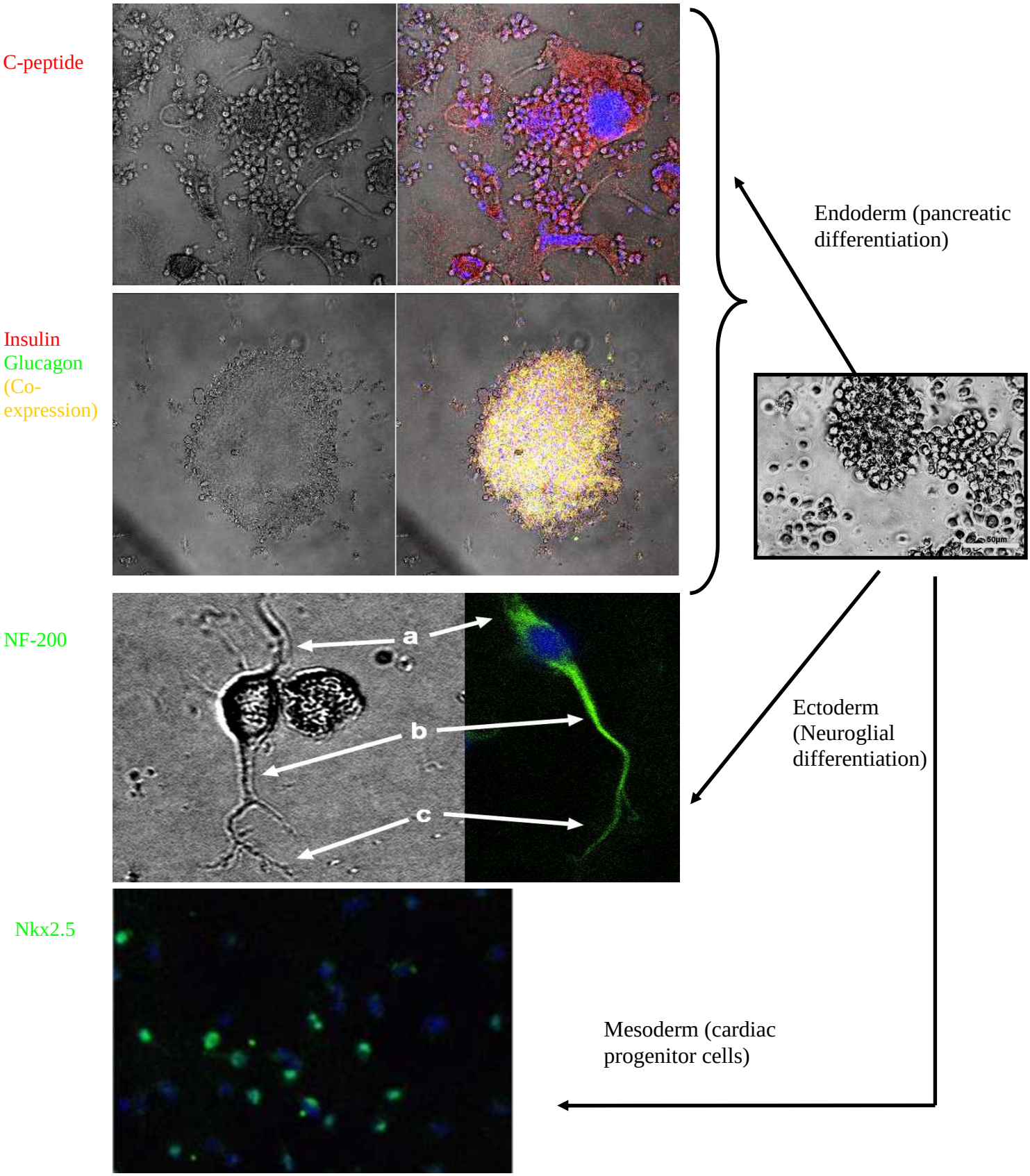


Figure 59. Multipotential capacity of cord blood stem/progenitor cells. These cells demonstrated the capacity to differentiate along ectoderm lineage (neuroglial differentiation) and mesoderm lineage (cardiac progenitor cell differentiation) in addition to endodermal fate. Images courtesy of Hamad Ali, Dr Christina Basford and Marko Strabad. Images were taken at magnification of x10, x20 and x40.

3.9 Chapter discussion

One of the goals of this study was the selection of stem/progenitor cell populations from umbilical cord blood. We have demonstrated here that not only can different stem/progenitor cell populations be isolated from cord blood units but have simultaneously compared the efficiency of the five most commonly used methods for cord blood processing. Since no one method came out consistenetly as best during analysis, a league table was devised and each method was rated for all the 7 analysis methods used. Based on the league table PrepaCyte was noted to be the best performer for many of the investigations. Additionally, PrepaCyte proved superior in terms of cost effectiveness. This closed separation system did not demand expensive laboratory equipment or specialized clean rooms. However, it is important to note that depending on the primary use of UCB, different processing methods may prove more applicable than others. Thereofore, the choice of UCB processing should be cautiuosly tackled with the view that cord blood can be readily transferred from the bench to the bedside in a suitable form for patient treatment.

We further investigated whether the desired stem/progenitor cells populations could be selectively isolated and concentrated from UCB. We compared our in-house Lineage negative selection protocol with the StemSep 'human primitive progenitor cell enrichment kit'. It was concluded that although our in-house protocol proved effective in the selection and isolation of early HSC populations, taking the overall results into view, StemSep proved to be the more efficient method. StemSep protocol was time effective, involving fewer steps, fewer washes with less manipulation of the cells. All these factors collectively contributed to greater cell viability and reduced the risk of contamination *in vitro*.

We further investigated the multi-potential capacity of the isolated stem/progenitor cell populations. These cells demonstrated the ability to differentiate into cells representing all three germ layers. Additionally these cells tested positive for a few ES cell markers.

This project also aimed to develop an *ex vivo* regime obeying the concepts of 'minimal manipulation' for the expansion of isolated UCB stem/progenitor cells. Our expansion system resulted in a very modest increase in cell number and was incapable of maintaining the cells in culture for long term applications. Hence, commitment of stem/progenitor cells towards endoderm lineage was undertaken simultaneously with the short-term expansion. These expanded cells demonstrated a normal karyotype on analysis. This dual culture system allowed modest expansion and commitment of stem/progenitor cells to endodermal fate.

Chapter 4

Processing of human umbilical cord- isolation of Mesenchymal stem cells

4 Introduction

A population of non-haematopoietic multipotent stem cells termed mesenchymal stem cells (MSCs) have previously been derived from the human umbilical cord (Romanov, Svintsitskaya et al. 2003; Wang, Hung et al. 2004; Sarugaser, Lickorish et al. 2005). These cells grow adherent to plastic and express specific pattern of cell surface determinants (CD105, CD90, CD73, and CD44). This cell fraction is distinct from the haematopoietic and pluripotent stem cells present in cord blood and do not express blood-cell determinants (CD34, CD45 and CD133). These Mesenchymal stem cells can be expanded very efficiently *in vitro*. Additionally, these multipotent cells (Kang, Zang et al. 2006) have unique immunoregulatory features that suppress lymphocyte proliferation *in vitro* (Gerson 1999) and exhibit high self renewal potential (Bartholomew, Sturgeon et al. 2002). MSCs have potential to differentiate into tissues of all three germ layers: including bone, cartilage, fat, muscle, endothelial cells, neuronal, glial cells and liver cells (Bruder, Jaiswal et al. 1997; Kopen, Prockop et al. 1999; Pittenger, Mackay et al. 1999; Erices, Conget et al. 2000; Woodbury, Schwarz et al. 2000; Campagnoli, Roberts et al. 2001; Horwitz, Gordon et al. 2002; Krampera, Glennie et al. 2003; O'Donoghue, Choolani et al. 2003; Lee, Kuo et al. 2004; Hong, Gang et al. 2005; Li, Qu et al. 2005; Chien, Yen et al. 2006; Lange, Bruns et al. 2006; Ong, Dai et al. 2006; Snykers, Vanhaecke et al. 2006; Lu, Teng et al. 2007; Paunescu, Deak et al. 2007; Tamagawa, Oi et al. 2007; Yen, Chien et al. 2007; Campard, Lysy et al. 2008; Lu, Dong et al. 2008).

4.1 Isolation of Mesenchymal stem cell (MSC)-like cells from human umbilical cord and cord matrix

One of the goals of this study was to isolate MSCs from umbilical cord. We were able to accomplish this task with a 100% efficiency (n=15). The manual method of umbilical cord dissection and culture proved more effective than the enzymatic technique of cord processing (Materials and Methods, section 2.3.1.1) which was carried out by incubating cord pieces with Collagenase (2mg/ml) overnight in an incubator. Cord pieces were subsequently washed with serum-free DMEM and incubated with Trypsin at 37◦C for 30 minutes with regular agitation. FBS was then used to inhibit the enzymatic reaction of Trypsin. Cord pieces were then washed in serum-free DMEM and cultured in DMEM-F12 10% FBS. The manual method of umbilical processing (Materials and Methods, section 2.3.1.2) was less time consuming and involved minimal manipulation. Cells appeared to migrate out of the cord pieces and grow in culture as early as day 3 post transfer of umbilical cord pieces into culture plates (Figure 60).

Isolated cells initially displayed a fibroblast-like morphology in culture, with the majority of the cells being flat with a wide cytoplasm within which were noted stress fiber patterns (Figure 60).

The abundant availability of umbilical cords as a biological by-product, the efficiency of isolation of this cell population and the reproducibility of this technique defines the umbilical cord as a valuable resource for regenerative medicine. We have indicated here that hospitals could achieve this task with clinical ease with no requirement for specialist labs to do this for them.

Conclusion

Mesenchymal stem cell-like cells were isolated from human umbilical cord matrix with 100% efficiency (n=15).

Manual dissection and transfer of umbilical cord pieces into culture media proved more effective than enzymatic digestion of umbilical cord samples.

Isolation and expansion of Mesenchymal stem cells (MSC) from human umbilical cord

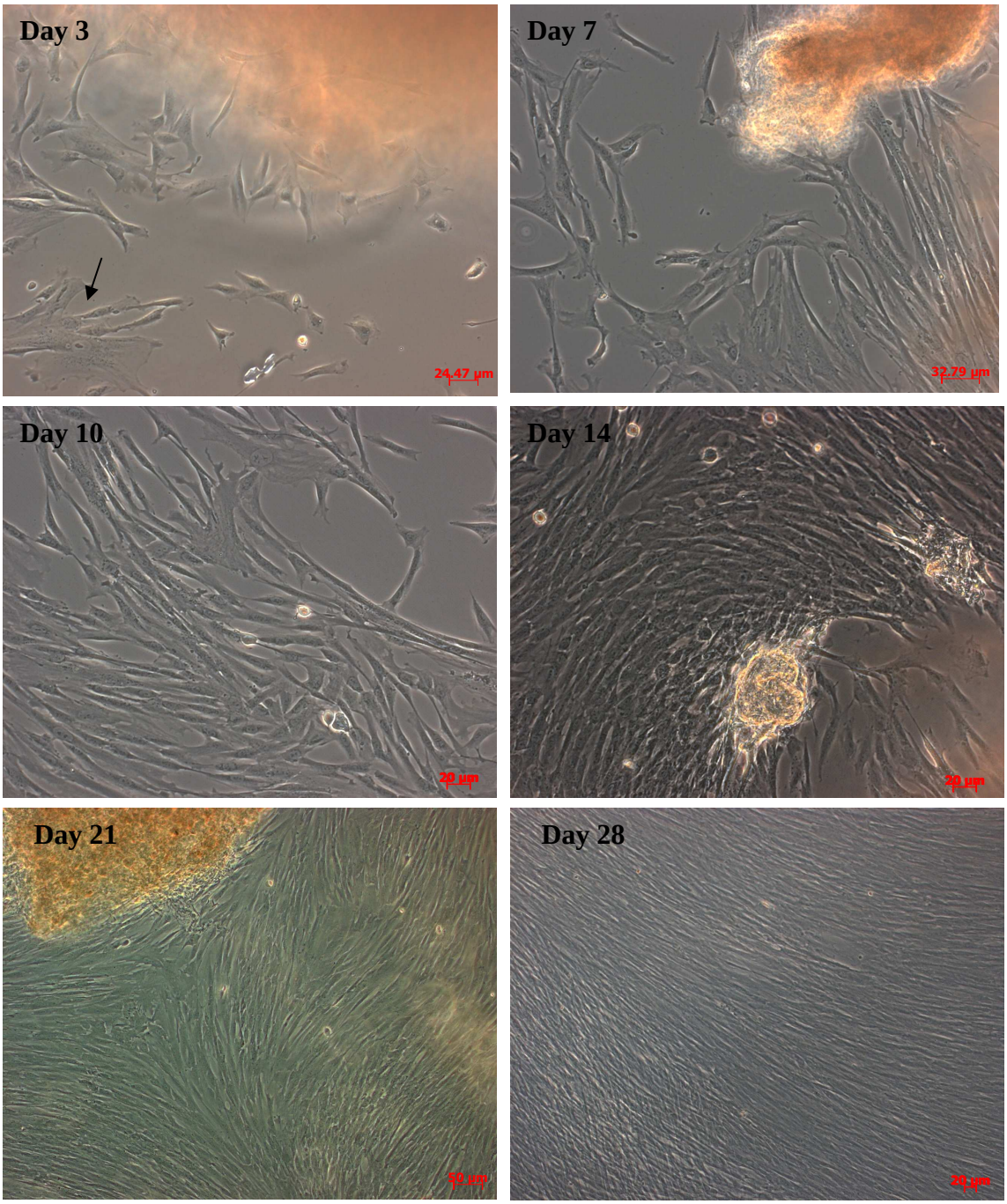


Figure 60. Progressive *in vitro* proliferation of MSCs isolated from umbilical cord matrix over duration of 4 weeks. Note that initially post isolation the fibroblastic cells display a flattened morphology with wide cytoplasm (⟵) but with progressive proliferation they become more spindle-like in phenotype with a narrow cytoplasm and a low nuclear to cytoplasmic ratio (*). Images were taken at magnification of x10 and x20.

4.2 Characterisation of isolated Mesenchymal stem cell-like cells from umbilical cord

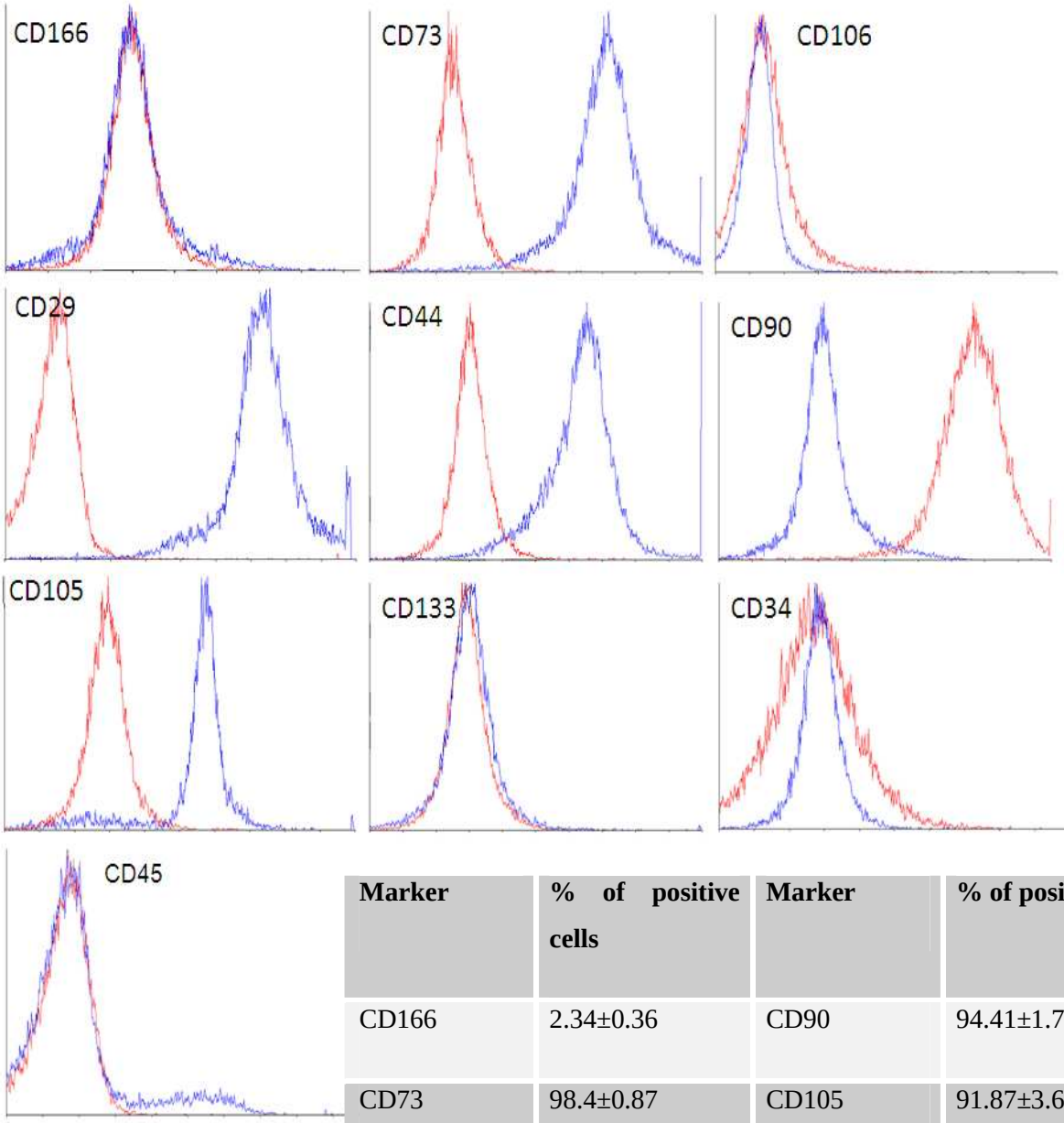
4.2.1 Flowcytometric analysis of umbilical cord Mesenchymal stem cell-like cells

Flow cytometric analysis of the isolated MSC-like cells demonstrated that these cells expressed most of the markers that have routinely been used to characterize MSCs in the past: matrix markers (CD44, CD105) and integrin markers (CD29), CD73, CD90 but not haematopoietic markers (CD45, CD34 and CD133). These cells, however, were negative for CD106 and CD166. Other reports have previously noted that the expression of some of these markers, for example CD106, might fluctuate between different studies and may depend on the precise location (perivascular or intervascular) in the cord where the cells are isolated (Sarugaser, Lickorish et al. 2005; Weiss, Medicetty et al. 2006; Campard, Lysy et al. 2008). The MSCs were also analysed for the expression of Major Histocompatibility Complex (MHC). In humans, MHC is called Human Leukocyte Antigen (HLA) system. The cells tested positive for major HLA class I antigens (A, B and C) but were negative for HLA class II (DR and DQ) and minor HLA class I antigen G. HLA class I antigens present peptides from inside the cell. These peptides are produced from digested proteins that are broken down in the proteasomes. HLA-A, B and C are major Class I proteins while HLA-E, F and G are minor class I proteins. HLA class II antigens present antigens from outside of the cell to T-lymphocytes. These particular antigens stimulate T-helper cells to multiply, and these T-helper cells in turn then trigger antibody producing B-cells to produce antibodies to that specific antigen. Self-antigens are suppressed by suppressor T-cells.

The data generated here confirmed previous reports that MSCs (from various sources) do not express immunologically relevant cell surface markers including HLA class II (Niemeyer, Krause et al. 2006) . This data identified MSCs as being immune privileged. *In vitro* studies have previously demonstrated that HLA-mismatch MSC do not provoke an immune response in the host and are even able to suppress allogenic lymphocyte proliferation (Le Blanc, Tammik et al. 2003; Le Blanc, Tammik et al. 2003). This phenomena seems to be independent of the major histocompatibility complex and mediated by production of cytokines, in particular interleukin-2 (IL-2) and IL-10 (Rasmusson, Ringden et al. 2003), HGF and Transforming growth factor-β-1 (TGF-β-1) and not by apoptosis (Di Nicola, Carlo-Stella et al. 2002). These features make MSCs an attractive candidate for HLA-independent therapeutic strategies. Their immunemodulatory features have already contributed to the application of MSCs in the treatment of severe graft-versus

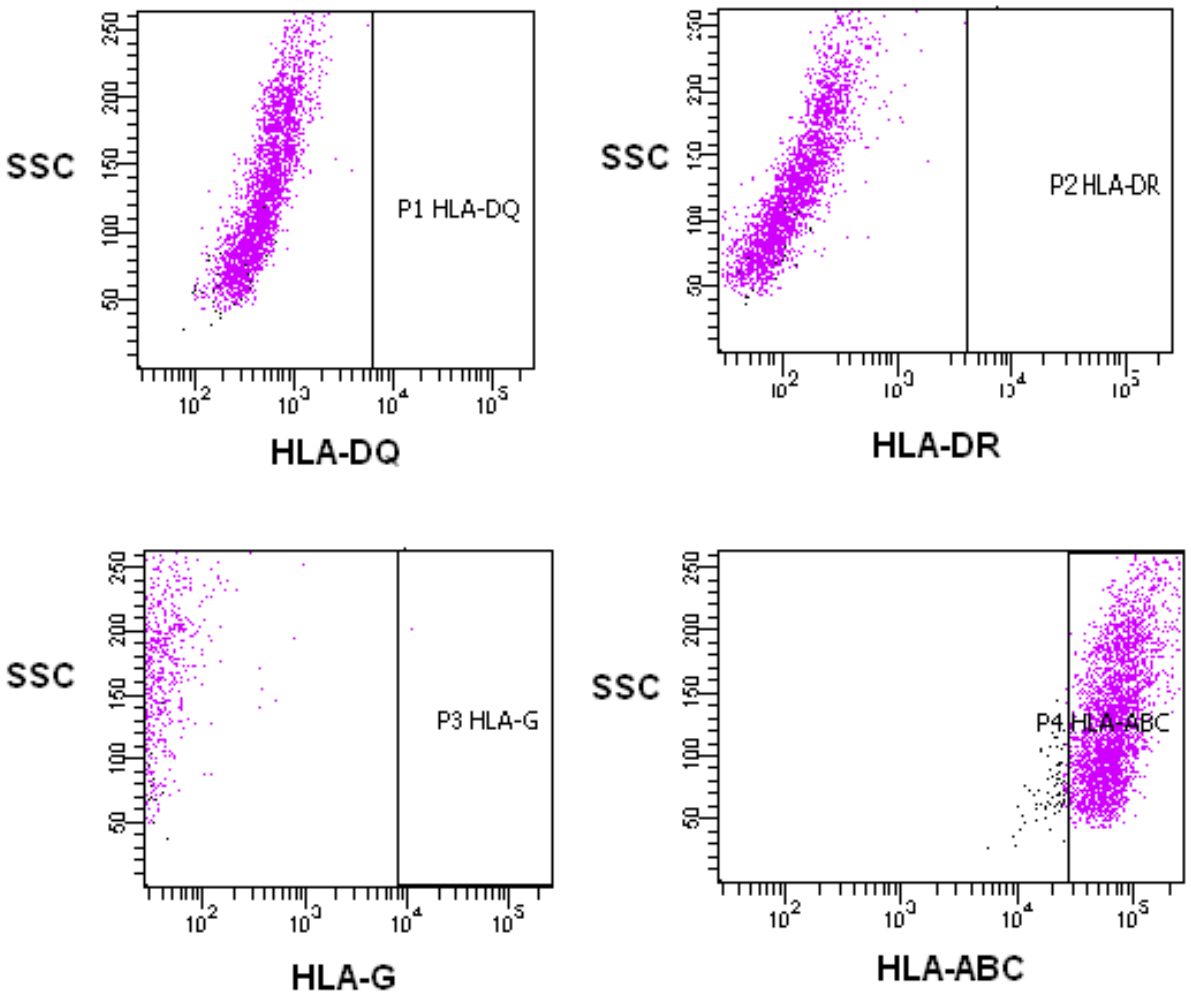
host disease (Le Blanc, Rasmusson et al. 2004). Additionally, several groups have already demonstrated that the immunosuppressive capacity of MSCs remain even after *in vitro* differentiation (Le Blanc, Tammik et al. 2003; Niemeyer, Seckinger et al. 2004; Niemeyer, Krause et al. 2006). This property is very important for tissue engineering procedures and HLA-mismatch transplantation. When considering allogenic approach, it is important to note that donor age and the disease state of the patient may considerably influence stem cell number and quality. Immune privileged MSCs would make it possible to use approved MSCs of high quality for transplantation purposes. However, it is important to note that the immunemodulating properties of MSCs may provoke potential side effects. In the face of allogenic transplantaion, the induced immune suppression may allow local malignant growths (Djouad, Plence et al. 2003). Thus, the possible adverse effects of immunemodulation caused by allogenic MSCs demand further investigation before these cells can be safely translated to the clinical.

Flowcytometric analysis of umbilical cord Mesenchymal stem cells



Marker	% of positive cells	Marker	% of positive cells
CD166	2.34±0.36	CD90	94.41±1.71
CD73	98.4±0.87	CD105	91.87±3.6
CD106	0.84±0.91	CD133	0.77±0.97
CD29	97.6±1.5	CD34	0.2±2.1
CD44	93.1±1.18	CD45	7.44±4.69

Figure 61. Characterisation of umbilical cord Mesenchymal stem cells by flowcytometry. The red graphs represent the negative control and the blue is the tested population. (N=4)



Marker	% of Positive cells
HLA-ABC	96.57±1.86
HLA-G	0
HLA-DR	0
HLA-DQ	0

Figure 62. Characterization of MSCs for HLA histocompatibility complexes by flowcytometry. The cells tested strongly positive for major HLA class I (A,B and C) but were negative for minor HLA class I antigen (G) as well as negative HLA class II (DR and DQ). (N=3)

4.2.2 Expression of embryonic stem cell markers in umbilical cord Mesenchymal stem cells

4.2.2.1 Immunocytochemistry

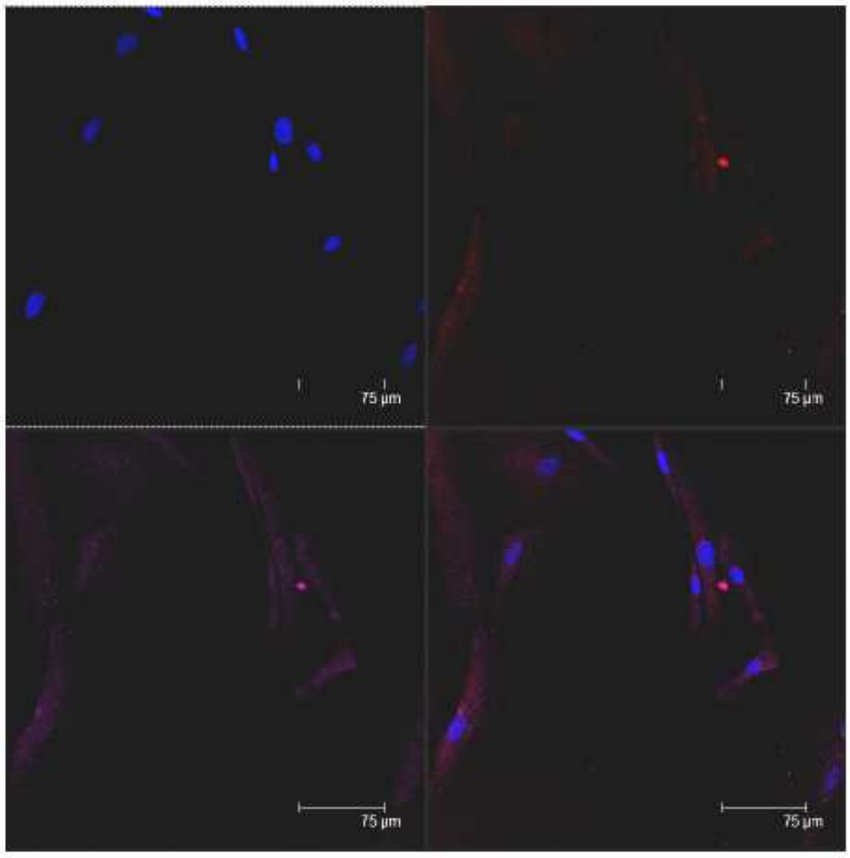
Undifferentiated umbilical cord MSCs tested positive for Oct4a and Sox-2 by immunocytochemistry, but failed to test positive for Nanog (Figure 63).

4.2.2.2 Flowcytometric analysis

To confirm the presence of these makers we examined these cells by flowcytometry. On FACS analysis, the isolated MSCs tested positive for various embryonic markers, including TRA-60, TRA-81, SSEA-4, Oct4a as well as Nanog (Figure 64).

Expression of Oct4 and Sox-2 in umbilical cord Mesenchymal stem cells

(a)



(b)

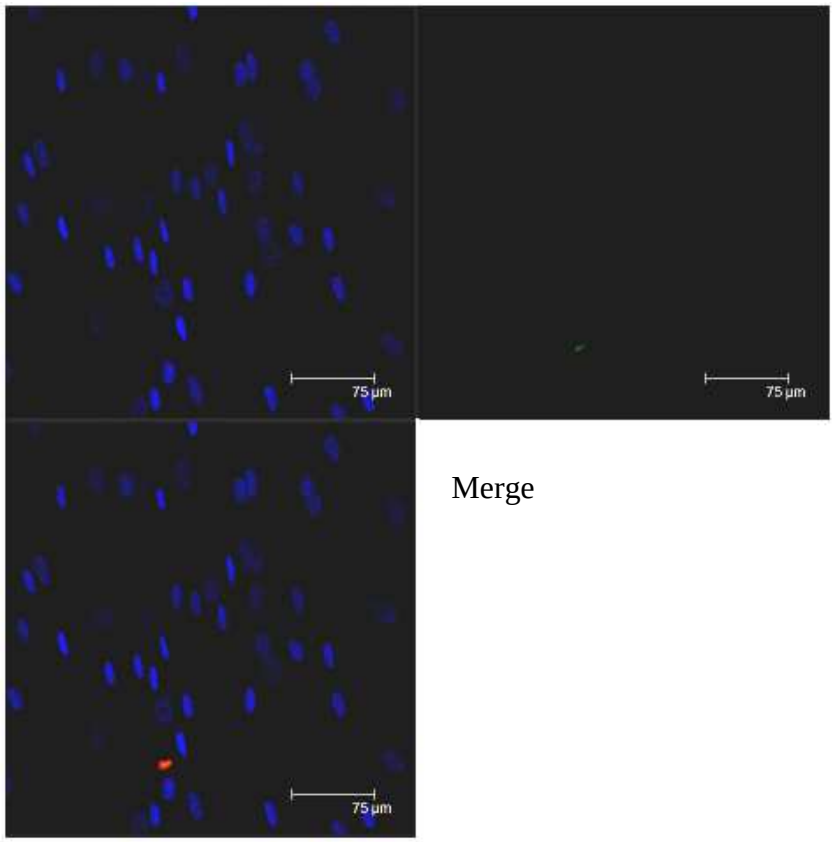
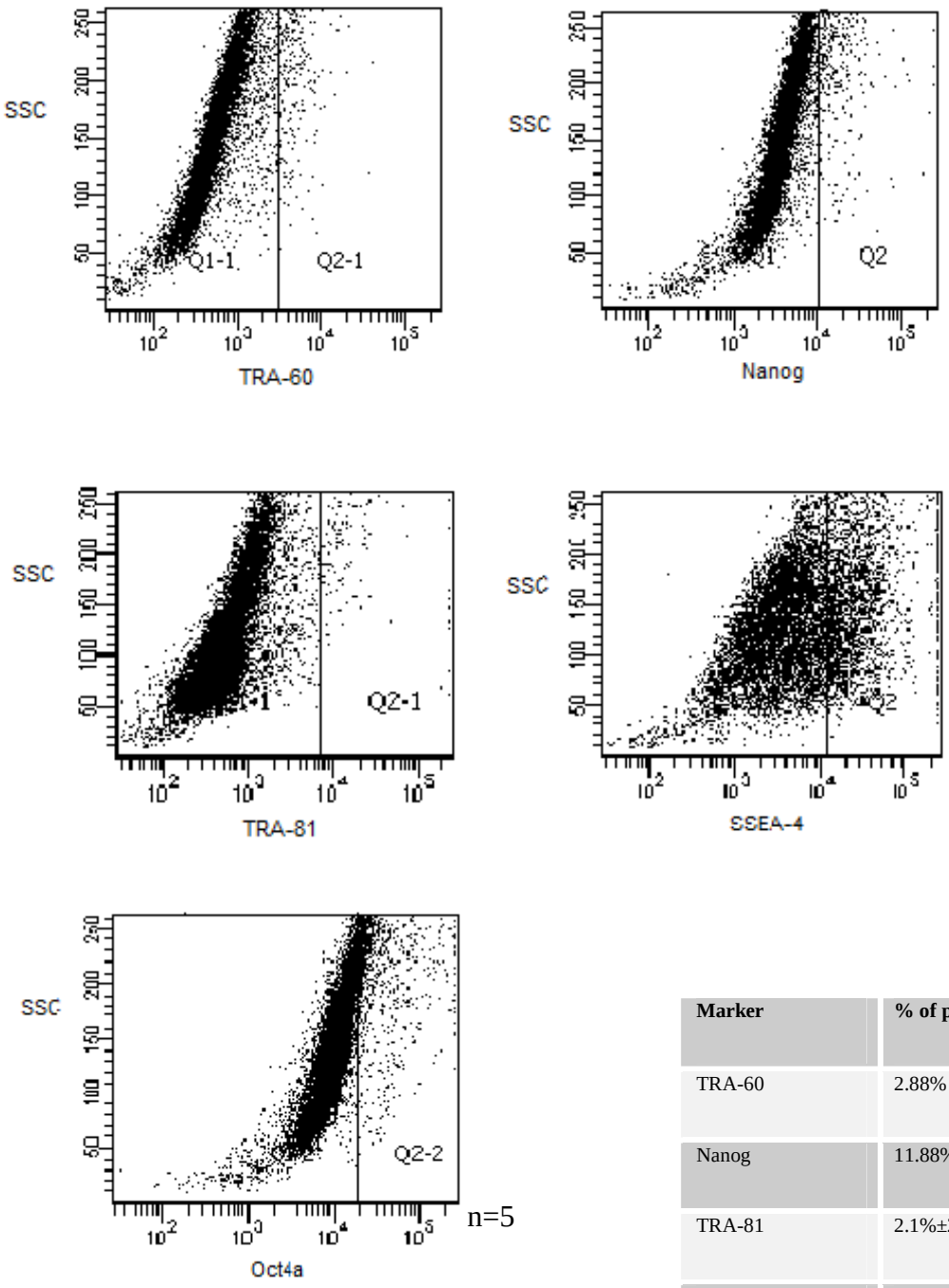


Figure 63. Expression of embryonic stem cell markers in MSCs. (a) Expression of Oct4 and Sox-2 in umbilical cord Mesenchymal stem cells. (b) Mesenchymal stem cells tested negative for the marker Nanog. Cellular nuclei was stained blue with DAPI. Red=TxRed, Purple=Cy5 and Green= FITC Images were taken at a magnification of x20. All positively stained images were acquired by setting negatives (secondary antibody only) as a control.

Flowcytometric investigation of Mesenchymal stem cells for embryonic stem cell markers



n=5

Marker	% of positive cells
TRA-60	2.88% ± 3.22
Nanog	11.88%±7.44
TRA-81	2.1%±2.72
SSEA-4	59.7%±29.19
Oct-4a	11.82%±11.23

Figure 64. FACS dot plots illustrating the expression of embryonic markers in cord matrix derived MSCs.

Conclusions

Human umbilical cord-derived Mesenchymal stem cell-like cells were characterized by flowcytometric analysis and expressed most of previously described Mesenchymal stem cell markers: CD73, CD29, CD44, CD90 and CD105

These cells tested negative for haematopoietic markers: CD45, CD34 and CD133

MSCs were positive for major HLA class I antigens (A, B and C) but tested negative for minor HLA class I antigen (G) and major HLA class II antigens (DR and DQ)

The cells expressed some embryonic stem cells markers: Oct4, Sox2, Nanog, TRA-60, TRA-81 and SSEA-4, as noted on immunocytochemistry and flowcytometric analysis

4.3 *In vitro* expansion of umbilical cord Mesenchymal stem cells

Umbilical cord MSCs posed no difficulty to propagation *in vitro*. Post isolation these cells were expanded primarily in Mesenchymal stem cell growth medium (MSCGM) 10% fetal bovine serum (FBS) (Invitrogen, 16000-036); MSCGM SingleQuots (Lonza, Cat. no. PT-4105, Walkersville, MD, USA) formulated in Mesenchymal stem cell basal medium (MSCBM) (Lonza, Cat. no. PT-3238, Walkersville, MD, USA) to MSCGM. The media was supplemented with Penicillin-streptomycin and Fungizone to evade bacterial and fungal contamination. The first medium change was carried out 4 days after initial plating of cells to allow the cells adequate time to adhere to the plate forming a monolayer. The cells were expanded in MSCGM until the first passaging of cells was undertaken, nearly two weeks post isolation (when cells were approximately 80% confluent). The cells were then transferred to DMEM-F12 (Gibco, Cat. no. 11039-021) supplemented with 10% FBS. Penicillin/streptomycin and Fungizone were again added to minimize the chances of bacterial and fungal contamination during the prolonged period of proliferation. In this culture media the cells underwent 10 successful passages with a doubling time of about a week before they reached a stage of growth arrest (Figure 63). During the period of proliferation, the cells acquired the morphology of small very spindly cells with small centrally located nuclei. At the stage of cell cycle arrest, a transformation from small spindle cells to large flat irregular, occasionally multinucleated cells with wide cytoplasm was noted. With regular media changes these cells could be maintained *in vitro* for several days but then gradually showed a decline in cell viability. Interestingly, it was observed that if these growth arrested cells were transferred back to Mesenchymal stem cell growth medium (MSCGM) (plus supplements) they could be pushed back into proliferation and re-acquire the small spindle morphology.

It may be argued that MSCs demonstrated a better *ex vivo* expansion and survival than cord blood stem/progenitor cells because these cells have been shown to express adhesive ligands and soluble factors that support their growth and self-renewal. MSCs have previously been used *in vitro* in co-cultures with unmanipulated cord blood cells and have been shown to support haematopoiesis and maintenance of cord blood stem/progenitor cells *ex vivo* (*Robinson et al, 2006*).

It must be noted, however, that MSCs from umbilical cord did not produce niche-like colonies. This could partly be due to the fact that these cells were a more homogeneous population and hence lacked the cellular heterogeneity that is essential for niche formation.

