Three dimensional platforms for ex vivo hematopoietic cell bioprocesses

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**Three Dimensional Platforms for *ex vivo*
Hematopoietic Cell Bioprocesses**

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SUMMARY

Umbilical cord blood (UCB) has been shown as a convenient source of hematopoietic stem cells (HSCs) for autologous or allogeneic HSC transplantation in the treatment of leukemia and many other hematological diseases. However, widespread application of cord blood is hampered by the low stem cell number in a single cord unit. One possible approach to solve this problem is *ex vivo* expansion. Three dimensional (3D) culture systems and bioreactors are developed to recapitulate the bone marrow microenvironment *in vitro* to enhance cell-cell and cell-niche interactions and mimic the biologic and mechanical function of bone marrow niche. Two different potential 3D platforms for culturing HSCs and abnormal hematopoietic cells are presented in this thesis; electrospun nanofiber scaffold (NFS) and hollow fiber bioreactor (HFBR).

Electrospun NFS created a nano-scale 3D architecture mimicking the native bone marrow structure *in vitro*. It effectively enhanced the adherence of both abnormal leukemia cells and normal HSCs. Yet NFS only proved to enhance growth of normal hematopoietic cells but inhibit leukemia cell proliferation. Nevertheless, cells cultured on NFS showed enhanced cell-matrix interaction, which demonstrate that NFS provides a better mimicry of bone marrow microstructure. Electrospun NFS is therefore useful as a matrix coating for enhancing HSC expansion and its potential application for leukemic cells can be further explored.

The HFBR showed excellent feasibility as a bone marrow mimetic *in vitro* system for hematopoiesis. Larger number of stromal cells cultured inside the HFBR formed a 3D and bone marrow mimetic architecture for hematopoietic cell culture. Successful and extensive expansion of both HSCs and leukemic cells are achieved in hollow fiber bioreactor while preserving the differentiation ability of HSCs. Interaction between hematopoietic cells and stromal cells is also significantly enhanced in the HFBR. *Ex vivo* expanded cord blood HSCs in the HFBR successfully engrafted into mice models, demonstrating its potential clinical utility in the future.

This thesis successfully demonstrates that NFS and HFBR can recreate a closer mimicry of the bone marrow microenvironment, which provide significant improvements for *in vitro* studies of leukemic and hematopoietic cell functions. The NFS and HFBR can provide different sets of tools to study insights of hematopoiesis *in vitro*.

PUBLICATIONS

JOURNAL ARTICLES

X Cao, K Kwek, J Chan, C K.H.C., M Lim. Electrospun nanofibers as a bioadhesive platform for capturing adherent leukemia cells. *Journal of Biomedical Materials Research: Part A*. 2014. 102: p. 523-531.

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Usuludin, S.B.M., X Cao, M Lim. Co-culture of stromal and erythroleukemia cells in a perfused hollow fiber bioreactor system as an *in vitro* bone marrow model for myeloid leukemia. *Biotechnology and Bioengineering*. 2012. 109(5): p. 1248-1258.

N Menon, X Cao, YJ Chuah, CK Ng, P Xue, B Cao, M Lim, Y Kang. Cell patterning near micro-grills using selective plasma treatment and a fake channel. Lab on chip. (Submitted)

P Kiruthika, X Cao, R Dalan, M Lim. A flow cytometric approach to evaluate endothelial function in patients with type 2 diabetes mellitus. (Manuscript in preparation)

CONFERENCE PRESENTATIONS

X Cao. (2011) *Ex vivo* expansion of Hematopoietic Stem Cells with bone marrow stromal cells in Hollow Fiber Bioreactor as an *In Vitro* Bone Marrow Model.

Poster presentation at stem cell society Singapore symposium 2011, Singapore.

X Cao. (2012) The hollow fiber bioreactor as an *ex vivo* expansion platform of stromal-supported and serum-free cord blood stem cell culture. *Poster presentation at 6th BES Scientific Meeting 2012, Singapore.*

X Cao. (2013) Utility of hollow fiber membrane bioreactors for an *ex vivo* bone marrow system. *Oral presentation at 6th annual biopharma asia convention 2013, Singapore.*

X Cao. (2013) Co-cultures in the Hollow Fiber Bioreactor: A Good Platform for *ex vivo* Expansion of Hematopoietic Stem Cells? *Oral presentation at TERMIS-EU, Istanbul, Turkey.*

ABBREVIATIONS

2D	Two-dimensional
3D	Three- dimensional
AGM	Aorta-gonad-mesonephros
ATCC	American type cell culture
A*STAR	Agency for Science, Technology and Research
BFU-E	Erythrocyte burst-forming units
BM	Bone marrow
BSA	Bovine serum albumin
CB	Cord blood
CFU	Colony-forming units
CFU-E	Erythrocyte colony-forming units
CFU-GEMM	Granulocyte/erythrocyte/macrophage/megakaryocyte colony- forming units
CFU-GM	Granulocyte/macrophage colony-forming units
CLPs	Common lymphocyte progenitors
CML	Chronic myeloid leukemia
CMPs	Common myelocyte progenitors
DMEM	Dulbecco's modified eagle's medium
ECL	Enhanced chemiluminescence
ECM	Extra cellular matrix

ECS	Extra-capillary space
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
FL	Flt-3 ligand
FT-IR	Fourier transform infrared spectroscopy
G-CSG	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMPs	Granulocyte-myelomonocytic progenitors
GVHD	Graft <i>versus</i> host disease
HFBR	Hollow fiber bioreactor
HFP	1,1,1,3,3,3,-hexafluoro-2-propanol
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSCs	Hematopoietic stem cells
IMDM	Iscoe's modified Dulbecco's medium
IL	Interleukin
Lin ⁻	Lineage depleted hematopoietic progenitor cells
LSCs	Leukemic stem cells
LSK	Lin ⁻ Sca-1 ⁺ c-kit ⁺
LT-HSCs	Long term- Hematopoietic stem cells
MEPs	Megakaryocytic erythroid progenitors

MNC	Mononuclear
MPPs	Multipotent progenitor cells
MSCs	Mesenchymal stem cells
MWCO	Molecular weight cut off
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide ademine dinucleotide
NFS	Nanofiber scaffold
NSG	NOD/SCID IL-2R $\gamma^{-/-}$
PBS	Phosphate-buffered saline
PCL	Poly poly (W-caprolactone)
PE	Phycoerythrin
PET	Polyethylene teraphthalate
PerCP	Peridinin-chlorophyll-protein
PGA	Poly (glycolic acid)
PI	Propidium iodide
PLA	Poly (lactic acid)
PLA-CL	Poly (lactic-co-caprolactone)
PLGA	Poly (lactic-co-glycolic acid)
SCF	Stem cell factor
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
ST-HSCs	Short term- Hematopoietic stem cells

TBS	Tris-buffered saline
TCP	Tissue culture polystyrene
TCPB	Tantalum-coated porous biomaterial
TNC	Total nucleated cells
TPO	Thrombopoietin
UCB	Umbilical cord blood
VCAM-1	Vascular cell adhesion molecule-1
VEGFR-2	Vascular endothelial growth factor receptor-2

CHAPTER 1

Introduction

The hematopoietic system controls all blood cell formation through our whole life and sustains the production of a vast number of blood cells every day. Hematopoiesis is initiated at the level of HSCs, which are regulated by the interplay of cytokines and external signals. The generation of mature blood cells from HSCs involves a highly regulated progression through successive stages of commitment to a specific cell lineage, terminal differentiation of lineage-restricted progenitor cells. Due to the self-renewal and differentiation ability of HSCs, they become an attractive cell source for many clinical applications, and in some cases, offer the only cure for certain diseases. Autologous or allogeneic HSC transplantation has been successfully implemented in the treatment of hematological diseases, neoplasia, primary immunodeficiency diseases, and metabolic disorders of children and adult patients. Historically, the bone marrow (BM) has been taken as the main source of HSCs in pediatric and adult individuals.

However, in many cases, a suitable donor is hard to find. This difficulty has led to a search for alternative sources of HSCs for use in human transplantation. Umbilical cord blood and peripheral blood have been shown to be good alternative sources of HSCs. Since UCB is not only easy to collect, but also has higher tolerance in mismatch of human leukocyte antigen (HLA); and there is a decreased incidence of graft *versus* host disease (GVHD) compared with that after bone marrow transplantation, UCB becomes an attractive potential therapeutic resource. Unfortunately, the recipient of UCB transplantation is restricted to pediatric patients because of limited number of HSCs in a single cord blood unit.