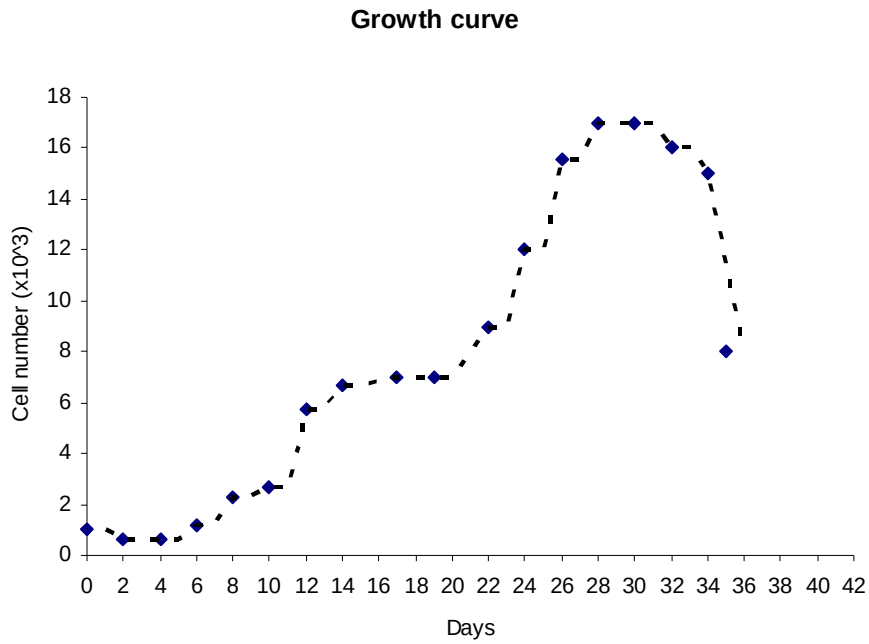
Conclusions

Mesenchymal stem cells demonstrated very efficient *in vitro* proliferation and viability

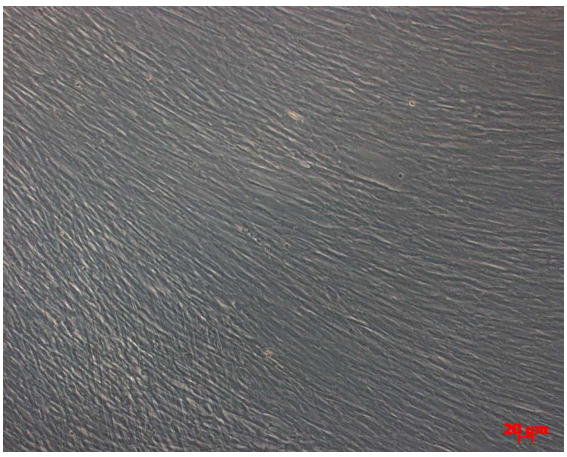
Mesenchymal stem cell growth medium (MSCGM) (Lonza) was essential for kick starting MSC proliferation *ex vivo*

With prolonged *ex vivo* expansion in DMEM-F12 10% FBS MSCs transformed from small spindle-shaped cells to large, flat, irregular and occasionally multinucleated cells with wide cytoplasm. At this stage the cells were noted to be in cell cycle arrest.

(a)



(b)



(c)

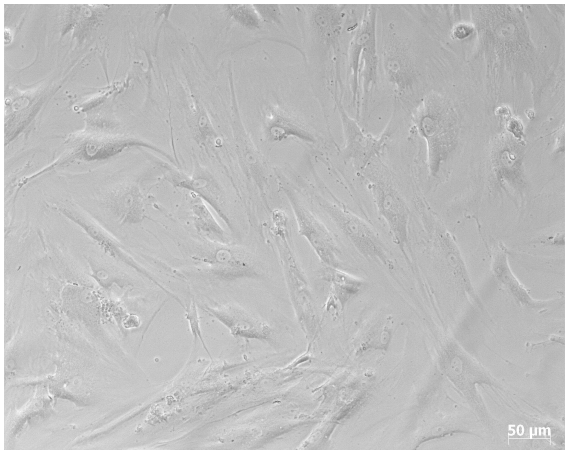
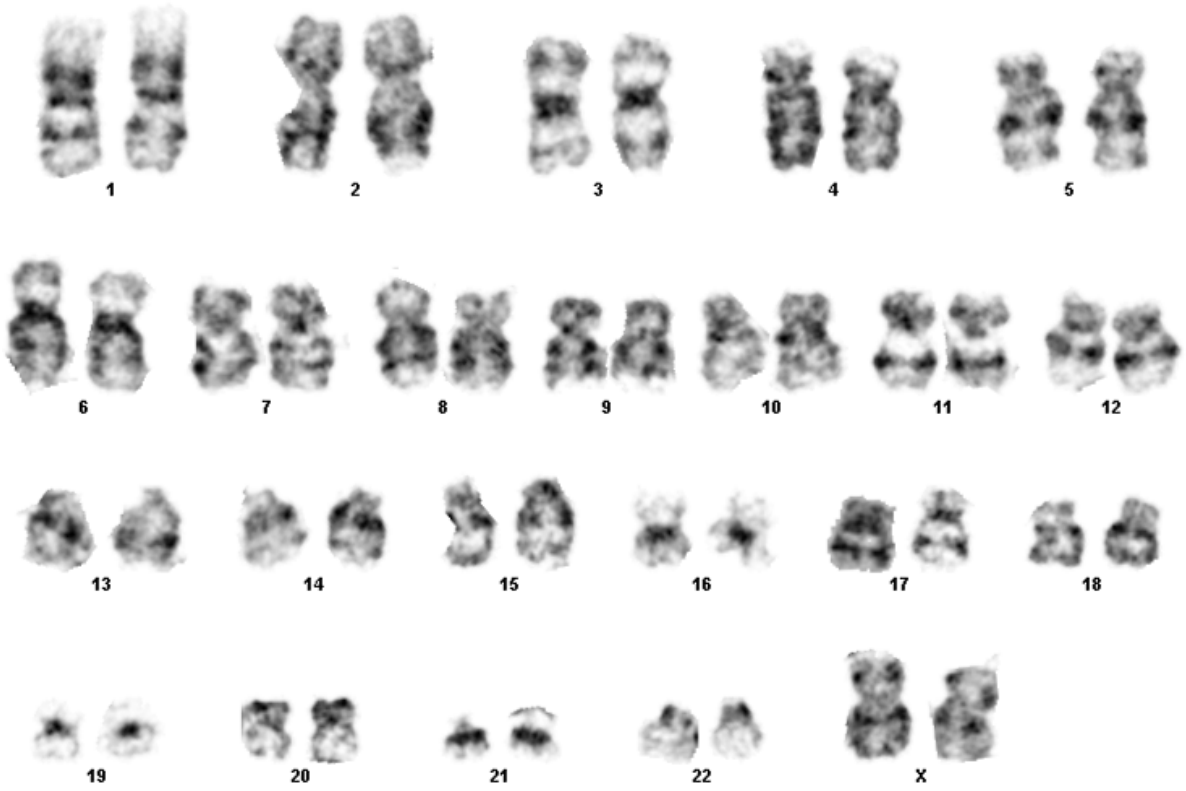


Figure 65. Growth curve for human umbilical cord-derived Mesenchymal stem cells during a 6 week proliferation assay. (a) Six week *in vitro* proliferation assay for human umbilical cord-derived MSCs. Average doubling time for the cells was one week . (b) Mitotically active MSCs were noted to be small, spindle shaped with small nuclei. Magnification x10 (c) Upon entrance into cell cycle arrest, the cells transformed into large, flat, irregular shaped cells with wide cytoplasm and were occasionally noted to be multinucleated. Magnification x20.

4.4 Cytogenetic analysis post expansion

Post prolonged *ex vivo* expansion of Mesenchymal stem cells, cytogenetic analysis was undertaken to determine the genetic status of the cells. The cells demonstrated a normal karyotype after 14 passages.



P=14

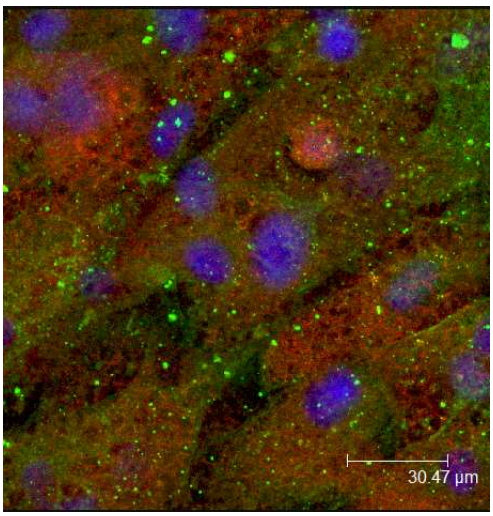
Figure 66. Karyotype of human umbilical cord Mesenchymal stem cells post prolonged *ex vivo* expansion. N=2

4.5 Multi-potential of isolated human umbilical cord Mesenchymal stem cells

In addition to hepato-bilary differentiation (endoderm), other members of the group were able to successfully differentiate umbilical cord-derived MSCs into cells representing the other two germ layers: Neuro-glial cells (ectoderm) and cardiac progenitor cells (mesoderm).

Multipotential characteristic of umbilical cord-derived Mesenchymal stem cells

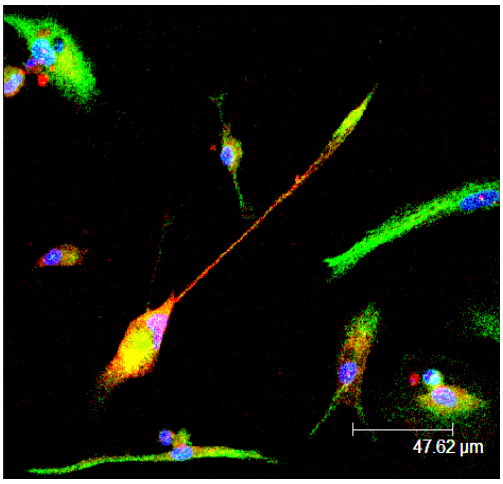
(a)



ALB
AFP

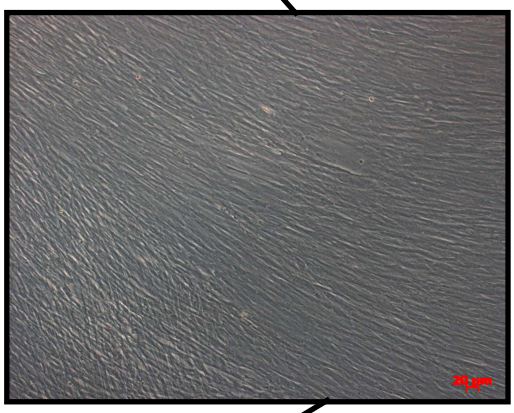
Endoderm

(b)

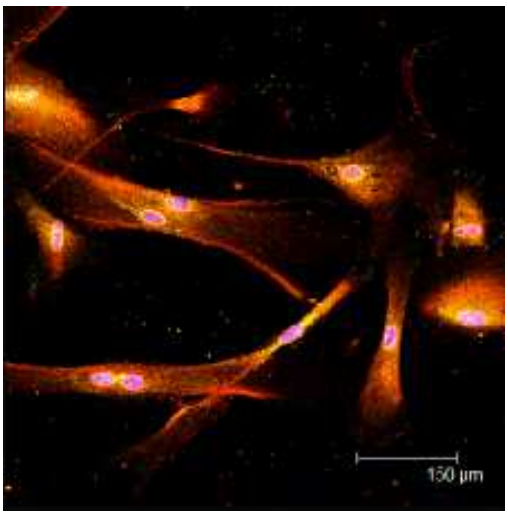


Nf-200
TUJ1

Ectoderm



(c)



Mesoderm

Nkx2.2
CARP

Figure 67. Multipotentiality of umbilical cord-derived MSCs. Images taken at magnification x10 and x20. Nuclei stained blue with DAPI, green= FITC, Red= TxRed. Image (b) courtesy of Mr Hamad Ali and image (c) courtesy of Dr Christina Basford.

4.6 Chapter discussion

One of the goals of this study was to isolate Mesenchymal stem cells from umbilical cord samples. We demonstrated this task with 100% efficiency. Manual dissection and transfer of umbilical cord pieces into culture media proved more effective than enzymatic digestion of umbilical cord samples.

The isolated MSCs tested positive for most of the markers commonly applied to characterize MSCs. These cells tested positive for major HLA class I antigens but were negative for HLA class II antigens. Lack of expression of HLA class II identified MSCs as being immune priveleged. This feature makes MSCs an attractive candidate for HLA-independent therapeutic strategies.

The isolated MSCs were investigated for the expression of some pluripotency factors and tested positive for a few ES cell markers. These MSCs demonstrated very efficient *in vitro* proliferation and expansion. Cytogenetic analysis of the cells post expansion demonstrated a normal karyotype.

We further investigated the multi-potential property of these cells. The isolated MSCs were able to differentiate into cells representing all three germ layers.

Thus, it may be concluded that the abundant availability of umbilical cords as a biological by-product, the efficiency of isolation and the reproducibility of the manual technique applied defines the umbilical cord as a valuable stem/progenitor cell source for regenerative medicine.

Chapter 5

Differentiation of human umbilical cord and cord blood stem/progenitor cells towards liver lineage

5 Introduction

This study endeavoured to exploit the multi-potential property of umbilical cord and UCB-derived stem/progenitor cells to reproduce liver cells *ex vivo*. Stimulation with a range of hepatic inducting signals resulted in successful commitment of UCB-derived undifferentiated stem/progenitor cells and Mesenchymal stem cells along hepatic and biliary lineages. **A three step protocol encompassing the following stages was applied to effect hepatic differentiation from undifferentiated primitive cells:**

- **Short-term expansion with meso-endoderm commitment**
- **Hepato-bilary differentiation**
- **Short-term maturation**

The selected stem/progenitor cell populations have tremendous potential in the management and treatment of liver disorders. However, translation from the bench to regenerative medicine clinical applications and clinical trials demands certain important concerns to be considered. The factors of particular importance include: (1) minimizing *in vitro* culturing time duration, which is important to reduce the risks of bacterial and fungal contamination and also to avoid chromosomal abnormalities which have occurred in other stem cell populations (Corselli, Parodi et al. 2008); (2) culture systems free of animal products in order to diminish zoonosis and the transmission of diseases across species; (3) use of well defined media (serum-free) to prevent any effect serum or serum components might have on the growth and differentiation pathways (Xu, Peck et al. 2005); (4) development of efficient 3-dimensional tissue engineering systems incorporating bio-degrable scaffolds. These factors constitute the core components of GMP standard and clinical grade tissue engineering SOPs (Standard Operational Protocols).

5.1 Serum versus serum-free culture systems

A few research groups have managed to successfully differentiate umbilical cord blood stem/progenitor cells and umbilical cord MSCs into hepato-bilary cells. However, the major limitation of their techniques was the use of un-defined culture systems that contained animal serum and occasionally prolonged *ex vivo* culture durations, both of which pose potential risk for the clinical applicability of these stem cells.

The system we applied to effect hepato-biliary differentiation of the selected stem/progenitor cell populations aimed to abide by the above mentioned GMP standards of tissue culture. We used a defined serum-free media during differentiation and maturation and a short duration of cell expansion *in vitro* to minimize the risks of infections and chromosomal aberrations. B-27 and N-2 were selected as serum substitutes. Both B-27 and N-2 have previously been defined as serum replacements suited for long-term cultures of primary embryonic hippocampal and other brain neurons (Brewer 1995). Also, Ali, Jurga *et a*l, recently demonstrated successful differentiation of umbilical cord blood stem/progenitor cells into neural cells using a defined serum-free culture system (Ali, Jurga et al. 2009). We examined the composition of both these media supplements (Table 33 and 34) (A.Richardson 2001) . The ingredients did not contain anything that based on literature review should be excluded or avoided in hepatic differentiation protocols. Thus we decided to use both B-27 and N-2 as serum replacements in our defined serum-free differentiation and maturation culture systems.

During the one week expansion of cells, media was supplemented with FBS 5%. This was carried out taking into account that physiologically blood is composed of 55% plasma in which the cells circulate. Thus, to avoid rapid changes in the microenvironment of cells, we decided to gradually introduce the cells to a serum-free environment. Additionally, serum would potentially enhance attachment of cells during initial seeding in culture plates. At the end of expansion, half of the media was replaced with our defined serum-free differentiation medium. The culture medium was refreshed on alternate days, every time replacing 50% of the old media with fresh serum-free medium, until eventually reaching 0% FBS by day1 of maturation.

Conclusion

A defined serum-free differentiation medium was formulated by replacing serum with B-27 and N-2 supplements.
Serum levels were gradually tapered by transferring cells from low-serum expansion medium to serum-free differentiation medium.

Table 33. Composition of B-27 supplement

Components	Final mg/dl
Hormones	
Corticosterone	0.02
Progesterone	0.0063
Retinol, acetate	0.1
Insulin	4.0
T3 (triodo-L-thyronine)	0.002
Vitamins	
Biotin	0.10
Antioxidants	
D, L-α-Tocopherol (Vit.E)	1.0
D, L-α-Tocopherol acetate	1.0
Catalase	2.5
Glutathione (reduced)	1.0
Superoxide dismutase	2.5
Others	
L-Carnitine	2.0
Ethanolamine	1.0
Putrescine	16.1
Selenium	0.016
Linolenic acid	1.0
Linoleic acid	1.0
Albumin, bovine	2500.0
Transferrin	5.0

(A.Richardson 2001)

(Wachs, Couillard-Despres et al. 2003)

Table 34. Composition of N-2 supplement

Component	Final mg/L
Insulin (bovine)	5mg/L
Progesterone	20nM
Putrescine	100μM
Na selenite	30nM
Transferrin (human)	100mg/L

(A.Richardson 2001)

(Wachs, Couillard-Despres et al. 2003)

5.2 Hepato-biliary differentiation culture system *in vitro*

5.2.1 Introduction

During embryonic development, early liver formation from embryonic endoderm requires a series of inductive signals from at least three different mesodermal cell types. Induction of hepatic genes requires fibroblast growth factor (FGF) signaling from the adjacent cardiogenic mesoderm, bone morphogenic protein (BMP) signaling from nearby septum transversum mesenchyme and interaction with endothelial cells (Schmidt, Bladt et al. 1995; Jung, Zheng et al. 1999; Duncan 2000; Duncan 2003; Lemaigre and Zaret 2004). When hepatic endoderm is specified and the liver bud begins to grow, the cells are referred to as hepatoblasts. These hepatoblasts are bipotent cells and eventually give rise to definitive hepatocytes and bile duct epithelial cells (cholangiocytes). Hepatoblasts have a phenotype, intermediate between hepatocytes and cholangiocytes. During and after the mid-stage of hepatogenesis, surrounding Mesenchymal cells secrete HGF and support the foetal hepatocytes (Kinoshita and Miyajima 2002; Zaret 2002). At the developmental phase where liver becomes the major site for haematopoiesis, haematopoietic stem cells produce Oncostatin M, that in the presence of circulating glucocorticoids promotes hepatic cell differentiation and maturation and simultaneously suppresses embryonic haematopoiesis (Schmidt, Bladt et al. 1995; Kinoshita and Miyajima 2002; Zaret 2002).

Post development and in adult life hepatoblasts are replaced by progenitor cells which form a reserve cell compartment, also called ‘progenitor cell compartment’, and reside in the smallest and most peripheral branches of the biliary tree, the ductules and canals of Hering (Roskams, Theise et al. 2004). These canals represent the anatomical and physiological link between the parenchyma (intra-lobular canalicular system of hepatocytes) and the portal tract mesenchyme (the biliary tree). This reserve compartment is activated when the mature epithelial cell compartment of the liver are damaged or inhibited in their replication.

5.2.2 Formulated differentiation system applied in this study

The differentiation system applied in this study reflected analogy to mechanisms involved in normal mammalian liver development. Additionally, the three-step protocol was deduced after extensive literature review into culture systems previously reported to have been successful in differentiation of stem/progenitor cells into hepato-biliary cells. The sequential approach for the introduction of cytokines to the culture system applied was not innovative in this field, groups like Snykers *et al* and others have tested its effectiveness before (Lee, Kuo et al. 2004; Hong, Gang et al. 2005; Chien, Yen et al. 2006; Ong, Dai et al. 2006; Snykers, Vanhaecke et al. 2006; Talens-Visconti, Bonora et al. 2006; Yoshida, Shimomura

et al. 2007; Campard, Lysy et al. 2008; Kazemnejad, Allameh et al. 2008; Lysy, Campard et al. 2008).

We were able to generate stem cell colonies from UCB similar to the liver 'stem cell niche' described by Roskam (Roskams 2006) using exogenous differentaiting cues that mimic physiological conditions needed for liver development.

It has been shown that exogenous FGF and HGF could mimic the hepatic inductive effects of cardiogenic mesoderm (Kakinuma, Tanaka et al. 2003; Lee, Kuo et al. 2004; Kang, Kim et al. 2005; Kang, Zang et al. 2005; Kang, Zang et al. 2005; Baharvand, Hashemi et al. 2006; Kang, Zang et al. 2006; Hay, Zhao et al. 2007) . Acidic-FGF (aFGF or FGF1) has been shown to constitute autocrine and paracrine regulation of liver development (Kan, Huang et al. 1989). Acidic FGF is protein of the FGF family. A-FGF shares 55% DNA sequence homology with Basic FGF. These two growth factors are ubiquitously expressed and exhibit a wide spectrum of similar biological activities with quantitative differences likely due to variation in receptor affinity or binding (Ledoux, Gannoun-Zaki et al. 1992). A-FGF interacts with all 4 FGF receptors. It is known that FGF is essential for the induction of endoderm towards liver lineage (Kawasaki, Tamura et al. 2005). Mouse embryonic stem cells grown in medium supplemented with FGF could differentiate into cells expressing hepatocyte-specific genes and antigens (Kan, Huang et al. 1989; Jung, Zheng et al. 1999; Ruhnke, Ungefroren et al. 2003).

HGF was first identified as a blood-derived mitogen for hepatocytes. HGF is a ligand of tyrosine kinase c-Met (Limaye, Bowen et al. 2008). HGF and its receptor c-Met are the key factor for liver growth and function and its actions are brought about through induction of all members of the TGF-β family (Michalopoulos, Bowen et al. 2003).

In addition to FGF and HGF, epidermal growth factor (EGF) was introduced later in the culture system. EGF is a ligand of tyrosine kinase receptor EGFR (Limaye, Bowen et al. 2008). EGF has been reported to increase the expression of all liver-specific genes and stimulates differentiation of foetal liver epithelial cells (Kawasaki, Tamura et al. 2005). Akaike *et al*, reported that EGF signaling was mediated through integrins. They noted that primary hepatocyte cultures only proliferated when cultured on collagen (an integrin) matrix (Kim and Akaike 2007). Thus, we used collagen IV in our differentiation and maturation medium. Previous reports have noted that EGF on its own and in association with HGF and dexamethasone induces biliary epithelial formation (Michalopoulos, Bowen et al. 2003; Limaye, Bowen et al. 2008). All three factors induce HNF-6 expression, which is important in biliary epithelial development.

Initial endoderm commitment of UCB-derived stem/progenitor cell populations was achieved using TPO, SCF, acidic-FGF and HGF in low-serum (5% FBS) culture conditions for a week. This was followed by differentiation using by a cocktail of acidic-FGF, HGF and EGF in serum-free culture conditions which allowed for tapering of serum levels to achieve an ultimate serum-free system.

Umbilical cord derived MSCs, in addition to illustrating very efficient propagation in DMEM-F12 and viability *in vitro,* also demonstrated markers specific to all three germ layers at day 0, i.e. in their undifferentiated state post isolation (Figure 71). Hence, endoderm induction of these cells was unnecessary. The aim, however, was to enhance and increase the expression of endodermal and liver specific markers in these cells during the period of differentiation and maturation.

Post cell differentiation we were able to generate bipotent liver progenitor cells from both UCB-derived stem/progenitor cells and MSCs. Our findings validated results of previous work done in this area (Lee, Kuo et al. 2004; Kang, Zang et al. 2005; Kang, Zang et al. 2005; McGuckin, Forraz et al. 2005). In addition to bipotent progenitors a heterogenous population of liver cells were also noted. Cells displayed a range of phenotypes from hepatocytes to biliary epithelail cells. The presence of EGF in the culture induced simultaneous biliary epithelial cell development. This action was synergistically enhanced by the introduction of corticosteroid (dexamethasone) during the subsequent maturation phase of the protocol.

Maturation of umbilical cord and UCB-derived hepatocyte progenitors was achieved using a combination of dexamethasone and Oncostatin M. Oncostatin M is a member of the interleukin 6 family cytokines and was originally identified by its ability to inhibit growth of A375 melanoma cells (Zarling, Shoyab et al. 1986). Later studies showed that primary cultures stimulated with oncostatin M showed a progression of hepatocytic development towards maturation (Kamiya, Kinoshita et al. 1999; Kinoshita, Sekiguchi et al. 1999; Miyajima, Kinoshita et al. 2000). Dexamethasone, by itself, suppresses growth and induces maturation of cells exclusively towards hepatic lineage. This action is exhibited through suppression of Il-6, CXC-chemokine receptor, amphiregulin, COX-2 and HIF; and induction of HNF4 and C/EBPα expression, both transcription factors needed for hepatocyte differentiation (Michalopoulos, Bowen et al. 2001; Michalopoulos, Bowen et al. 2003). But, in combination with HGF and EGF, it induces biliary epithelial formation.

In the presence of maturation inducers, retraction of the elongated end of MSCs membranes was observed and the polygonal morphology of hepatocyte-like cells became more apparent with increased length of culture (Figure 71). Under similar hepatogenic conditions the

rounded morphology of UCB-derived colony cells gradually progressed towards the polygonal morphology of hepatocytes in a time-dependent manner and became apparent by 2 weeks post-differentiation (Figure 70)

Conclusion

Short-term *ex vivo* expansion and simultaneous endoderm commitment of UCB-derived stem/progenitor cells was executed before exposing the cells to differentiation

Umbilical cord MSCs already illustrated commitment to hepatobiliary lineage post isolation and hence stimulation of endoderm commitment during *ex vivo* expansion was not necessary.

The differentiation culture system was designed to further differentiate the endoderm committed cells towards hepato-biliary progenitor cells (hepatoblasts) with inclination towards hepatocyte differentiation primarily.

The presence of EGF in the culture system induced simultaneous biliary epithelial cell formation. This action, however, was synergistically enhanced by the introduction of dexamethasone during maturation.

5.3 Temporal significance of cytokine introduction in the differentiation system

Liver development is accomplished by a sequential array of biological events, although it is important to note that overlap between the stages is greatly possible. Phenotypic transformations during liver development are preceded by a chronological cascade of molecular events that are tightly regulated and determine the ultimate fate of cells. Various extracellular signals, including cytokines and growth factors, as well as cell autonomous mechanisms are involved in this sequence of events. The cytokine, EGF, demonstrated this phenomena during our *ex vivo* differentiation protocol.

Early introduction of EGF in the culture system, i.e. during expansion, resulted in 60% of the cells expressing markers of ectodermal lineage (Nestin and GFAP). Additionally cells demonstrated neuronal-like morphology. In the absence of EGF, however, the expression of these markers was minimal post expansion and the cells illustrated a more rounded morphology with propensity towards aggregate formation, cell density allowing (section 3.6, Figure 57). This illustrated that early introduction of EGF favoured ectoderm lineage commitment *ex vivo*. However, when EGF was added to the media later in the protocol, i.e. during differentiation, it provided signaling cues that enhanced hepato-biliary differentiation.

To study the influence of EGF on hepato-biliary differentiation *in vitro* two parallel identical differentiation experiments (week one of the differentiation protocol) were set up with the only deferring factor being the presence or absence of EGF. Post differentiation the corresponding cells were examined by immunocytochemistry and at the mRNA level. Although the cells failed to demonstrate remarkable phenotypic differences on immunocytochemistry examination, interesting differences were noted on molecular examination.

In the absence of EGF (media supplemented with aFGF and HGF as well) UCB stem/progenitor cells demonstrated an up regulation in genes associated with hepatocyte progenitor (Gata-4, CLDN2) and hepatoblast (AFP) phenotypes. The cells also expressed some CYP genes at this stage. In paralleling EGF supplemented experiment no Gata-4 and AFP expression was detected and CLDN2 was measured at a lower level. CYP genes if detectable were expressed at lower levels as well (Section 5.4.7, Figure 85-88). In comparison, when EGF was introduced into the media at a later stage in the differentiation protocol (week two) a different gene expression profile was noted. Endodermal marker,

Gata-4, was no longer detectable; hepatic stem cells marker, CLDN2, was down regulated and hepatoblast related genes, HNF4α and AFP, were up regulated. Some CYP genes were no longer expressed, whilst others showed an increase in expression.

Here we have shown that early introduction of EGF into the culture media does not enhance hepatocyte differentiation of cord blood stem/progenitor cells. On the other hand, supplementation of the differentiation system with EGF at later stage (after initial hepatocyte progenitor induction) favours further hepatocyte development and maturation. It has previously been reported that bile epithelium formation is dependent on EGF (Nishikawa, Doi et al. 2005) and here we demonstrate that EGF signalling early in hepatocyte differentiation system does not favour hepatocyte formation.

MSCs responded differently to the presence of EGF *in vitro*. Early introduction of EGF into the differentiation system resulted in an up regulation of hepatocyte progenitor genes (CLDN2 and HNF4α) and down regulation of CYP genes. In contrast, later introduction of EGF during week two of differentiation was associated with down regulation of CLDN2 and AFP whilst HNF4α and all the studied CYP genes were up regulated (Section 5.4.7, Figure 85-88) illustrating a more mature hepatocyte phenotype. In appears that in MSC cultures early influence of EGF signalling pathway caused either dedifferentiation of cells already committed to hepatobiliary fate causing them to revert to a more primitive hepatic stem cell phenotype, or stimulated hepatocyte differentiation towards biliary epithelial cells through a transdifferentiation mechanism. If this phenomenon were proven to be true it would demonstrate phenotypic plasticity of hepatocytes *in vitro*. However, more work needs to be carried out to enable a better understanding of the role of EGF in hepato-bilary differentiation.

Here we have shown that although EGF plays a pivotal role in hepato-bilary differentiation, the determination of the exact time of cytokine introduction into the differentiation protocol is of prime importance.

Likewise, introduction of maturing factors, dexamethasone and Oncostatin M during differentiation, would expectedly hinder the activated differentiation pathways and cause premature maturation of cell in culture.

Conclusion

The timing of EGF introduction into the differentiation medium was of prime importance.

Early introduction of EGF during expansion promoted cells to commit along ectoderm lineage.

Later introduction of EGF into the differentiation medium (after endoderm commitment) promoted differentiation towards hepatobiliary epithelial cells.

In vitro characterization assays on differentiated cells was performed at regular intervals post induction and the results are noted in the sections below.

5.4 Periodic analysis of stem/progenitor cells during differentiation

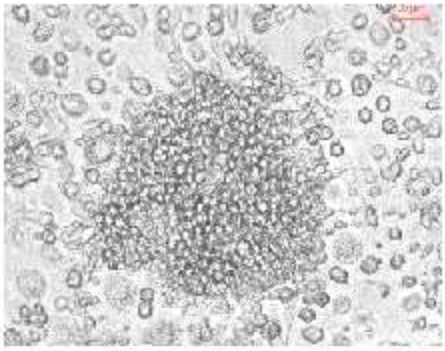
One of the challenges of identifying and characterizing non-liver derived hepato-bilary cellular populations *in vitro* is the lack of definite cell markers to classify these cells. Taken individually the markers that are frequently used for hepatocyte characterization are not limited to the liver. Studies have shown expression of cytokeratins, often used for hepato-bilary characterization, have also been detected in the gastric cardia, the lung, the skin and in the umbilical cord. For example, Ck18, commonly used as a mature hepatocyte marker, was observed in the lung. Ck8, also a mature hepatocyte marker, is apparently one of the most expressed genes in umbilical cord MSCs. Furthermore, mRNAs for albumin, AFP and α-1AT (Alpha-1Antitrypsin) were detected in human pancreas. Cytochrome 3A4 has recently been identified in the brain (Robertson, Field et al. 2003). Consequently, we decided to use a combination of markers to characterize our umbilical cord and cord blood-derived differentiated stem/progenitor cells.

5.4.1 Phenotypic analysis during UCB-derived stem/progenitor cellular colony differentiation

After 1 week of *ex vivo* expansion, UCB-derived primitive cells were stimulated with a range of differentaitive hepatic cues for 2 weeks. Cells were examined and images taken at different stages of differentiation and maturation. Results demonstrated that initial exposure to differentiating signals caused a reduction in the size of the cellular aggregates (Figure 68a and b). This phenomenon was probably due to the fact that not all cells are able to withstand the drastic change in the culture conditions and underwent apoptosis (programmed cell death). The ‘niche-like’ structures appeared to be more tolerant to the change in culture conditions. With increased length in culture during differentiation, the cell clusters increased in size and demonstrated a heterogeneous population of cells that probably function in a synergistic way to provide signals that determine the fate of stem cells (Figure 68c). We noted that the small rounded cells were positioned centrally and were surrounded by spindle-shaped cells that occasionally overlay large multinucleated osteoclast-like cells (Figure 68c and d). These structures were similar to the niches described by Sun *et al*, 2007; rounded cellular colonies formed on large multinuclear cells (osteoclast-like cells), surrounded by spindle-shaped cells. With differentiation the cells displayed an increase in size and cytoplasmic granularity (Figure 68c). Contact with maturation inducers caused a

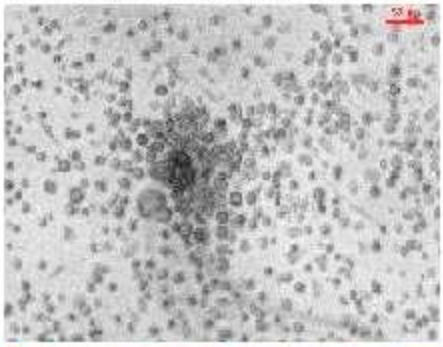
further increase in size with an increase in nucleus to cell size ratio and cells began to take on a polygonal phenotype (Figure 68d). These UCB-derived cells did not survive *in vitro* after 2 weeks of *ex vivo* differentiation and maturation.

(a) Day 1



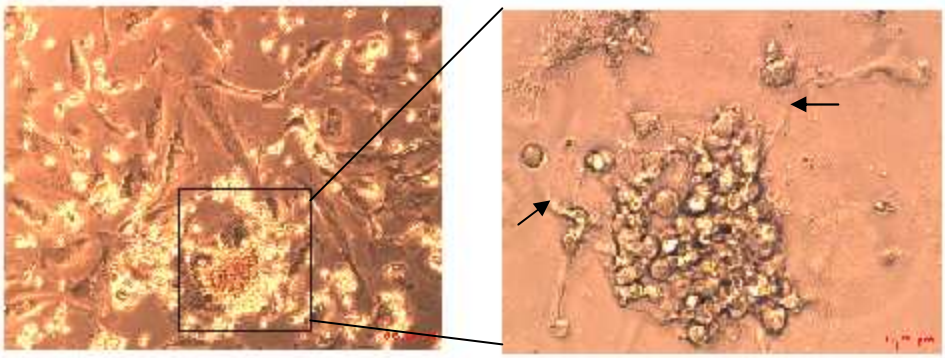
Post *ex vivo* expansion and endoderm commitment

(b) Day 7

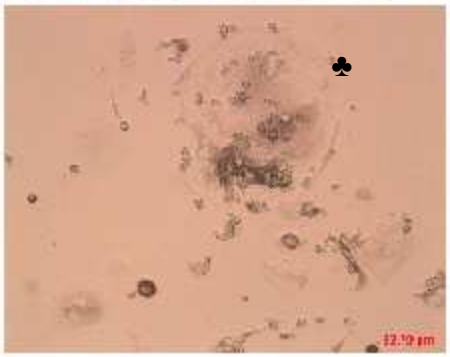


Post 1 week *ex vivo* differentiation

(c) Day 10



(d) Day 14



(e) Day 16

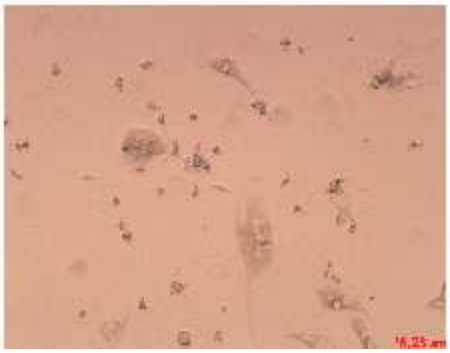


Figure 68. DIC images of UCB-derived stem cell colonies at different stages of the differentiation protocol. (a and b) UCB stem/progenitor cell colonies after expansion. (b) An initial loss in colony size post exposure to differentiation medium. (c and d) During differentiation cells retained their rounded morphology but demonstrated an increase in nuclear to cell size ratio. Differentiated colonies were supported by large multinucleated osteoclast-like cells (♣) on which the colonies were assembled and surrounded by occasional spindle shaped cells. Introduction of maturation factors resulted in a further increase in cell size and the appearance of polygonal cellular morphology. (e) The differentiated colonies failed to survive *ex vivo* more that 2 weeks. Apoptosis of the large supporting osteoclast-like cells preceded eventual loss of the differentiated colonies. Images were taken at magnifications of x5, x10 and x20. UCB (Umbilical cord blood)

5.4.2 Phenotypic analysis during umbilical cord-derived MSC differentiation

After *ex vivo* expansion, UC-derived MSCs were stimulated with a similar range of differentaitive hepatic and biliary cues for 1 week and then transferred to maturation medium for a further 1-4 weeks. Cells were checked and images taken at different stages of differentiation and maturation. Results demonstrated that initial exposure to differentiating signals caused a reduction in cell confluency (Figure 67a and b). This phenomenon was probably due to the fact that not all cells were able to withstand the drastic change in the culture conditions from expansion to differentiation and underwent apoptosis. The cells that did survive were the ones that went on to differentiate into hepatic progenitors. With differentiation MSCs illustrated a phenotypic transformation. Retraction of cellular appendages was noted and cells demonstrated a transformation in morphology from spindle shape to polygonal shape (Figure 69b and c). These transformed cells were noted to form dense cellular aggregates surrounded by the yet untransformed spindle shaped MSCs (Figure 69c). These MSCs did not display a change in morphology most likely due to lack of response to differentaitive signals in culture. With introduction of maturation factors and prolonged culture duration these untransformed cells eventually underwent apoptosis. Additionally, the cells in the cellular aggregates displayed a more pronounced polygonal morphology (Figure 69d) with increased cellular granularity and appearance of intracyplasmic inclusion bodies (Figure 69e and f) which may be interpreted as a sign of increased metabolic activity. The MSCs were a lot more robust in culture and were able to endure the *in vitro* culture environment for much longer. We were able to maintain these cells in culture for 4-5 weeks.

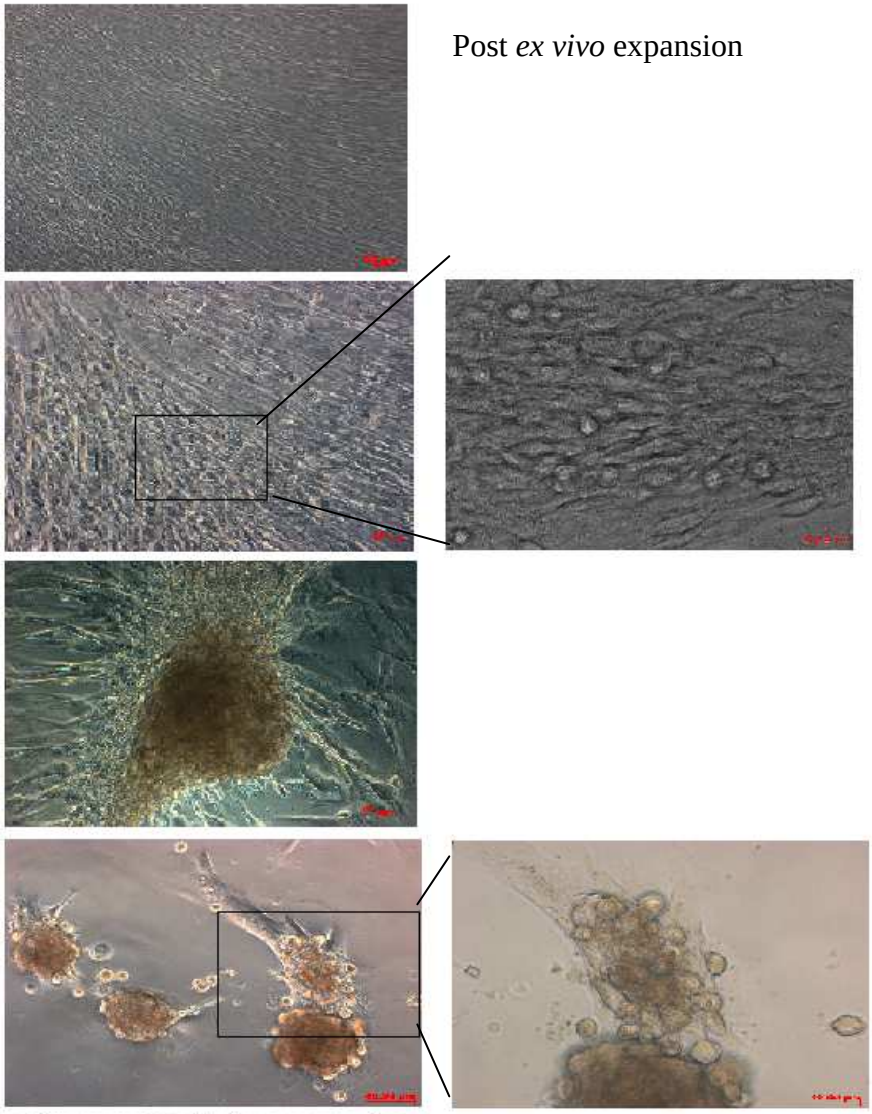


Figure 69. DIC images of UC-derived MSC cells at different stages of the differentiation protocol. (a and b) Exposure to hepatocyte differentaitive signals was associated with a slight initial loss in cell confluency. (b) Cells were exposed to differentiating factors for 1 week. At the end of differentiation cells displayed a noticeable change in morphology. Retraction of appendages and appearance of polygonal contour could be appreciated. (c) Introduction of maturation factors was associated with aggregation of transformed cells into cellular colonies surrounded by untransformed spindle shaped MSCs. (d-f) The cells were maintained *ex vivo* in maturation medium for 3 weeks. Prolonged culture during maturation resulted in eventual reduction in the size of aggregated cellular masses and appearance of intracytoplasmic granules and inclusion bodies were noted. Images were taken at magnifications of x5, x10 and x20

5.4.3 Antigenic analysis- immunocytochemistry on differentiated UCB-derived stem/progenitor cells

The differentiated stem/progenitor cells were examined for a number of liver related proteins by immunocytochemistry.

Differentiated cells exhibited expression of both albumin and AFP. Albumin is one of the earliest hepatic markers. AFP is closely related to albumin both genetically and structurally and is normally expressed in the foetal liver. As albumin synthesis increases during later foetal development, AFP concentrations begin to decline, reaching a trace concentration. Further characterization of differentiated cells was performed by using cytokeratins. These intermediate filament proteins are cytoplasmic structural proteins represented in human epithelial tissues by at least 20 different polypeptides. They range in molecular weight between 40 kDa and 68 kDa. The individual human cytokeratins are designated 1 to 20. The various epithelia in the human body usually express cytokeratins which are not only characteristic of the type of epithelium, but also related to the degree of maturation or differentiation within an epithelium. Therefore cytokeratins are considered to serve as good lineage markers in identifying epithelia and in tracing cell-of-origin in tumours. Differences in the expression of cytokeratins subtypes between human hepatocytes and biliary cells have been well studied (Osborn, van Lessen et al. 1986; Ramaekers, Huysmans et al. 1987; Van Eyken P 1987). Hepatocytes express Ck-8 and -18, and intrahepatic bile duct epithelial cells also express these, in addition to Ck-7 and -19. Ck-7, -8, -18 and -19 were used to characterize differentiated cells in this study. Ck-7 is expressed in hepatic stem cells and in bile ductular epithelial cells and is often co-expressed with Ck-19 (Sasaki, Nio et al. 2001). Ck-19 is expressed by bi-potential hepatic progenitor cells but is lost as these cells differentiate along hepatic lineage. However, expression of Ck-19 should be persistent when progenitor cells develop along biliary epithelium (Haque, Haruna et al. 1996). Ck-19 is expressed in immature as well as remodeled bile duct cells (Sasaki, Nio et al. 2001).. Sasaki *et al* reported that Ck-20 is another keratin detected only in developing bile duct cells in human foetal liver and is absent in late foetal stages (Sasaki, Nio et al. 2001).

Results obtained indicated that the differentiated cells from UCB were liver progenitor cells, expressing Ck-7, Ck-8 and Ck-18 and low levels of Ck-19 post differentiation and maturation *ex vivo* (Figure 70*)*. These findings suggest that differentiated cells can be characterized as bi-potential hepatic progenitors expressing both hepatocyte and biliary cytokeratins but not yet fully committed to either lineage. These cells were predominantly located in the centre of the niches. These cells are similar in morphology and immunophenotype to the ‘intermediate cells’ described by Roskam and the ‘small

hepatocytes' characterized by Kon *et al* (Kon, Ooe et al. 2006; Roskams 2006). The so-called 'small hepatocytes' are a sub-population of rat hepatocytes that have a high growth potential in culture and are two times smaller than normal mature hepatocytes, expressing both hepatocytic and biliary characteristics. The importance of the society of liver cells was also demonstrated in our culture system by the fact that once the surrounding supporting cells of the niche were loss some of the centrally located differentiated stem cells escaped from the colony and became suspended in the medium or underwent apoptosis.

Immunocytochemistry of 2 week differentiated UCB-derived stem/progenitor cells

2 weeks Differentiation

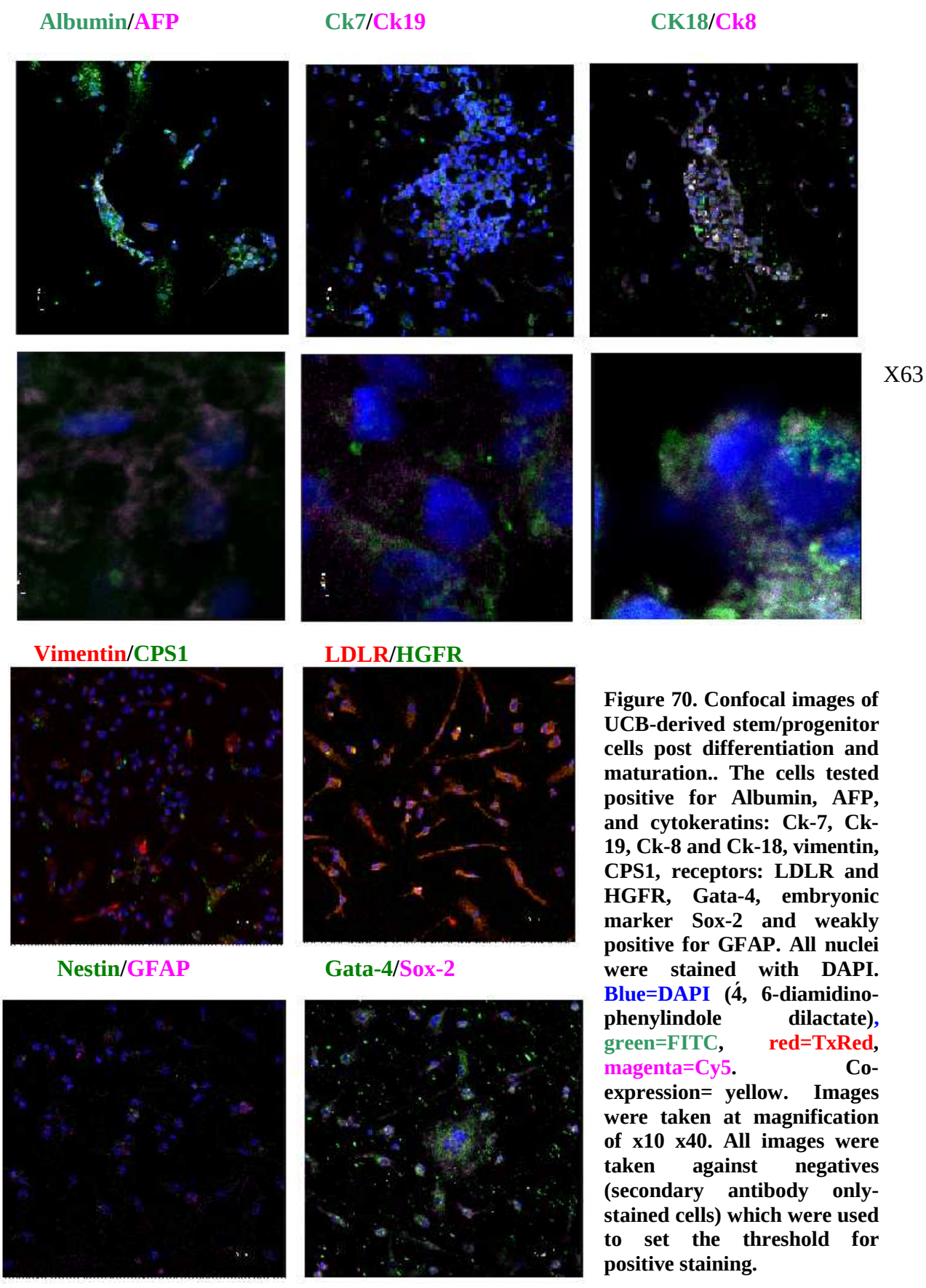


Figure 70. Confocal images of UCB-derived stem/progenitor cells post differentiation and maturation.. The cells tested positive for Albumin, AFP, and cytokeratins: Ck-7, Ck-19, Ck-8 and Ck-18, vimentin, CPS1, receptors: LDLR and HGFR, Gata-4, embryonic marker Sox-2 and weakly positive for GFAP. All nuclei were stained with DAPI. Blue=DAPI (4́, 6-diamidino-phenylindole dilactate), green=FITC, red=TxRed, magenta=Cy5. Co-expression= yellow. Images were taken at magnification of x10 x40. All images were taken against negatives (secondary antibody only-stained cells) which were used to set the threshold for positive staining.

5.4.4 Antigenic analysis- immunocytochemistry on differentiated umbilical cord-derived Mesenchymal stem cells

Characterisation of Mesenchymal stem cells indicated that these cells already displayed signs of lineage commitment upon isolation. These cells already expressed some hepatic markers exhibiting a partial hepatocyte identity. This suggested hepatogenic potency of umbilical cord MSCs. MSCs also tested positive for expression of some embryonic stem cell markers and illustrated capacity to differentiate into tissues representing all three germ layers. Therefore, it might be safe to assume that these cells occupy a lower position in the stem cell hierarchy and must be best referred to as progenitor cells with multipotent capacity rather than pluripotent stem cells.

In addition to testing positive for some liver markers at day 0: albumin, Ck-7, Ck-8, Ck-18, CPS1 and Gata-4, undifferentiated MSCs also tested positive for markers of other germ-layer specific tissues, such as nestin and TUJ (ectodermal) and Nkx2.5 and myocardin (mesoderm) (data generated by other members of the group and not shown here). It is interesting to note that at day 0 MSCs failed to illustrate expression of AFP, LDLR and HGFR.

The aim of the differentiation protocol was to try to amplify the expression of liver markers and down regulate expression of other germ layer markers. Nestin was used as an ectodermal marker during immunocytochemistry analysis.

MSCs were analysed at weekly time points during the three week differentiation and maturation protocol. At the end of the first week of differentiation the cells showed an increase in the expression of: gata-4 (endodermal marker), albumin (hepatocyte marker), Ck-18 (hepatocyte marker) and Ck-19 (hepatic stem cell marker; expression of which persists when cells differentiate along biliary epithelium lineage). Expression of Ck-8 (hepatocyte marker), Ck-7 (hepatic stem cell marker) and nestin (ectodermal marker) was reduced. Cells tested negative for AFP (hepatoblast marker), LDLR and HGFR (Figure 71 and 72). At this stage the antigenic profile of cells showed an increase in the expression of some liver markers and demonstrated a heterogeneous liver cell population with propensity towards hepatic stem cell phenotype.

Analysis of the same antigens post two weeks of differentiation and subsequent to introduction of maturation markers illustrated the following changes: further increase in the expression of albumin, Ck-18 and Ck-19. Expression of Ck-8 also showed an increase at

this stage. Expression of gata-4 and CPS1 appears unchanged. Ck-7 was not expressed and nestin showed a significant down regulation. AFP, LDLR and HGFR were still negative at this stage (Figure 71 and 72). Again antigenic analysis illustrated a heterogeneous population consisting of a lower percentage of hepatic stem cells (demonstrated by reduction in Ck-7 expression), mature hepatocytes (albumin, Ck-8, Ck-18 and CPS1) and biliary epithelium progenitors (albumin, Ck-18, Ck-8 and Ck-19).

Analysis at week three after prolonged exposure to differentiation and maturation factors was associated with remarkable changes in cellular phenotype. The cells were no longer homogenously spindle shaped and in monolayer. Cellular aggregates of polygonal cells surrounded by spindle shaped cells were noted. The expression of markers in the cells was notably different than the previous phases of culture. Expression profiles were no longer uniform and generalized. The intensity of expression of albumin in the cells remained the same. AFP, which was not expressed earlier, was detected at this stage. The expression pattern of this marker, however, was not uniform in the cells. Only the cells located in the centre of the cellular masses tested positive for this marker. Ck-7 expression was strikingly up regulated at this stage and once again the expression was localized to cells in the centre of the cellular aggregates. Ck-8 expression was unchanged compared to week two but was only detected in spindle shaped cells situated at the peripherally of the cellular masses. Again, Ck-19 expression was not noticeably different than week two but was focused in the centrally positioned cells surrounded by Ck-18 positive cells. CPS1 expression was up regulated as well. LDLR and HGFR, which were negative thus far, were expressed at this stage. Nestin expression was absent at this phase of the protocol demonstrating commitment of cells to meso-endodermal lineage and not ectodermal lineage (Figure 71 and 72).

At week three, cells displayed ‘niche-like ‘structures composed of a heterogeneous cellular population. Cells located at the centre of the niches displayed characteristics of primitive progenitor cells: small, rounded and illustrated high expression of albumin, Ck-7 and Ck-19 (hepatic stem cell markers) and AFP (hepatoblast marker). Once again, these cells could be compared to the ‘intermediate cells’ described by Roskam and the ‘small hepatocytes’ characterized by Kon *et al* (Kon, Ooe et al. 2006; Roskams 2006). Cells at the periphery of the niches tested positive for more mature markers: Ck-8 and Ck-18 (hepatocyte markers). Although gata-4 expression was still present, the intensity of expression was reduced compared to earlier stages.

It could concluded that at the end of the protocol we generated a heterogeneous population

of liver cells, with progenitor cells (hepatic stem cells and hepatoblasts) located at the centre of the niches, surrounded by more mature cells. In our culture system these cells displayed characteristics of mature hepatocytes (Ck-8, Ck-18, CPS1, LDLR, and HGFR). Although, biliary epithelium progenitor cells were present in culture (Ck-19 and Ck-7 positive cells), our formulated culture system supported preferred commitment along hepatocyte lineage rather than cholangiocytes.

Figure 71a. Expression of hepatic and biliary markers at different stages of Mesenchymal stem cell differentiation *ex vivo*. Day zero (post expansion), day 7 (one week post differentiation), day 14 (one week post maturation), day 21 (two weeks post maturation). Confocal images were obtained at magnification x10 and x20. Cellular nuclei have been stained blue with Dapi (4', 6-diamidino-phenylindole dilactate). AFP, Ck-7 and Ck-19 were stained magenta with Cy5. Albumin, Ck-7 and Ck-18 were stained green with FITC. Dual staining (co-expression) is represented by yellow staining. All images were taken against negatives (secondary antibody only-stained cells) which were used to set the threshold for positive staining.

Immunocytochemistry of differentiated UC-derived MSCs

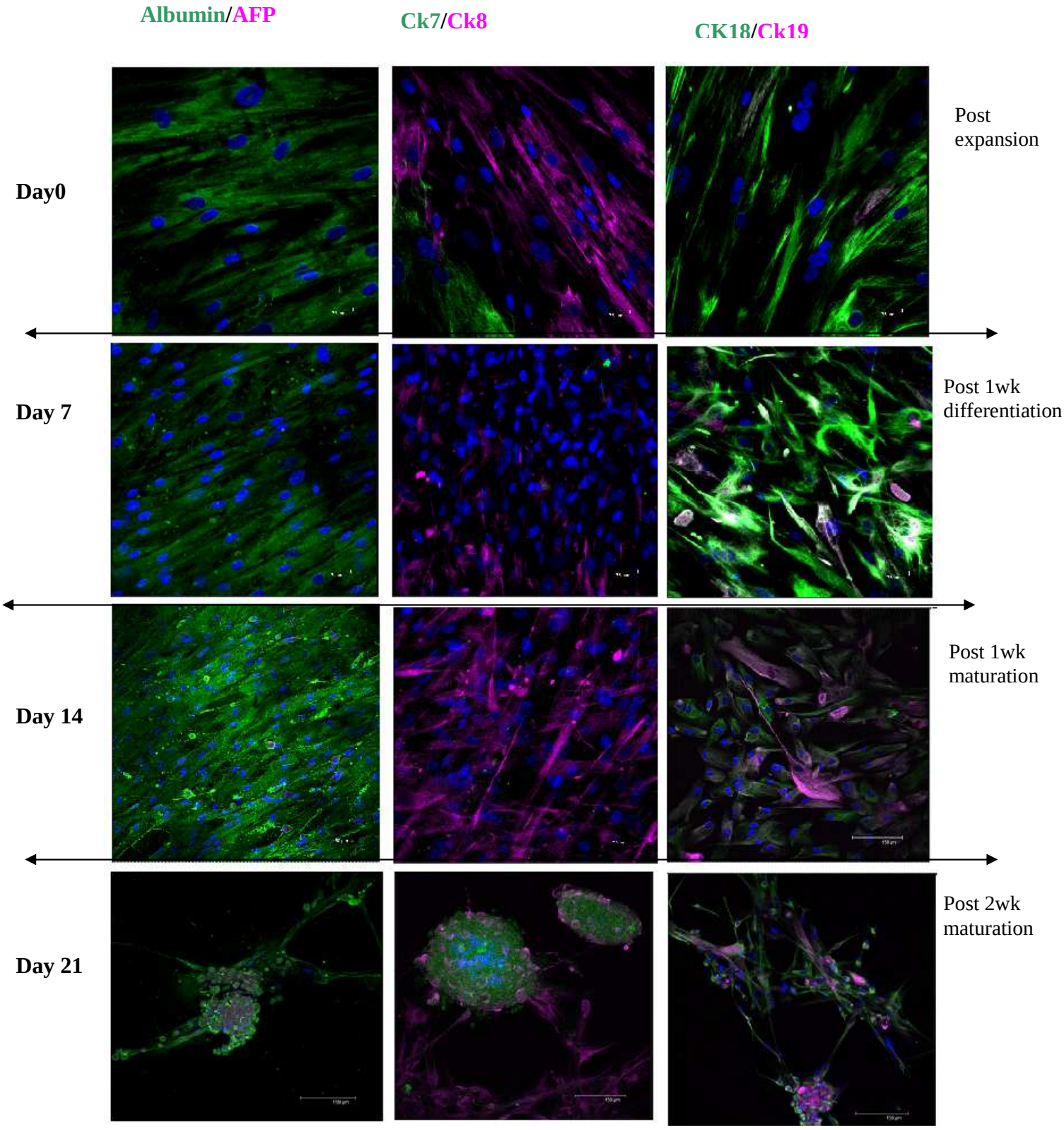
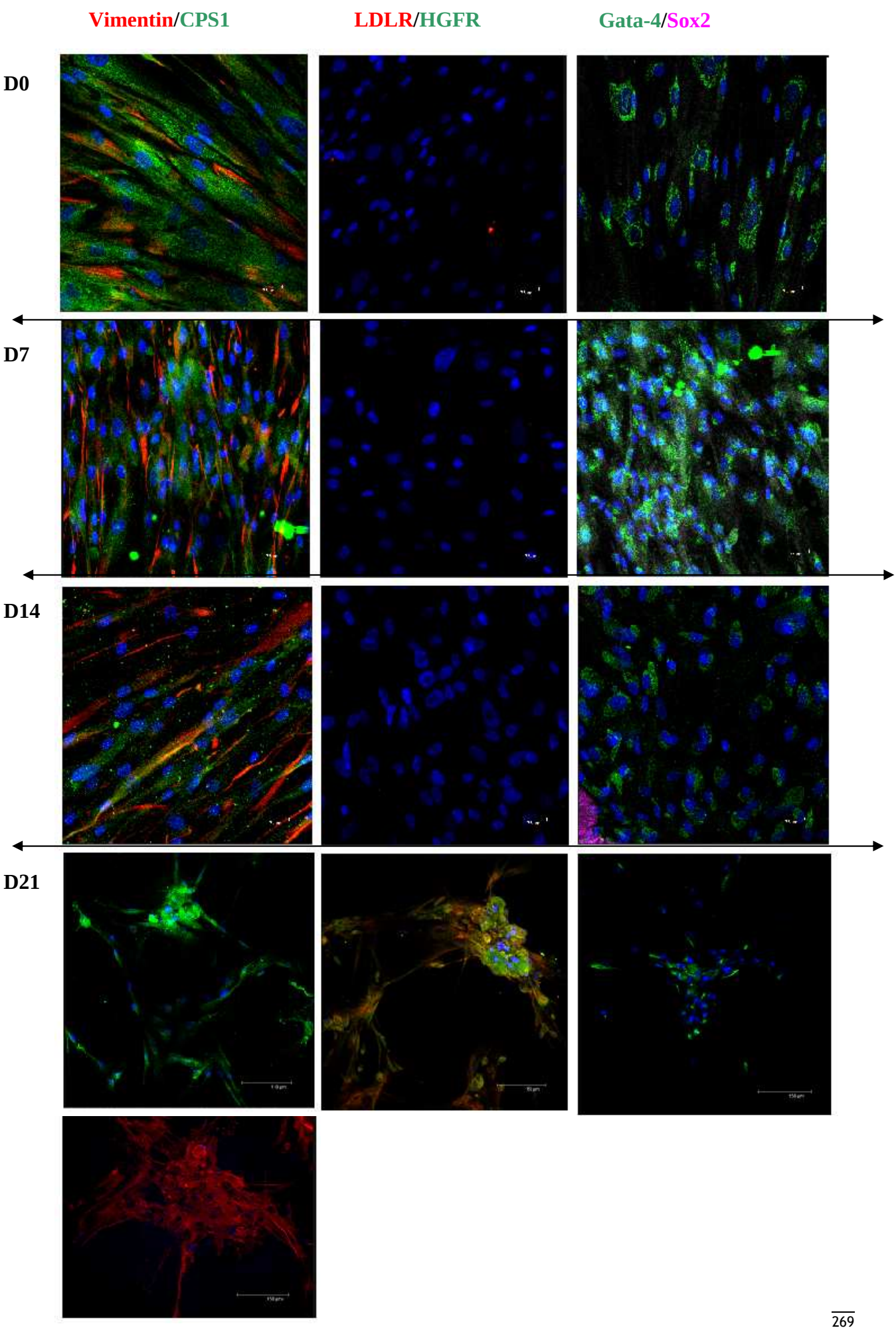


Figure 71b. Expression of hepatic and biliary markers at different stages Mesenchymal stem cell differentiation *ex vivo*. Day zero (post expansion), day 7 (one week post differentiation), day 14 (one week post maturation), day 21 (two weeks post maturation). Confocal images were obtained at magnification x20. Cellular nuclei have been stained blue with Dapi (4', 6-diamidino-phenylindole dilactate). Vimentin and LDLR were stained red with TxRed. CPS1, HGFR and gata-4 were stained green with FITC. Sox-2 was stained magenta with cy5. Dual staining (co-expression) is represented by yellow staining. All images were taken against negatives (secondary antibody only-stained cells) which were used to set the threshold for positive staining.


Vimentin/CPS1
LDLR/HGFR
Gata-4/Sox2
D0
D7
D14
D21

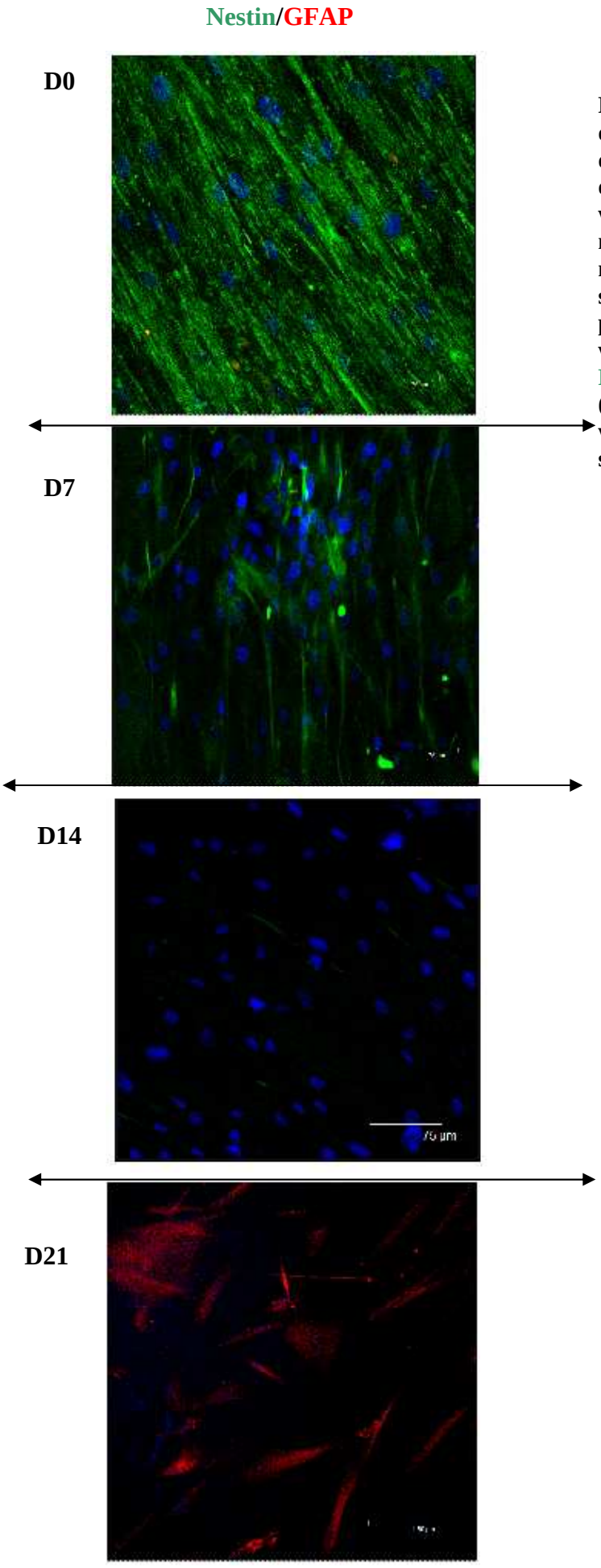


Figure 72. Expression of Nestin and GFAP at different stages of Mesenchymal stem cell differentiation *ex vivo*. Day zero (post expansion), day 7 (one week post differentiation), day 14 (one week post maturation), day 21 (two weeks post maturation). Confocal images were obtained at magnification x20. Cellular nuclei have been stained blue with DAPI (4', 6-diamidino-phenylindole dilactate). GFAP was stained red with TxRed. Nestin was stained green with FITC. All images were taken against negatives (secondary antibody only-stained cells) which were used to set the threshold for positive staining.

5.4.5 Positive controls for immunocytochemistry

To verify the quality and scope of our *ex vivo* differentiation system positive controls were considered essential. We were able to get hold of three types of cells that have previously been used as positive controls in hepatocyte differentiation protocols:

- HepG2 cell-line

- Normal foetal liver parenchyma at 9 weeks of gestation (tissue sections on slides)

- Normal adult liver parenchyma (tissue sections on slides)

All three cell types were used as controls for immunocyto/histochemistry. Positive control for functional analysis of differentiated cells was restricted to HepG2 cell-line.

5.4.5.1 HepG2

The HepG2 cell line has been isolated from a liver biopsy of a male Caucasian aged 15 years, with a well differentiated hepatocellular carcinoma. These cells are epithelial in morphology, have a model chromosome number of 55 and are not tumorigenic in nude mice. The cells secrete a variety of major plasma proteins e.g. albumin, alpha 2-macroglobulin, alpha 1-antitrypsin, transferrin and plasminogen. They have been grown successfully in large scale cultivation systems. HepG2 cells are used routinely for a variety of biochemical and cell biological studies, including studies of hepatocyte functions. HepG2 is the most commonly used cell line for examining the regulation of hepatic protein synthesis by cytokines. In this study HepG2 cell line was used as a control for the UCB- and umbilical cord-derived differentiated liver cells. All the investigations performed on our tested cell populations in culture were repeated on the HepG2 cell line for comparison.
HepG2 cells were kindly provided by Catherine Mowbray and Professor Barry Hirst (Institute of Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, UK). The cells provided were at passage 69. We sub-cultured the cells in a well defined medium composed of DMEM-F12 supplemented with 10% FBS and penicillin/streptomycin and fungizone only. Samples were cryopreserved and subsequently thawed and re-cultured for all further analysis during the course of this study. One characteristic of these cancer cells was to form mounds or piles during *ex vivo* expansion. This phenomenon is due to the reduction in cell-cell and cell-extracellular matrix adhesion that allows masses of cells to form. Cancer cells do not exhibit contact inhibition and thus are able to continue growing

even when surrounded by other cells. This alteration in cell adhesion also facilitates cancer cells to move and migrate in order to metastasize.

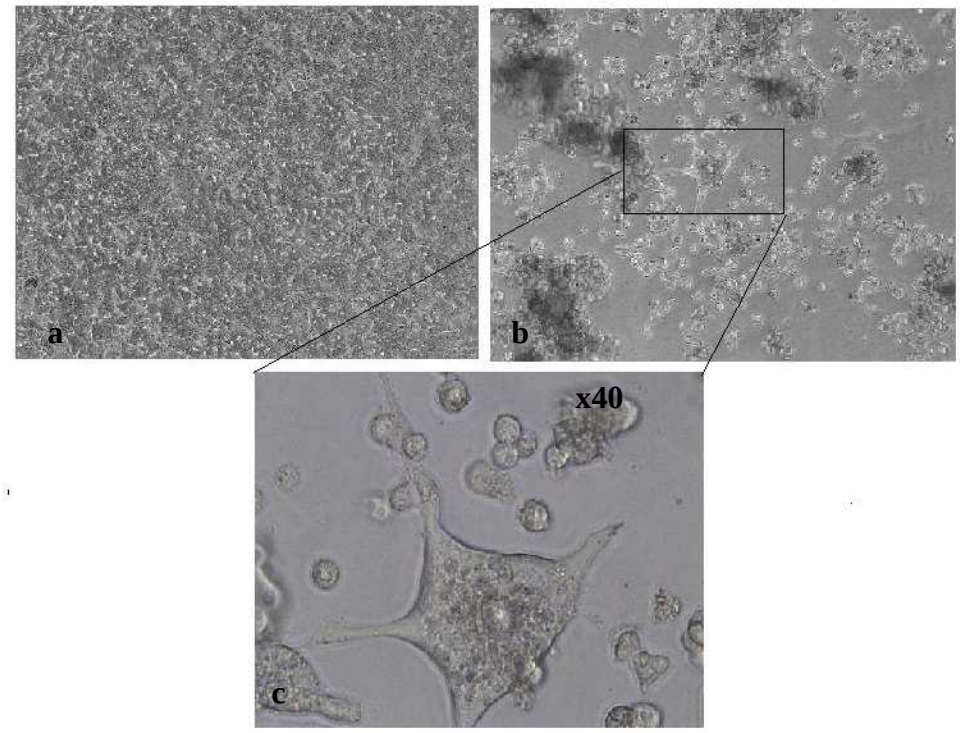


Figure 73. HepG2- well differentiated hepatocellular carcinoma culture. (a) A confluent low magnification image of HepG2 cells in culture. The cells have a tendency to form piles or mounds as they grow (b), rather than growing as monolayers and even when they get confluent, the cell are still very piled-up. (c) High magnification image of an individual cell exhibiting classical hepatic cell morphology: polygonal and a high nucleus to cytoplasmic ratio and increases granularity. DIC images were acquired at magnification of x10, x20 and x40.

Antigen analysis of HepG2 cells by immunocytochemistry

HepG2 cells were examined for the same antigens as UCB- and umbilical cord cells. These cells tested positive for all the studied liver markers: Gata-4, albumin, AFP, Ck-7, Ck-8, Ck-18, Ck-19, HGFR, LDLR, CPS1 and vimentin. These cells were also positive for embryonic markers: Oct4, Sox-2 and Nanog, albeit some at lower levels than others as detected on immunocytochemistry. Nestin and GFAP were also positive in the cells.

The expression of AFP and CK-19 was noted to be predominantly in the centre of the cellular mounds or piles that were structured during *ex vivo* culture of this cell-line (Figure 74). The pattern of expression of other liver markers in HepG2 cells was very similar to that noted in umbilical cord-derived Mesenchymal stem cells post maturation with the important difference that the differentiated MSCs showed very low if any expression of Nestin, and were negative for the embryonic marker Sox-2. The expression of embryonic markers in these cells was not surprising (Figure 74 and 75). It has previously been noted that cancer cells possess traits reminiscent of those ascribed to normal stem cells. Ben-Porath *et al*, 2008 reported previously an unknown link between genes associated with embryonic stem cell identity and the histopathological traits of tumours. They claimed that this probably supports the possibility that these genes contribute to stem cell-like phenotypes shown by many tumours (Ben-Porath, Thomson et al. 2008).

It is worth noting that these HepG2 cells display a phenotype intermediate between hepatocytes and biliary epithelial cells (cholangiocytes). These cells might be comparable to early liver progenitor cells; hepatic stem cells and hepatoblasts but not to mature hepatocytes. Additionally, the abnormal cell kinetics of these cancer cells should be a factor of concern when it comes to interpretation of biomedical studies.

Conclusion

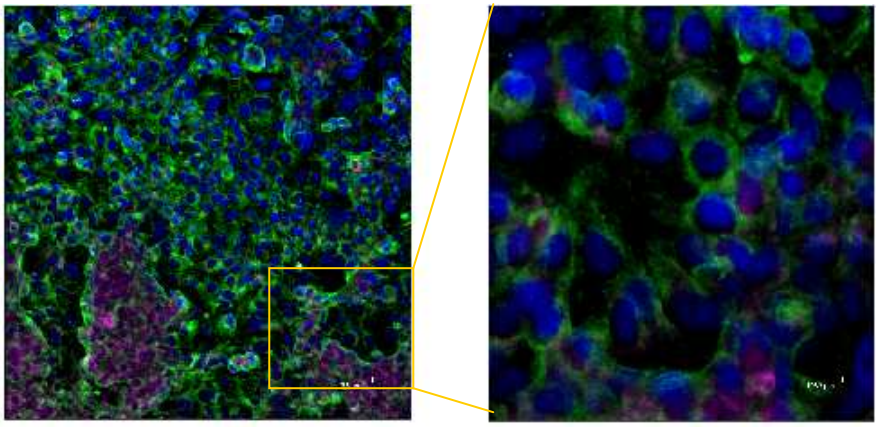
On characterisation HepG2 cells displayed a phenotype comparable to hepatocyte progenitor cells and not mature hepatocytes.

Lack of mature hepatocyte features suggested that this cell line was not an ideal representative for mature hepatocytes and thus not the best *in vitro* model for biochemical and cell biology studies.

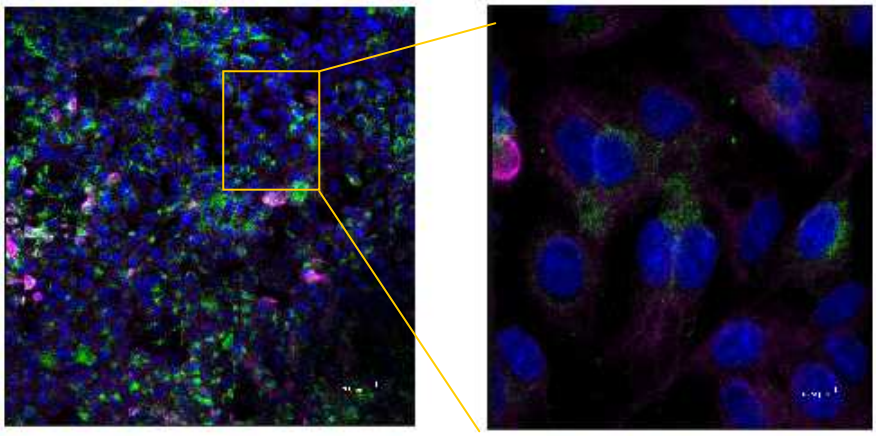
Figure 74. Expression of hepatic, biliary and embryonic stem cell markers in HepG2 cells. Confocal images were obtained at magnification x20 and x63. Cellular nuclei have been stained blue with DAPI (4', 6-diamidino-phenylindole dilactate). AFP, Ck-8, Ck-19, and Sox-2 were stained magenta with Cy5. Albumin, Ck-7, Ck-18 and gata-4 were stained green with FITC. All images were taken against negatives (secondary antibody only-stained cells) which were used to set the threshold for positive staining. Scale 50μm

Immunocytochemistry of undifferentiated HepG2 cells

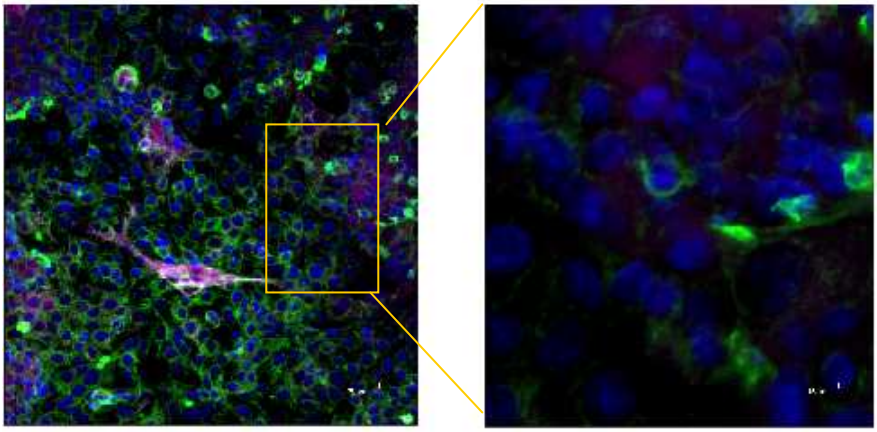
(a) ALB/AFP



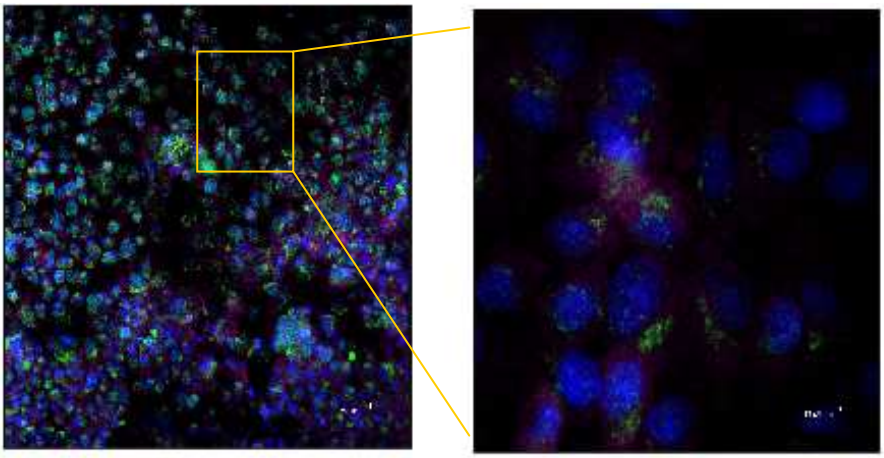
(b) Ck7/Ck8



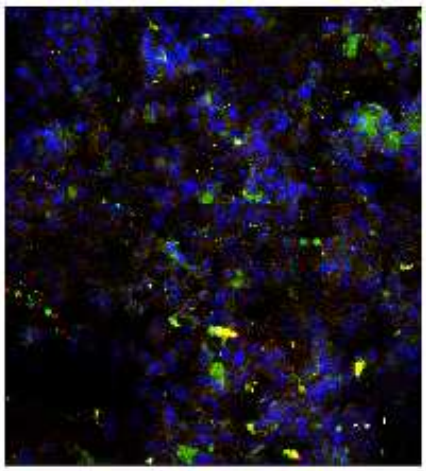
(c) Ck18/Ck9



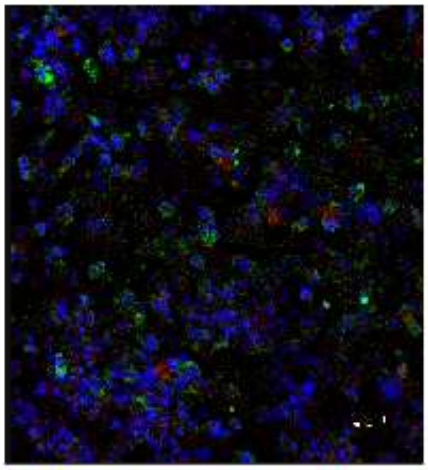
(d) Gata-4/Sox-2



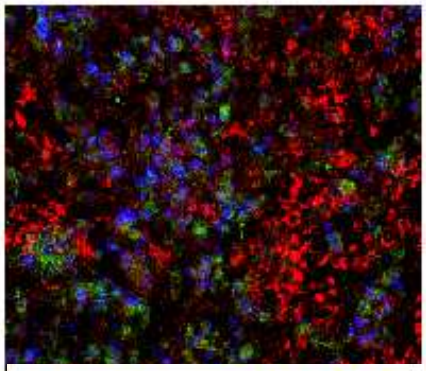
(e) HGFR/LDLR



(f) Nestin/GFAP



(g) CPS1/Vimentin



(h) Nanog/Oct4a

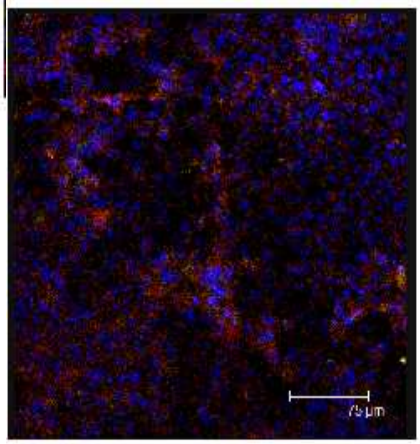


Figure 75. Expression of hepatic and some embryonic stem cell markers in HepG2 cells. Confocal images were obtained at magnification x10 and x20. Cellular nuclei have been stained blue with DAPI (4', 6-diamidino-phenylindole dilactate). HGFR, Nestin, CPS1 and Nanog were stained green with FITC. LDLR, GFAP, vimentin and Oct4 were stained red with TxRed. Co-expression is depicted by yellow staining. All images were taken against negatives (secondary antibody only-stained cells) which were used to set the threshold for positive staining. Scale 75μm

5.4.5.2 Foetal liver tissue sections

Paraffin embedded normal foetal liver tissue sections were provided courtesy of Steve Lisgo, Human Development Biology Resource (HDBR) department, a Wellcome Trust/MRC-funded resource run at the Institute of Human Genetics, Newcastle University and Institute of Child Health, University College London. The liver sections were from human embryos at 9 weeks of gestation.