Over decades, substantial progress has been made towards the goal of *ex vivo* expansion of HSCs. Many *ex vivo* culture systems have been developed, such as cytokine supplied culture systems and stromal cell dependent culture systems. However cytokines supplied culture systems rely on cytokines addition which are unable to sustain the stemness of HSCs and alter cellular function of HSCs; and stromal cell dependent culture systems only provide a two dimensional (2D) monolayer culture, which cannot fully represent the native structure of bone marrow. Due to limitations faced in 2D culture systems, many 3D culture systems and bioreactors have been developed to recapitulate the bone marrow HSCs microenvironment *in vitro*. Compared to 2D culture systems, 3D models, which provide a bio-mimetic microenvironment, can enhance cell-cell and cell-extra-cellular matrix (ECM) interaction to mimic the biologic and mechanical function of bone marrow niche. Different materials have been employed to create an optimal 3D environment for *ex vivo* expansion of HSCs utilizing 3D bone marrow

mimicking scaffolds [12], such as collagen, cellulose, and synthetic materials polyethylene terephthalate (PET) and poly(lactic-co-glycolic acid) (PLGA). Moreover, many different bioreactor systems are widely used for large-scale expansion of HSCs, such as perfusion chambers [1], stirred bioreactors [2, 3], rotating wall bioreactors [4], membrane based bioreactors [5], and packed or fluidized bed reactors [6, 7]. Scientists believe that replicating the bone marrow microenvironment is the best solution for designing an optimal system for HSCs *in vitro*. This thesis will give an overview of development of *ex vivo* expansion systems for HSCs in decades and some of the problems currently faced in HSC bioprocessing. We aim to build improved culture platforms that better mimic the bone marrow microenvironment for HSCs expansion and use these platforms to study the differences between normal HSCs and leukemic cells.

CHAPTER 2

Background

2.1 HEMATOPOIESIS

Blood performs a vital role in the human body by delivering oxygen and nutrients to cells while removing carbon dioxide and metabolic waste products away, and is responsible for immunological functions and the regulation of body temperature. Blood cells consist of two main classes, myeloid cells (monocytes, granulocytes, megakaryocytes, and erythrocytes) and lymphoid cells (B, T and natural killer cells) (Figure 2.1) [8, 9]. Life span for blood cells in circulation is limited and varies from several hours for granulocytes to a few years for memory T cells. Due to the high mortality of blood cells, there is a continuous need of blood cells to be replenished for a multitude of tasks, and this process requires a system, the hematopoietic system, to continuously generate new blood cells to maintain the

survival of our body. For an adult human, the bone marrow is the main site for hematopoiesis, approximately 10^{11} - 10^{12} blood cells are generated every day to replenish the blood cell number in peripheral circulation [10]. At the core of a complex hematopoietic system sits a relatively small and rare fraction of HSCs, together with bone cells, stromal cells, ECM and growth factors [11]. These components form a 3D complex structure called HSC/bone marrow niche, which keeps HSCs in a highly regulated microenvironment. Each component within the hematopoietic niche plays an important role in maintaining a balance and the regulation for hematopoiesis. Among the blood-related cells, stromal cells provide physical support and biological function to HSCs *via* cell adhesion, cell-cell interaction and secretion of cytokines/growth factors, which act as key regulators of HSCs. The role of ECM in the bone marrow include localization of primitive stem cells, keeping cells in quiescence, and signaling for cell fate decision. The generation of blood cells from HSCs proceeds through successive stages of commitment to a specific cell lineage, terminal differentiation of lineage-restricted progenitor cells, growth arrest and apoptosis. HSCs hold unique properties such as self-renewal and differentiation, which make them the most essential component within the hematopoietic system.

Fig 2.1 Schematic representation of the differentiation of HSCs into cell-lineage restricted pathways (myeloid, megakaryocytic, lymphoid, monocytic/macrophage, erythroid). Long term HSCs converted to short term HSCs in which HSCs lost long term self-renewal ability and turned to short term self-renewal. And short term HSCs further differentiated into progenitor cells. Multipotent progenitor cells (MPPs) gave rise to mature blood cells *via* differentiation along certain lineage restricted pathways. Different growth factors play important role in regulation mature blood cell differentiation, which is shown in figure. Adapted from [12].

Self-renewal is the most important characteristic of HSCs as it maintains the HSC number in an adequate pool by symmetric or asymmetric divisions. Symmetric division means two daughter cells are both HSCs with full stem cell characteristics, whereas in asymmetric divisions, one daughter cell retains a stem cell and the other one loses stemness to differentiate into mature cells [13]. Secondly, self-renewal maintains the ability of HSCs to supply the blood tissue and ensures sufficient number of HSCs and mature blood cells over a lifespan. Finally,

self-renewal is also an absolute requirement in HSC transplantation as self-renewal ability provides the possibility to reconstitute the hematopoietic system in recipients thus determining the long-term recovery of HSC transplantation [11, 14].

The ability to differentiate coexists with self-renewal in HSCs. Differentiation implies that HSCs lose their self-renewal ability and undergo a series of maturational cell divisions with lineage commitment. HSCs ultimately differentiate into committed progenitors and mature cells of either lymphoid or myeloid cell lineages (Figure 2.1) [15-19]. Differentiation ensures sufficient number of mature blood cells supplied under homeostatic conditions throughout life, and HSCs can consistently meet the body's demand for continuous replenishment of blood cells.

2.1.1 The developmental origin of HSCs

The embryo is believed to provide specific environments for HSC development during embryogenesis. It is found out there are multiple waves of hematopoietic generation in several embryo tissues including yolk sac, aorta-gonad-mesonephros (AGM) region, placenta and liver (Figure 2.2) [10, 20].

Short-lived primitive erythrocytes are first wave of blood generation in the embryo, which can deliver oxygen through rapidly growing conceptus. This wave begins at embryonic day E7.5 in mouse conceptus [21, 22]. And some primitive macrophages and megakaryocytes are produced in this first wave. These primitive erythrocytes are generated in the yolk sac blood islands and formed from aggregates of mesodermal precursors or "hemangioblasts".

The second wave initials directly after the first wave at E8/8.5 in mouse, has overlaps with first wave [20]. In this wave, hematopoietic progenitors and cell clusters of hematopoietic cells show in major vasculature at E9.5 with more complex functions. These cells own differentiation ability to produce erythroid myeloid cells, but there is no self-renewal potential showed on these cells and they are not long-lived HSCs.

B

B

Fig 2.2 Hematopoiesis development in mouse embryo. (A) First wave of HSCs generation in mouse aorta. (B) The AGM (aorta-gonad-mesonephros/aorta and urogenital ridges) region (red rectangle) in mouse embryo. (C) A close up of cluster formation of HSCs. Adapted from [20, 23].

The third wave generates the normal adult type HSCs. Generally it is believed adult type HSCs are generated from yolk sac, but hematopoietic clusters are found from vascular endothelial cells at E10.5 (Figure 2.3 C) [15]. At the same time, AGM region is also believed to be the position to generate adult HSCs in mouse. The HSCs generated in this wave is considered to be long-live self-renewing HSCs, which will reside in bone marrow through whole life.

At 6th and 7th week, fetal liver and spleen are found out with hematopoietic colonies. These two organs are the site of hematopoiesis during early embryonal development. Started from 14 weeks to 20 weeks, HSCs in fetal liver and spleen migrate through blood circulation to bone marrow, bone marrow with its intersinusoidal spaces becomes the main site for hematopoiesis [21].

2.1.2 Hematopoiesis in adult

In the bone marrow, about 2,600g active bone marrow with approximately 1.3×10^{12} marrow cells generates about 70 billion neutrophilic leukocytes and 200 billion erythrocytes [10, 24]. However, even with such great productivity, the number of HSCs relative to mature blood cells is very low. HSCs are believed to sustain at a stable and quiescent state. In the presence of external stimuli, HSCs respond to various conditions by entering cell cycle from quiescence (G0 phase) [17, 25]. HSCs replicate themselves and give birth into progenitor cells and mature blood cells that get released into the blood stream to replenish the lost cells. At the end of productive lifespan, HSCs migrate back to bone marrow and are replaced by younger HSCs; this ensures that the bone marrow contains a certain number of HSCs through a lifespan [26].

Long term HSCs (LT-HSCs) have the self-renewal ability for the life of the host, while short term HSCs (ST-HSCs) retains self-renewal capacity for about 8 weeks. ST-HSCs cannot convert back to LT-HSCs by going backwards in the development due to unidirectional reprogramming of these cells to maturation. ST-HSCs give birth to MMPs, which will then generate common myelocyte progenitors (CMPs) and common lymphocyte progenitors (CLPs). Granulocyte-myelomonocytic progenitors (GMPs) and megakaryocytic erythroid progenitors (MEPs) are generated from CMPs and CLPs. These different progenitor cells go further into lineage committed differentiation pathways to generate all different kinds of mature blood cells for our daily requirement [13, 27].