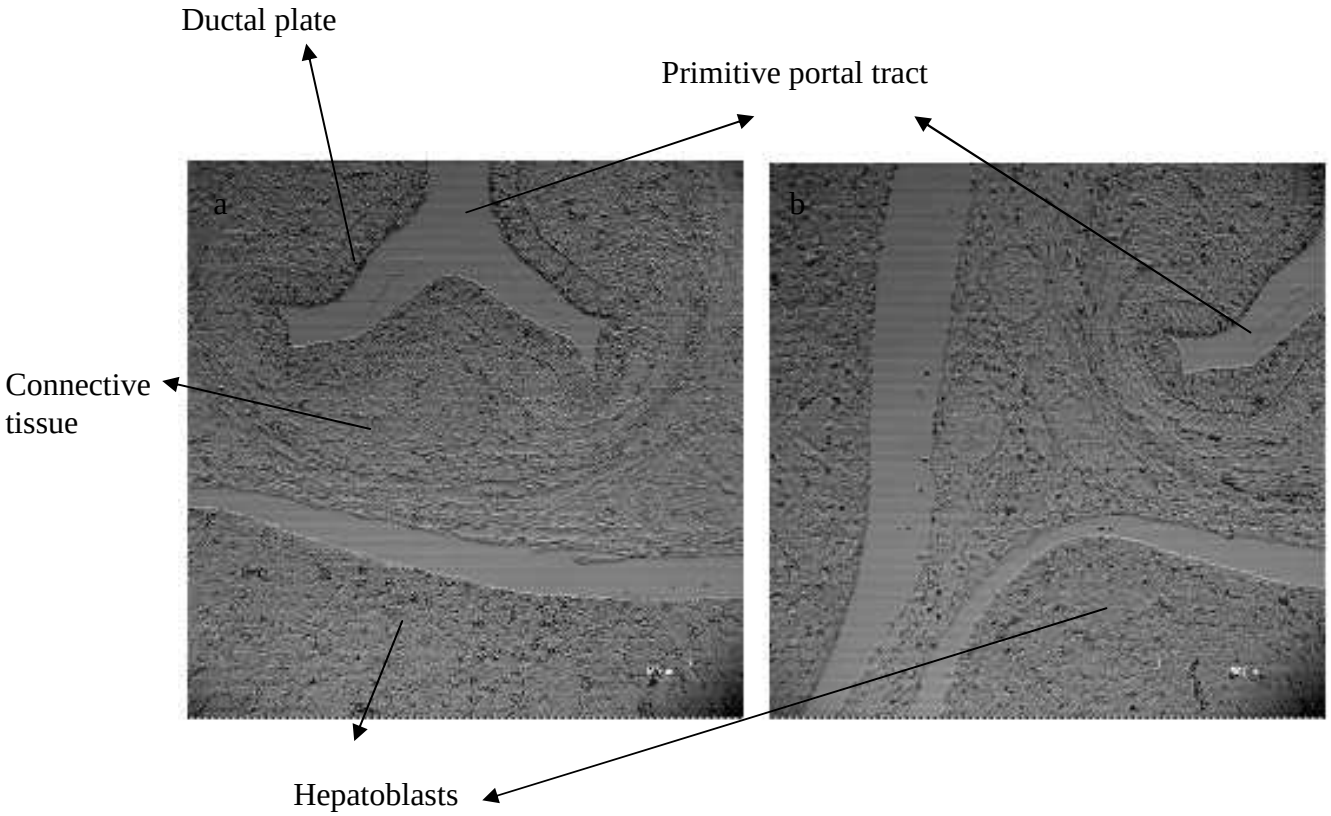


Figure 76. Transverse sections of human embryonic liver at 9 weeks gestation. DIC images were taken at magnification of x20 and illustrate the primitive portal tract, ductal plate and hepatoblasts. Ductal plate is a network of primitive biliary epithelium developing in the mesoderm following the portal vein branches and progressive towards the periphery of the hepatic parenchyma. The ductal plate is thought to give rise to the intra-hepatic biliary system (Godlewski et al, 2004). Scale 150µm

Immunohistochemistry of normal human foetal liver tissue sections

The foetal liver tissue sections from human embryos at 9 weeks of gestation were examined for most of the antigens used to characterize the differentiated umbilical cord and UCB-derived differentiated stem/progenitor cells.

The foetal liver cells at this gestational stage exhibited most of the characteristics of bi-potent hepatic progenitor cells (hepatoblasts). The cells tested positive for albumin, AFP, Ck-7, Ck-8, Ck-19, CPS1 and HGFR. Ck-7 (hepatic stem cell marker) was notably strong and expressed in all of the cells examined in the tissue sections (Figure 77b). AFP (hepatoblast marker) expression was localized predominantly to the ductal plate (origin of the intra-hepatic biliary tree). Ck-19 (another stem cell marker which persists when hepatoblasts differentiate into biliary epithelium) displayed limited weak expression (Figure 77c). Ck-8 and Ck-18 (mature hepatocyte marker) were only barely positive in a small percentage of the cells (Figure77b). CPS1 (rate limiting enzyme of the urea cycle) illustrated weak expression in the ductal plate cells and in the hepatoblasts. Gata-4 (endoderm marker) was weakly positive. This was expected as the endoderm cells at this developmental stage have already committed to hepatobilary lineage. Vimentin and LDLR were also negative at this stage of liver development. Weak HGFR expression was noted in the cells.

The albumin^{+}, Ck-7^{+}, Ck-19^{+}, Vimentin^{-} cells in foetal liver represent bipotent progenitor. These cells are comparable to HepPar1^{+}, Ck-14^{+}, Ck-19^{+}, Vimentin^{-} cells that Haruna *et al*, 1996, proposed as bipotent progenitors in human foetal liver samples (Haruna, Saito et al. 1996).

The phenotype of our *ex vivo* differentiated UC and UCB stem/progenitor cells were comparable to the foetal liver hepatic progenitor cells. Our differentiated cells, however, also tested positive for Gata-4 (endodermal marker) and Vimentin (mesodermal marker) expression demonstrating that they were at a more primitive developmental stage than the foetal hepatic progenitors at 9 weeks of gestation which tested negative for both of these markers.

We were unable to obtain primary foetal embryonic liver cell cultures to use as functional models in our study. It should, however, be noted that these progenitor cells, being at an early developmental stage are highly unlike to serve as good hepatic functional models for biochemical and biomedical studies.

Conclusion

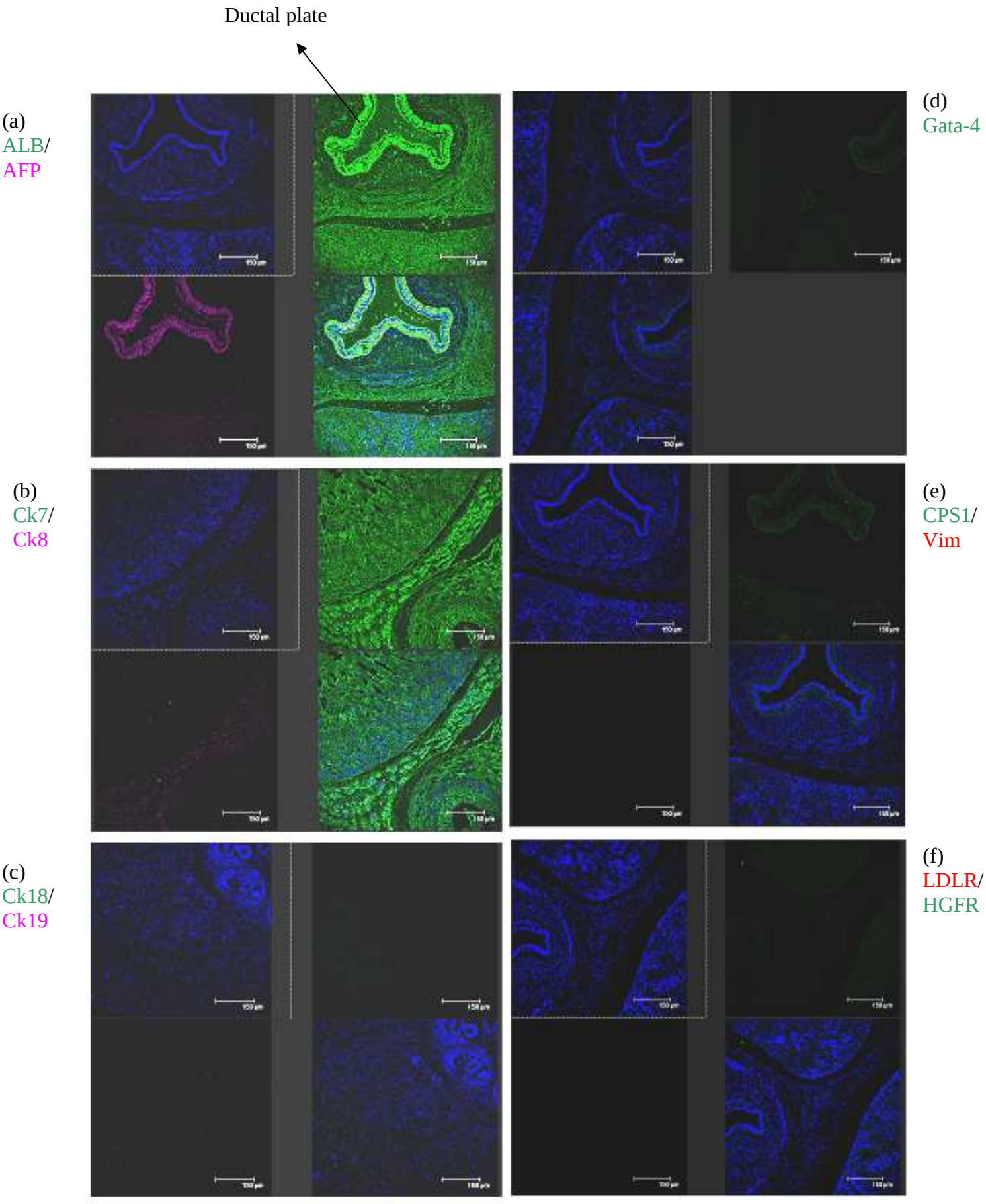
Foetal liver tissue sections at different developmental stages serve as a good model to check the phases of *in vitro* differentiation by identifying the acquisition or loss of specific protein.

In this study, human liver tissue sections from embryos at 9 weeks of gestation were the youngest developmental stage that we could get hold of.

The phenotype of our *ex vivo* differentiated umbilical cord and UCB stem/progenitor cells were comparable to 9 week foetal liver hepatic progenitor cells albeit, at a slightly earlier developmental stage (Gata-4 and Vimentin positive).

The results obtained were only used to represent hepatobilary cells at an embryonic developmental stage. These results collectively with the results obtained from adult liver tissue sections were used as models to compare our results with.

Figure 77. Confocal immunocytochemistry images of human foetal liver tissue sections at 9 weeks gestation. (a) Albumin expression was noted throughout the liver section but stronger expression in the ductal plate. AFP was expressed predominantly in the ductal plate with weaker expression in the hepatoblasts. (b) Strong Ck-7 expression was noted both in the hepatoblasts as well as in the ductal plates. Ck-8 expression was weak and localized mainly to the hepatoblasts. (c) Ck-18 was very weakly expressed at this stage. Ck-19 expression was very weak and only expressed in a few hepatoblasts. (d) Gata-4 was weakly positive with greater expression in the ductal plate. (e) CPS1 was positive in both hepatoblasts and ductal plate with once again stronger expression in the ductal plate. Vimentin was negative. (f) HGFR was noted to be expressed both in hepatoblasts and ductal plate but LDLR was negative. Images were taken at magnification of x20. All nuclei were stained blue with DAPI (4', 6-diamidino-phenylindole dilactate). Albumin, Ck-7, Ck-18, Gata-4, CPS1 and HGFR were stained green with FITC. AFP, Ck-8 and Ck-19 were stained magenta with Cy5. Vimentin and LDLR were stained red with TxRed. Dual staining and co-expression appeared as whitish staining on imaging. All images were taken against negatives (secondary antibody only-stained cells) which were used to set the threshold for positive staining. Scale 150μm


Ductal plate
(a)
ALB/
AFP
(b)
Ck7/
Ck8
(c)
Ck18/
Ck19
(d)
Gata-4
(e)
CPS1/
Vim
(f)
LDLR/
HGFR

5.4.5.3 Adult liver tissue sections

Paraffin embedded normal adult liver tissue sections were provided courtesy of Dr Mathew Wright, School of Biomedical Sciences, Newcastle University. Similar to the foetal liver tissue sections, these adult tissue sections were used as positive controls for our differentiated stem/progenitor cells.

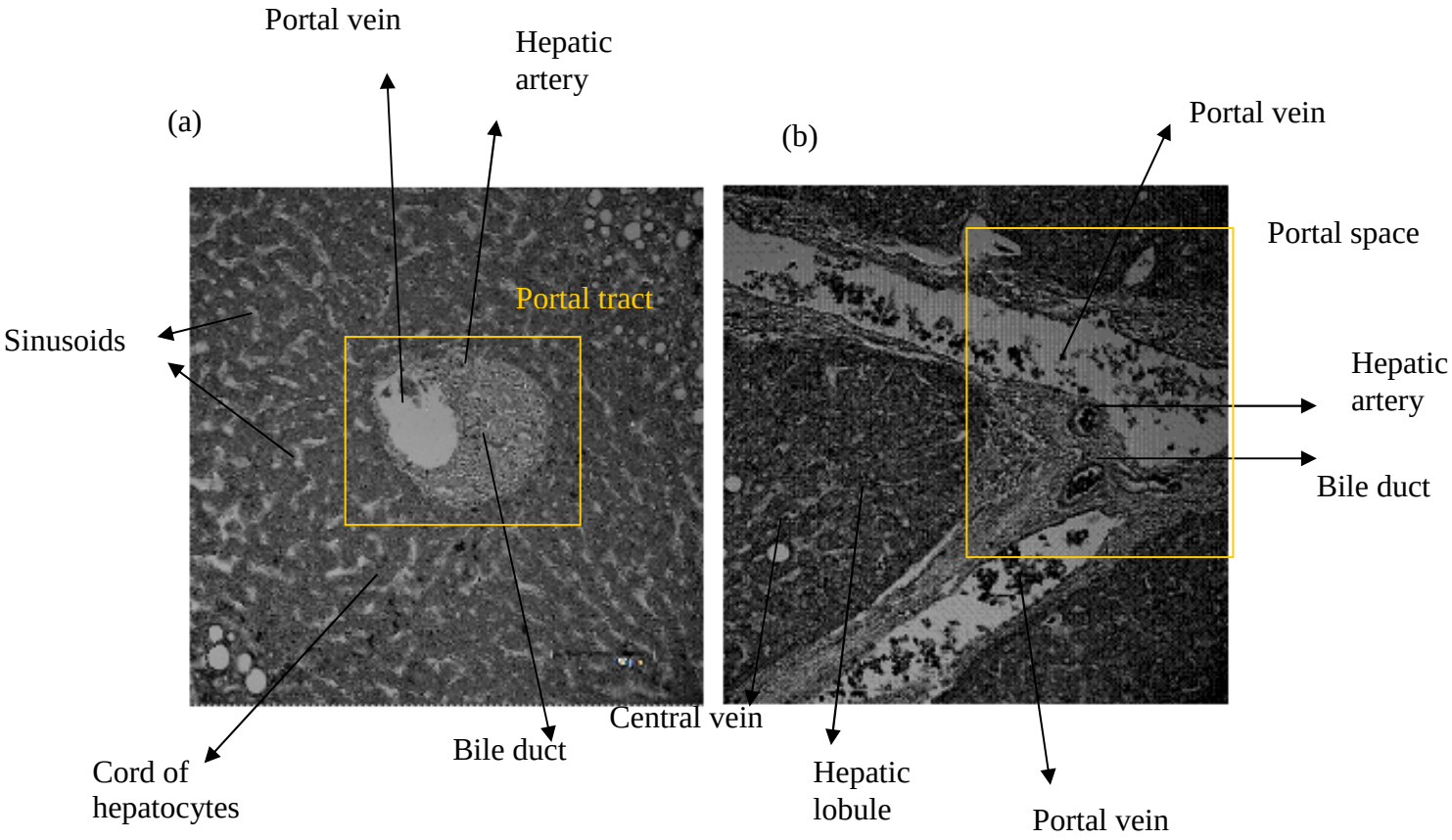


Figure 78. Confocal immunohistochemistry images of adult liver transverse sections. DIC images were taken at magnification of x20 and illustrate the portal triad (portal tract): portal vein, hepatic artery and bile duct. (b) Hepatic lobule surrounded by the portal space. Arteries, veins and bile ducts occupy the portal spaces. Nerves, connective tissue and lymphatic vessels are also present but not shown in this image. Scale 150µm

Immunohistochemistry of adult liver tissue sections

The tissue sections from human adult liver samples were examined for the same antigens used to characterize the differentiated umbilical cord and UCB-derived differentiated stem/progenitor cells and the foetal liver tissue sections.

The hepatocytes in the paraffin-embedded tissue section tested positive for albumin, Ck-8 Ck-18 and CPS1; all of which are indicated markers of mature hepatocytes. Ck-18 expression was notably stronger in hepatocytes in and surrounding the limiting plates (distinct row of hepatocytes joined together and forming a row which surrounds the portal tract) (Figure 79). Ck-7 was also positive but expression was once again limited to a few cells in and in close vicinity to the limiting plate (Figure 79). Based on their location these Ck-7^+ cells, could either represent a potential hepatic stem cell compartment located within the intra-hepatic biliary tree or could be intra-hepatic biliary epithelial cells (cholangiocytes). It was expected that the $Ck7^+$ cell population would also test positive for Ck-19 but surprisingly Ck-19 expression was negative. The cells were negative for Gata-4 and vimentin.

At the end of our *ex vivo* differentiation protocol, the resulting heterogeneous cellular phenotype displayed a percentage of cells that resembled mature hepatocytes and some that were comparable to the Ck-7^+ cells noted in the adult liver. This cellular population in our differentiation system, however, was also positive for Ck-19.

Conclusion

Adult human hepatocytes represent the ideal morphological and phenotypical positive control for *ex vivo* hepatic differentiation systems.

Resource for this tissue is limited to cadaveric supply or from post hepatectomy specimens, hence making control samples not readily accessible.

Primary mature hepatocytes would be the ultimate functional control for *in vitro* differentiation systems. These cultures, however, are very difficult to maintain in culture and have a propensity to undergo phenotype transformations.

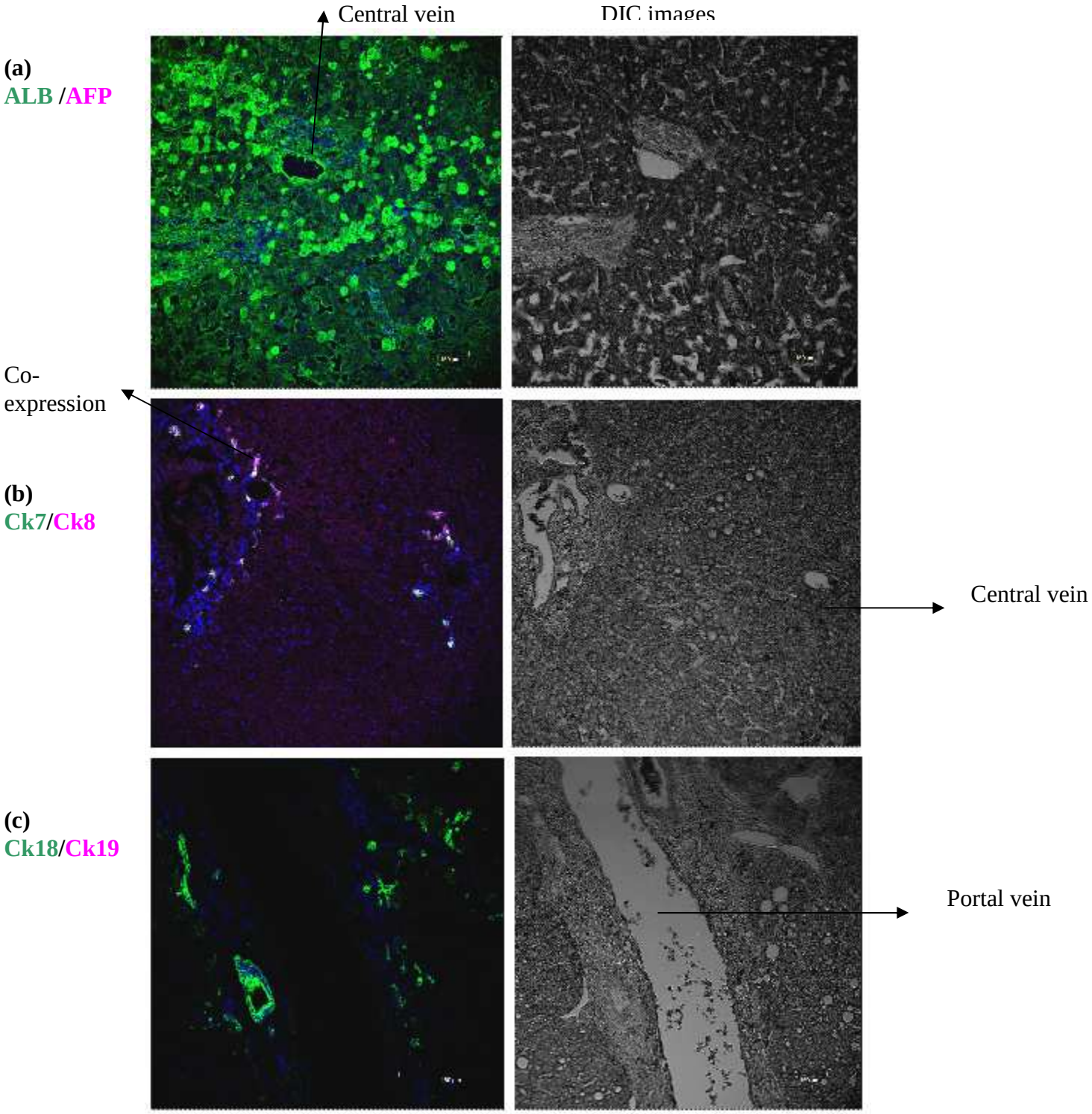


Figure 79. Confocal immunohistochemistry images of adult liver tissue transverse sections. (a) Hepatic lobular cells positive for albumin but negative for AFP. (b) Hepatocytes were diffusely positive for Ck-8 but Ck-7 expression was localized to areas of the limiting plate (distinct row of hepatocytes joined together forming a row which surrounds the portal tract). (c) Ck-19 was not expressed. Ck-18 expression was similar to Ck-7 expression. All cellular nuclei were stained blue with DAPI (4', 6-diamidino-phenylindole dilactate). Albumin, Ck-7 and Ck-18 were stained green with FITC. AFP, Ck-8 and Ck-19 were stained magenta with Cy5. Images were taken at magnification of x20. Dual staining and co-expression appeared as whitish staining on imaging. All images were taken against negatives (secondary antibody only-stained cells) which were used to set the threshold for positive staining. Scale 150µm

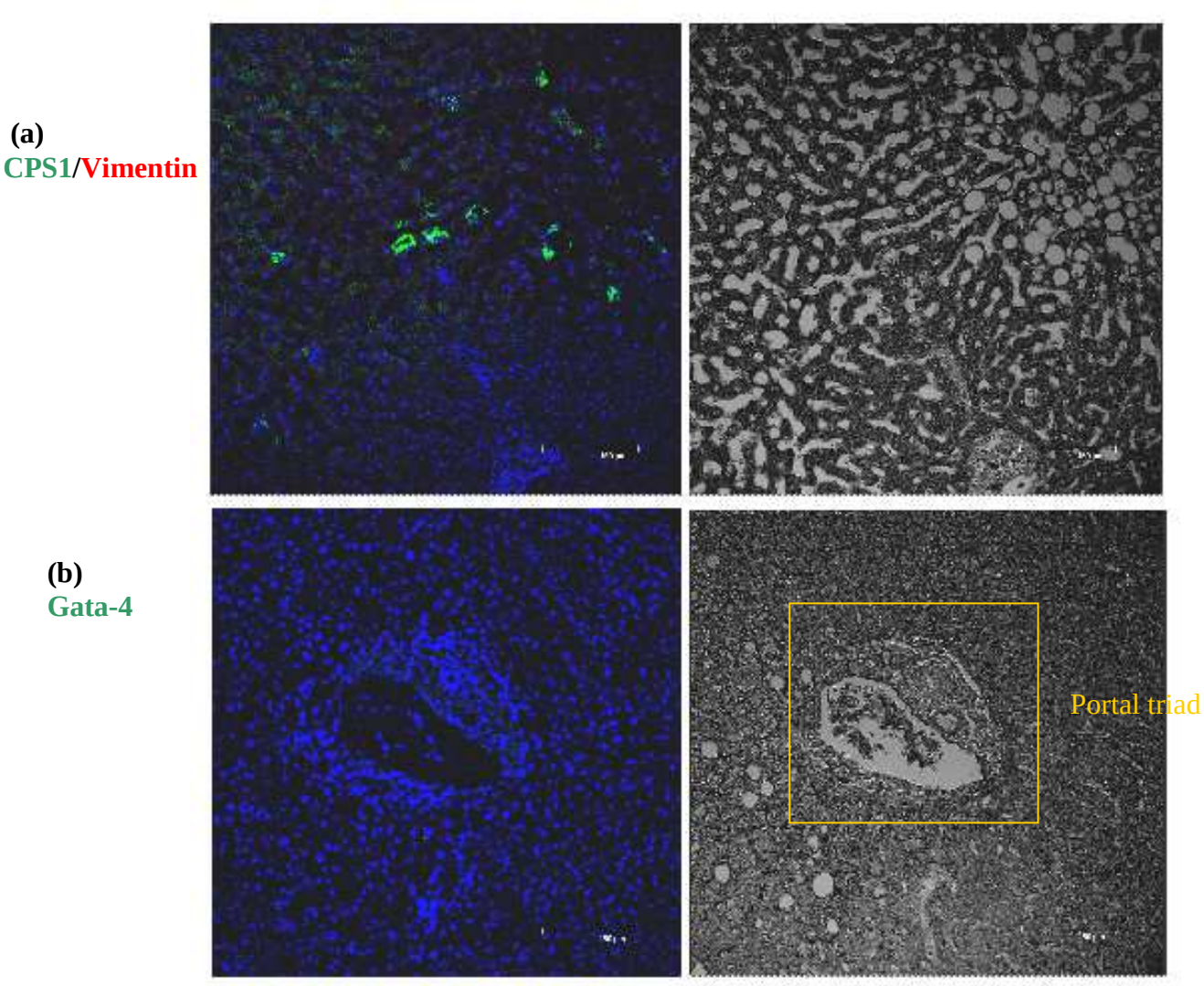


Figure 80. Confocal immunohistochemistry images of adult liver tissue transverse sections. (a) A small percentage of hepatocytes in the hepatic lobule imaged tested positive for CPS1. Vimentin was negative. (b) Hepatocytes were negative for Gata-4. No Gata-4 expression was noted in the portal tract either. All cellular nuclei were stained blue with DAPI (4', 6-diamidino-phenylindole dilactate). CPS1 and Gata-4 were stained green with FITC. Vimentin was stained red with TxRed. All images were taken against negatives (secondary antibody only-stained cells) which were used to set the threshold for positive staining.

5.4.6 Protein analysis of differentiated cells

Western bloting was drawn on to verify the presence of the liver related proteins detected on immunocyto/histochemistry. This test, however, could only be carried out on umbilical cord-derived Mesenchymal stem cells. This was due to the fact that this assay was less sensitive than immunocyto/histochemistry and hence required a comparatively large amount of protein for antigen detection which could not be met by the low cell counts yielded from umbilical cord blood. Efficient *ex vivo* expansion of Mesenchymal stem cells, on the other hand, provided sufficient cell numbers that could meet the protein requirements of this test. In each case, 5-10mg of protein was used for the detection of each antigen. In each run the amount of protein from each test sample loaded was the same.

Protein extracts from cultured cells at different stages of the differentiation protocol were used for this technique.

Undifferentiated Mesenchymal stem cells and embryonic stem cells were used as negative controls. Human embryonic stem cells were provided courtesy of Professor Majlinda Lako, NorthEast England Stem Cell Institute, Centre for Life, Newcastle University. HepG2 cells and adult liver protein were used as positive controls. Adult liver protein extracts were provided courtesy of Dr Matthew Wright.

ß-Actin was used as a control for integrity of cell in the western blot runs. ß-Actin is one of the two non-muscle cytoskeletal actins. Actins are highly conserved proteins that are involved in cell motility, structure and integrity.

The proteins examined by western bloting included: AFP, albumin, Ck-19, C-k7, Ck-8, HGFR, CPS1 and CYP450 enzymes (CYP3A4, 2A and 2E).

AFP was detected only in adult liver and very low levels could be appreciated in HepG2 cells as well. However, AFP expression in adult liver tissue sections was not noted on immunohistochemistry analysis. This may have been because the area of the tissue examined microscopically was not periportal where potential progenitor cell populations are believed to reside in. AFP expression was not detected in any of the differentiation phases of the MSCs, although, on immunocytochemistry AFP expression was noted at week 3 of the protocol. A likely explanation is that immunocytochemistry is a more sensitive assay for protein detection and the use of a larger quantity of protein extract may have made AFP detection possible on western bloting. The negative controls were negative for AFP expression (Figure 81).

Albumin was detected at all the stages of MSC differentiation as well as in the undifferentiated MSC control. Highest albumin expression was noted in adult liver as expected. This result corresponds to the results obtained on immunocytochemistry. Embryonic stem cell negative control was negative for albumin (Figure 81)

Ck-19 was positive in undifferentiated MSCs, as well as all other stages of differentiation except the final stage (week 3-post maturation). This result once again corresponds with the results from immunocytochemistry, except for Ck-19 not being detected at week 3. It is worth noting here that the cell number at week 3 of the differentiation system was considerable lower than the cell density at earlier stages of differentiation. The lower cell count and hence consequential lower protein content could explain the lack of detection of Ck-19 at the final stage of differentiation. Ck-19 expression was noted in both positive controls with adult liver demonstrating a lower expression compared with its corresponding ß-Actin. This is because Ck-19 expression in the liver post development is restricted to biliary epithelium and not hepatic parenchyma. Embryonic stem cells were positive for Ck-19. This result was not very surprising as embryonic stem cells have previously been reported to test positive for various germ layer markers (Figure 82)

Ck-8 expression was noted in undifferentiated MSCs as well as all the stages of MSC differentiation. These results corresponded to the immunocytochemistry results obtained for this protein. HepG2 and adult liver both tested positive for Ck-8. Protein level was slighter lower in adult liver but this reflected the comparatively smaller protein content of this sample as was depicted by ß-Actin (Figure 83). Embryonic stem cells were positive for Ck-8.

CPS1 was detected in undifferentiated MSCs as well as all the stages of MSC differentiation. These results yet again corresponded to the immunocytochemistry results obtained for this functional protein. Both positive controls tested positive as well. Embryonic stem control also demonstrated expression of this protein (Figure 83).

The results acquired for Ck-7 did not reflect the immunocytochemistry results for this protein. Undifferentiated MSCs were positive for Ck-7 on both assays. Differentiated MSCs at week 1 and week 2 of differentiation tested positive for Ck-7 on western blotting, but were negative for Ck-7 on immunocytochemistry. Similarly, at week 3 of differentiation Ck-7 was negative on western analysis but was strongly positive on immunocytochemistry. HepG2 cells tested negative for Ck-7 whilst the protein displayed a positive expression on immunocytochemistry. Adult liver illustrated expression of this cytokeratins, albeit at low

levels. Embryonic stem cell controls were also weakly positive for Ck-7 (Figure 82).

HGFR expression on western blot analysis did not demonstrate complete concurrence with immunocytochemistry results either. HGFR was detected in undifferentiated as well as differentiated MSCs at week 1, 2 and 3 of the differentiation protocol. On immunocytochemistry, however, HGFR expression was only noted at week 3 of MSC differentiation and was negative at the earlier stages. HepG2 was negative for HGFR on western but tested positive on immunocytochemistry. Adult liver cells were also negative for HGFR but there was no corresponding immunocytochemistry data to compare this with. Embryonic stem cell controls were weakly positive for this protein (Figure 82).

The presence of CYP450 enzymes were not tested on immunocytochemistry. On western blot protein analysis all the test samples were negative for CYP3A4, except for adult liver. CYP2A was positive in undifferentiated MSCs and all stages of differentiated MSCs, albeit at low levels. HepG2 and adult liver were positive for this CYP enzyme. CY2E demonstrated a more selective pattern of expression- very low levels were noted in undifferentiated MSCs as well as week 1 of differentiation and subsequently no more protein was expressed. Both positive controls demonstrated CYP2E expression. Embryonic stem cells were negative for CYP3A4 but demonstrated low levels of CYP2A and CYP2E protein expression (Figure 84).

Conclusion

Western blot does not provide quantitative data and hence did not add addition information to the data already obtained from immunocyto/histochemistry.

This technique is dependent on adequate protein concentration for detection.

Embryonic stem cells did not prove to be good negative controls for this assay.

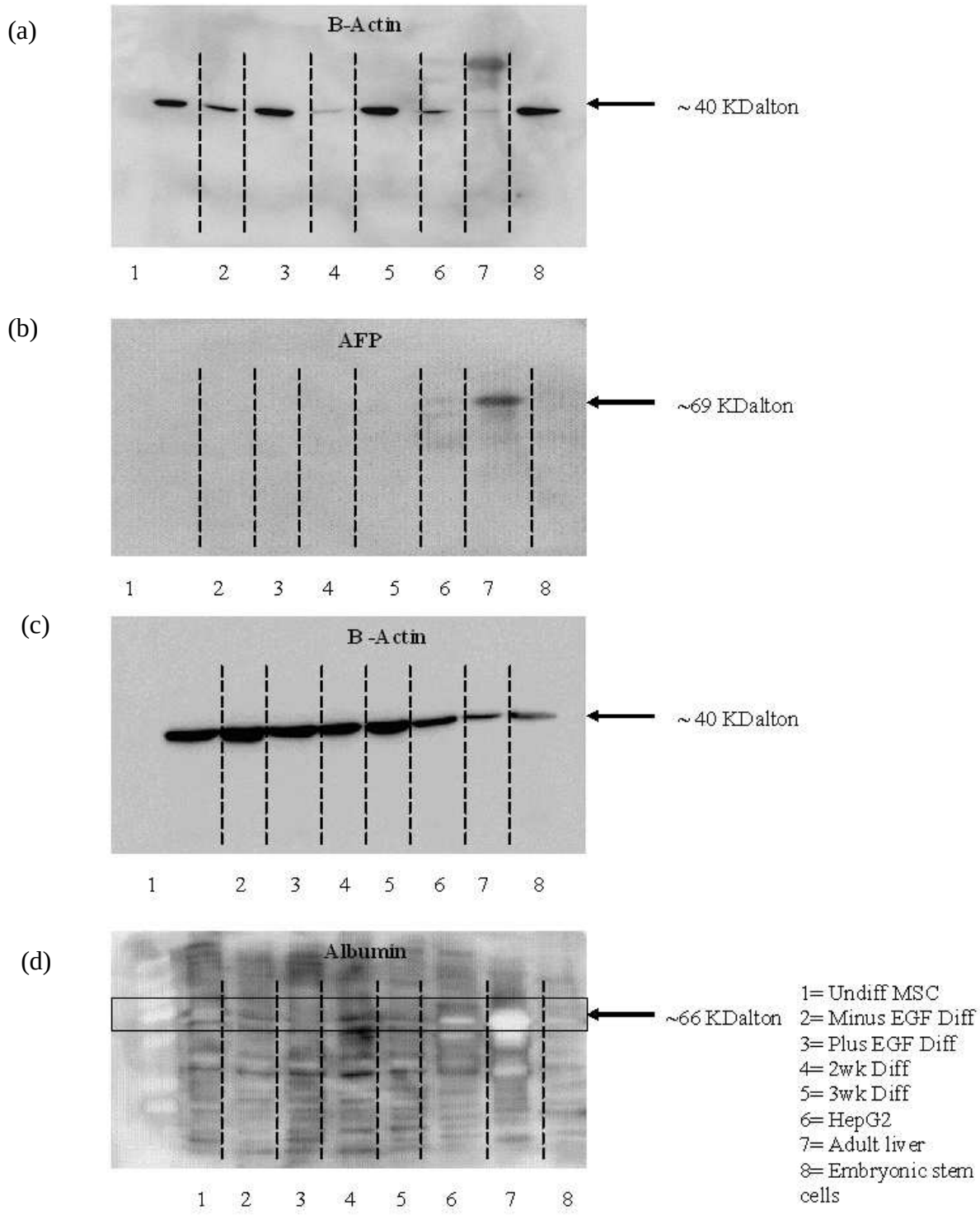


Figure 81. Western blot assays- chemiluminescent protein detection in differentiated MSCs. HepG2 and adult liver protein extracts were used as positive controls. Undifferentiated MSCs and Embryonic stem cells were used as negative control. 10mg of protein was loaded into each well for protein detection, except for albumin where 5mg was used. Higher concentration of this protein resulted in very bright optical density on luminescence, making interpretation difficult. All images were obtained after 1-3 minutes of exposure. ß-actin was used as a control for cellular integrity in all the runs. Sample numbers are decoded in the legend provided.