To characterize HSCs in *vitro*, surface markers are commonly used in flow

cytometry. Various markers whose expression is gained (or lost) through stages of hematopoietic differentiation have been identified. The presence of CD34, AC133, and the absence of CD38, HLA-DR and other lineage associated markers are used to define HSCs [28, 29]. CD34 is the first differentiation marker to be used for recognizing HSCs and is still widely used to enrich HSCs and progenitors for research and clinical application. In bone marrow cells, 1-3% of the mononuclear fraction express CD34 antigen while in peripheral blood and cord blood, the percentage of CD34⁺ cells is much lower and more likely in the range of 0.1-0.2% and 0.8-1.2% respectively [10]. It is believed that CD34⁺ cells consist of both stem cells and progenitor cells (lineage-committed hematopoietic cells) [13, 30].

For murine, Lin⁻Thy1.1^{lo}Sca-1⁺ cells are normally taken as HSCs, which is found within the "LSK" (Lin⁻Sca-1⁺c-kit⁺) fraction, and HSCs with most primitive self-renewal ability and long-term reconstituting activities (LT-HSCs) are also within this LSK fraction. LT-HSCs could be in the CD34⁻, CD38⁺, or Thy1.1^{lo} fraction of LSK population. In contrast, even the LSK population with CD34⁺, CD38⁻, or Thy1.1⁻ phenotype could be capable of only transient reconstitution, which means it only contains MMPs or ST-HSCs. However, some studies showed that CD34⁺Lin⁻ cells from bone marrow also preserve the HSC stemness, and are capable of engraftment and differentiation into CD34⁺ cells [10, 30].

Another surface marker, which can be used to define HSCs, is AC133 (also known as CD133). AC133 is found to express on HSCs and progenitor cells derived from the bone marrow, fetal liver, and blood, but AC133 is not found on other mature blood cells and can be used to characterize HSCs as a alternative to

CD34 [8].

Approximately 0.1-0.5% of CD34⁺ cells in BM express KDR which is vascular endothelial growth factor receptor-2 (VEGFR-2, also known as KDR). It has been showed that CD34⁺ KDR⁺ cells also represent HSC stemness and preserve good repopulating ability in mice and fetal sheep, whereas CD34⁺KDR⁻ cells appear to have lost their engrafting ability [29]. A summary of markers used to distinguish the HSCs from mature cells is given in table 2.1 [31].

However, this separation based on the surface marker expression may not be as absolute as earlier, since the surface phenotype of HSC is poorly defined, researchers have yet to find a single molecular marker that is expressed exclusively by HSCs.

Table 2.1 Markers of HSCs

Name	Function
CD34 ⁺ or-	Sialomucin, possibly involved in cell-cell adhesion
Thy 1 ⁻ (CD90)	Possibly invovled in cell-cell interaction
CD38 ⁻	ADP-ribosyl cyclase 1, synthesizes cydic ADP-ribose; indicates differentiation to both erythroid and myeloid progenitors
c-Kit ⁻ (CD117)	Tyrosine kinase receptor for stem cell factor (SCF)
AC133- (CD133)	Prominin-like protein, function unkown
Lin ⁻	Generic designation for several markers of blood cell lineage

Colony-forming unit (CFU) assay is a widely used method for identifying and quantifying the type of progenitors and their ability to form distinct colonies of mature cells in semisolid agar or methylcellulose after 7 to 14 days *ex vivo* culture. These semisolid media reduce cell mobility and allow individual cells to develop into cell clones that are identified as single clusters (< 50 cells) or colonies (> 50

cells). The composition of the colonies determines which CFU is being assessed. Granulocyte/macrophage colony-forming units (CFU-GM) and erythrocyte colony-forming units (CFU-E) are downstream committed progenitors of granulocyte/erythrocyte/macrophage/megakaryocyte colony-forming units (CFU-GEMM). Erythrocyte burst-forming units (BFU-E) are the more commonly measured precursor of the CFU-E [13].

2.2 LEUKEMIA

Dysregulation of HSCs leads to bone marrow cancers and hematopoietic disorders, such as leukemia and anemia. It has been discovered that leukemia originates from leukemia stem cells (LSCs), which are self-renewal, multipotential and highly proliferative as the normal HSCs [11, 32-37]. LSCs not only share similar properties with normal HSCs, they also give rise to leukemic blasts which grow without restrictions as transformed immortalized cells and become maturation arrested *via* a differentiation block (Figure 2.3) [38-40].

Fig 2.3 Schematic representation of the development of LSCs. Mutations can either take place in stem or progenitor cells. Leukemic transformation often takes place in stem cells due to their high level of self-renewal. LSCs give rise to leukemic progenitors (light grey cells) and leukemic cells (black cells). Adapted from [22, 41].

LSCs are first time defined 14 years ago in John Dick's seminal paper in Nature [42]. LSCs share the same morphological markers with normal HSCs, which is found in the immature $CD34^+CD38^-$ cells [11, 40]. It is found out that the number of HSCs ($CD34^+$) declined in leukemic mice, and HSCs cannot be mobilized into peripheral blood with cytokine stimulation [11, 38]. As the leukemic cells grow in the bone marrow, normal HSCs niche is disrupted and an aberrant environment is created by leukemic cell growth [43]. This LSCs 'niche'

can cause dysfunction of HSCs *via* disturbing the normal HSC environment [22, 32].

B) Leukemia

Fig 2.4 Signaling pathways in normal (A) and abnormal (B) hematopoietic niche. Illustration of signaling pathways involved in the hematopoietic stem cells (A) and leukemic stem cells (B) fate in their niche. The differences in the signaling are most likely initiated by chromosomal translocations that are frequently observed in leukemias, such as BCR-ABL and AML1-ETO, or mutations in RAS or tyrosine kinase receptors such as FLT3 AND c-KIT. Adapted from [39].

This picture (Figure 2.4) [39] shows the differences in regulatory molecules for the regulation of HSCs and LSCs in chronic myeloid leukemia (CML). The dissimilarities between HSCs and LSCs are initiated by chromosomal translocations, such as BCR-ABL or tyrosine kinase receptors [22, 35, 38, 40, 44]. The karyotypic abnormalities seen in Table 2.2 among other similar events have been found to encode fusion chimeric proteins that represent transcription factors affecting the stem cell fate, differentiation and growth potential as well as ability to senesce or tolerance apoptosis [22, 39, 40].

Table 2.2 Molecular genetic and cytogenetic abnormalities detected in human leukemias[22].

Human leukemia type	Cytogenetic abnormalities	Molecular genetic abnormalities
CML	t(9;22)	Bcr-Abl tyrosine kinase
AML	t(8;21)	AML1/ETO AML/MTG8
APL	t(15;17) t(11;17)	PML-RARa
ALL-children(B-cell involved)	t(9;22) t(12;21) t(8;14) t(2;8) t(8;22)	Bcr-Abl tyrosine kinase TEL-AML 1 MYC MYC MYC
ALL-adult	t(1;19) t(9;22) t(12;21) t(8;14) t(2;8) t(8;22)	E2A-PBX1 Bcr-Abl tyrosine kinase TEL-AML 1 MYC MYC MYC
MLL	t(1;19) t(4;11) t(11;19) t(9;11)	E2A-PBX1 N.D. N.D. N.D.

2.3 CLINICAL APPLICATION OF HSCS

HSCs have been used clinically for transplantations since 1959 [45-47]. To date, HSCs are successfully and widely used for treating many hematologic diseases, such as acute leukemia, chronic myeloid leukemia, myelodysplastic syndromes, severe aplastic anemia, Fanconi's anemia, homozygous-thalassemia and sickle cell anemia[10, 37, 48-52]. Blood cancers results in an uncontrolled

proliferation of cells, high dosages of chemotherapy drugs are needed to effectively kill the cancerous cells, and the injured bone marrow is then replenished by transplantation of HSCs to reconstitute hematopoiesis in the recipients [48]. Allogeneic stem cell transplantation of HSCs restores normal hematopoiesis after bone marrow failure or after myeloablative doses of chemo-radiotherapy [53-56]. For HSC transplantation, the bone marrow remains the main site for HSCs. There is a low number of HSCs circulating in peripheral blood. And injection of a variety of natural or molecularly engineered cytokines such as granulocyte macrophage colony-stimulating factor (GM-CSF) can mobilize HSCs and progenitor cells from the bone marrow into circulation, which makes the peripheral blood a possible source of HSCs. Another good alternative source of HSC is human umbilical cord blood, which can be easily collected during child birth (Figure 2.5).

Fig 2.5 Schematic representation of HSCs harvest from bone marrow (A), peripheral blood (B) and cord blood (C).

2.3.1 Bone marrow

Bone marrow is the classical source of HSC with about one in every 100,000 cells shown to be the long-term repopulating HSCs. The collection of bone marrow cells is an invasive and painful procedure, which requires puncture of the femoral bone through the hip using syringe and a large needle, followed by separation of stem cells (Figure 2.5 A) [52]. In this procedure, the donor must be hospitalized. Moreover, the bone marrow transplantation only works for HLA-matched recipients, which is not only an important requirement for successful engraftment, but also for avoiding severity of GVHD and recipient's overall recovery [49]. GVHD is a systemic disorder characterized by immune deregulation, immunodeficiency, impaired organ function, and the development of symptoms of various autoimmune disorders, thus often results in decreased survival [57, 58]. It is common amongst recipients with allogenic bone marrow or peripheral derived HSCs transplants.

2.3.2 Peripheral blood

Studies have showed that stem cells from the circulating peripheral blood were able to fully reconstitute a hematopoietic system [51, 59-62]. Harvest of HSCs from the peripheral blood is a practical procedure (Figure 2.5 B). Injection of cytokines such as GM-CSF allows large numbers of HSCs mobilize into peripheral blood. Mobilized peripheral blood is normally passed through a separator that collects cells expressing CD34, while the remaining blood cells are returned to the donor immediately. Cells harvested peripherally have twice the amount of HSCs as

compared to cells harvested from the bone marrow. Normally, a successful transplantation requires no less than 2×10^6 CD34⁺ cells/kg body weight, which usually can be collected in one to three consecutive leukapheresis sessions [63-67].

Peripheral blood has several advantages over bone marrow collection in that HSCs harvested from peripheral blood does not require donor to be hospitalized for the collection procedure. Moreover, transplantation of peripheral blood HSCs facilitates a more rapid hematopoietic recovery, thus leading to reduced overall cost [57, 68-70]. Furthermore, with minimal pain and no need for anaesthesia, peripheral blood is an accepted and preferred source for HSC transplantation today. However, the harvest of HSCs from peripheral blood requires cytokine mobilization, not every donor will have equivalent response to cytokines thus cell number harvested from peripheral blood will vary between individuals. Also, in some cases, donors' splenic sizes increased in both length and width after injection of granulocyte colony-stimulating factor (G-CSF). Therefore, concerns about donor safety have been apprehended because of splenic rupture during G-CSF application [71].

2.3.3 Cord Blood

Traditionally, umbilical cords were taken as discarded by-product during a baby delivery and have not been utilized clinically. However, the therapeutic use of UCB cells grew rapidly since Gluckman *et al.* reported the first successful UCB transplant in children with Fanconi's anemia [72, 73]. Today, UCB can be collected either before the placenta is delivered (*in-utero* collection) or after the placenta is

delivered (*ex-utero* collection) (Figure 2.5 C) [74], and the whole collection procedure is harmless and non-invasive to donors. Moreover, even though UCB has a limited amount of harvested cells, its frequency of progenitor cells (CD133⁺/CD34⁺ progenitor stem cells) per volume is higher than BM or peripheral blood [72, 75, 76], and cord blood has less pro-inflammatory cytokines. Also, the key requirement for a successful bone marrow transplantation is to have a full match of 6 HLA (HLA-A, -B, and -DR); for cord blood transplantation, only 4 out of 6 HLA match is sufficient for HSC transplantation, cord blood transplantation shows higher tolerance in HLA mismatch [77-79], thus a disparity of only two of less antigens can be tolerated. In addition, high levels of Interleukin-10 (IL-10) expression and decreased expression of beta-2 micro globulin found in cord blood resources reduce chance of disease relapse [76]. Moreover, cord blood stem cells are more readily available, taking less time to find an unrelated donor when compared with bone marrow transplantation. The event of GVHD in cord blood stem cell transplantation is also less likely to occur. Other advantages of UCB include ease of availability, which is of importance to high-risk patients with an unstable disease, and greater proliferative capacity as compared to HSCs from BM. All these advantages make cord blood an attractive alternative source to peripheral blood or bone marrow. In the clinic, patients can get UCB units directly from the cord-blood bank, where UCB is frozen after collection from donors and thawed prior to use [80-84].

However, since the volume of cord blood is limited, the cell number in cord blood is very low and not sufficient for adult patient, or even for some pediatric

patients. Transplantation with lower cell numbers generally causes delayed engraftment of neutrophils and platelets [82]. Also, cord blood is collected from cord banks prior to use, and requires freeze and thaw procedure that will reduce survival of HSCs in cord blood. When thawed cord blood containing clumps of lysed cell debris and dead cells are directly infused to patient, this may affect HSC homing into bone marrow and slow down recovery of the transplanted patient [85-87]. One solution to compensate for the low cell number in cord blood is to use multiple UCB units for transplantation. Survival rates and long-term recovery of hematopoiesis are equivalent to a single unit UCB transplantation, but use of multiple UCB units complicates HLA matching, which increases risk of GVHD in patients [88-93]. The limited cell number in cord blood also means patients cannot obtain backup cells in case of relapses, but for bone marrow or peripheral blood transplantation, the patient is able to get a second donation from same donor. In the case of a cord blood transplant, this option is not available.

2.4 THE HEMATOPOIETIC STEM CELL NICHE

The maintenance and regulation of HSC self-renewal and differentiation critically depends on their specific microenvironment called the stem cell niche. Schofield first put forth the concept of a HSC 'niche' to describe the unique microenvironment in which HSCs reside in 1978 [94]. HSC niche with a putative cellular scaffolding structure can support the localization, and has important role in survival and self-renewal of HSCs. So far, two distinct hematopoietic niches have

been proposed: the osteoblastic niche and the vascular niche (Figure 2.6). It has been shown that the stem cell niches have important roles in HSC fate decision, such as self-renewal and differentiation [95-98]. It has already been shown that stromal cells in the niche produce growth factors such as G-CSF, GM-CSF, SCF, IL-6 and SDF1 (CXCL12), which have an influence on hematopoiesis both *in vitro* and *in vivo* [22, 99-101]. Also it is hypothesized that the osteoblastic niche maintains stem cells in quiescence, while the vascular niche induces proliferation and differentiation of HSCs [31, 102, 103].

Fig 2.6 Osteoblastic niche and vascular niche in HSCs' quiescence, mobilization and homing. (a) Osteoblasts lining the endosteal surface keep HSCs in quiescence. (b) HSCs home back to niche, which is initiated by growth factors and chemokines in concert with a variety of molecules, including adhesion and cell-surface molecules, enzymes and ions. (c) HSCs mobilize to vascular niche and start to differentiate at the vascular niche. (d) HSCs reside in the vascular niche which makes HSCs respond to hematopoietic stress rapidly. Adapted from [104]

2.4.1 Osteoblast niche

Many studies focused on bone marrow myofibroblasts, fibroblasts, and endothelial cells to identify them as the postnatal niche, however the bone forming cell-osteoblasts lining the endosteal surface was found to secrete high levels of cytokines, including IL-6, GM-CSF, G-CSF, TNF α and TGF β ; which are important growth factors in HSCs self-renewal [95, 105-107]. Moreover, Heissig B showed that regenerating myeloblasts in 5-fluorouracil chemotherapy treated mice appeared in direct proximity to the trabecular osteoblasts first [108]. *In vivo* and *in vitro* evidences proved HSCs are located and induced in direct contact with osteoblasts.

Further, transplantation of bone marrow HSCs with murine osteoblasts enhanced lymphohematopoietic reconstitution and engraftment in lethally irradiated mice, but dendritic cells failed in co-transplantation [109-111]. This genetic model supports the idea that osteoblasts are a regulatory component in HSC niche [31, 112]. Additionally, ablation of osteoblasts by gancyclovir caused loss of osteoblasts, and number of lymphoid, erythroid progenitors and myeloid decreased in bone marrow [103, 113-115]. These results showed that osteoblasts provide support for HSCs *in vitro* and *in vivo*.

While these results highlight the significance of osteoblastic cells in HSC regulation, it is still not clear what is the differentiation state of osteoblasts that exist in bone marrow niche, differentiated and undifferentiated osteoblastic cells can be an important component in bone marrow niche involved in HSC regulation [115, 116].

2.4.2 Vascular niche

Hematopoietic and endothelial cells have a close relation originating from early hematopoietic development in the yolk sac [116]. Moreover, it is widely accepted that endothelial cells regulate differentiation of HSCs [117]. Functionally, vascular endothelial cells have been showed to maintain stemness of HSCs both *in vitro* and *in vivo* [118]. While the evidence of an endothelial-cell-based vascular niche residing in the neural system has been found, there is no study that suggested its existence as a HSC niche. Until recently, it was not clear how one could identify a hematopoietic vascular niche [31, 103, 119].

The main role of the osteoblastic niche is to maintain quiescence of HSC while the vascular niche promotes proliferation and further differentiation into a series of mature cells into the circulatory system (Figure 2.6). Oxygen level is the major physiological difference between the two niches, and is higher in the vascular niche than in the hypoxic region of the osteoblastic niche [31, 119]. It has been accepted that HSCs located in the hypoxic region of the osteoblastic niche are quiescent, and HSCs move to the vascular niche due to inducement. HSCs are induced by increased oxygen levels to undergo series of differentiation in order to replenish peripheral blood with mature blood cells [118]. When blood cells are fully replenished, HSCs migrate back to the osteoblastic niche and become quiescent again [120]. This process can not only maintain HSCs in a constant pool, but also supply mature blood cells in the peripheral blood.