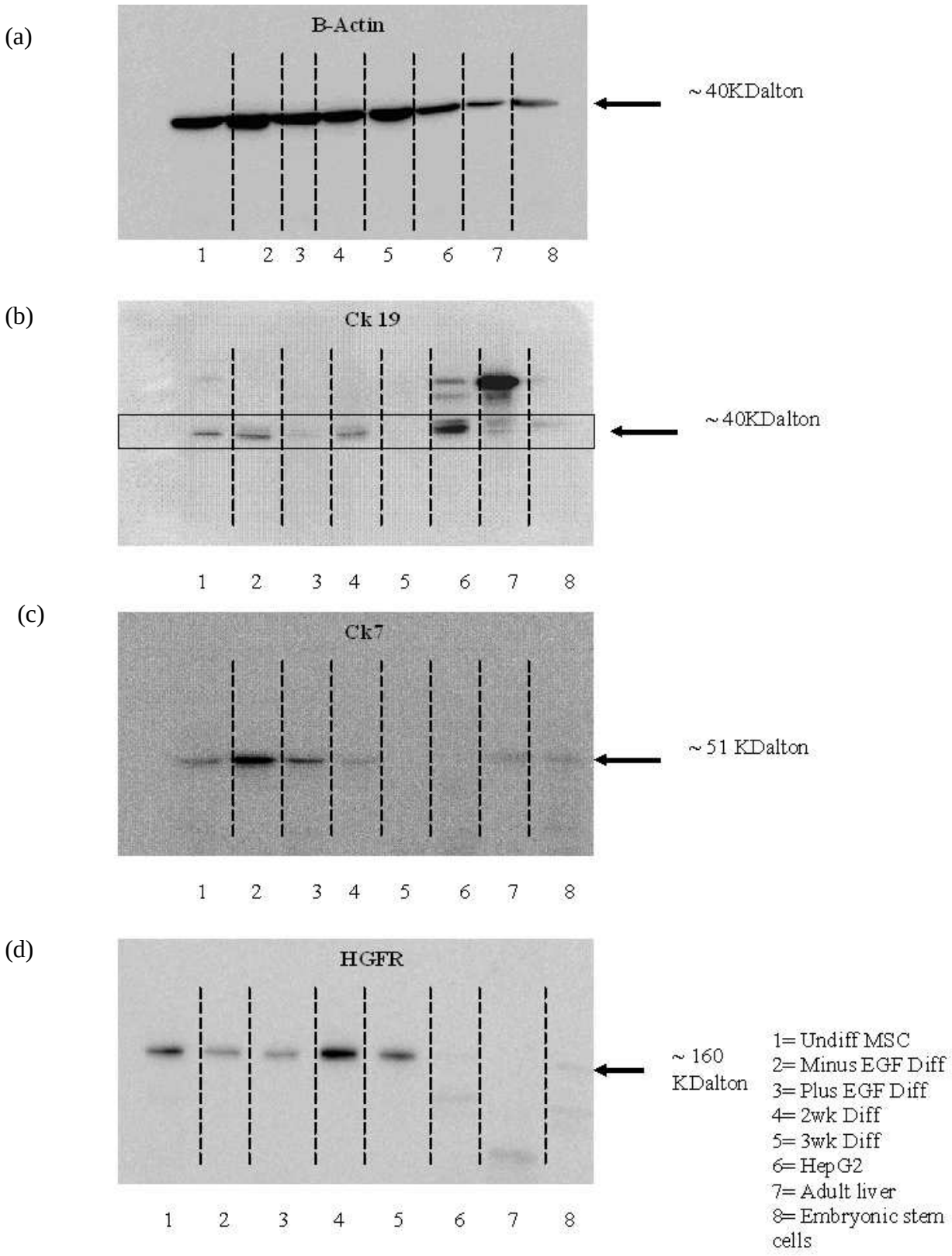


Figure 82. Western blot assays for protein detection in differentiated cells. HepG2 and adult liver protein extracts were used as positive controls. Undifferentiated MSCs and Embryonic stem cells were used as negative control. 10mg of protein was loaded into each well for protein detection. Sample numbers decoded in the legend provided.

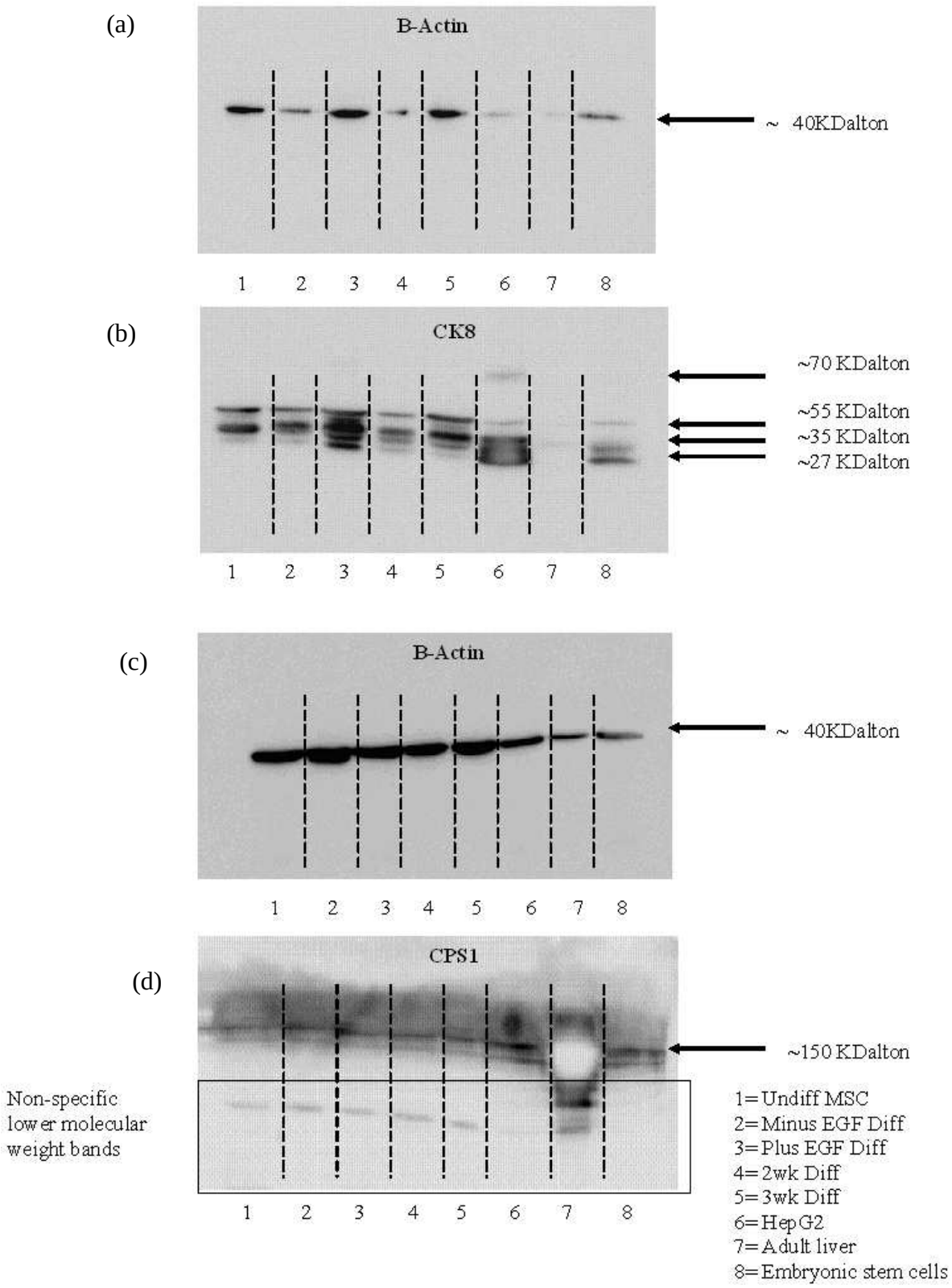


Figure 83. Western blot assays for protein detection in differentiated cells. HepG2 and adult liver protein extracts were used as positive controls. Undifferentiated MSCs and Embryonic stem cells were used as negative control. 10mg of protein was loaded into each well for protein detection. Sample numbers decoded in the legend provided. (b) There are two types of cytokeratin 8: Acidic (Type 1) ~ 40-55 KDalton; Neutral to basic (Type II) ~ 56-70 KDalton. We were interested in acidic Ck-8 (~45 KDaltons).

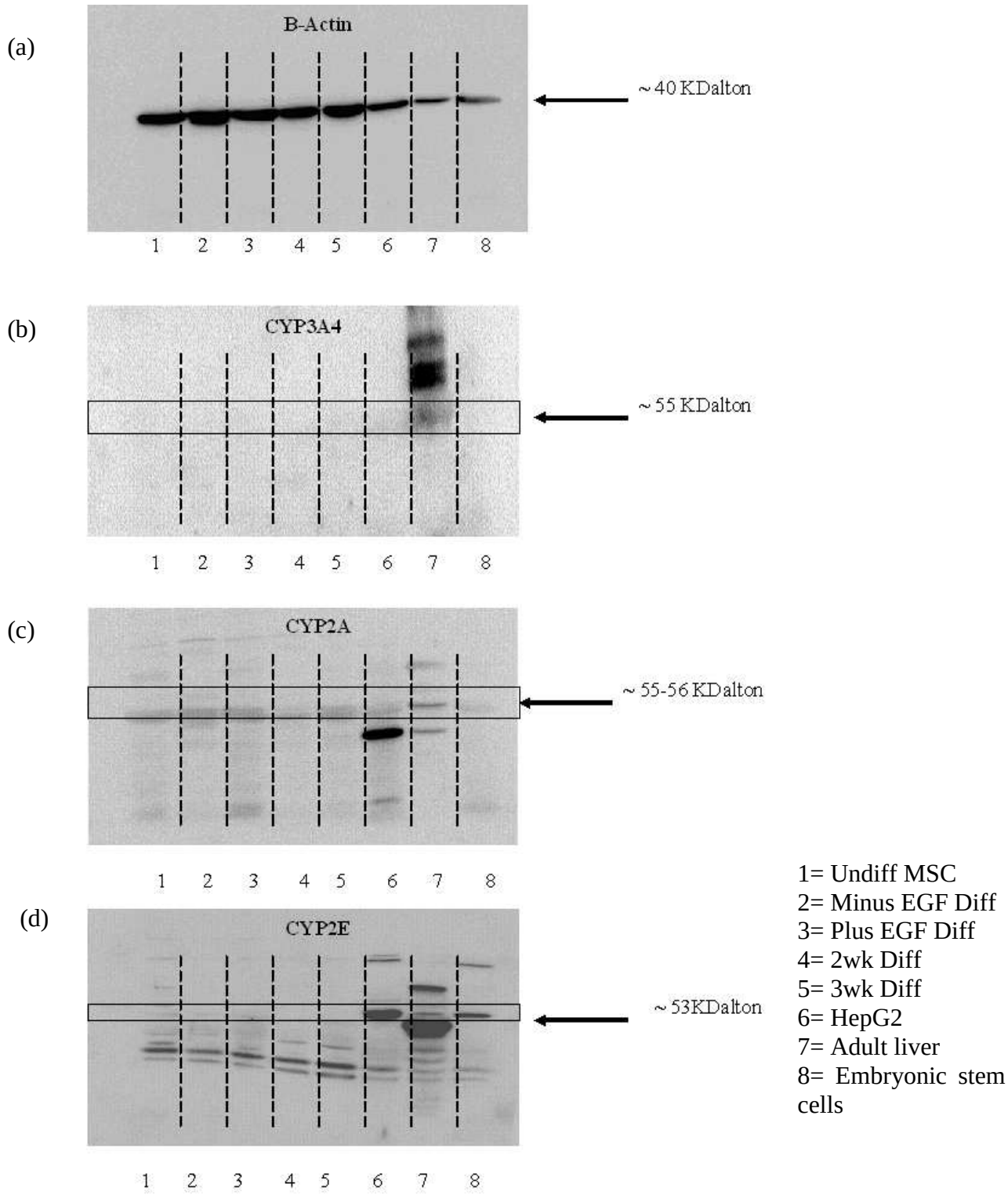


Figure 84. Western blot assays for protein detection in differentiated cells. HepG2 and adult liver protein extracts were used as positive controls. Undifferentiated MSCs and Embryonic stem cells were used as negative control. 10mg of protein was loaded into each well for protein detection. Sample numbers decoded in the legend provided.

5.4.7 Molecular analysis of isolated stem/progenitor cells

Immunocytochemistry and western blot analysis are useful technique for studying protein expression in cells, providing valuable information about the presence and the cellular localization of proteins within the cells. However, a limitation of these techniques is that they fail to provide quantitative information. Since, a lot of the proteins investigated were expressed throughout the various stages of the differentiation protocol (in the case of MSCs even prior to differentiation); we decided to study the gene expression profiles of some of these proteins at the different stages. We used real-time PCR to quantify the studied genes. All the values generated were relative quantities (normalised to house-keeping genes). This enabled examination of changes in gene expression profiles throughout the differentiation and maturation protocol. HepG2, foetal liver mRNA (7weeks and 12weeks) and adult liver cDNA were used as positive controls for all the tested genes.

Undifferentiated umbilical cord MSCs prior to differentiation expressed all the genes studies,albeit, some at low levels ♣. This verified our finding at the protein level with immunocytochemistry and western blotting. After the first week of differentiation, we documented an up regulation of the majority of the genes examined (Gata-4, AFP, CLDN2, HNF1α, Albumin and all the studied CYP450 genes). However, HNF4α, HGFR and LDLR were noted to be down regulated (Figure 85-87).

Post week two of differentiation, the expression of all the genes noted was down regulated, in some cases to a level even below post expansion. One probable explanation for this could be the introduction of a new cytokine (EGF) to the culture media, which may have altered the cell kinetics, directing the cell machinery to produce proteins needed for the newly activated signalling pathway at the expense of some other genes (Figure 85-87).

Week three of the protocol, demonstrated the maximum expression of the examined genes. This observation reflected an increase in the level of maturity of the cells after exposure to dexamethasone and Oncostatin M

♣ Week 1 (1wk) of the protocol was carried out in two parallel experiments, with and without EGF. This has been represented as 'minus EGF' and 'plus EGF' in the graphs. This was done to determine the role of EGF in hepatobiliary differentiation *ex vivo*. This has been discussed in Chapter 5 section 5.3 . In this section, however, all references to week 1 are with regards to week 1 of differentiation without EGF (minus EGF 1wk).

The differentiation system applied to cord blood stem/progenitor cells was of a shorter duration taking into account the limited viability of these cells *in vitro*. Undifferentiated haematopoietic stem/progenitor cells also tested positive for the expression of all the genes examined, suggesting that they too have hepatogenic potential. The corresponding proteins, however, did not test positive on immunocytochemistry analysis.

After one week of lineage commitment and modest expansion the cells demonstrated down regulation of all of the genes, except CLDN2, Gata-4 and CYP2E1. Up regulation of Gata-4 (endodermal marker) confirms the ability of the culture system to establish an endoderm fate. Up regulation of CLDN2 (Hepatic stem cell marker) was suggestive of a hepatic stem cell phenotype. The simultaneous up regulation of CYP2E1, however, which is a more mature hepatocyte marker, reflected the heterogeneous nature of the cultures (Figure 85-87).

After differentiation (4 days) and maturation (4 days) we documented an increase in the expression profiles of AFP (hepatoblast marker), HNF4α (transcriptional factor necessary for hepatocyte differentiation) and CYP20A1. All the other examined genes demonstrated a down regulation. Simultaneous suppression of Gata-4 and CLDN2 expression meant that at this stage the cells demonstrated a phenotype more mature that hepatic stem cells and less mature that hepatocytes; they reflected similarity to hepatoblasts.

The probability test, Anova two-factor with replication, was used to statistically analyse the significance of the changes noted in the level of gene expression during the different stages of differentiation. A p value of 0.005 was regarded as statistically significant. We documented that the trend of gene expression level changes was significant (n=3) ($p<0.0001$). The Anova test also enabled us to study the level of variability in gene expression profiles between different cord blood samples. We reported that even though the trend in gene expression profiles during the differentiation protocol was more or less consistent, the variability between the cord blood samples was very significant ($p<0.00001$). This significant difference may be attributed to the many parameters that have previously be shown to influence cord blood quality: gestational age, age of the mother, number of pregnancy, sex of the baby etc (McGuckin, Basford et al. 2007).

To ensure the specificity of the primers used, end-point PCR gel electrophoresis was performed to determine the sizes of the products picked up by the primers (appendix C).

Conclusion

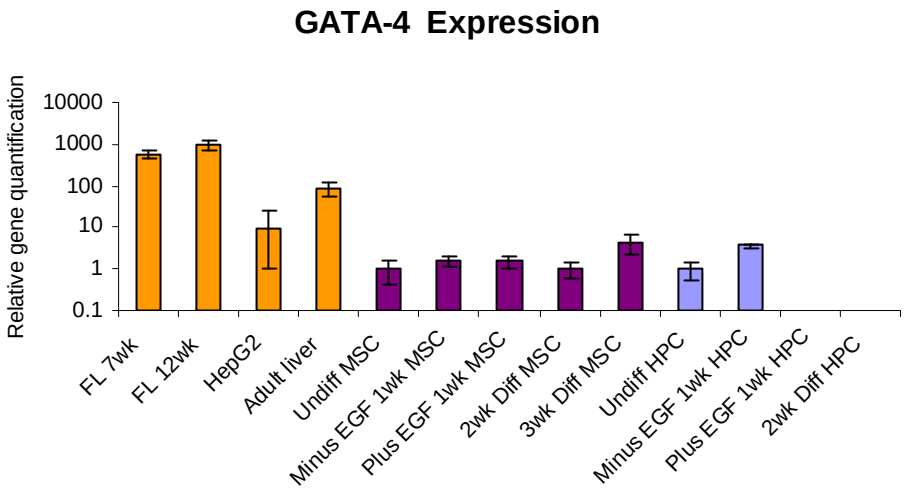
Both MSCs and UCB haematopoietic stem/progenitor cells tested positive for the examined hepatocyte related genes. This reflected the hepatogenic potential of these cells

Hepatocyte differentiated MSCs displayed highest level of hepatocyte gene expression post maturation. A heterogeneous population of hepato-biliary cells was generated with cellular phenotype ranging from hepatic stem cells to mature hepatocyte-like cells.

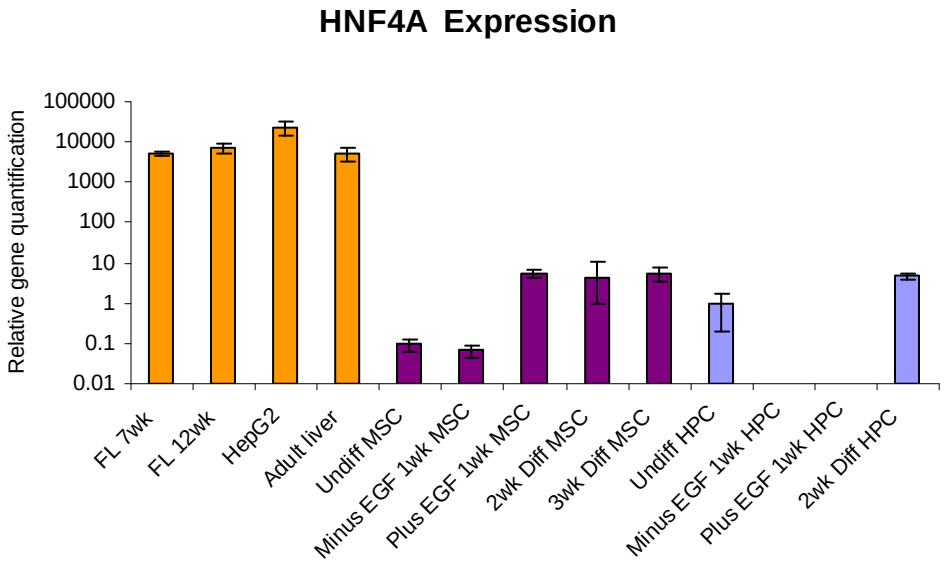
Haematopoietic stem cell-derived differentiated cells illustrated a phenotype analogous to hepatoblasts at the end of the differentiation protocol

Figure 85. Real-time PCR analysis of MSCs and haematopoietic stem cells (HPCs) at different stages of differentiation. Graphical data is in Logarithmic scale. HepG2, foetal liver at gestation age of 7 weeks (FL7wk) and 12 weeks (FL12wks) and adult liver cDNA were used as positive controls. RNase-free water was used as negative control. Week 1 (1wk) of the protocol was carried out in two parallel experiments, with and without EGF. This has been represented as 'minus EGF' and 'plus EGF' in the graphs. This was done to determine the role of EGF in hepatobiliary differentiation *ex vivo*. This has been discussed in Chapter 5 section 5.3. In this section, however, all references to week 1 are with regards to week 1 of differentiation without EGF (minus EGF 1wk). (a) Highest level of Gata-4 expression was noted in foetal liver at gestational age of 12weeks (FL12wk). Both undifferentiated MSCs and HPCs tested positive for Gata-4. In MSCS expression of this gene reached maximum expression at week 3. In HPCs, an up regulation was noted after 1week but thereafter the cells were negative for Gata-4. (b) Highest level of HNF4α was noted in HepG2, followed by foetal liver. Undifferentiated MSCs were positive for HNF4α but undifferentiated HPCs were negative. MSCs demonstrated a gradual increase in gene expression, attaining maximum expression at week 3. In HPCs an initial down regulation was noted at week 1 followed by a noticeable up regulation. (c) Highest CLDN2 expression was noted in adult liver. Both undifferentiated MSCs and HPCs tested positive for this gene. At week 3, MSCs demonstrated highest expression of this gene.

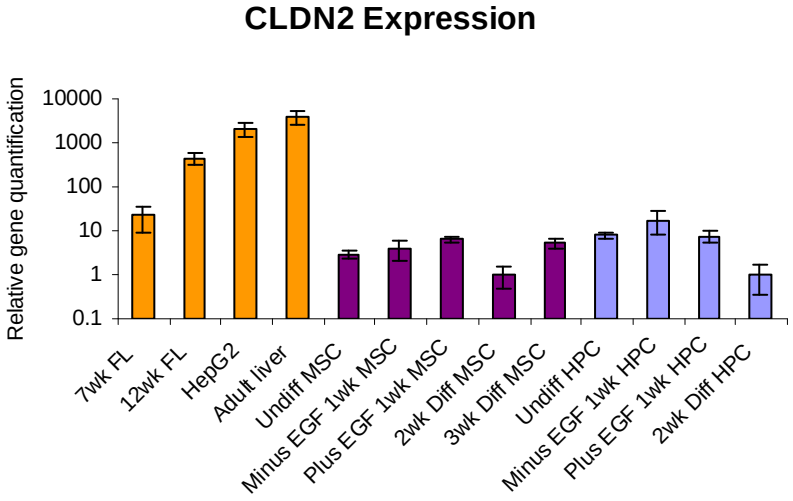
(a)



(b)



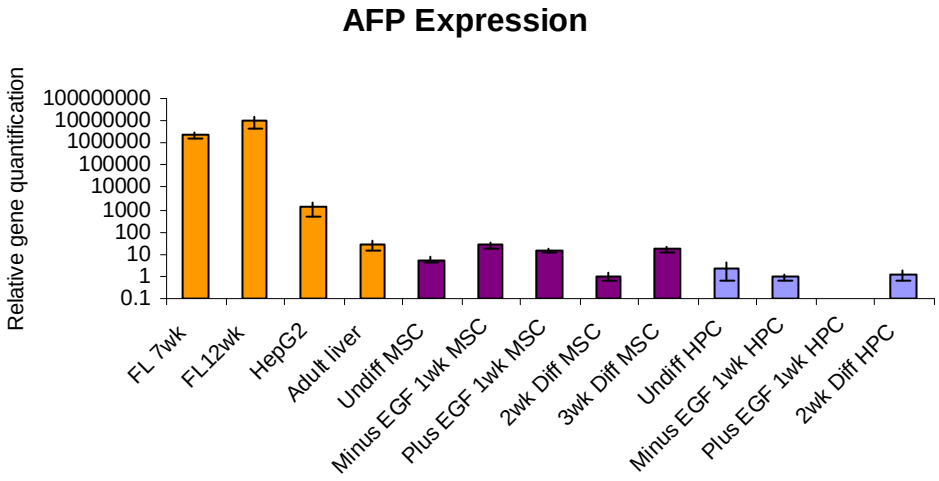
(c)



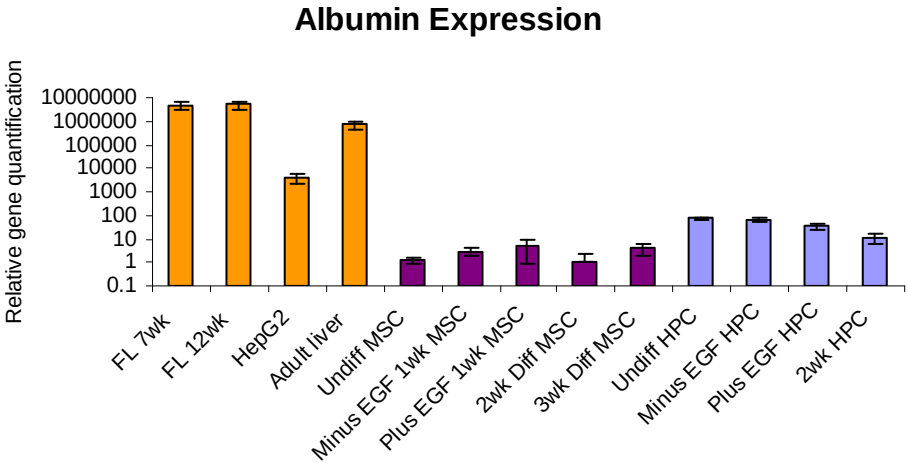
FL=foetal liver
Undiff= undifferentiated
HPC= Haematopoietic stem cells
MSC= mesenchymal stem

Figure 86. Real-time PCR analysis of MSCs and haematopoietic stem cells (HPCs) at different stages of differentiation. Graphical data is in Logarithmic scale. HepG2, foetal liver at gestation age of 7 weeks (FL7wk) and 12 weeks (FL12wks) and adult liver cDNA were used as positive controls. RNase-free water was used as negative control. Week 1 (1wk) of the protocol was carried out in two parallel experiments, with and without EGF. This has been represented as 'minus EGF' and 'plus EGF' in the graphs. This was done to determine the role of EGF in hepatobiliary differentiation *ex vivo*. This has been discussed in Chapter 5 section 5.3. In this section, however, all references to week 1 are with regards to week 1 of differentiation without EGF (minus EGF 1wk). (a) Highest level of AFP expression was noted in foetal liver at gestational age of 12weeks (FL12wk). Both undifferentiated MSCs and HPCs tested positive for AFP. In MSCs the highest level of this gene was expressed at week 1 (post differentiation). This was followed by a slight down regulation but expression picked up again at week 3. In HPCs, AFP expression demonstrated a subtle down regulation post differentiation, after which levels remained constant. (b) Highest albumin expression was noted in foetal liver samples (FL7wk and 12wk). Both undifferentiated MSCs and HPCs tested positive for albumin. In MSCs, albumin levels illustrated an up regulation at week 1, then a slight reduction in expression level, picking up again and attaining maximum expression at week 3. In HPCs, albumin expression remained unchanged after differentation but then was slightly down regulated at week 2. (c) Highest HGFR expression was noted in foetal liver (FL12wk). Both undifferentiated MSCs and HPCs tested positive for HGFR. MSCs demonstrated a down regulation at week 1 and 2 but levels picked up at week 3. In HPCs, after differentiation HGFR showed a subtle down regulation at week 1 followed by a further reduction in expression at week 2.

(a)



(b)



(c)

FL=foetal liver
Undiff= undifferentiated
HPC= Haematopoietic stem cells
MSC= mesenchymal stem cells

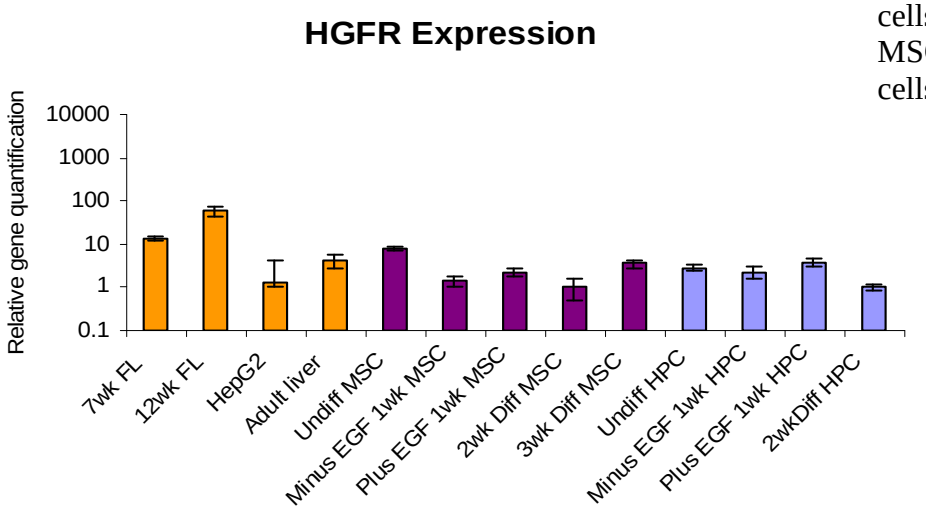
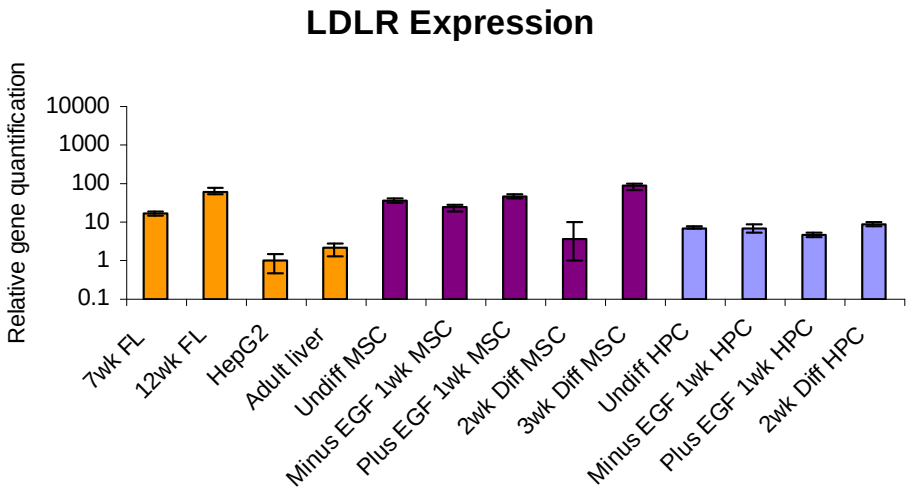
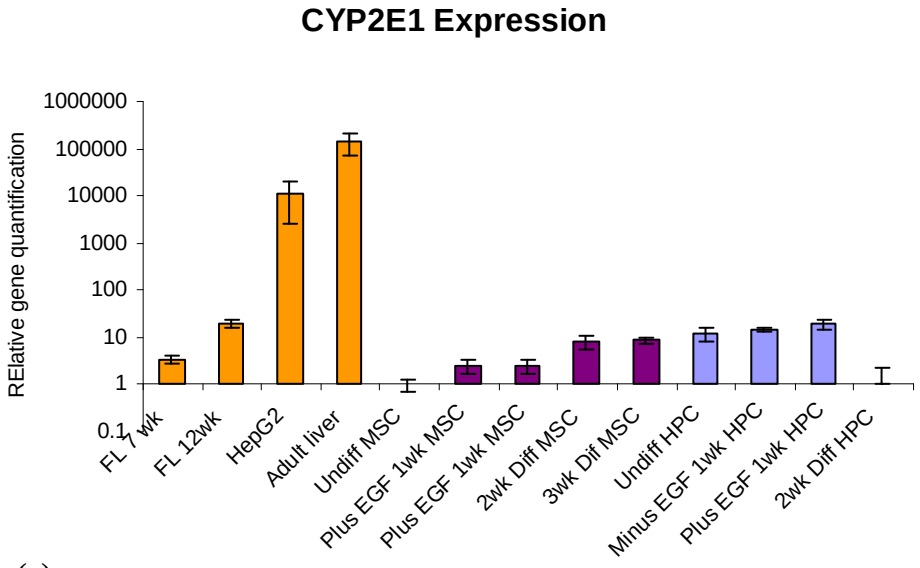


Figure 87. Real-time PCR analysis of MSCs and haematopoietic stem cells (HPCs) at different stages of differentiation. Graphical data is in Logarithmic scale. HepG2, foetal liver at gestation age of 7 weeks (FL7wk) and 12 weeks (FL12wks) and adult liver cDNA were used as positive controls. RNase-free water was used as negative control. Week 1 (1wk) of the protocol was carried out in two parallel experiments, with and without EGF. This has been represented as 'minus EGF' and 'plus EGF' in the graphs. This was done to determine the role of EGF in hepatobiliary differentiation *ex vivo*. This has been discussed in Chapter 5 section 5.3. In this section, however, all references to week 1 are with regards to week 1 of differentiation without EGF (minus EGF 1wk). (a) LDLR demonstrated highest level of expression in foetal liver (FL12wk). Undifferentiated MScs and HPCs, both tested positive for LDLR gene. In MSCs, LDLR expression decreased during the first two weeks of differentiation but demonstrated an upregulation at week 3. This was the highest level of expression noted during the differentiation protocol. HPCs, also illustrated a very slight increase in LDLR expression at week 2. (b) Highest level of CYP2E1 was noted in adult liver cells. Undifferentied MSCs were negative for this gene. Undifferentiated HPCs, on the other hand tested positive for this CYP enzyme. In MSCs, CYP2E1 expression was triggered at week 1 post differentiation followed by a gradual increase in expression levels reaching maximum expression at week 2 and expression was maintained thereafter. HPCs, demonstrated a very subtle increase in expression at week 1 but expression was switched off at week 2. (c) Highest CYP3A4 expression was appreciated in FL12wk. Both undifferentiated MSCs and HPCs demonstrated CYP3A4 expression. In MSCs, this CYP gene was upregulated post differentiation at week 1 and thereafter expression level was maintained. In HPCs, CYP3A4 expression at week 1 was unchanged from undifferentiated state but at week 2 a down regulation was noted.

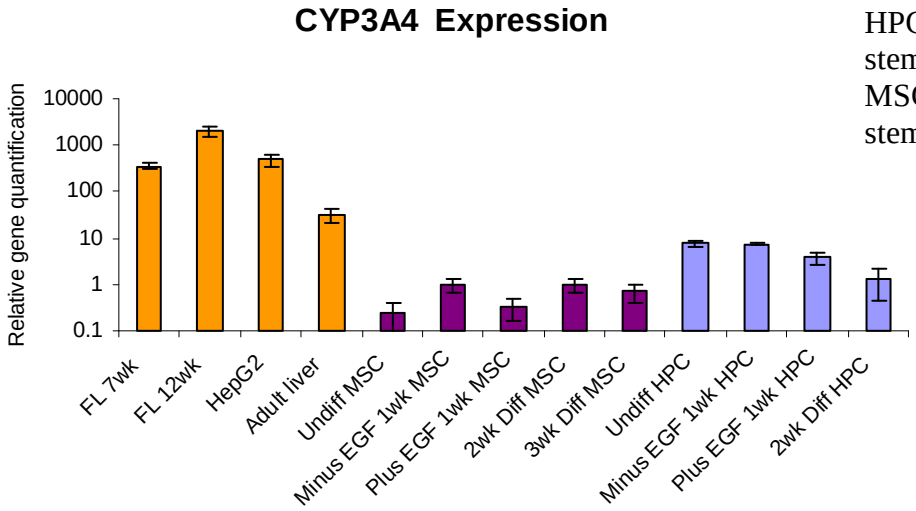
(a)



(b)



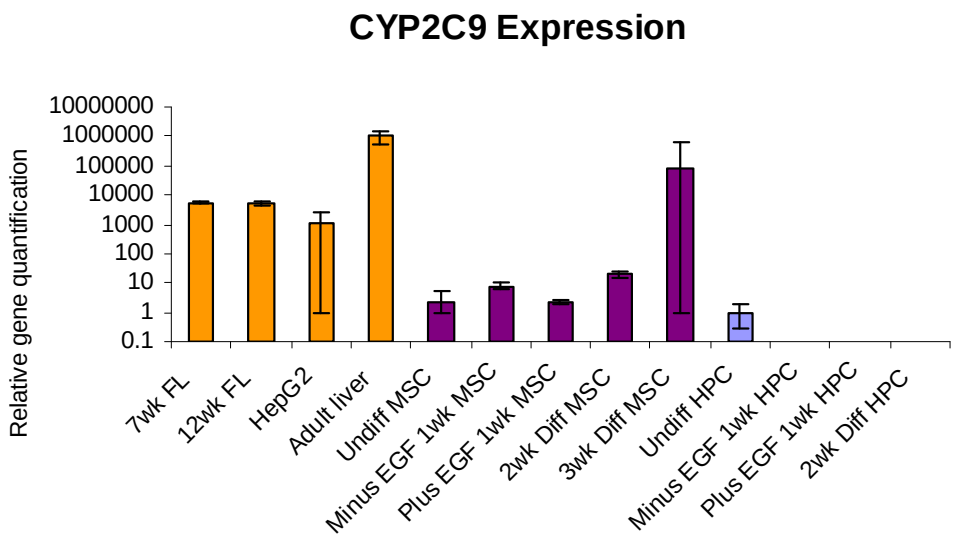
(c)



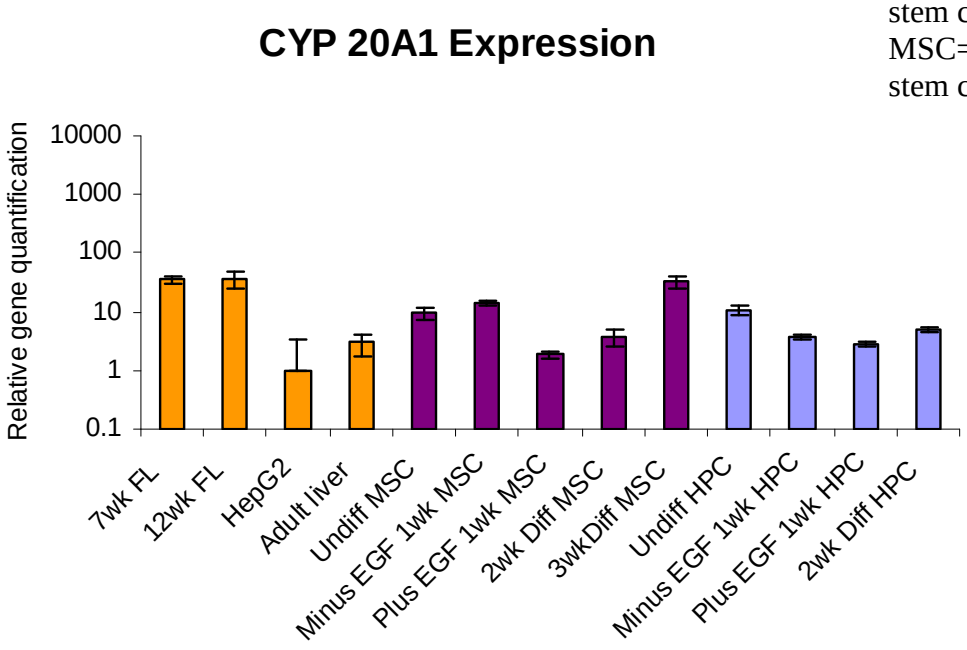
FL=foetal liver
Undiff= undifferentiated
HPC= Haematopoietic stem cells
MSC= mesenchymal stem cells

Figure 88. Real-time PCR analysis of MSCs and haematopoietic stem cells (HPCs) at different stages of differentiation. Graphical data is in Logarithmic scale. HepG2, foetal liver at gestation age of 7 weeks (FL7wk) and 12 weeks (FL12wks) and adult liver cDNA were used as positive controls. RNase-free water was used as negative control. Week 1 (1wk) of the protocol was carried out in two parallel experiments, with and without EGF. This has been represented as 'minus EGF' and 'plus EGF' in the graphs. This was done to determine the role of EGF in hepatobiliary differentiation *ex vivo*. This has been discussed in Chapter 5 section 5.3. In this section, however, all references to week 1 are with regards to week 1 of differentiation without EGF (minus EGF 1wk). (a) Highest level of CYP2C9 expression was noted in adult liver cells. Both undifferentiated MSCs and HPCs demonstrated CYP2C9 expression. In MSCs, the expression of this gene demonstrated an up regulation throughout the differentiation protocol reaching the highest level at week 3. HPCs, on the other hand, demonstrated complete switch off of this CYP gene post differentiation. (b) Highest CYP20A1 levels were noted in foetal liver cells (FL7wk and FL12wk). Undifferentiated MSCs and HPCs tested positive for this CYP gene. In MSCs, CYP20A1 was slightly up regulated at week 1 but levels decreased at week 2. At week 3, however, expression picked up reaching maximun level of expression comparable to foetal liver cells. HPCs, demonstrated a down regulation of CYP20A1 at week 1 but levels increased slightly at week 2, however, not surpassing the expression level at day zero (undifferentiated state).