Furthermore, blood cells can easily penetrate sinusoids which consist of a single layer of endothelial cells, residence in the vascular niche makes HSCs

respond to hematopoietic stress rapidly (Figure 2.6 d) [121], and HSCs can be immediately released into the bloodstream upon chemokine or cytokine treatment. Moreover, the vascular niche can serve as an alternative hematopoietic site in extramedullary organs like the liver and spleen [122, 123].

In vitro transplantation study showed co-transplantation of HSCs with highly purified CD31⁺ microvascular endothelial cells enhanced the hematopoiesis reconstitution in recipients after bone marrow lethal doses of radiation. Furthermore, in these transplanted recipients, the endothelial cells restore host HSC activity in a non-cell-autonomous manner. All these evidences showed that endothelial cells have the ability to support reconstitution of irradiated HSCs [47, 124, 125].

2.5 EX VIVO CULTURE PLATFORMS FOR HSCS

The success in HSC transplantation depends on both the cell number and pluripotency of HSCs transplanted. Normally, a successful transplantation would require no less than 2×10^6 CD34⁺ cells/kg body weight. For adult patient, the cell number needed for successful transplantation will take one to three sessions to collect enough cells from peripheral blood. And for cord blood, the number of stem cells in a single unit is usually insufficient for adult patient thus the application of cord blood for adult patient is restricted. *Ex vivo* expansion systems provide the potential to increase HSC number from a small population of cells *in vitro*, and have shown successful results in the past. This accelerated the development of

many different *ex vivo* expansion systems which are widely used for *in vitro* culture of HSCs. Two main classifications in *ex vivo* culture systems are: 2D culture systems and 3D culture systems. Long-term 2D culture of HSCs was first shown by Dexter and is now the most popular and standard system in HSC expansion. 2D culture systems depend on either extrinsic addition of cytokine cocktail or stromal cells support. 2D systems provide an easy handling platform for *ex vivo* expansion of HSCs. However, 2D culture systems have obvious limitations as static monolayer cultures which are different with the native bone marrow microstructure and suffer from concentration gradients due to the static culture environment. As the important role in which the bone marrow plays in HSC regulation is noted, researchers realize that bio-mimic 3D systems hold greater potential in controlling cell growth and differentiation of HSCs *ex vivo*. Several 3D culture systems, such as collagen scaffolds, electrospinning nanofibers and hydrogels, were explored to support HSC culture and have gotten successful results. These culture platforms provide 3D architecture that favored HSC growth *in vitro* and overcome the limitations of 2D culture systems. To fulfill the large number of HSCs required for clinical applications, scale up systems are needed to expand cell numbers for transplantation. Different bioreactors such as the rotating wall vessel and stirred bioreactors have shown to support extensive expansion of HSCs. In comparison to flask culture systems, bioreactors are more readily scalable and controllable units for bioprocessing. Overall, 3D systems showed many outstanding advantages over 2D culture systems in providing a better mimicry to the native 3D HSC microenvironment and providing larger surface areas for cell

attachment or growth. Cellular interactions and cell-ECM interactions can also be enhanced by the 3D architecture.

2.5.1 2D culture systems with cytokine addition

Many investigations have shown that the expansion of HSCs can be achieved using an appropriate cocktail of cytokines. SCF, thrombopoietin (TPO), Flt-3 ligand (FL), IL-1, IL-11, IL-6, and soluble IL-6 receptor are widely used cytokines for *ex vivo* expansion of HSCs (Table 2.3) [126]. Purified subpopulations of cells cultured in serum-free or serum-containing medium with various combinations of cytokines addition showed high proliferation results [126, 127]. In general, cytokine dependent culture reduces the complexity of the *ex vivo* culture system, however, cell number decreases after the maximum expansion at 2 to 3 weeks which indicates a limitation in stem cell expansion capabilities under such conditions[128]. Moreover, required concentrations of cytokines in *ex vivo* expansion are much higher than those required in stroma-supported cultures, therefore cytokine supported cultures can be an expensive process.

Table 2.3 Cytokines for *ex vivo* culture of HSCs [129]

Cytokines	Function
Flt-3, FL	Potentiates the effects of other cytokines; promotes survival of HSCs
G-CSF	Mobilization of HSCs to peripheral blood
IL-3	Together with IL-6, promotes proliferation of HSCs
IL-6	Together with IL-3, promotes proliferation of HSCs
IL-10	Helps proliferation of HSCs
IL-11	Shortens the G0 phase of cell cycle of HSCs
Jagged-1	Regulates HSC self-renewal
PDGF	Mitogen for connective tissue cells
SCF	Growth factor for HSC progenitor cells
TPO	Stimulator of megakaryocytopoiesis

2.5.2 Co-culture systems with stromal cells

In co-culture systems, it is generally accepted that stromal cells enhance HSC expansion by two mechanisms. Firstly, stromal cells stimulate HSC proliferation by producing cytokines and growth factors that support hematopoiesis. Secondly, they provide an appropriate physical support for HSC localization and promote cell-cell interaction. Furthermore, direct contact with stromal cells is hypothesized as a requirement for sustaining stemness of HSCs during *ex vivo* expansion. Adhesive interactions between HSCs and stromal ligands transduce growth regulatory signals that regulate hematopoiesis. Many murine stromal cells have been successfully used in co-culture system, such as M2-10B4, MS-5, OP9, or HESS-5 cells, which is a conventional method for the expansion of HSCs [130]. However, using murine stromal cell lines in co-culture system forms a xenogeneic system, which may not be safe for human transplantation.

Several *ex vivo* expansion methods with mesenchymal stem cells (MSCs) have been reported. It is shown that *ex vivo* expansion systems with MSCs acting as a feeder layer are highly effective. Nael A *et al.* reported that direct cell-cell contact in co-culture system supported *ex vivo* expansion, migratory potential, and maintained pluripotency of CD133⁺ HSCs [131]. Other groups have shown that indirect co-culture with MSCs enhanced the generation of CFU-GEMM, CFU-C, and CD34⁺ cells [132]. Due to differences in experimental procedures and cell culture practice, it is difficult to deduce either direct or indirect contact culture is better.

The major disadvantage in utilizing direct contact cultures with feeder cells is the introduction of allogeneic human or animal antigens to a graft product meant for clinical use. Autologous feeders may be used, but exposure of the stroma-donor to chemo-therapy and/or radiation prior to harvest of the autologous stroma feeder-cells may have rendered the cells ineffective or the stromal cells may have inherent defects as a result of the disease process itself [133-135].

2.5.3 3D culture systems

In an effort to improve current limitations faced in 2D culture systems and recapitulate the intricate 3D interactions that regulate hematopoiesis *in vivo*, Several 3D culture systems (Table 2.4) have been developed. Most 3D culture models present highly porous scaffolds that command a higher surface area than that of conventional static culture and facilitate higher cell densities [136]. The 3D growth and distribution of stromal and HSCs facilitates the construction and

development of functional tissue units, akin to hematopoietic niches, within the scaffolds that mimic the biologic and mechanical function of the ECM [137]. Manipulation of the scaffold may enhance these niche-units in structure as well as function [138].

Table 2.4 3D culture systems used for hematopoietic cells *ex vivo* culture [139, 140].

Scaffold material	Culture system
Natural material	
Porous cellulose microspheres	Packed-bed bioreactor (murine BM and human BM MNCs)
Macroporous collagen carries	Packed-bed bioreactor (murine BM and human CD34 ⁺ cells in co-culture with the murine S1/S1 stromal cell line)
Synthetic material	
PVF	Static (murine BM cells)
PET fabric	Static (human CD MNCs and CD34 ⁺ cells)
TCPB	Static (human BM CD34 ⁺ cells)
Nylon filtration screen	Static (human BM MNCs)
Porous gelatin microspheres	Stirred flask (human BM stroma and MNCs)

2.5.3.1 Natural material scaffold

Collagen, an important structural protein in the ECM, is widely used in fabricating scaffolds. Joachim O *et al.* fabricated fibrillar collagen gel as a scaffold to culture cord blood CD34⁺ cells. Their results showed CD34⁺ cell numbers from fibrillar collagen scaffold is lower than that on surface coated with nonfibrillar tropocollagen after 7 days of expansion, but the CFU assay results showed the number of clonogenic progenitors is higher after *ex vivo* expansion on fibrillar

collagen scaffold; this indicates that direct cell-collagen contact enhances the number of clonogenic progenitors in CFU assays. The Scanning electron microscopy (SEM) micrograph also showed HSCs intermingling with the fibrillar collagen matrix (Figure 2.7 B-D), and HSCs seeded on a planar surface did not show the full coverage from collagen as those observed on fibrillar collagen matrix (Figure 2.7 A) [141].

Fig 2.7 SEM images of HSCs. Control group (A):HSCs on a collagen coated surface; test groups (B-D): HSCs in contact with fibrillar collagen [141].

Collagen was also made into porous microbeads of average diameter, 400-700 µm (pore size, 40-50 µm; porosity, 85%) (Figure 2.8) for culturing CD34⁺ cells for up to 12 days. This study showed a significant increase in both CD34⁺ cell numbers and total CFU numbers compared with 2D culture [142]. The 3D microporous structure presented a microarchitecture that could mimic a bone

marrow microenvironment and presented greater surface area for cell attachment (Figure 2.8 B).

Fig 2.8 SEM micrographs of collagen microbeads (A) and UCB CD34⁺ cell growing in a pore (B). Adapted from [142].

Natural materials generally have good biocompatibility and are safe to use as cell-supporting scaffolds, but some disadvantages they face include weak mechanical properties and high degradation speed, wherein synthetic materials can make up for.

2.5.3.2 Synthetic material scaffold

Various synthetic materials have been explored for HSC expansion. A 3D tantalum-coated porous biomaterial (TCPB) was used for *ex vivo* expansion of HSCs to support HSC maintenance and pluripotency in long-term culture. This study showed that TCPB can support *ex vivo* expansion of HSCs for up to 6 weeks without cytokine addition. Furthermore, the results demonstrated that TCPB

enhanced a 6.7-fold increase in CFU numbers after expansion. Under the same conditions, the 2-D culture system as control group was unable to support pluripotency and progenitor viability [143].

Li *et al.* used a nonwoven PET fabric to construct a 3D culture system to expand cord blood progenitors. To improve cell adhesion, PET surface was modified by hydrolysis. One advantage of PET scaffold over 2D culture system is that cells in PET scaffold were distributed spatially whereas 2D culture system forms only a monolayer. The 3D culture produced 30-100% higher total cells and CD34⁺ cells than 2D culture without cytokines addition. In the presence of TPO and FL, the 3D culture supported two to three fold higher total cell numbers, CD34⁺ cells and CFUs for 7-9 weeks [144].

Polyester nonwoven fabric porous disc carriers (Fibra-cell) may be better at supporting primary murine stromal cell development as well as at supporting expansion of hematopoietic colony-forming units when compared with those of 2D murine cultures and of 3D cultures comprised of porous cellulose micro beads (pore size 100 μm) and micro cubes (pore size 500 μm). The superiority of this microenvironment was found to be at least partially due to the increased secretion of laminin Q5 but not by an increase in the soluble cytokines SCF and TPO [145]. Stromal cell attachment on 3D structures may have promoted the ECM production.

2.5.3.3 Bioreactors

Static cultures also do not provide the proper 3D microenvironment in the bone marrow thus it may significantly hamper and alter cellular functions [126,

146]. However, 2D culture systems lack of mixing, there is critical concentration gradients for dissolved oxygen, metabolites, pH, cytokines; and it is difficult for online monitoring; the process reproducibility is low; cell number also is limited by the given surface area [129]. Bioreactors are developed since large number of HSCs is needed for the transplantation with the desire of large surface area and high cell density. Successful *ex vivo* expansion of HSCs with different bioreactors has been showed, including perfusion chambers, rotating wall vessel and stirred bioreactors (Figure 2.9).

Fig 2.9 Bioreactors for *ex vivo* culture of HSCs. (A) Perfusion chamber; (B) Grooved surface of perfused chamber; (C) stirred bioreactor; (D) air-lift fixed-bed; (E) Fixed bed or fluidized bed bioreactor. Adapted from [129].

Stirred bioreactors have been successfully used in *ex vivo* expansion of HSCs. Stirred bioreactors can provide a homogeneous microenvironment from mixing. This system is easy to control and take online sample, which supports

online monitoring and generally more scalable culture. Mixing inside stirred bioreactor eliminates the nutrient concentration gradient in the static culture systems. However, stirred systems can impede HSCs to attach to any surface for HSC growth. HSCs are also affected by shear stress, thus the mixing speed can affect HSC survival and proliferation. Moreover, addition of stromal cells is more challenging in stirred systems and the use of microcarriers or microencapsulation [147] techniques may be necessary.

The rotating wall vessel was applied in HSC expansion. Rotating wall vessel supported high proliferation of total cells, CD34⁺ cells and increase of CFU-GM number. Rotating wall vessel provides a low shear stress and homogenous environment for cell growth. There is no nutrient gradient in the rotating wall vessel as the culture medium is constantly being mixed by the rotation action. Co-cultures can also be developed for the rotating wall vessel system [4].

Akon Higuchi *et al.* used polyurethane foaming membranes to develop a direct *ex vivo* expansion method after the filtration of UCB through polyurethane foaming membranes. Most of the mononuclear cells and red blood cells flowed through the membranes while HSCs were retained on the membranes. This method saves working time to less than 30 minutes before culture, while normal purification methods such as using the Ficoll–Paque procedure and magnetic-activated cell sorting of HSCs would take about 5 to 8 hours to perform. Following filtration of the UCB and washing of the membranes, HSCs that remained on the membranes can be expanded in a 3D environment mimicking bone marrow niche.

Direct *ex vivo* expansion of HSCs from UCB in this filtration unit and 3D culture system increased cell number (*i.e.*, 6.6- to 45.7-fold), and the expanded cell populations showed good colony forming ability [148].

Cho *et al.* fabricated porous chitosan scaffolds immobilized N-desulfated heparin to culture CD34⁺ cells isolated from UCB in a perfusion bioreactor system. Cells cultured in chitosan scaffolds showed higher proliferation results compared to cells on static culture. Cells in 3D scaffolds also generated more colony forming units. The perfusion bioreactor system showed to maintain cell proliferation and sustain the stemness of HSCs *in vitro* [149].

2.5.3.4 Electrospinning nanofiber scaffolds

Nanomaterials and nano-structured scaffolds have become of interest due to the possibility of replicating complex architecture of natural systems such as the ECM. Amongst many different methods to produce nano scale materials, Electrospinning is the easiest method for fabricating nanofibers, and is a widely used technique. A typical electrospinning setup consists of a high voltage source, a syringe pump and a grounded collector (Figure 2.10).

In the electrospinning process, an electrical field is applied to the polymer solution at the end of the syringe. A syringe pump forces the polymer solution through the needle. When the electric field intensity is small, the polymer solution cannot form any fiber. As the electric field intensity increases, the surface of the solution at the tip of the syringe forms a conical shape called the Taylor cone. When the force produced by the electric field is big enough to overcome surface

tension, a fiber jet is emitted from its apex. When the jet travels from the tip of syringe to the collector, as the solvent evaporating, charged fibers accumulate on collector [150-154].

With the application of a number of different collectors such as a rotating mandrel and stationary plate, different kinds of fiber can be fabricated. Randomly oriented fibers can be produced by using a stationary collector while aligned fibers are generated with a rotating collector. The rotation speed determines the degree of anisotropy. Fibers with a more porous structure can be obtained with non-conductive collectors[155-158].

Fig 2.10 Schematic of a typical electrospinning system. Adapted from [158].

A wide range of synthetic and natural polymers is available for electrospinning and they are capable of forming fine nanofibers for various

applications [159]. Natural polymers, such as collagen, gelatin, chitosan, chitin, cellulose, and starch are widely used in electrosinning process. It is safe to use natural materials for making scaffolds. The main problem with natural materials is the weak mechanical property and rapid degradation rate.

Synthetic polymers include frequently used biodegradable polymers such as poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (lactic-co-glycolic acid) (PGLA), poly (W-caprolactone) (PCL) and poly (lactic-co-caprolactone) (PLA-CL), all of which are approved by the US Food and Drug Administration (FDA) for biomedical applications. PLA has good biodegradability and biocompatibility, thus it is frequently used in the electrospinning process. PGA is a hydrophilic polymer with higher biodegradation rate than PLA both *in vivo* and *in vitro*. PLGA, as the copolymer of PLA and PGA, its mechanical strength and biodegradation rate depend on the ratio of LA and GA units.

Electrospun nanofiber scaffolds have demonstrated different potential in many applications, such as for drug delivery and tissue engineering. NFS generates nanometer scale architecture which can mimic the native ECM and affect cell behavior (adhesion, morphology and cytoskeletal arrangements). It is showed by Jeremy J.Mao that PLGA NFS supported a continuous differentiation of hMSCs into chondrogenic and osteogenic cells in 14 days[160]. Electrospun nanofiber scaffolds have been explored for drug delivery over decades. It showed a sustained release manner for various agents including antibiotics, proteins, DNA and anti-tumor agents. PCL and PEO mixed NFS supported controlled release of lysozyme in certain period with reduced initial bursts, which can be modulated by the

PCL/PEO blending ratio[161]. A double layer NFS was successfully used to enhance the cytochrome P450 activity in primary rat hepatocytes, which demonstrated the new application of NFS as both adhesive substrate and a delivery vehicle for bioactive molecules[162].