(a)



(b)



FL=foetal liver
Undiff= undifferentiated
HPC= Haematopoietic stem cells
MSC= mesenchymal stem cells

5.4.8 Functional analysis

The umbilical cord and UCB- derived hepatobiliary-like cells displayed morphological and immunophenotypical characteristics similar to human liver cell populations. However, as Hengstler *et al* put it, the expression of a few hepatic markers by stem/progenitor cells does not make them mature hepatocytes. More importantly, the cells should exhibit signs of metabolic activity. Therefore, we examined the differentiated cellular populations for a range of hepatocyte functions: glycogen storage, uptake and excretion of indocyanine green dye and cytochrome P450 activity.

(A) Periodic acid Schiff staining

Liver is a prime organ involved in carbohydrate metabolism in the human body. It plays a pivotal role in maintaining blood sugar levels during periods of starvation and post-feeding. To assess this crucial function of liver cells Periodic Acid Schiff (PAS) staining was used to examine glycogen deposits in differentiated stem/progenitor cells. A staining kit from Sigma was utilized and the staining procedure was adapted from kang *et al* (Kang, Zang et al. 2005) and according to the manufacturer's instructions.

Post differentiation both MSC and UCB stem/progenitor cells displayed evidence of functional activity (Figure 89 and 90). Glycogen stores were stained magenta pink with periodic acid Schiff staining kit (Materials and Methods, section 2.16.1).

Positive PAS staining in differentiated cord blood stem/progenitor colonies

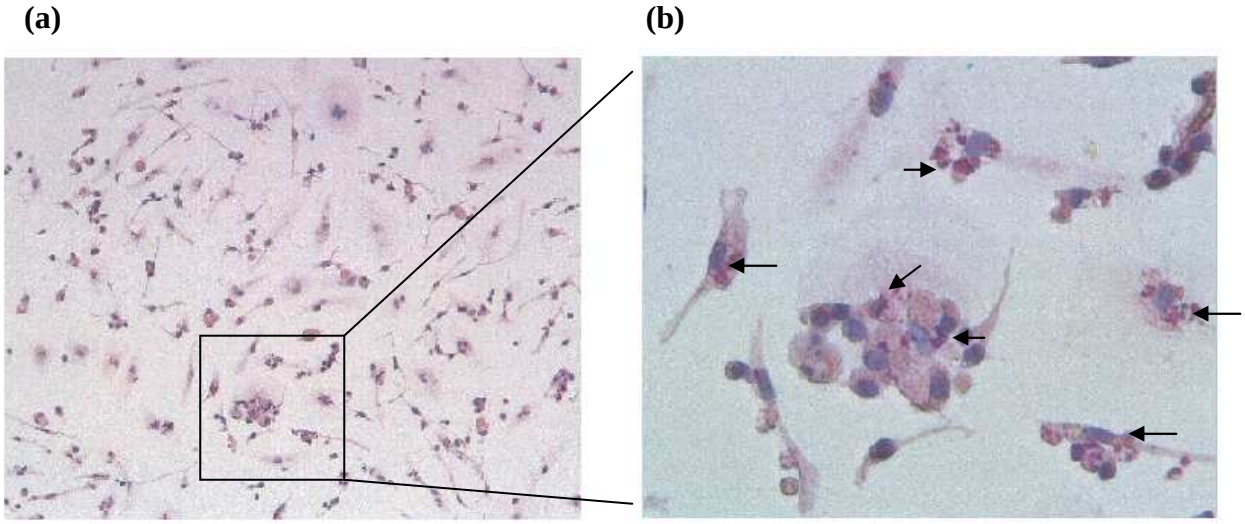
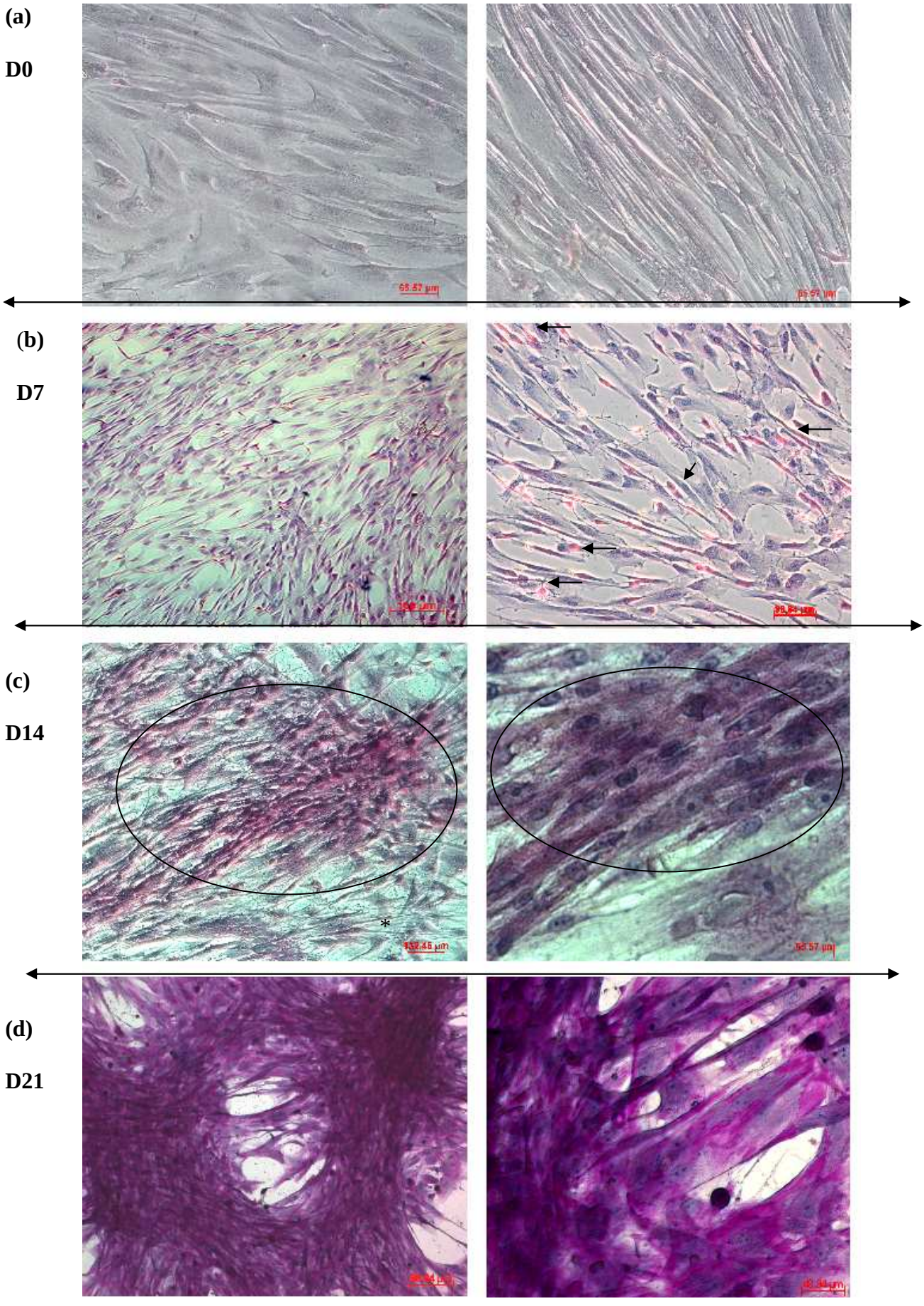


Figure 89. Periodic acid schiff staining of intracellular glycogen stores in UCB-derived hepatocyte-like cells. (a) Differentiated stem/progenitor cells in cellular niches demonstrated the presence of intracellular glycogen stores (arrow). Cellular nuclei were stained bluish purple with haematoxylin. (b) Higher magnification of image (a). DIC images taken at magnification of x10 and x20 respectively. Scale 50µm.

Figure 90. Periodic acid schiff staining of intracellular glycogen stores in differentiated Mesenchymal stem cells. Glycogen stores were depicted by magenta pink intracellular staining. Cellular nuclei were stained bluish purple with haematoxylin dye. (a) Undifferentiated MSCs were negative for glycogen stores. (b) After one week of differentiation a few cells demonstrated intracellular glycogen granules (arrow). (c) After introduction of maturation factors into the culture system, MSCs illustrated notable retraction of cellular appendages accompanied by amassing of cells into aggregates. These cellular aggregates were positive for glycogen stores (circled area). Surrounding cells were noted to be negative for glycogen (*). (d) After three weeks of *ex vivo* differentiation, Periodic Acid Schiff staining was strongly positive. All the cells in culture were stained for intracellular glycogen. DIC were taken at magnification of x10 and x20.

Positive PAS staining in differentiated Mesenchymal stem/progenitor cells



HepG2 cells demonstrate positive Periodic acid Schiff staining

HepG2 cells were used as a positive control for all functional investigations applied to UCB-derived stem/progenitor cells and Mesenchymal stem cells. Primary cultures of this hepatocelluar carcinoma cell line were tested for PAS staining (Figure 91). The intensity of PAS staining in the HepG2 cells was comparable to PAS staining in differentiated MSCs after maturation.

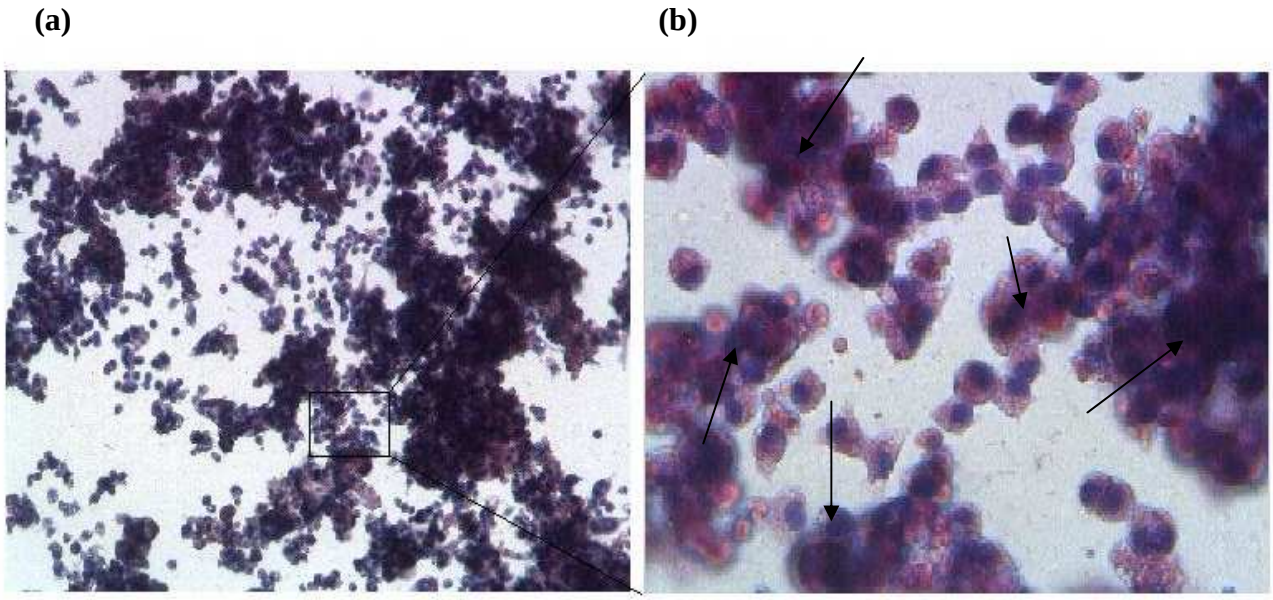


Figure 91. Glycogen storage demonstrated in HepG2 cells. A high fraction of the cells exhibit glycogen storage as demonstrated by magenta staining (arrow) with Periodic Acid Schiff (a and b). Estimation of the precise percentage of positivity with PAS was difficult due to the growth pattern of the cell line in mounds. It was noted that not all the cells in culture tested positive for glycogen storage. Nuclei are counter stained with haematoxylin. Scale 50µm.

(B) Indocyanine green dye uptake and release test

Another major function of the liver is elimination of various endogenous and exogenous compounds from the circulation. For many of these substances, the clearance process involves at least three steps: hepatic uptake, conjunction and biliary excretion (Berk and Chamovitz 1969) (Scharschmidt, Waggoner et al. 1975) . This clearance process involves basolateral (sinusoidal and lateral) membrane transport systems that mediate the hepatocellular uptake of bile acids, organic anions, and organic cations (Meier PJ 1997) (Muller and Jansen 1998).

Indocyanine green (ICG) is an organic anion that is clinically used as a test to evaluate liver function since it is non-toxic and eliminated exclusively by hepatocytes (Branch 1982) (Berk PD 1986; Meijer DKF 1988). In this study Indocyanine green dye uptake and release by cultured cells was used to identify functionally differentiated hepatocytes within the culture system. The ICG solution (1mg/ml) was loaded to the cell culture dish and incubated for 20 minutes at 37° C. After, the culture plate was rinsed three times with (x1) PBS and cellular uptake of ICG was examined stereomicroscopically.

Both MSCs and UCB-derived differentiated cells exhibited the ability to take up and excrete Indocyanine green dye back into the culture media. Within 24 hours of loading the dye in culture, cells exhibited ability to excrete most of the dye back into the culture media.

Indocyanine green dye uptake by differentiated cord blood stem/progenitor colonies

(a) Prior to ICG treatment **(b)** Post 25minutes of ICG

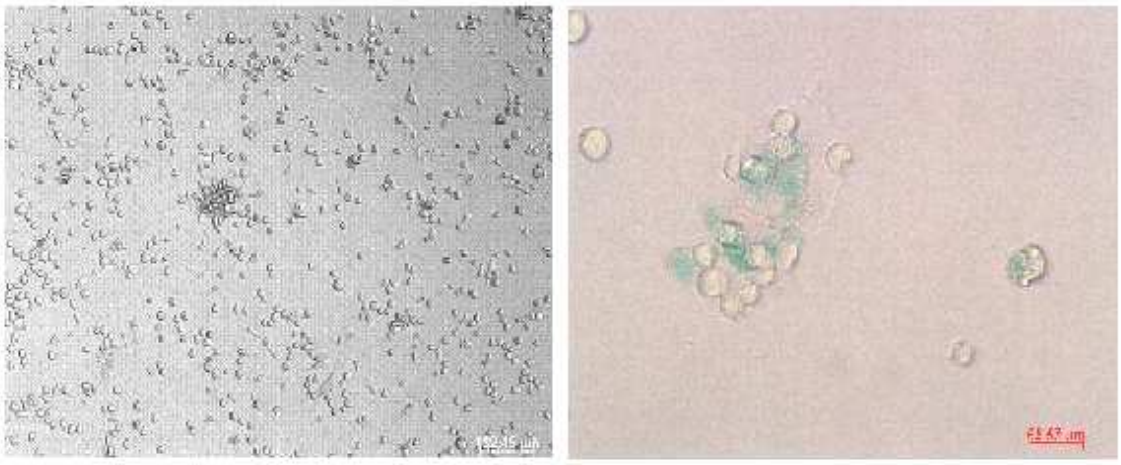


Figure 92. Indocyanine green dye uptake and release test in differentiated cord blood stem/progenitor cellular colonies. (a) Stem/progenitor cellular colonies prior to exposure with Indocyanine green dye. (b) Green coloration of cells represented Indocyanine uptake. Note that the dye uptake is selective to only a few cells. The majority of isolated cells not in colonies have been lost during the assay washes and the remnant ones did not illustrate very efficient dye uptake. DIC images were taken at magnification x10 and x20 respectively.

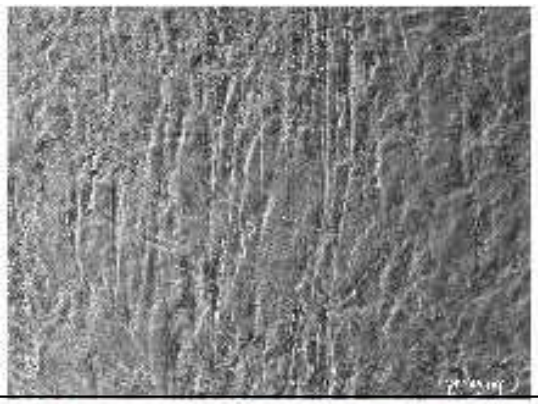
Figure 93. Indocyanine green dye uptake and release test in differentiated Mesenchymal stem cells at various stages during the differentiation protocol. (a) Undifferentiated MSCs demonstrate weak dye uptake. (b) After 1 week of differentiation, dye uptake by cells had increased notably. It should be noted here that similar to cord blood stem/progenitor cells, dye uptake was not generalized but selective to some cells. (c) Post week 2 of differentiation Indocyanine green dye uptake was just as efficient. Additionally, changes in cellular morphology of MSCs were noted. (d) Post maturation, green dye uptake was at its maximum intensity and cellular transformation from spindle shaped MSC to polygonal cells was appreciated. Once again Indocyanine uptake was selective. DIC images were taken at a magnification of x10 and x20.

Indocyanine green dye uptake by differentiated Mesenchymal stem cells

Higher magnification

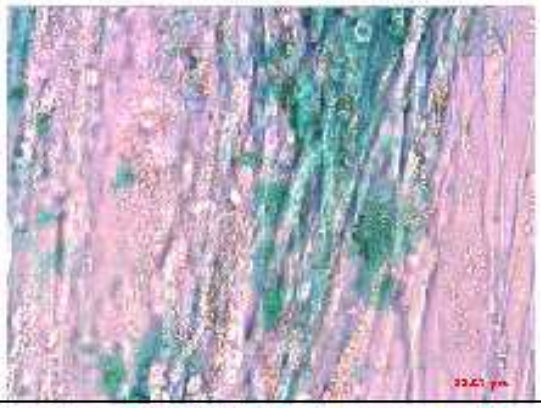
(a)

D0



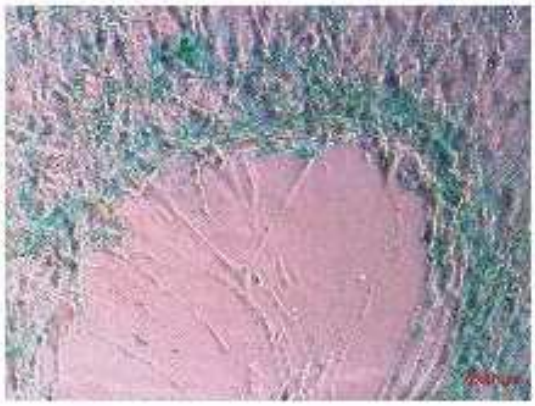
(b)

D7



(c)

D14



(d)

D21

HepG2 cells demonstrate positive Indocyanine green dye uptake

HepG2 primary cultures demonstrated ability to take up Indocyanine green dye from culture. Uptake of this dye was not universal in all the cells in culture but localized to cells localized in cellular colonies (Figure 94). The cells also demonstrated the ability to excrete the dye back into the culture medium after several washes. Uptake of Indocyanine dye by UCB-derived differentiated stem/progenitor cells and Mesenchymal stem cells were comparable to HepG2 cells. In both cell types, uptake of the dye was also not universal and limited to cells contained in celluar aggregates or colonies.

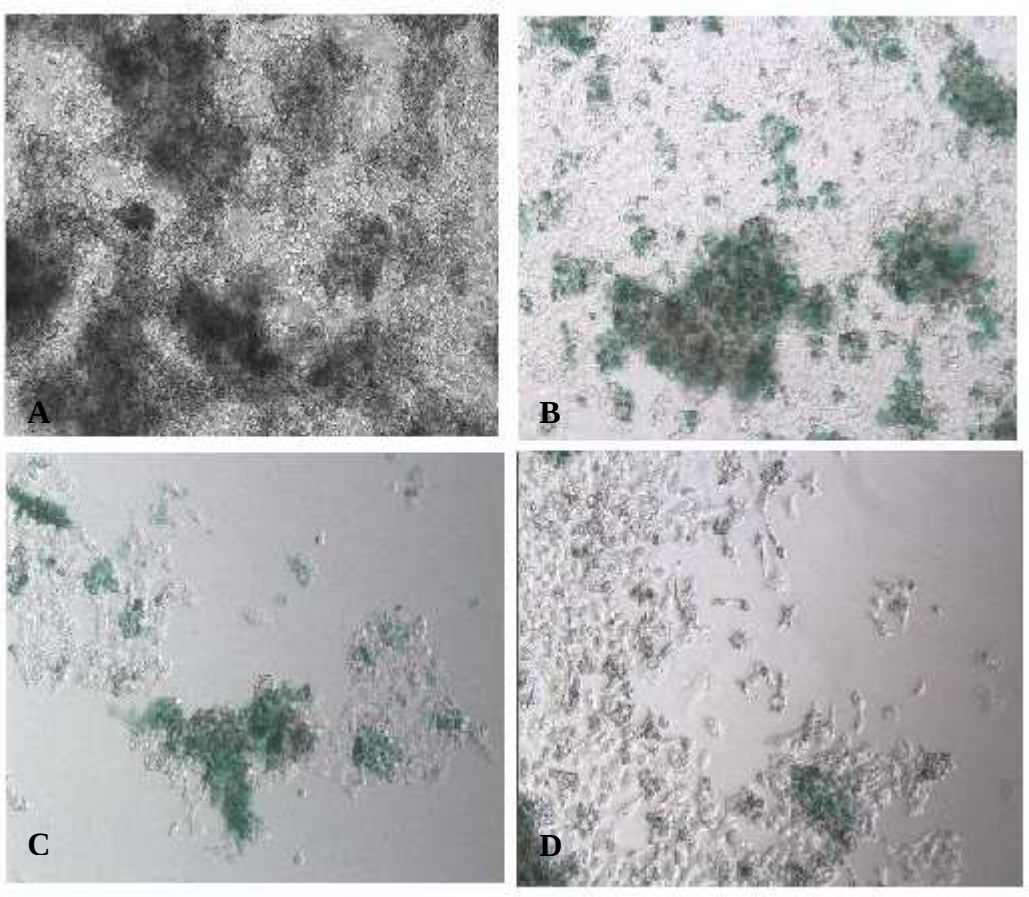


Figure 94. HepG2 cells exhibited ability to take up Indocyanine green dye. (A) Before exposure to green dye. (B) Not all the cells were positive for this functional assay. One hour post assay and washing the cells with (x1) PBS. (C) Note most of the cells not in cellular colonies were lost during cell washing. 24 hours post assay and washing most of the cells had excreted the green dye (D). Images acquired at magnification of x10 and 20. Scale 50µm

(C) Cytochrome P450 Glo Assay

Human Cytochrome P450 (CYP450) are membrane-associated proteins located either in the inner membrane of the mitochondria or in the endoplasmic reticulum of cells. Cytochromes are involved in the metabolism of thousands of endogenous and exogenous chemicals. CYP450 enzymes are present in most tissues of the body, and play a pivotal role in hormone synthesis and breakdown, cholesterol synthesis, and vitamin D metabolism. CYPs also function to metabolize potentially toxic compounds, including drugs and endogenous metabolites such as bilirubin, principally in the liver. CYP enzymes account for approximately 75% of the total drug metabolism (Guengerich 2008). The Human Genome project has identified 57 human genes coding for the various cytochrome P450 enzymes and 59 pseudogenes divided among 18 families of CYP450 genes and 43 subfamilies (Nelson 2002).

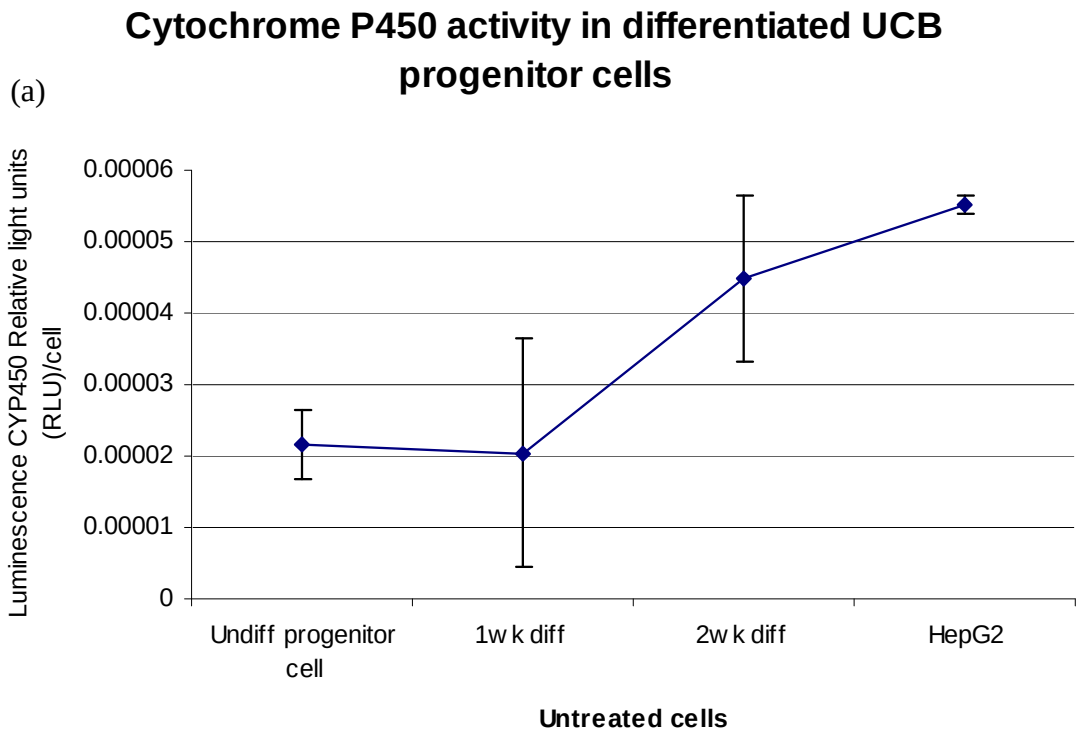
In the liver, Cytochrome P450 activity is one of the metabolic functions used as an indication of hepatocyte maturity. Thus, to access the level of maturity in differentiated cells we measured CYP enzymes using a non-selective cytochrome (CY) P450 Enzyme Assay. This assay uses a bioluminescent probe substrate that cross-reacts with multiple P450 enzymes. Enzyme activity was studied both in untreated differentiated cells as well as post treatment of the cells with CYP enzyme inducers. Omeperazole and Rifampicin were used as inducers resulting in selective upregulation of certain CYP enzyme subfamilies (Material and Methods, section 2.16.4). HepG2 was used as a positive control for both UCB-derived differentiated stem/progenitor cells as well as umbilical cord differentiated MSCs. Cell-free cultures were used as negative control.

The results demonstrated that undifferentiated cord blood cells display CYP activity. This probably was the effect of the expression of the prominent CYP subfamily, CYP3A, in blood neutrophils and lymphocytes. These blood cells are one of the various extra hepatic tissues that express CYP enzymes (Starkel, Sempoux et al. 1999). A point, however, that is worth noting is that both these cell lineages are not inducible, contrary to hepatocyte CYP enzymes. Gallagher *et al*, also reported cytochrome P450 mRNA expression in foetal liver haematopoietic stem cells . These $CD34^+$ cells are very similar to $CD34^+$ cells circulating in blood and serve as the primary organ of haematopoiesis during foetal liver development. CYP1A1, CYP2E1, CYP3A4 and CYP3A5 mRNA were measurable in these cells. The CYP3A4/5 mRNA expression was accompanied by detectable CYP3A protein as well (Shao, Stapleton et al. 2007). Similarly, it may be argued that the selected cord blood stem/progenitor cells in this project could also display evidence of CYP enzyme activity.

Upon differentiation, cells failed to illustrate an increase in CYP activity after week one of *ex vivo* simulation with signalling cues, but demonstrated a significant increase in CYP levels at week two. This increase in enzyme expression is attributable to greater maturity of differentiated cells post introduction of maturation factors (Figure 95a). HepG2 demonstrated greater evidence of CYP activity (x1.14 fold) compared to our differentiated UCB cells.

Undifferentiated MSCs, although constitutively expressing markers of hepatic lineage, did not illustrate significant CYP activity (Figure 95b). After differentiation, measurement of CYP enzymes failed to show an increase in enzyme levels during the first two weeks of differentiation *in vitro*. Upon introduction of maturation factors, however, a significant increase in enzyme levels was noted on measurement. This was suggestive of increase in cell maturity and subsequent metabolic activity in differentiated cells. Upon maturation differentiated MSCs demonstrated a greater level of CYP (x3.27 fold) activity than the HepG2 control.

(a)



(b)

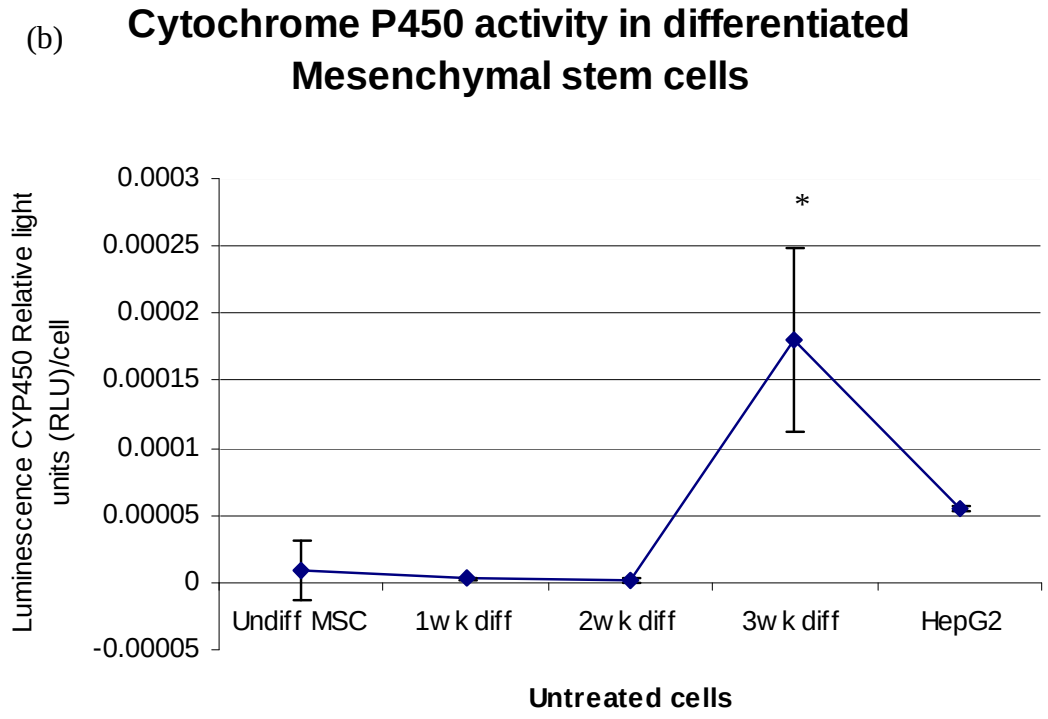
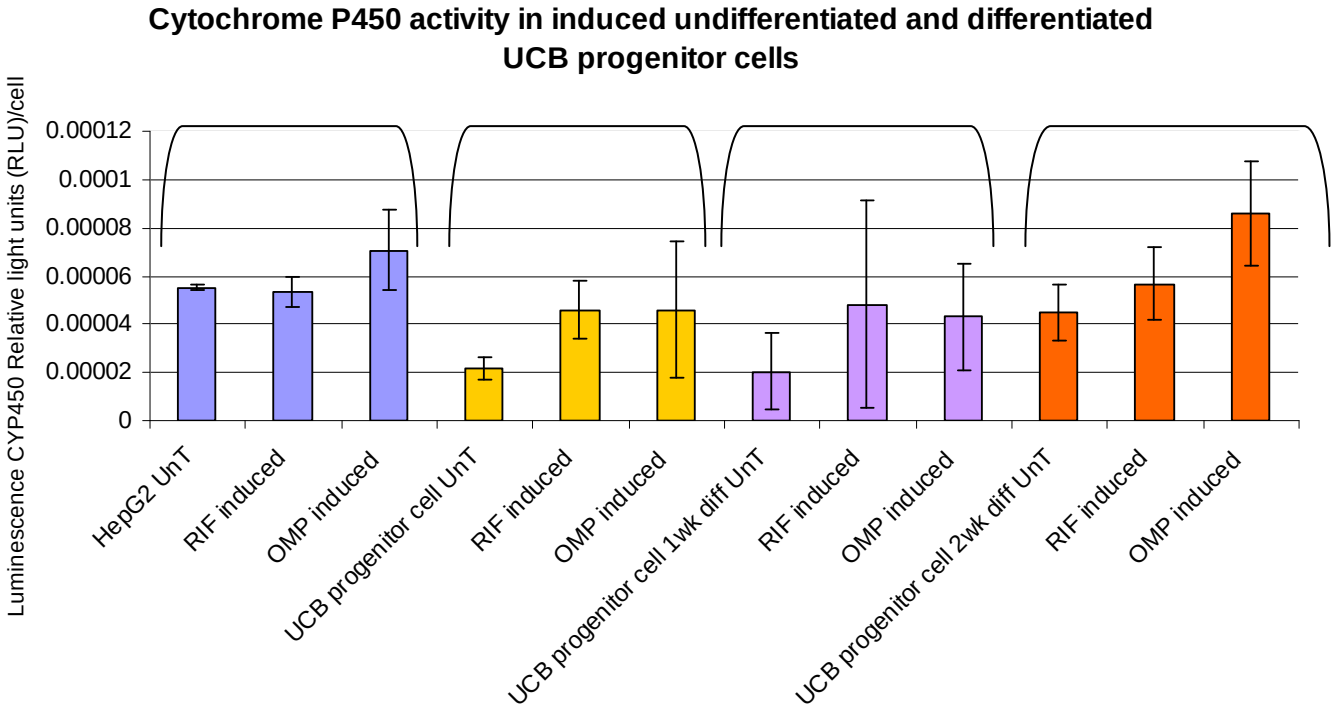


Figure 95. Measurement of cytochrome P450 proteins in differentiated UCB-derived and MSC-derived hepatocyte-like cells. (a) Undifferentiated cord blood stem/progenitor cells displayed evidence of low levels of CYP activity. Post differentiation and maturation a notable increase in CYP levels was appreciated on measurement with a luminometer. (b) Undifferentiated MSCs displayed very low if at all CYP activity. After differentiation and maturation a remarkable up regulation in CYP protein levels was noted at week 3. HepG2 was used as a positive control for both cell lineages. Cell-free cultures were used as negative control allowing measurement of background luminescence which was taken into account during calculation of net luminescence of each cell sample. (*) MSC CYP activity x3.27 folds greater that HepG2 control.

After measurement of net luminescence (CYP activity) in differentiated cells, we carried out CYP gene induction studies. Cells were treated with CYP enzyme inducers (Rifampicin and Omperazole) for 72hrs prior to treating the cells with the MultiCYP luminogenic substrate (Materials and Methods, section 2.16.4). Fold changes in net signal from the inducer-treated samples as compared to the untreated control samples represented the change in CYP activity upon enzyme induction *ex vivo*. Once again, HepG2 was used as a positive control for this assay.

Results obtained showed that cord blood-derived cells responded to induction positively displaying an up regulation in CYP levels post treatment (Figure 96a). It was interesting to note that CYP proteins in undifferentiated cord blood cells were also inducible. This result was contrary to that reported by Starkel, Sempoux *et al,* who showed that CYP3A in blood neutrophils and lymphocytes was not inducible. It should be pointed out that Strakel *et al* looked at only one of the CYP subfamilies (CYP3A) and it may be that other CYP subfamilies detectable in haematopoietic cells are capable of induction by CYP gene inducers. Although prior to induction HepG2 cells exhibited a greater level of CYP activity compared with cord blood-derived hepatocyte-like cells; post induction CYP levels in differentiated cells were comparable to HepG2 expression levels with the highest level being detected in UCB differentiated cells post maturation.

Undifferentiated MSCs failed to show a positive response to induction. Differentiated MSCs, however, did respond to induction and displayed an up regulation in CYP levels (Figure 96b). This reaction was most appreciated in differentiated MSCs post maturation. HepG2 cells also responded to induction and displayed a net increase in CYP levels after treatment with the inducers.