CHAPTER 3

Goal and Objectives

3.1 MOTIVATION

Because of the self-renewal and differentiation ability possessed by HSCs, its utility in hematopoietic reconstitution offers potential cures for many blood related disorders, diseases and malignancies. Amongst the various cell sources, UCB drew great attention to researchers due to its easy collection and low GVHD occurrences. However, HSCs transplantation demands larger number of cells for adult patient, the challenge for UCB application is how to get large number of cells from the limited cell numbers in a single UCB unit. *Ex vivo* expansion systems can contribute in expanding the utility of UCB cells in the clinic.

Many different *ex vivo* expansion systems have been built up over decades for HSC proliferation from 2D culture flasks to 3D scaffold and bioreactors.

However, most of these culture systems failed to preserve the stemness of HSCs during culture, which means HSCs lost the self-renewal ability and led to a failure in further transplantation. As we gain more and more knowledge about the microenvironment in bone marrow, the important role of HSCs niche played in HSC fate decisions is widely accepted by researchers, this also explained the failure in 2D culture. Bio-mimic architecture can give more promising *ex vivo* expansion results compared to 2D culture environment.

It is found out that normal HSCs reside in bone marrow with good adherent ability, and the differentiation of HSCs is only committed to certain lineage, which is strictly regulated by the HSC niche. However, leukemic cells show different behavior from normal HSCs. Leukemic cells have unlimited proliferation ability to generate blast of abnormal blood cells; and with leukemic cell growth, HSC niche is destroyed by leukemic cells, leukemic cells become un-adherent cells and intend to circulate in blood vessel rather than in quiescent state. The reason for these different behaviors between normal HSCs and leukemic cells is still unknown. 3D biomimic systems give the potential to study the differences between HSCs and leukemic cells.

3.2 GOAL AND OBJECTIVES

The overall goal of this thesis is to develop bio-artificial bone marrow models and *ex vivo* systems that would better support large-scale expansion of HSCs. As a secondary objective, the *ex vivo* systems would also enable us to study

hematopoietic cell interactions. To this end, we seek to develop two independent culture systems: one to mimic the bone marrow ECM and the second one that focused on replicating the bone marrow stroma. In the first culture system, we utilized electrospun NFS to create a bio-artificial bone marrow ECM environment for promoting hematopoietic cell attachment. In the second system, we utilized the hollow fiber bioreactor to create a cell dense stromal supported culture. Under each system, we investigated the influences of 3D cultures mimicking the native HSC microenvironment in enabling *ex vivo* expansion of HSCs while maintaining their pluripotency. We also investigated the different behaviors between normal HSCs and leukemic cells with 3D culture system. This study will provide insight into cellular interactions with the ECM of HSC and allow us to develop the design of artificially engineered microenvironment for mimicking ECM *in vivo*.

The specific objectives are:

1. To develop bio-mimetic ECM-like scaffold and bioreactor for HSCs expansion.
2. To fabricate nano-scale biomimetic scaffold for capturing HSCs/leukemic cells to study the adherent differences between captured and un-captured cells.
3. To characterize and evaluate functionality of expanded HSCs in the HFBR co-culture model.
4. To demonstrate improved *in vivo* reconstitution of expanded HSCs in mice models.

CHAPTER 4

Materials and Methods

4.1 CELL SOURCE

4.1.1 Leukemia cell line

The human erythroleukemia K562 cell line was purchased from the American Type Cell Culture (ATCC, Manassas, VA) thawed and cultured per manufacturer's instruction. Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Life technologies, NY) was used for K562 cell culture. Medium containing 4.5 g/L glucose were supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 1.5 g/L sodium bicarbonate (Sigma, Aldrich) and 1% penicillin/ streptomycin (A&E Scientific, PAA Laboratories GmbH, Austria) to constitute the complete medium.

4.1.2 Bone marrow stromal cell line

An immortalized human bone marrow stromal cell line, HS- 5 (ATCC) was used in this study. Cells were routinely propagated in tissue culture polystyrene

(TCP)- flasks in complete medium at 37°C in a humidified incubator with 5% CO₂. Cells were passaged every 3–4 days on standard 2D TCP-flask cultures. Dulbecco's modified eagle's medium (DMEM) (Invitrogen) was used for HS-5 cell culture. Medium containing 4.5 g/L glucose were supplemented with 10% FBS (HyClone Laboratories, Logan, UT), 3.024 g/L sodium bicarbonate (Sigma, Aldrich) and 1% penicillin/ streptomycin (A&E Scientific, PAA Laboratories GmbH, Austria) to constitute the complete medium.

4.1.3 Cord blood Lin⁻ cells isolation

UCB was obtained from the placentas of normal full-term infants during delivery on the consent of donor patients (CIRB 2010/618/D). The UCB was collected in blood bags containing heparin. Cord blood was transferred from blood bag to 50 mL tubes using 50 mL syringe and 18G needle. Cord blood was mixed with IMDM containing 30% (v/v) FBS and centrifuged at 180g for 10 mins. Supernatant was discarded using a sterile pasteur pipette, cell pellets were re-suspended in IMDM with 30% (v/v) FBS. Washing step was repeated twice and cells were mix with IMDM with 2% (v/v) FBS. Ficoll–Paque Plus (1.077 g/mL; Amersham-Biosciences, Uppsala, Sweden) was gently layered under cell solution, the tube was centrifuged at 400g for 35 minutes. The middle buffy coat layer was collected with pasteur pipette as mononuclear (MNC) fraction.

After separation over Ficoll–Paque Plus, the low-density mononuclear cells were washed in phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin (BSA) and 2 mM Ethylenediaminetetraacetic acid (EDTA).

Lineage depleted hematopoietic progenitor cells (Lin⁻ cells) were isolated *via* magnetic bead cell isolation using the Lineage Negative Selection Kit (Miltenyi Biotec GmbH, Germany) (Figure 4.1) per manufacturer's protocol. MNCs were mixed with lineage cocktail and incubated for 15mins, and washed with PBS containing 0.5% BSA and 2mM EDTA once. Cells were incubated with microbeads for 10mins. After incubation, unstained cells were separated using high-gradient magnetic field in the MidiMACS (Miltenyi Biotec GmbH). Cell clumps were removed by 70 µm nylon mesh, cells were applied into MACS column. Cell selection was performed twice through the magnetic columns to ensure high purity of Lin⁻ cell population.

Fig 4.1 HSCs separation using magnetic nanoparticles. (A) Cell conjugates with magnetic beads; (B) Process of cell separation under magnetic field.

4.2 NFS AS A BIOARTIFICIAL BONE MARROW ECM

4.2.1 Fabrication of nanofiber scaffolds

To fabricate collagen blended PLGA NFS, the PLGA(75:25) copolymer (Sigma-Aldrich, St. Louis, MO) at a molecular weight of 90,000-126,000 and a medical grade type-I collagen powder (Koken Pte. Ltd, Japan) were mixed at a ratio of 1:1 (wt:wt) overnight at room temperature with 6%, 8% and 10% (w/v) in HFP (1,1,1,3,3,3,-hexafluoro-2-propanol; Sigma-Aldrich). The polymer solution was placed in a syringe with a needle tip diameter of 0.21 mm. The solution was dispersed by a syringe pump at a feeding rate of 1.2 mL/h at a humidity of 55%-60% and temperature of 20-24°C. An electrospinning voltage of 18kV DC was applied to the needle using a high voltage power supply (Gamma high Voltage Research, Ormond Beach, FL). The distance between tip of syringe and collector was 15 cm. The resultant NFS were collected on glass cover slips. The nanofiber-coated cover slips were then placed in a vacuum dryer overnight to remove any remaining solvent.

4.2.2 Materials characterization

Various methods can be employed for material characterization of electrospun NFS [145]. In this study, we used scanning electron microscopy to define its architecture, performed Fourier transform infrared spectroscopy (FT-IR) analysis to determine its chemical composition and conducted water contact angle measurements to characterize its surface properties.

SEM analysis (FEI Company, Hillsboro, OR, USA) was performed to observe the topography of electrospun collagen blended PLGA NFS and morphology of cells adhering on NFS. To prepare the samples, electrospun NFS with/without captured cells were gently rinsed with PBS, and fixed with 2% paraformaldehyde. Samples were dehydrated using a graded series of ethanol (50%, 70%, 85%, 90% and 100%) and finally gold sputter-coated. Diameters of fabricated nanofibers were manually measured from SEM images using an image analysis software.

To confirm the chemical composition of our electrospun nanofibers, we performed a FT-IR (Thermo Nicolet 5700 FT-IR, Thermo Electron Corp., Madison, WI) analysis on the fabricated NFS. The spectral range recorded was from 4000 nm to 600 nm and the test resolution was at 1 cm^{-1} .