(b)

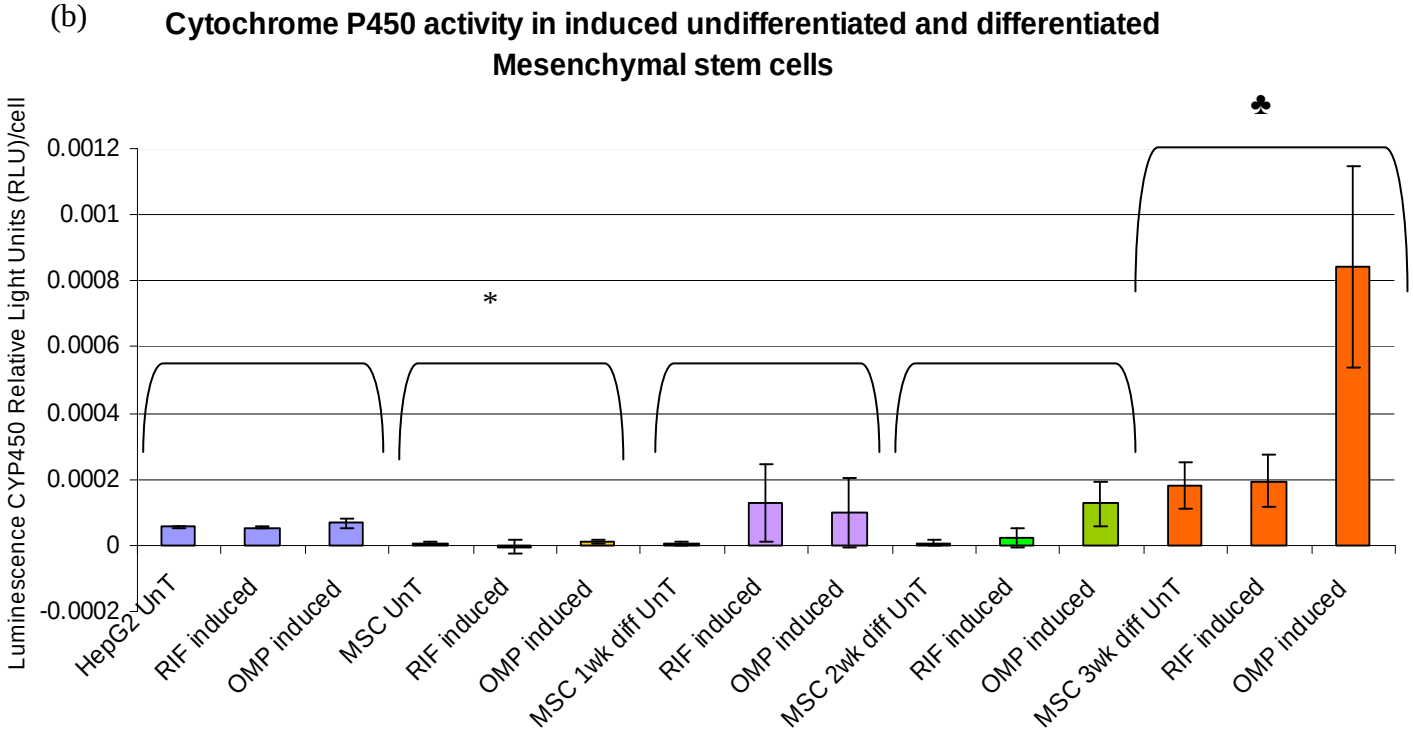


Figure 96. Induction of cytochrome P450 proteins in differentiated UCB-derived and MSC-derived hepatocyte-like cells. (a) Both undifferentiated and differentiated UCB-derived stem/progenitor cells were inducible and displayed a net increase in CYP protein levels upon induction. (b) Undifferentiated MSCs were not induced by the CYP gene inducers (*). Differentiated MSCs displayed a net increase in CYP luminescence upon induction with Rifampicin and Omperazole with maximum results being obtained after maturation of the cells *in vitro* (♣).

Conclusion

Undifferentiated UCB-derived stem/progenitor cells displays evidence of CYP activity.

UCB-derived differentiated hepatocyte-like cells express cytochrome P450 proteins after *ex vivo* differentiation and maturation.

Both undifferentiated and differentiated UCB-derived stem/progenitor cells were inducible and displayed a net increase in CYP protein level.

Undifferentiated MSCs showed minimal to no CYP activity.

MSC-derived differentiated hepatocyte-like cells express measurable cytochrome proteins only after maturation.

Cytochrome P450 levels were quantitatively higher in MSC-derived hepatocytes compared to UCB-derived hepatocytes post maturation.

CYP levels post maturation were higher in differentiated MSCs compared with the positive control used (HepG2).

Undifferentiated MSCs were not induced by the CYP gene inducers.

Differentiated MSCs displayed a net increase in CYP luminescence upon induction.

5.4.9 Progression to three-dimensional (3D) culture system

The low cost and high speed of testing have made two-dimensional (2D) cell culture a key component of *in vitro* research. However, a major limitation of 2D cell culture is the unnatural geometric and mechanical constraints that are imposed on the cells. 2D culture systems provide only approximate properties of normal tissue. A possible reason for this is that cells in monolayer cultures on 2D substrates do not realistically mimic the 3-dimensional morphologies of cells in tissues. The dynamic and complex interactions of cells with their supporting extracellular materials seen in 3D morphologies of cells is also absent. Furthermore, 2D cell monolayer cultures are generally exposed to a uniform environment (Albrecht, Underhill et al. 2006; Griffith and Swartz 2006). Development of 3D polymeric scaffolds and rotatory cell culture systems (RCCS) has paved the way for 3D tissue engineering that overcomes the limitation of 2D cell culture. These synthetic scaffolds are engineered to provide a surface chemistry that facilitates cell attachment and migration. Additionally, scaffolds deliver and retain cells and biochemical factors; enable diffusion of vital cell nutrients and expressed products as well as exert certain mechanical and biological influences that modify the behavior of the cell phase. Biodegradability is an essential property of scaffolds that allows them to degrade leaving behind only the bioengineered tissue. The rotatory cell culture provides a laminar flow of medium and minimal shear force inside the culture vessel. The cells are maintained in suspension by the resolution of the centrifugal, gravitation and coriolis forces. Thus the cells experience minimal mechanical stresses and high mass transport of nutrients, oxygen etc and thus are able to assemble into tissue-like aggregates (http://www.synthecon.com/bibliography/index.p). 3D cell culture systems mimic normal physiological environments for cell development.

In this study we attempted at reproducing our 2D differentiation in a 3D culture system. We used a variety of scaffolds (Materials and Methods, section 2.12.3.2) but only macrophorous hydrogel (Fibrinogen-gelatin) scaffolds proved effective. These scaffolds were provided through collaboration with Protista International AB Company (http://www.protista.se/?id=736). These scaffolds were used either on their own or in combination with the rotatory cell culture system or bioreactor provided by Synthecon (http://www.synthecon.com/bibliography/index.p).

Results obtained from cord blood stem/progenitor cells showed that these cells failed to survive *in vitro* post seeding into the scaffolds. This could be due to the low cell number generated post processing of cord blood units that limited cell-cell interaction within the scaffolds. Mesenchymal stem cells, on the other hand, responded very well to the 3D culture

environment. These cells were noted to form cellular aggregrated within 24 hours post seeding into scaffolds (Figure 97). In 2D monolayers, these cellular aggregates were not noted until 3 weeks post differentiation. When scaffolds were used on their own in culture plates, cells propagated both within the scaffold (as cellular aggregates) as well as outside the scaffold in the culture plate (Figure 97). This feature was avoided when scaffolds were cultured in the bioreactor (Figure 98). Post differentiation (1 week), cells within scaffolds were investigated for the presence of liver cell markers by immunocytochemistry. The cells tested positive for albumin, Ck-7, -8 and -18 (Figure 99). The pattern of expression of the various hepatic cell markers at week 1 in 3D culture was similar to that noted in 2D experiments at week 3 of the differentiation protocol. This clearly showed that the 3D culture environment facilitated cell differentiation. The Protista scaffolds were provided to us in limited numbers thus the only functional test that was performed on the differentiated cells in the 3D system was Indocyanine green uptake and release test. Periodic acid schiff could not be performed on the scaffolds due to their polysaccharide structure. There were not enough scaffolds to allow Cytochrome P450 assays to be carried out. The results of Indocynaine green test illustrated that the cells were able to take up the green dye from culture (Figure 100) and eventually excrete it back into the medium within 24 hours.

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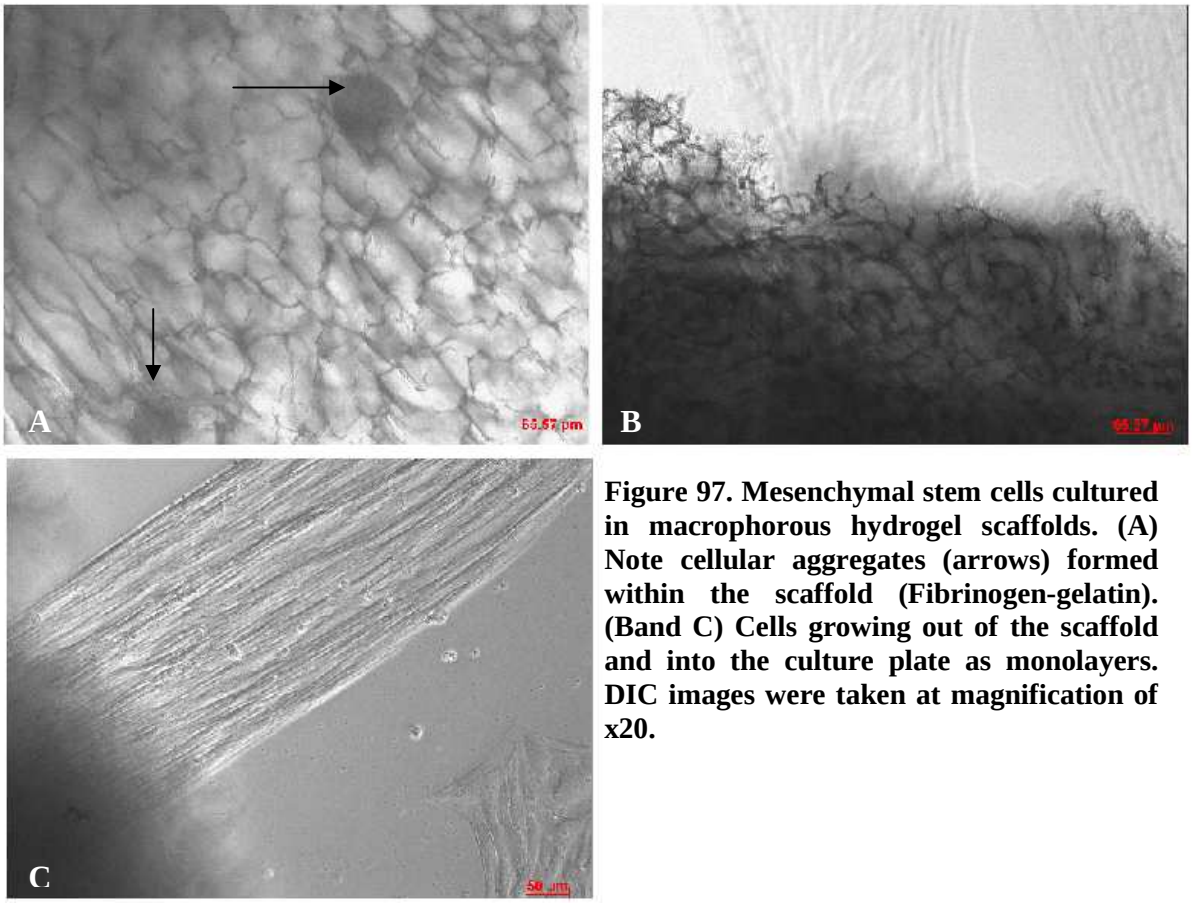


Figure 97. Mesenchymal stem cells cultured in macrophorous hydrogel scaffolds. (A) Note cellular aggregates (arrows) formed within the scaffold (Fibrinogen-gelatin). (Band C) Cells growing out of the scaffold and into the culture plate as monolayers. DIC images were taken at magnification of x20.

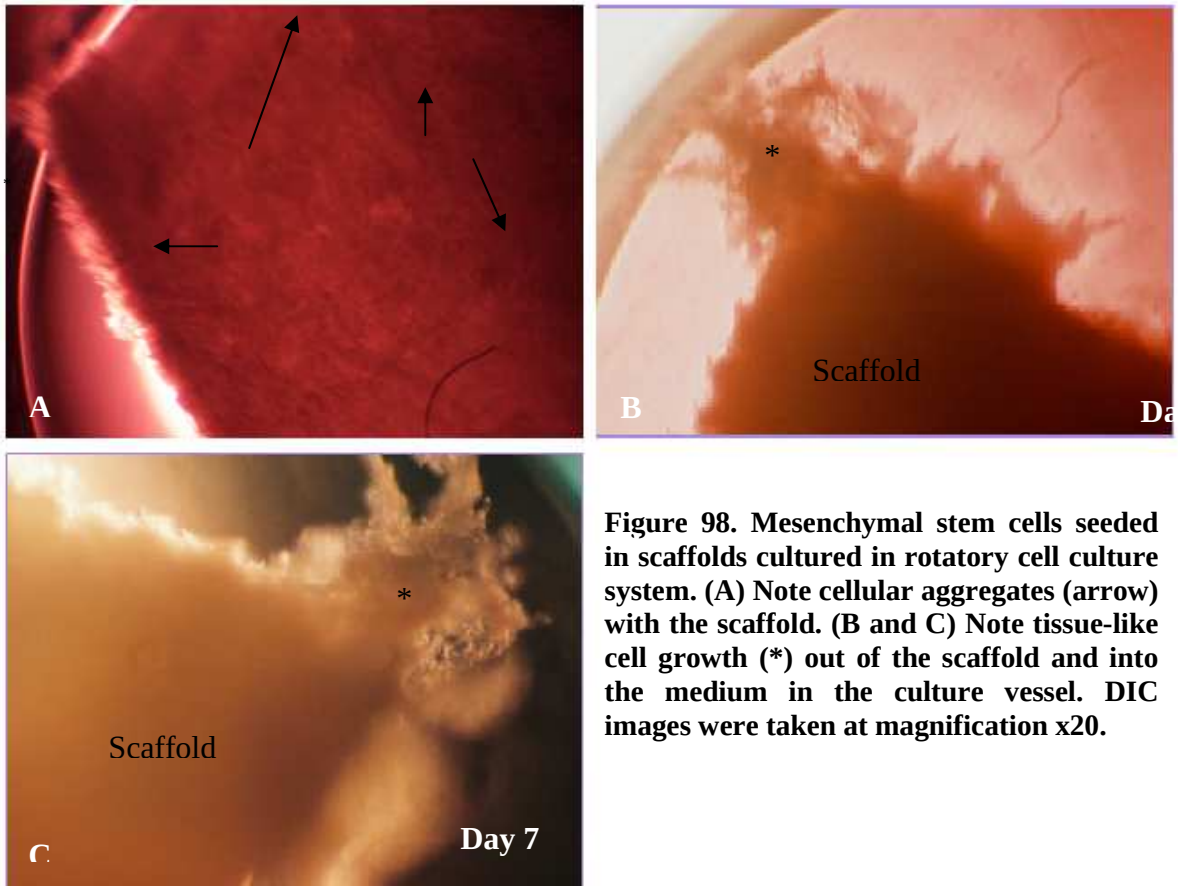


Figure 98. Mesenchymal stem cells seeded in scaffolds cultured in rotatory cell culture system. (A) Note cellular aggregates (arrow) with the scaffold. (B and C) Note tissue-like cell growth (*) out of the scaffold and into the medium in the culture vessel. DIC images were taken at magnification x20.

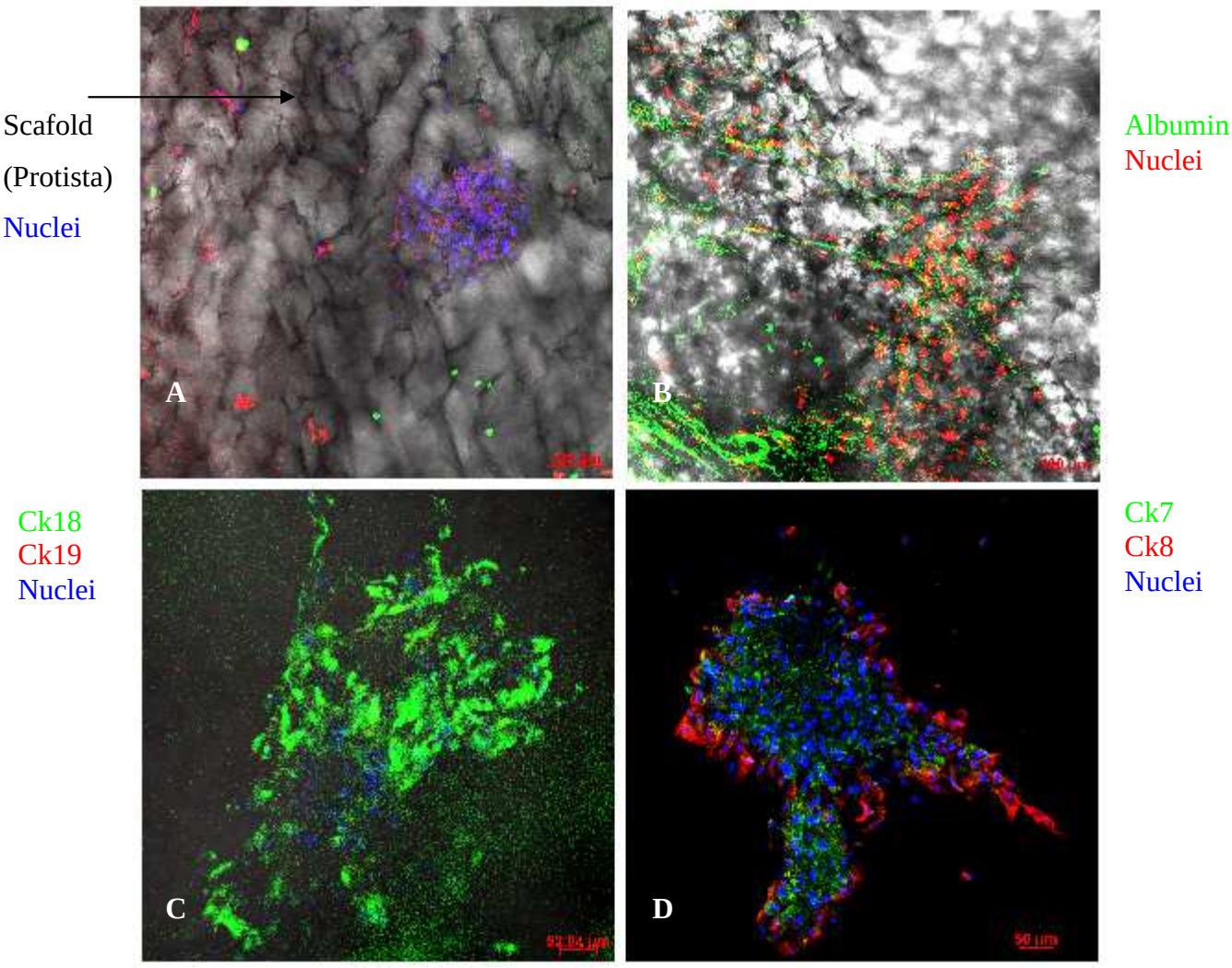


Figure 99. Confocal immunocytochemistry images of MSCs differentiated in 3D culture system. Cellular nuclei were stained blue or red with DAPI (4', 6-diamidino-phenylindole dilactate). Cells tested positive for albumin (B) Ck-7, -8 (D) and Ck-18 (C). Green= FITC, Red=TxRed. Images were taken at magnification x10.

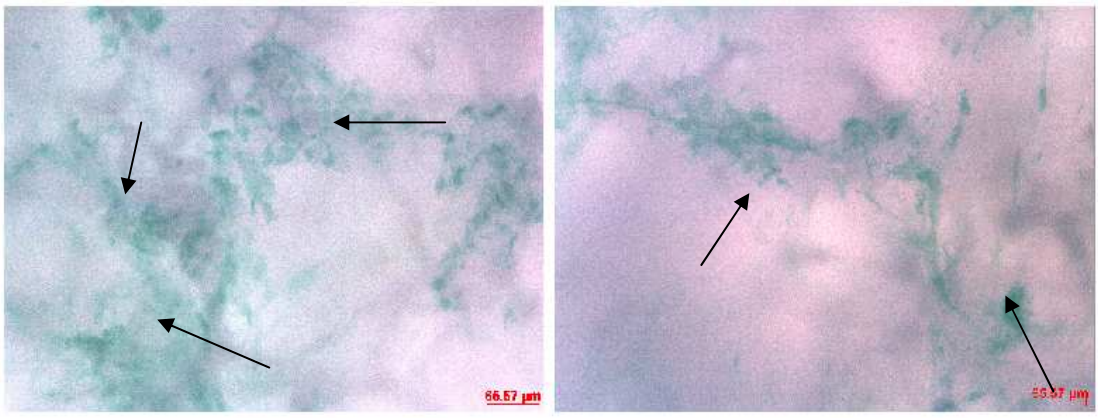


Figure100. Indocyanine green dye uptake by MSCs differentiated in 3D culture system. Cells demonstrated the ability to take up the green dye from culture medium (arrow) and excrete it back into the medium within 24 hours.

5.4.10 Specificity of differentiation protocol

To confirm the specificity of our differentiation system towards meso-endodermal lineage we examined our differentiated cells for markers of ectodermal germ layer lineage. As pointed out early, some of the isolated stem/progenitor cell populations; specifically Mesenchymal stem cells expressed markers of different germ layers prior to induction for hepatobiliary differentiation. To certify that our proposed differentiation system restricted further development of these progenitor cells only along endodermal and hepatic lineage we examined them for ectodermal/neural markers post *ex vivo* differentiation. This would also prove that differentiation was induced by the *in vitro* provided signaling cues and not the result spontaneous differentiation in culture. Foetal brain mRNA was used as a positive control in all the experiments. Also, cord blood-derived neurone differentiated cells (produced by other members of the group) were used to compare with our hepatobiliary differentiated cells. Foetal and adult liver mRNA was used as negative controls.

Differentiated cells were investigated for the following genes: Paired box gene 6 (Pax6), T-box brain 1 (TBR1) and T-box brain 2 (TBR2).

- Pax6 gene is involved in the development of ectodermal tissues (Callaerts, Halder et al. 1997).
- TBR1 is a putative transcription factor that is highly expressed in glutamatergic early-born cortical neurons and its expression is largely restricted to the cerebral cortex (Hevner, Shi et al. 2001).
- TBR2 is specifically expressed in the intermediate (basal) progenitor cells of the developing cortex (Sessa, Mao et al. 2008).

On examination, both UCB-derived and MSC-derived differentiated cells tested negative for Pax6. This illustrated that these cells had committed to meso-endodermal lineages (Figure 101a). Adult liver was also negative for this gene.

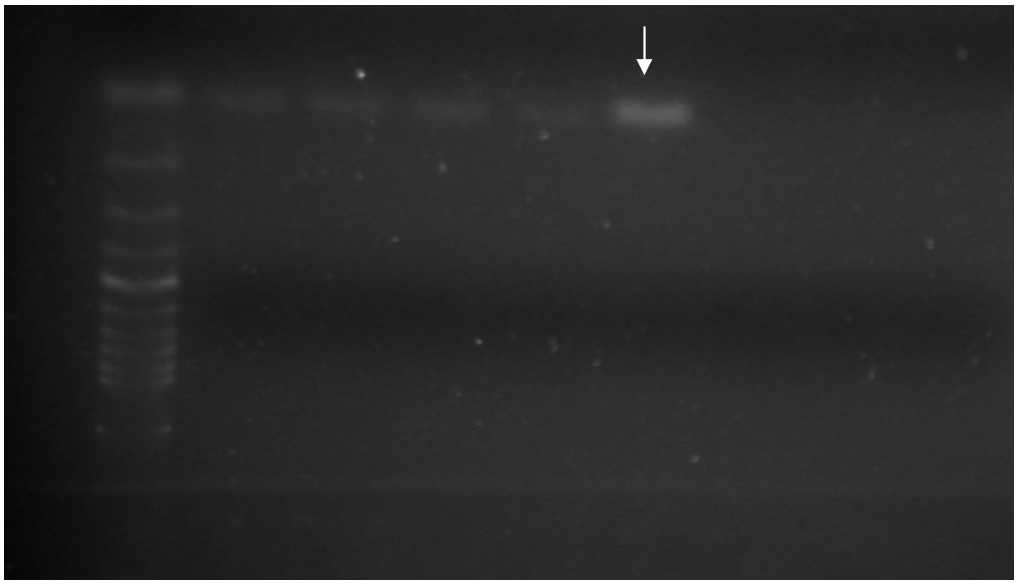
Interestingly, investigation for TBR2 revealed that adult liver tested positive for this gene, whilst foetal liver was negative. This phenomena could be explained by the theory of plasticity and differentiation potential of adult liver cells that has been reported previously in literature (Koenig, Krause et al. 2006). MSC differentiated cells displayed a weak expression of this gene but UCB-derived differentiated cells were negative (Figure 101b).

TRBR1 expression was negative in both foetal and adult liver cells but weakly positive in MSC differentiated cells. Differentiated UCB cells tested negative for TBR1.

The expression of neural progenitor markers in MSCs post differentiation suggested that even after differentiation along a specific developmental pathway these cells had maintained some of their original multipotent phenotype. One of the limitations of our proposed

differentiation system was that we succeeded in producing a heterogeneous society of liver cells with majority displaying a bi-potent liver progenitor cell phenotype rather than mature hepatocytes. Further development and maturation of these progenitor cells may lead to a more mature hepatobiliary cell phenotype and may cause a down regulation in the expression of progenitor markers from other germ layers.

(a) Pax6



Cord blood differentiated neural cells

Foetal brain tissue

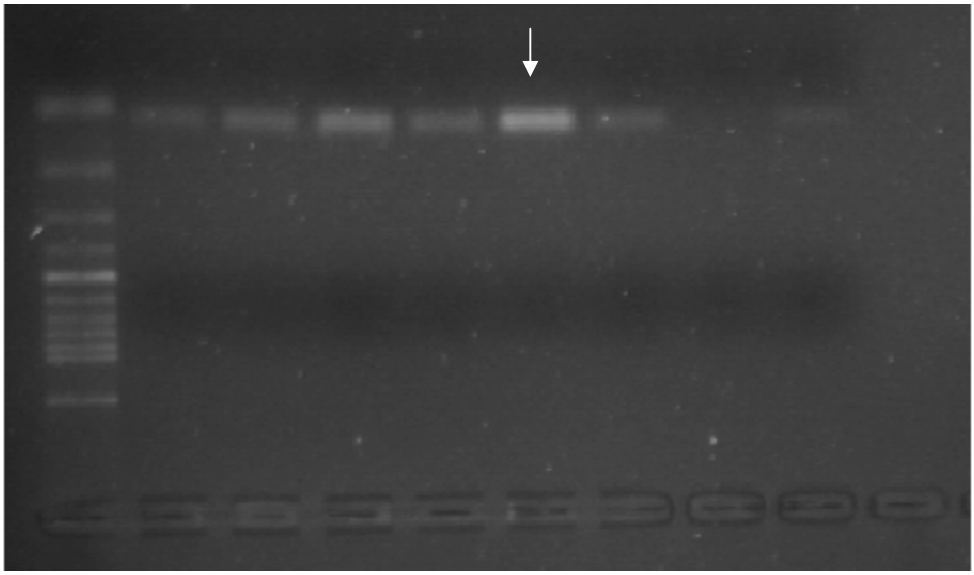
Adult liver tissue

MSC differentiated hepatocytes

UCB differentiated hepatocytes

(b) TBR2

100bp



Cord blood differentiated neural cells

Foetal brain tissue

Adult liver tissue

Foetal liver tissue

MSC differentiated hepatocytes

UCB differentiated hepatocytes

(c) TBR1

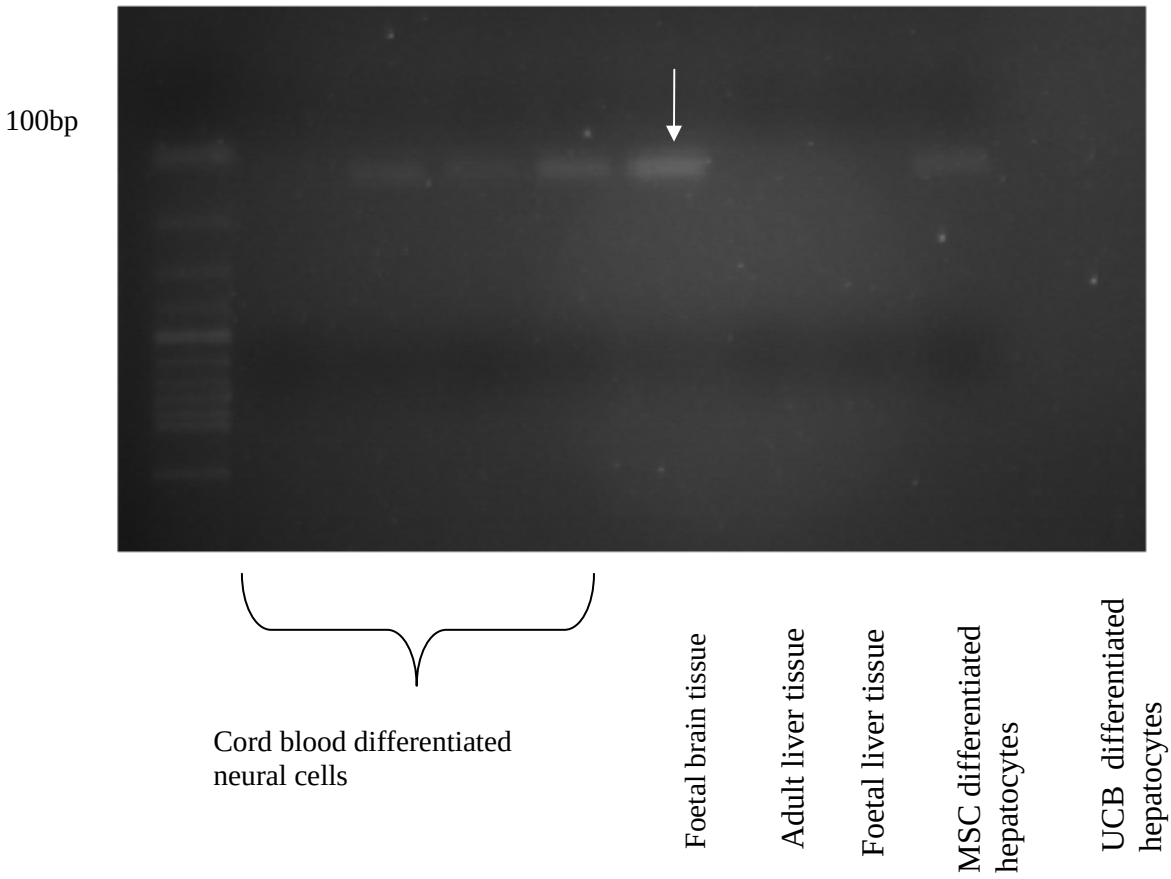


Figure 101. Establishment of the specificity of the proposed liver differentiation system. Foetal brain mRNA was used as a positive control (←). Foetal and adult liver mRNA was used as negative controls. Cord blood-derived neurone differentiated cells were used to compare with our hepatocyte differentiated cells. (a) Both adult liver cells and our differentiated cells tested negative for Pax6, ectodermal marker. (b) TBR2 expression was detected in adult liver tissue, but negative in foetal liver. MSC-derived differentiated cells also tested weakly positive for TBR2. (c) TBR1 was negative in both foetal and adult liver but once again weakly expressed in differentiated MSCs.

5.4.11 Discussion

The first clinical application of UCB for treatment of acute lymphoblastic leukaemia in 1970 fuelled a lot of interest in cord blood as a valuable stem cell resource. 20 years later, the value of cord blood was internationally appreciated and the concept of cord blood banks came into existence in 1990. The majority of research implemented was, however, focused on the whole mononucleated cell fraction of cord blood and experiments were predominantly peformed *in vivo* in animal models (Ishikawa, Drake et al. 2003; Newsome, Johannessen et al. 2003; Wang, Ge et al. 2003; Almeida-Porada, Porada et al. 2004; Sharma, Cantz et al. 2005). Little work was done to characterize the cells in cord blood and very few *in vitro* data was reported. In 2005, McGuckin *et al*, reported for the first time, the selective isolation a stem/progenitor cell population within cord blood that expressed embryonic stem cell markers (McGuckin, Forraz et al. 2005). They further went on to characterize these cord blood cells (Forraz, Baradez et al. 2006) and demonstrated their multipotential property *in vitro*.

Taking into account the importance of cord blood banking, one of the goals of this study was to compare the most commonly used cord blood processing techniques and to identify the most efficient way forward for cord blood banks. Identification of the most effective and economical processing and storage methods is of prime importance to some cord blood banks that are struggling financially. The two key determining factors for engraftment post transplantation are the number of nucleated cells (total nucleated cell count) and $CD34^+$ cells infused and the number of HLA differences (Gluckman, Rocha et al. 2004; Kamani, Spellman et al. 2008). Total nucleated cell counts (TNC) and haematopoietic ($CD133^+$; $CD34^+$) and non-haematopoeitic stem/progenitor cell contents of cord blood immediately post processing and after cryopreservation were compared for the various processing techniques. Here we have demonstrated that when cord blood samples are aimed for immediate transfusion/transplantation then PrepaCyte-CB would be the technique of choice. PrepaCyte demonstrated very efficient TNC recovery post processing, as well as very effective RBC depletion. Recoveries of Various stem/progenitor cells were also notably superior to the other techniques tested. An added advantage with PrepaCyte-CB was that it provided a completely closed system for cord blood processing. This allowed processing of cord blood units to be performed in class II laminar flow hoods rather than specialized GMP grade clean rooms. This may prove of considerable importance for hospitals and transplantation centers where the cost of installation of specialized GMP grade processing units is a financial burden.

For cord blood banks, where blood units are to be cryopreserved for possible future use then Sepax would be a better alternative. Sepax demonstrated the highest TNC post freezing and subsequent thaw. Recoveries of both HSC and non-haematopoietic stem/progenitor cells were also efficient. The single use kits provided also allowed controlled separation of cellular products in cord blood in a fully automated and closed environment. No other equipment was required during the processing procedure. The major limitation of this technique was the suboptimal RBC depletion and volume reduction compared with the other techniques. This is an important issue for cord blood banks.

Density gradient method of cord blood processing was probably the best option for *in vitro* research in the laboratory. This method demonstrated very effective RBC depletion but was the least effective in terms of TNC and stem/progenitor cell recoveries. However, the time and cost effectiveness of this technique makes it an attractive method for regular laboratory based research.

The next phase of this study was aimed at demonstrating the selective isolation of a stem/progenitor cell population within cord blood that expressed embryonic stem cell markers. Isolation of a homogenous stem/progenitor cell population could prove to be of great clinical importance. Thorough characterization of isolated cell fractions may contribute to better engraftment as well as reduce the chances of tumour formation in the patient. Characterization of cell therapy products is becoming increasingly important to translate pre-clinical research into clinical trials, as most regulatory agencies want guarantees that investigators understand the characteristics of the cells applied in therapy.

We did this by using our in-house ‘lineage negative selection protocol’ that was previously designed by our group (McGuckin, Forraz et al. 2005) and a commercial ‘human primitive progenitor cell enrichment kit’ from Stem Cell Technologies. Our in-house protocol proved more effective in the selection of early stem/progenitor cells. However, the labour intensive nature of this procedure and the extensive cell manipulation involved concluded in opting for the StemSep kit for practical reasons and subsequent results were similar to the ones obtained with the in-house protocol.

One of the main restricting factors that deem cord blood units of modest volume unsuitable for transplantation is the low yield of stem/progenitor cells. Several attempts have been made previously by research groups to achieve self-renewal of stem/progenitor cells *in vitro* but the results have so far been modest (Sorrentino 2004). As a part of this study we formulated an *ex vivo* expansion system for UCB stem/progenitor cells that abided by the

concepts of minimal manipulation. The results obtained, however, were unimpressive. The cells only demonstrated one doubling in culture after which the cell viability deteriorated. Thus, we decided to perform a short (1 week duration) expansion and simultaneous germ layer (meso-endoderm) commitment. This would allow any further procedures (eg differentiation) to be carried out while the cells were still viable. We demonstrated that at the end of this protocol the cells expressed endoderm specific markers (Gata-4) as well as some early hepatic markers (albumin, Ck-7 and Ck-19). Cytogenetic analysis on these cells demonstrated that the cells had a stable karyotype post treatment. Genome stability is of key importance in order to translate these findings into clinical settings.

The complexicity of cord blood processing and the resulting scarcity of stem/progenitor cells generated forced us to look into the umbilical cord for an alternative stem/progenitor cell source. We were able to successfully isolate Mesenchymal stem cells from umbilical cord matrix (Wharton's jelly) with ease and 100% efficiency. We have reported that in addition to the conventional MSC markers these cells also expressed some embryonic stem cell markers. These cells also demonstrated very efficient *in vitro* proliferation and viability. Karyotype analysis post prolonged *in vitro* expansion demonstrated that the cells were genetically stable.

This study also endeavoured to exploit the multi-potential property of UCB-derived stem/progenitor cells and umbilical cord Mesenchymal stem cells to reproduce the liver 'stem cell niche' *ex vivo*. These cells could provide an ideal *in vitro* model that could serve as a platform to improve our understanding of the developmental biology of the liver as well as be exploited in toxicology studies and in bio-artificial liver devices.

Stimulation with a range of differentaitive hepatic signals resulted in successful commitment of UCB-derived undifferentiated progenitor cells and Mesenchymal stem cells along hepato-biliary lineage. **A three step protocol encompassing: (I) short-term expansion with meso-endoderm commitment, (II) hepato-bilary specific differentiation and (III) short-term maturation**; was applied to effect hepatic differentiation from undifferentiated primitive cells (Figure 103). We were able to successfully generate niche-like cellular colonies when cord blood stem/progenitor cells were cultured. These 'niches' were initially composed of colonies of small rounded cells, 4-5µ in size, surrounded by spindle-shaped cells. As the colonies enlarged, the heterogeneous nature of the niches became more evident and large multinuclear cells were noted on which the colonies rested.

Initial endoderm commitment of UCB-derived stem/progenitor cell populations was achieved using acidic-FGF, HGF and SCF in low-serum culture conditions for a week

followed by differentiation using a cocktail of acidic-FGF, HGF and EGF in low serum culture conditions which was eventually tapered to achieve a serum-free system. Post endoderm induction and cell differentiation we were able to generate bipotent liver progenitor cells from both MSCs and cord blood primitive cells. Maturation of hepatocyte-like differentiated cells was achieved using a combination of dexamethasone, Oncostatin M and ITS+3 (Insulin 1mg/ml, transferrin 0.55mg/ml, sodium selenite 0.5μg/ml, linoleic acid 470 μg/ml, oleic acid 470 μg/ml, bovine serum albumin 50 mg/ml). In the presence of maturation inducers, a retraction of elongated end of MSCs membranes was observed and the polygonal morphology of cord blood-derived hepatocyte-like cells became more apparent with increased length in culture. *In vitro* characterisation assays of differentiated cells illustrated that most of the liver markers showed a slightly higher ratio of expression in the MSC cultures compared to the cord blood culture systems. The phenotypic profile of our differentiated cells was heterogeneous with a phenotype spectrum ranging from hepatic stem cells, hepatoblast and mature hepatocytes and biliary epithelial cells. The majority of the cells, however, could be characterized as bipotential hepatic progenitors expressing both hepatocyte and biliary cytokeratins but not yet fully committed to either lineage (Figure 103). This is of particular importance as production of umbilical cord MSCs is highly reproducible and less cumbersome than isolating MSC from UCB. 5-10mm^3 of the UC can produce as much as 1 billion MSCs in 30 days and three passages. Since the average UC measures 50cm, this MSC source is likely to become increasingly relevant for cell therapy research and applications (Forraz 2010).

Both differentiated cell lineages in addition to illustrating morphological and immunophenotypical characteristics of liver progenitor cells, also exhibited signs of functional activity. The cells illustrated active carbohydrate metabolism (positive Periodic acid Schiff staining); the ability to take up and excrete Indocyanine green dye and cytochrome P450 system activity.

As a control for our *in vitro* differentiated cells the following cell sources were used: a well known human hepatocellular carcinoma cell line (HepG2); human foetal liver tissue (gestational ages 7, 9 and 12 weeks) and human adult liver tissue. The immunocytological assays performed on the umbilical cord and UCB- derived cells were repeated on these cell lines. Estimation of an exact ratio of marker expression in HepG2 cell line proved difficult due to the nature of the growth of the cell line in piles and mounds instead of monolayer. However, it could still be appreciated that in this well differentiated liver cell line the degree of expression of liver markers was not 100%. The majority of the cells displayed a phenotype comparable to bipotent hepatoblast. Thus, the efficiency of differentiation in our

primary cultures was comparable to the data generated from this liver carcinoma cell line. However, lack of a mature hepatocyte phenotype in this cell line meant that it was not an ideal representative control. This information is of prime importance to drug companies and biochemical industries where these cells are frequently resorted to as *in vitro* hepatocyte models for toxicology studies and drug testing.

Immunocytochemistry data generated from human foetal liver tissue sections (9weeks gestation) demonstrated that the phenotype of foetal liver hepatic progenitor cells were comparable to the phenotype of our *ex vivo* differentiated cells. Again, due to the lack of mature hepatocyte phenotype, foetal liver tissues at different developmental stages would serve as a good model to check the phases and extent of *in vitro* stem/progenitor cell differentiation but do not represent an ideal mature hepatocyte control.

The ideal control for this liver differentiation study was human adult liver tissue sections. Mature hepatocytes represent the perfect morphological, phenotypical and functional positive control for *ex vivo* hepatic differentiation. However, resource for this tissue is limited to cadaveric supply or from post hepatotectomy specimens, restricting its accessibility.

mRNA samples from the three control specimens were used as controls for molecular analysis of our differentiated cells. This supplemented qualitative protein analysis (immunocytochemistry/immunohistochemistry and western blots) with quantitative gene level data.

We were unable to attain primary cultures of human foetal liver cells and human adult hepatocytes, thus primary HepG2 cell cultures were used as the sole control for functional assays performed. Our UCB and umbilical cord-derived hepatocyte differentiated cells displayed a degree of metabolic and functional activity that was comparable to HepG2 cells. It may be concluded that the three step protocol utilized in this study permitted the umbilical cord and UCB-derived pluripotent/multipotent stem cells to commit to meso-endodermal germ layer lineage early in the course of *ex vivo* culture thus ensuring a higher efficiency of *in vitro* differentiation of these stem cells into liver cell populations. We successfully generated bipotent liver progenitor cells similar to *in vivo* hepatoblasts that expressed both hepatic and biliary markers.

Our staged differentiation protocol was devised in 2D cell culture experiments. This provided essential phenotypic, protein and molecular level information throughout the protocol. However, the 2D monolayer configuration of the cells in culture did little justice in mimicking the physiological 3D environment of cells *in vivo*. Thus, we ventured to investigate the use of scaffolds and rotatory cell culture systems to provide a 3D *in vitro*

culture environment more closely resembling the natural cell environment in the human body. Here we report that the 3D culture system enhanced *ex vivo* cell differentiation through improved cell-cell and cell-extracellular matrix interactions. Clinically, these 3D engineered tissue constructs may allow direct delivery of cells to the site of tissue damage/loss and eventually degrade leaving behind the bioengineered tissue. This route of administration may prove more beneficial and effective than intravenous transfusion of stem cells and may reduce the dose of immunosuppressants required to dampened graft rejection.

The scarcity of donor livers and other organs is the driving force behind the emerging field of tissue engineering and regenerative medicine. Efforts to build tissues from the ground up have proven very challenging and have yet to be translated into transplantable replacement organs. The liver, in particular, poses a great challenge for this task. Each hepatocyte functions as a metabolic factory demanding constant, direct contact with the vascular system. One of the limitations of artificial *in vitro* tissue engineering so far has been the lack of an established vascular system associated with the cultured cells. Attempts at co-culturing with endothelial cells have been made but this still did not provide cells with stable vascular supply (Talamini, McCluskey et al. 1998). Powerful developments in the multidisciplinary field of tissue engineering have yielded a novel set of tissue replacement parts and implementation strategies A Latest advancement in the field of tranplantational medicine has developed a technique that may someday translate into growth of transplantable replacement organs. In 2008, Ott *et al* performed decellularization of rat hearts leaving behind an intact vascular system that facilitated repopulation of the structural matrix and subsequent survival and function of introduced cardiac and endothelial cells (Ott, Matthiesen et al. 2008). This technique was refined in 2010 and applied to rat liver (Uygun, Soto-Gutierrez et al.). This work is a break through in the field regenerative liver therapy. Decellularized liver matrix may provide a biocompatible, natural scaffold on which stem/progenitor cells can be reseeded. Having the detailed microvasculature of the liver is a major advantage to growing liver tissue in a synthetic environment. These results provide a proof of principle for the generation of a transplantable liver graft as a potential treatment for liver disease. It must, however, be emphasized that although there are very promising advancements in the field of liver regeneration, we are still a long way from translating research into clinical application.

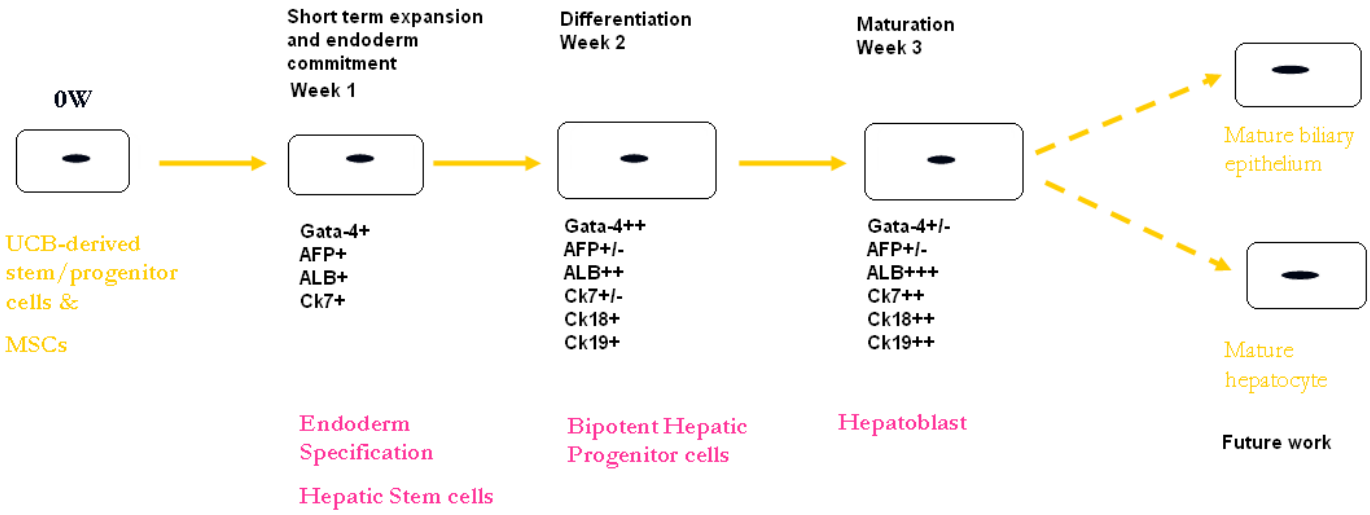
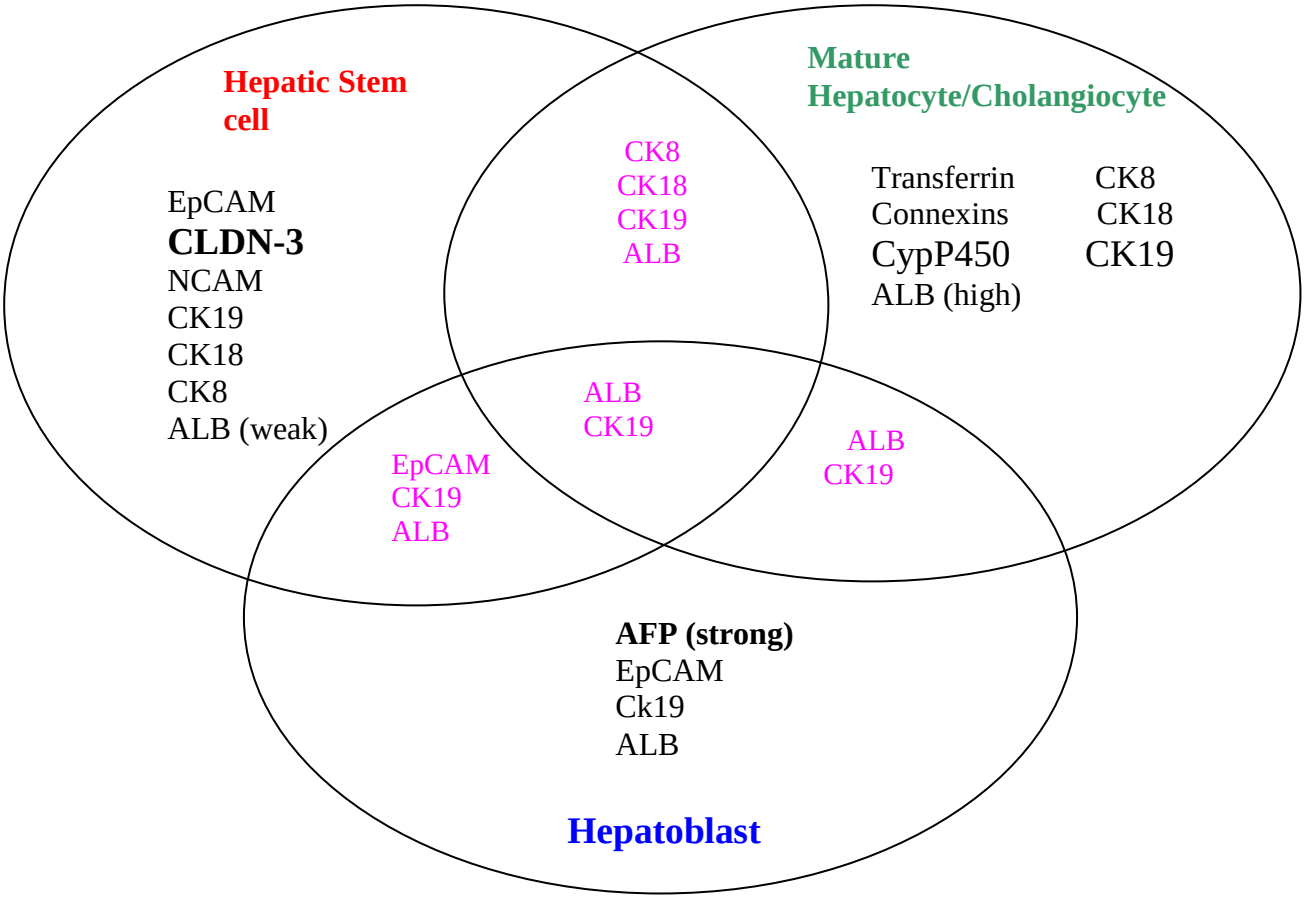


Figure 102. Three stage serum tapering *in vitro* differentiation system and resulting cellular phenotype at each stage.



(Schmelzer, Zhang et al. 2007)

Figure103. A schematic diagram illustrating phenotypic overlap between different liver cells. *In vitro* characterization of our differentiated cells displayed a cellular phenotype expanding over a society of liver cells with features in common with: hepatic stem cells, hepatoblasts, hepatocytes and cholangiocytes. Thus, it may be safely concluded that we succeeded in producing a population of bi-potent progenitor cells displaying both hepatocyte and biliary epithelium features but not fully committed to either lineage.

6 Summary and Future work

6.1 The following conclusions were deduced from this study:

Cord blood processing:

- PrepaCyte-CB is an appropriate choice for cord blood processing in hospitals and transplantation centers where UCB units are demanded for immediate transfusion/transplantation.

- The fully automated sterile system provided by Sepax is an ideal method for processing of cord blood units aimed for cryopreservation and long term storage.

Isolation and characterization of stem/progenitor cells from cord blood

- We were ably to successfully isolate and characterize a population of primitive cells within the mononucleated cell fraction of cord blood that expressed early haematopoietic stem cell markers as well as some embryonic stem cell markers.

- We were also able to identify a non-haematopoietic stem cell population within cord blood.

Expansion and Commitment of stem/progenitor cells

- We devised a short term protocol that achieved a very modest increase in stem/progenitor cell number but allowed simultaneous commitment of cells to meso-endoderm lineage.

Isolation and characterization of umbilical cord-derived MSCs

- We managed to successfully isolate MSCs from umbilical cord matrix (Wharton's jelly) with 100% efficiency. These cells expressed the conventional MSCs markers as well as some embryonic stem cell markers. MSCs demonstrated very efficient *in vitro* proliferation and propagation. MSCs demonstrated commitment to hepatic lineage upon isolation

***Ex vivo* differentiation of UCB-derived stem/progenitor cells and umbilical cord MSCs along hepatobiliary lineage**

- At the end of a three-step differentiation protocol we reported a heterogeneous population of differentiated cells that resembled natural human hepatobiliary cells in phenotype, protein level as well as genetic profile. These differentiated cells spanned the

spectrum of hepatobiliary lineage: hepatic stem cells, hepatoblasts, mature hepatocytes and cholangiocytes. The majority of the cells, however, resembled bipotent hepatoblast, expressing both hepatocyte and cholangiocyte markers. These cells also demonstrated signs of functionality and metabolic activity.

Progression from 2D to 3D culture system

- 3D culture environment enhanced and promoted liver differentiation *ex vivo*.

6.2 Futher developments

The results generated in this study are very promising for possible future application as translational cell therapy of end-stage liver disease. Upon delivery of these differentiated hepatic progenitor cells/hepatoblasts to the site of damage, the local microenvironment might determine the ultimate fate of the progenitor cells. It may be argued, however, that the heterogeneous nature of the *ex vivo* differentiated cell population may prove to be a matter of concern in terms of graft rejection ad possible malignant transformation. Thus, the need for refinement of the differentiation protocol to produce a homogenous population of either hepatocytes or cholangiocytes is demanded. Additionally, a homogenous population of hepatocytes will provide an ideal platform for *ex vivo* toxicology studies as well as in bio-artificial liver-assist devices.

Incorporation of vital liver supporting cells (stellate cells), sinusoidal cells and endothelial cells in a co-culture system may provide a more physiological environment for liver cell development and hence enhance further maturation of hepatic progenitor cells.

The ultimate aim for transplantation medicine would be to produce bioengineered liver tissue constructs that represent normal liver infrastructure. Although this may appear as a mammoth task at present, intricate advancements achieved by groups like (Uygun, Soto-Gutierrez et al.; Ott, Matthiesen et al. 2008) may lessen the complexity of future studies.

In vitro developed bioengineered liver tissue constructs will need to be tested in animal models of liver disease with detailed critical evaluation of subsequent results to determine their complete potential. Only then should such research be translated to clinical trials in humans.

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Appendix A- Patient information sheet and consent forms

Umbilical cord and cord blood units were collected from elective caesarean section deliveries at the maternity unit in the Royal Victoria Infirmary, Newcastle upon Tyne. Fully explained informed consent was obtained from expecting parents prior to delivery. All the ethical requirements required by hospital, university and National Health Service have been fulfilled.

Patient information sheet

Newcastle University
North East England Stem Cell Institute

Newcastle Centre for Cord Blood
Institute of Human Genetics
International Centre for Life
Central Parkway
NE1 3BZ, United Kingdom
Phone : 0191 241 8817
e-mail: cord.blood@newcastle.ac.uk

International Centre for Life

Participant Information Sheet

Evaluation of Cord Blood and Related Tissue Stem Cells for processing, preservation and tissue engineering.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Ask us if there is anything that is not clear or if you would like more information. Please take time to decide whether or not you wish to take part.

PART 1

What is the purpose of the study?

The purpose of the study is to learn more about the best ways to collect, process, and store cord blood and related tissue for transplantation and research.

This study will evaluate and develop products for the optimal processing of cord blood samples in order to improve the ways cord blood is separated into different blood cell groups and then stored in a freezer.

We will further investigate the potential of cord blood and related tissue stem cells for 'tissue engineering' whereby stem cells are grown in our laboratory and instructed by chemicals and materials to produce defined tissue structures such as blood or liver.

Basic information about Cord Blood and related tissues:

- Umbilical cord blood is the blood present in the umbilical cord which connects the baby to the placenta.
- Several research studies, including at the University of Newcastle, have shown that cord blood contains stem cells - the origin of all tissues and organs in the body – that can be turned into specific tissues in a laboratory offering high prospect for the advancement of medicine.
- Other related tissues including the cord, placenta and membranes also provide a source of stem cells
- Cord blood has been used clinically for over 20 years for the treatment of certain blood disorders (including leukaemia, lymphoma, and sickle cell disease) and immune deficiencies.

By donating your umbilical cord blood and related tissues to our research programme you will contribute the development of technologies and scientific discovery to better understand how umbilical cord blood and related tissue stem cells can help repair the human body.

Why have I been chosen?

You have been chosen because you are in the second or third trimester of your pregnancy. You may therefore wish to participate in this study by donating your cord blood and related tissues at the time of birth.

Do I have to take part?

No. It is up to you to decide whether or not to take part. **Cord blood and related tissue donation for this research is entirely voluntary**. If you decide to take part, you will be given this information sheet to keep and be asked to sign a consent form. You will be free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you will receive or the delivery of your baby.

What will happen to me if I take part?

- If you decide to take part in this study, you will be asked:

  -to read this patient information sheet carefully and ask us any question you might have with regard to this study.

  -to sign a consent form for cord blood and related tissue donation.

- Cord blood collection will occur **after birth with no harm to your baby or yourself**. The umbilical cord will be clamped when it is of no further use to your baby. Any samples will be taken after delivery of the placenta and cord and in a separate room by a member of staff. Blood will be drawn from the umbilical cord and placental vein. Samples of related tissues (such as cord, placenta and membranes) may sometimes be taken at this time.

- Donated cord blood and related tissue will be anonymised before it is used for the research study at the University of Newcastle. Laboratory researchers will not have access to your name, clinical records or any confidential information about your baby or yourself.

- As part of your routine clinical care you would have been or will be tested for sexually transmitted diseases. You may donate cord blood and related tissue only if you have been tested for sexually transmitted diseases. If any evidence of infection is found, you will receive advice and support from the NHS carers. Please ask a member of staff for more information and advice if you have any question on this matter.

- There is no cost to you for participating in this study. You will not receive payment for your participation in this study.

What do I have to do?

If you decide to take part in this research study by donating your cord blood and related tissue please:

- Read this Participant Information Form carefully.
- Complete and sign the consent form and return to:

Dr Majlinda Lako/Dr Lyle Armstrong, Newcastle Centre for Cord Blood, Institute of Human Genetics, International Centre for Life, Central Parkway, Newcastle Upon Tyne, NE1 3BZ

Phone: 0191 241 8817

Or to the Research Nurse during your next clinic visit..

What is the device / procedure that is being tested?

- In our laboratory at the University of Newcastle, we will investigate different products and methods to collect, to process and to store cord blood and related tissue for research and clinical applications.
- We will further develop techniques and methods to grow and transform cord blood and related tissue stem cells into different tissues including blood, brain and liver tissue.

What are the alternatives?

- The placenta, umbilical cord, and cord blood will be discarded as per hospital policy.
- You may also choose to arrange prior to your admission to the hospital, with a private company who will charge a fee to process and store your baby's cord blood. Please note that this is not currently facilitated by the NHS.

What are the side effects of any treatment received when taking part?

- No known side effects or discomforts are anticipated when taking part in this study.
- Cord blood and related tissues will be collected after birth with no harm to you or the baby. Your care and the delivery of your baby will not be altered if you decide to take part, or not, in this research study.

What are the other possible disadvantages and risks of taking part?

- You and your family will not be able to retrieve or access the cord blood, related tissue or derived-cells for personal or clinical use at a later stage.

What are the possible benefits of taking part?

- There are no direct benefits to you or your baby other than that the knowledge gained from this study may assist researchers in the discovery of improved methods for the collection, processing, and storage of cord blood and related tissue for transplant and research.
- The study may also lead to new ways of using umbilical cord blood and related tissue stem cells to detect or treat a wide variety of diseases in the future.

What happens when the research study stops?

- Donated cord blood and related tissue not used will be discarded at the end of the study.
- The information from this study will be collated with a view to understanding Cord

Blood and related tissue better.

What if there is a problem?

- Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?

- Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2. However, if you wish to discuss your participation in the study with others you are free to do so.

Contact Details:

For further information please contact, Dr Majlinda Lako or Dr Lyle Armstrong

Newcastle Centre for Cord Blood, Institute for Human Genetics, International Centre for Life, Central Parkway, Newcastle Upon Tyne, Ne1 3BZ

Phone: 0191 241 8817 E-mail: cord.blood@newcastle.ac.uk

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

PART 2

What if relevant new information becomes available?

If new information about this study becomes available prior to your consent or donation, you will be approached by a member of staff with an up-to-date participant information sheet.

What will happen if I don't want to carry on with the study?

If you withdraw from the study we will not collect your cord blood or any other related tissues. You may then withdraw from the study until your sample has been anonymised. We will then dispose of your identifiable sample as per hospital policy. You will not have to give any reason for withdrawing from the study and it will in no manner affect your care or the delivery of your baby.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with Dr Majlinda Lako or Dr Lyle Armstrong.

If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

NHS Indemnity does not offer no-fault compensation i.e. for non-negligent harm, and NHS bodies are unable to agree in advance to pay compensation for non-negligent harm. They are able to consider an ex-gratia payment in the case of a claim where the injury probably resulted from any test or procedure you received directly as part of this study. Any payment would be without legal commitment. (Please ask if you wish more information on this).

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against the Newcastle Hospitals NHS Trust. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

Will my taking part in this study be kept confidential?

Cord blood and related tissue will be anonymised before being used by laboratory research staff that will not have access to your name, your baby's name or your medical records.

Anonymised data regarding your delivery date, number of weeks in your pregnancy, the sex of your child, your age at delivery may be stored and used for statistical comparison only.

Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

Authorised University researchers, sponsors and regulatory authorities may have access to these data as granted by the Chief Investigator, Dr. Lako. Data will be stored for a maximum of 10 years on a university PC and secured server.

However, any information which is collected about you or your baby during the course of the research will be kept strictly confidential and be anonymised so that you and your baby cannot be recognised from it.

Handling, processing, storage and destruction of your data are compliant with the Data Protection Act 1998.

What will happen to any samples I give?

Cord blood will be drawn from the umbilical cord vein after your baby is born with no harm to you or your baby. Samples of placenta, cord or membranes may also be taken. Remaining tissues will be disposed of as per usual hospital policy. Your sample will be anonymised by a trained member of staff. Your cord blood and related tissue sample will then be dispatched to the Newcastle Centre for Cord Blood research staff.

Your donated cord blood and related tissue samples will be considered as a 'Gift' to the University of Newcastle in accordance with the Medical Research Council proposition.

Donated Cord Blood and related tissue will be used to evaluate techniques and commercial products to collect, process, and store cord blood for transplantation and research.

Cord blood and related tissue cells may also be tested for 'tissue engineering' (that is exposing cord blood cells to a range of chemicals and materials to direct the way they grow) applications and product development growing cells into specific tissue types for instance blood, liver or neural tissues.

Will any genetic tests be done?

Anonymised donated cord blood and related tissue cells may be tested for gene expression levels as required by the study, in order to monitor cells response to different growth conditions.

What will happen to the results of the research study?

The results and cells derived from the study may be used for academic scientific publications, commercial product development or validation, clinical application development and / or regulatory authorities' evaluation.

Results and progress of the study will be communicated through peer-reviewed scientific journals, international and regional conferences and also be disseminated to the general public with local seminars, media and the research group website (www.ncl.ac.uk/cordblood or www.iscbrm.org). Because your cord blood and related tissue sample will be anonymised, you will not be identified in any report, publication or communication.

Who is organising and funding the research?

Our current research is organised by the University of Newcastle and funded by the University of Newcastle, OneNorth East, BioE Inc, StemCyte Inc and the Fondation Jerome Lejeune. This list may change during the study.

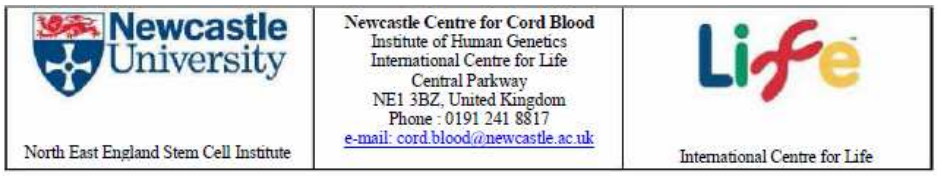
Who has reviewed the study?

This study was given a favourable ethical opinion for conduct in the NHS by the Newcastle and North Tyneside Local Research Ethics Committee.

You will be given a copy of the information sheet and a signed consent form for you to keep.

We thank you for considering taking part or taking time to read this sheet

Consent form



Consent Form

Patient Identification Number for this trial:

Title of Project: Evaluation of Cord Blood and Related Tissue Stem Cells for processing, preservation and tissue engineering.

Name of Researcher:

1. I confirm that I have read and understand the information sheet dated 16/01/2009 (version 9) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from the University of Newcastle, from regulatory authorities or from the NHS trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐
4. I agree to take part in this study. ☐

Name of Participant	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

When completed and signed please retain one copy (1 for participant) and return the other copy (1 for researcher file) to: Dr. Majlinda Lako/Dr Lyle Armstrong Newcastle Cord Blood Centre, Institute of Human Genetics, International Centre for Life, Central Parkway, NE1 3BZ, Newcastle Upon Tyne
Phone: 0191 241 8817 Email: cord.blood@newcastle.ac.uk

Or return the signed consent form to the Research Nurse at your next clinic visit.

Newcastle Centre for Cord Blood
Researching Adult Stem Cells for Human Therapy

REC Ref 06/Q0906/51 Consent version 8 16/01/2009

Appendix B- Details of Flowcytometric analysis

Flow cytometry is a semi-automated system that allows for the investigation of single cells in a continuous fluid stream, enabling simultaneous measurements of multiple extra- and intra-cellular characteristics.

The objective of flow cytometry is very simple: measurement by quantification of photon release, components of the membrane, cytoplasm and nucleus of a particular cell or group of cells. Cell surface and cytoplasmic measurements are usually cellular antigen expression, and the most commonly used probes are monoclonal antibodies directed to specific antigens on or in the cell. Dyes may be used that are specific for DNA, such as Hoechst 33342; for RNA, such as Pyronin-y; or other intracellular constituents, such as Indo-1 for calcium.

Multiple antibodies can be used simultaneously within the same sample, provided each antibody possesses a spectrally different attached fluorescent reporter molecule. The resultant data will give an antigenic profile of each cell, predetermined by the specificity of the antibodies used. For the dyes, these are fluorochromes in their own right and will bind to the cell constituents specific to each.

Principles of the flow cytometer – Becton Dickinson LSRII

A. Flow cell

The flow cell is where the cellular investigation takes place. Introduction of a single cell suspension in combination with hydrodynamic focusing and laminar flow ensures optimal excitation of fluorochromes allowing optimal scatter characteristics from cells to be acquired. As the cells are introduced into the flow cell they should pass the centre of the interrogation point of the laser beam. Once the sample is analysed they go to waste, along with the fluid sheath.

B. Laser investigation at the flow cell

A 488nm laser beam focuses on the flow cell inducing emission of scattered and/or fluorescent lights. The intensity of the light scattered in the forward direction (forward scatter light- FSC) is proportional to the square radius of the analyzed sphere hence representing the cell size. The 90° side scatter (SSC) is refracted in proportion of the nuclear and granular cell content giving indications of the internal cellular complexity and surface topography. In addition to providing essential information about physical characteristics of cells, flowcytometry also allows measure of fluorescent probes. Currently a single instrument is capable of detecting 18 fluorescent probes, allowing a large amount of data to

be generated from relatively small number of cells. The excitation and/or emission spectra of chosen fluorochromes have to be distinct in order for them to be used simultaneously. Insertion of multiple lasers into flow cytometers enables more excitation lines and hence widens the choice of fluorochrome.

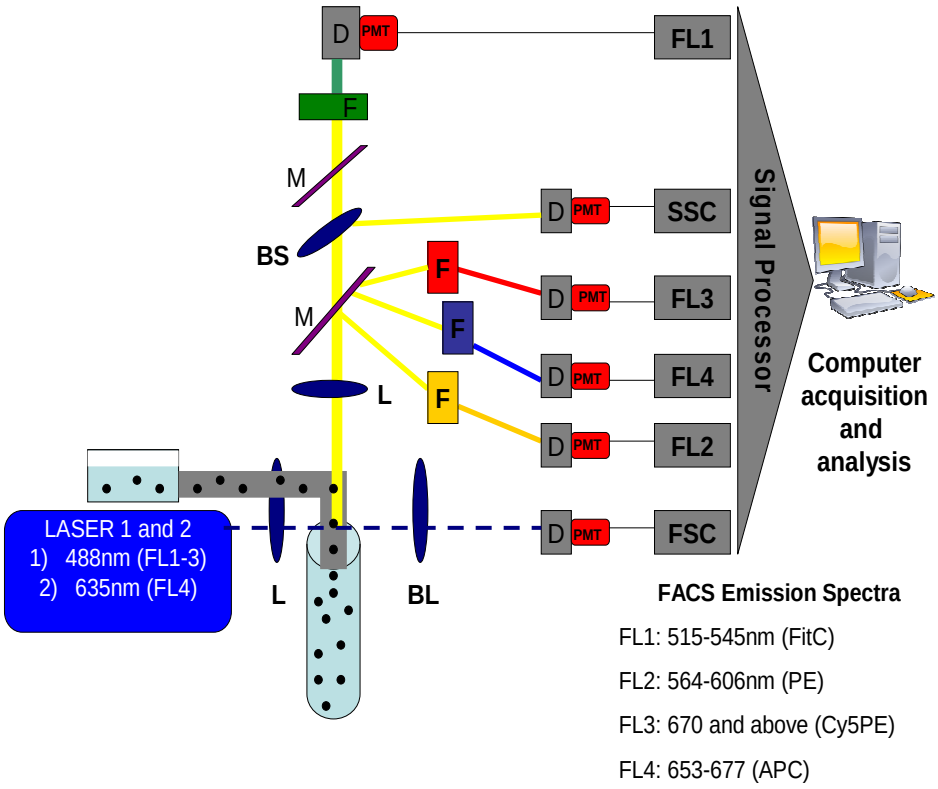


Figure 1. Principles of Flow Cytometry. Cells are aspirated into the flow cytometer and transported in a flow of sheath fluid to the flow cell chamber. The argon-ion laser produces a 488nm monochromatic light beam which is condensed by a lens (L) focussing the beam through the cells. The primary light beam passes through the cell and is blocked by a specific lens (BL) causing forward scatter light (FSC) which is collected by the detector (D). The 488nm side scatter (SSC) as well as fluorescence lights are diffracted at a 90º angle and further focussed through a lens. A series of relevant beam splitters (BS), dichoric mirrors (M) and filters ((F) enable specific collection of SSC as well as green, orange and red fluorescence lights. FSC, SSC and fluorescence lights falling upon their respective detectors are converted in electrical signals themselves amplified by a set of photomultiplier tubes (PMT) and received in five channels (FSC, SSC, FL1, FL2, and FL3). Another laser producing a 635nm beam is also used in the same manner and is in the end received by FL4 channel. These signals are all processed by an analogue-to-digital converter, which allows acquisition and analysis of the quantity and intensity of these signals on an attached computer. The signals are then represented on visual display unit as frequency of distribution.

Various fluorochromes used allow data to be generated provided that the appropriate fluorescent detector (laser) is available. The fluorochromes absorb light from an excitation source, which is the laser beam. This allows the electrons of the flurochrome to enter the next electron orbit which is an unstable state. For the excited molecule to reach a stable conformation, the absorbed energy is dispelled by heat and vibration, resulting in the emission of a longer wavelength of photons. This is the emission wavelength for the molecule. A point of note is that the maximum excitation of laser required need not be exact, for example, 488nm. The excitation of each fluorochrome is in the form of a spectrum, thereby enabling lasers of specific wavelengths to excite areas of the excitation spectrum away from the peak, although with sub-optimal results. Also, independent of excitation wavelength, the emission spectra of each fluorochrome will remain the same.

It should also be noted that different fluorochromes have different 'brightness', determined by the extinction coefficient and quantum fluorescence yield of each molecule. In principle, the weaker the antigen expression, the fluorochrome applied should be brighter.

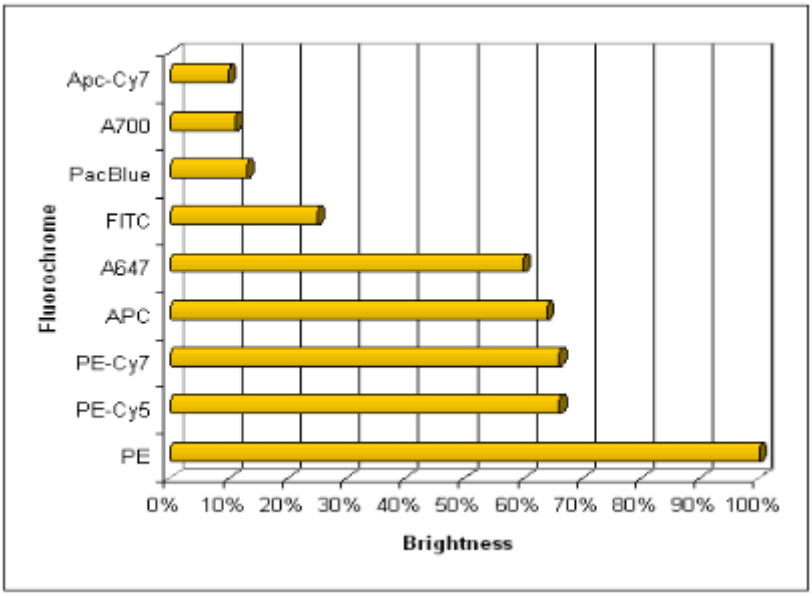


Figure 2. Grading of brightness of the fluorochromes utilized during the course of this research. Becton Dickinson LSRII.

For any given fluorochrome, the intensity of staining is directly proportional to the number of antigenic sites available. Any non-specific binding, which is at a much lower intensity than the specific binding, is due to complement receptors binding and cross-reactivity of the antibody with other antigens.

C. Accounting for non-specific staining

Detection of negative and positive populations can be done either by the use of an isotype control or the use of totally unstained cells.

In isotype controls, cells are incubated with the same protein concentration, fluorochrome and immunoglobulin subtype as the specific test antibody. The drawback, however, is that occasionally isotype controls can have very different staining characteristics to the specific antibody in terms of non-specific binding. Thus, it may be better to use unstained cells or the negative population of the positively-stained sample, to adjust the settings of the photomultiplier tube (PMT).

D. Spectral compensation

In the event of using more than one fluorochrome, any spectral overlap between the fluorochromes has to be removed. This ensures that each fluorochrome signal represents only the antigens marked by that particular fluorochrome. It is also important to measure accurately those cells that express more than one fluorochrome, by virtue of more than one antigen being expressed on the cell surface.

Appendix C- PCR gel electrophoresis

To ensure the specificity of the primers used for real-time PCR analysis, end-point PCR gel electrophoresis was performed to ensure that the sizes of the products picked up by the primers were accurate. A positive control (Adult liver cDNA) was used for this analysis. Product bands are illustrated in the figure below.

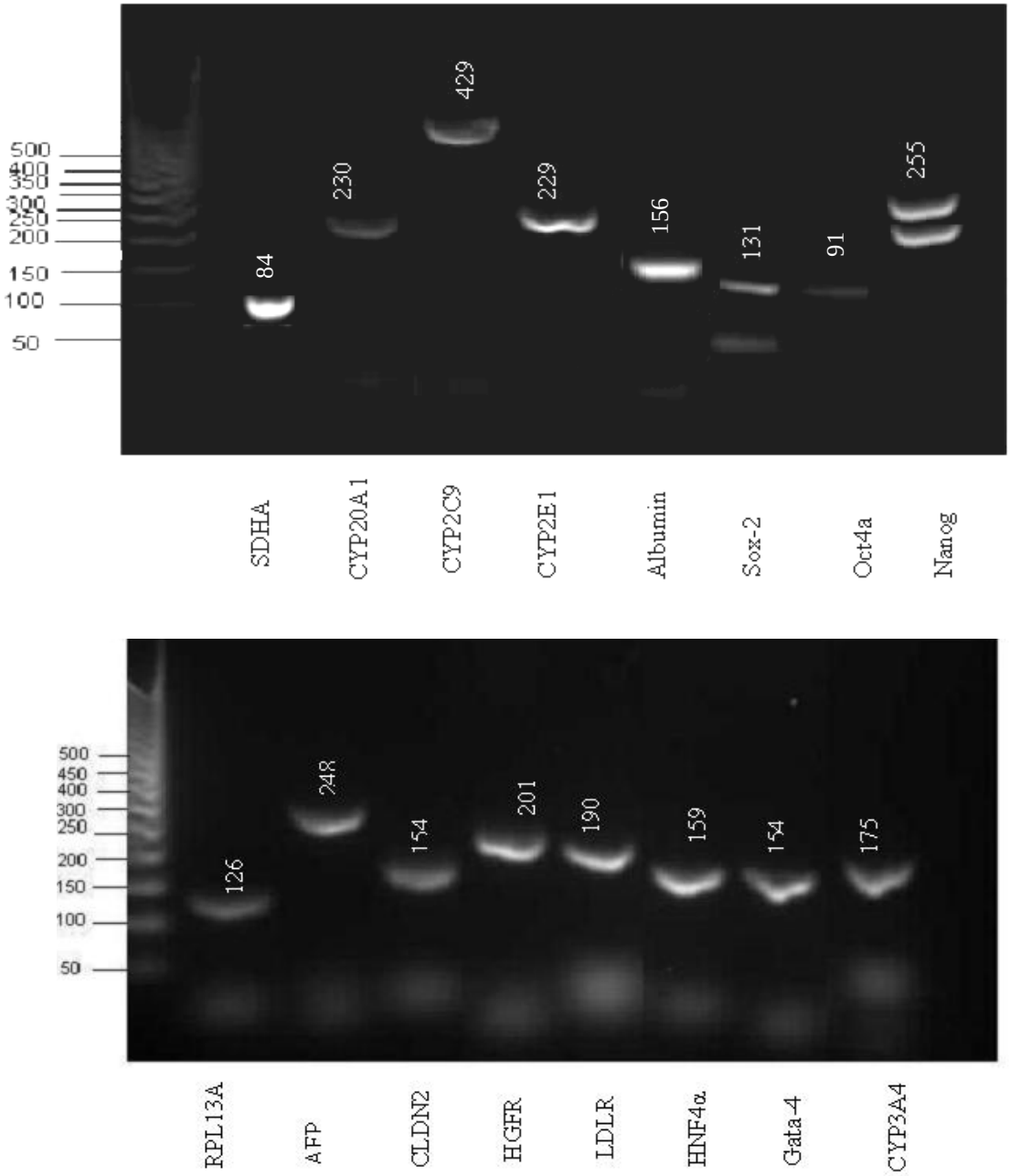


Figure 3. PCR gel electrophoresis illustrting product sizes for the primers examined. Furthest Left hand demonstrates the 50bp ladder applied. 1.5% agarose gel was used. Primers were tested on poitive control (Adult liver cDNA from Primer design). SDHA and RPL13A are house keeping genes.