Another material property that would be of interest and relevance to this study is the measurement of hydrophilicity/hydrophobicity of the electrospun scaffold. Hydrophilicity of the scaffold was analyzed by water contact angle measurement using a FTA stage controller. The sessile drop method was used to measure water contact angles for samples of pure PLGA NFS and blended NFS. Drops of distilled water (0.5 mL/min) were deposited onto the surface of NFS samples and a direct microscopic measurement was done with the computer software. Ten measurements were taken for final average reading.

4.2.3 Surface modification of NFS

NFS surfaces were modified by coating with 2 µg/mL fibronectin (Sigma-Aldrich) or 2 µg/mL E-selectin/IgG1 Fc chimeras (R&D Systems, Minneapolis, MN) in 20 mM Tris-buffered saline (TBS; pH 7.4) with 1 mM CaCl₂ at 4°C overnight. Coated nanofibers were subsequently washed three times with PBS (Sigma-Aldrich) supplemented with 0.1% BSA and one time with IMDM (Life Technologies Corporation, Carlsbad, CA) supplemented with 1% BSA (Sigma-Aldrich).

4.2.4 Capture of K562 and Lin⁻ cells on NFS

To quantify capture efficiency of hematopoietic cells on NFS, erythroleukemic K562 cells (ATCC) (0.5 mL) with an initial cell density of 200,000 cells/mL and Lin⁻ cells (0.5 mL at 40,000 cells/mL) were seeded into each well in a 24-well plate covered with different substrates: (1) The control substrate is TCP; (2) collagen blended PLGA NFS; (3) collagen blended PLGA NFS coated with E-selectin; (4) collagen blended PLGA NFS coated with fibronectin. The cell suspension was exposed to these substrates at 37°C for 1 hour, 2 hours or 6 hours and gently washed twice with PBS to remove any unattached cells. The unattached cells were subsequently counted using a hemacytometer. Attached cells were trypsinized before they were counted.

4.2.5 Cell growth of NFS-captured hematopoietic populations

Short-term culture of K562 cells and Lin⁻ cells on NFS was performed in appropriate supporting media on NFS.

4.2.5.1 Culture of K562 cells on NFS

For K562 cells, the culture media consisted of IMDM (Life Technologies Corporation) with 10% FBS (Hyclone Laboratories Inc., Logan, Utah). Captured population of K562 cells on different substrates and unattached cells were incubated in 24 well-plates (Corning Incorporated, Corning, NY) for up to 7 days at 37°C in a fully humidified 5% CO₂ incubator. Cells were harvested at day 7, and cell viability was assessed by Trypan blue dye exclusion.

4.2.5.2 Culture of Lin⁻ cells on NFS

Captured and un-captured Lin⁻ cells were cultured in 0.2 mL of serum-free expansion medium, which consisted of 1% BSA, 0.01 mg/mL recombinant human insulin (Sigma Aldrich), 0.2 mg/mL human transferrin (Sigma Aldrich) in IMDM, supplemented with 100 ng/mL SCF (stem cell factor) (R&D systems, Minneapolis, MN), 100 ng/mL FL (R&D systems), 50 ng/mL recombinant human TPO (R&D systems) and 20 ng/mL IL-3 (R&D systems). Cells were cultured at 37°C in an atmosphere containing 5% CO₂ for 7 days. Similar cultures were also performed on TCP, which served as a positive control. Cells were harvested after 7 days of expansion. All NFS substrates were trypsinized for 5 mins. A manual cell count and viability assessment was performed using the hemacytometer.

4.3 CO-CULTURE SYSTEM SET UP IN HFBR

We describe in detail the protocol developed and optimized in our laboratory for establishing a serum-free co-culture suited for HSCs derived from human umbilical cord blood stem cells. Figure 4.2 outlines the steps involved in this process and this includes the priming step, cell seeding, serum dilution step, cell culturing and harvesting from the HFBR. The human stromal cell line HS-5 (ATCC) was used in this study to develop the cell culture protocol.

Fig 4.2 HFBR set up and detailed process protocol. An overview of cell culture procedure from initiation, seeding, and harvesting of cells in the HFBR.

4.3.1 Priming: preparation for culture

The HFBR used in our studies was purchased from FiberCell Systems (FiberCell Systems Inc., Frederick, MD) and the chosen cartridge was a medium sized cartridge (C2008) with a 5 kDa molecular weight cut off (MWCO) and total surface area of 2100 cm². The material of the membrane used in the cartridge is Low Flux PS. For different applications, there are different cartridges for selection from FiberCell system. The cartridge we chose is C2008, which the material of membrane is hydrophilic fiber, Low Flux PS, with low molecule weight cut off (5kd @ 50%). It makes sure all cytokines and proteins produced by stromal cells are retained in cartridge for future cell co-culture. Figure 4.3 shows a schematic of a typical fiber cartridge and access ports to either the intra-capillary or extra-capillary spaces of the bioreactor. Media is continuously perfused through the lateral end ports, which access the intra-capillary spaces of the membrane fibers. Nutrients and metabolites are continuously exchanged across the membrane *via* diffusion. The horizontal side ports, on the other hand, act as sample ports for seeding and harvesting of cells. The bioreactor system was assembled under sterile conditions in a laminar flow biological cabinet. All components of the bioreactor were carefully wiped down with 70% ethanol and dried before placing it inside the CO₂ incubator. Prior to the seeding of cells, we performed two pre-culture cycles through the HFBR. The first pre-culture cycle consisted of sterile PBS (Sigma-Aldrich) perfused through the HFBR for up to 24 hrs. The second pre-culture cycle circulates the complete HS-5 culture medium, which is made up of 10% FBS in DMEM, and was perfused through the HFBR for up to 24 hrs. The second pre-

culture cycle serves to provide a protein coating on the membrane fibers to promote attachment of stromal cells.

Fig 4.3 Schematic Structure of HFBR cartridge.

4.3.2 The stroma culture

The HS-5 stromal cells were first thawed and expanded on T75-flasks in 10% FBS and DMEM for 4-6 days. To initiate cell culture in the medium sized cartridge, it was recommended that a minimum of 1×10^8 cells be seeded; this translated to an initial seeding density of 5×10^4 cells/cm². Once the required cell number was achieved in the flask culture, HS-5 stromal cells were transferred into the HFBR. Cells trypsinized from the flask culture were resuspended in 15 mL complete HS-5 medium. The cell suspension was introduced into the extra-capillary space (ECS) of the hollow fiber cartridge using a 20 mL syringe through one of the horizontal side ports, while the other side was connected to another 20 mL syringe. A gentle flushing action was performed between the two syringes; this was done to ensure even distribution of cells during seeding throughout the ECS. During this step, the lateral end ports were placed in the closed position. Once this was completed,

excess medium in the syringe was forced through the capillaries into the reservoir by opening one of the lateral end ports so that all the cells were completely inoculated into the bioreactor.

The bioreactor was connected to a 100 mL media reservoir and continuously circulated the medium during the cell culture. Every two/three days, the reservoir medium was replaced with 100% fresh medium based on the glucose consumption rate. At the same time, the reservoir medium was increased by doubling its volume at each change up to a maximum volume of 400 mL to accommodate for the increasing cell density (Figure 4.4 A). The bioreactor cell culture was maintained in a 37°C, 5% CO₂ fully humidified incubator.

4.3.3 Serum dilution step

After 6 days of culture in 10% serum-supported medium, glucose consumption of HS-5 would reach a plateau; the dilution of serum was then gradually introduced. This was achieved *via* a step-wise dilution procedure from 10% to 5% to 2% and finally serum-free (0%) condition (Figure 4.4 B). Each dilution step was introduced every 6 days to allow the stroma cells enough time to stabilize under each “new” condition. Under serum-free conditions, the metabolite levels are checked prior to the addition of HSCs. Serum dilution step was not applied to leukemic cell co-culture in HFBR.

Fig 4.4 Medium volume and FBS percentage for HFBR and TCP. (A) Medium volume in HFBR reservoir and TCP; (B) FBS percentage for cell culture medium in HFBR and TCP during 33 days culture period.

4.3.4 Initiation and maintenance of leukemia cell co-culture

To establish hematopoietic co-culture in the HFBR, 5×10^5 K562 cells were inoculated into the ECS of HFBR, then the reservoir medium was switched to IMDM complete media (IMDM with 10% (v/v) FBS). Full ECS medium replacement was done on day 4 of co-culture onwards. Spent ECS medium containing loose cells were centrifuged and inoculated back into the ECS. The

K562 co-culture was maintained for 14 days. Static TCP suspension culture (control) and co-culture were established and used as a comparison.

4.3.5 Initiation and maintenance of HSC co-culture

Freshly isolated Lin⁻ HSCs and progenitors were introduced into the ECS compartment of HFBR *via* the horizontal side ports using a gentle flushing action (as previously described in section 4.3.2). The medium used to support HSC co-culture was a serum-free IMDM (Invitrogen) based media, supplemented with 4 mg/mL BSA, 0.71 µg/mL human insulin, 27.8 µg/mL human transferrin and 1% pen/strep. Cytokines added into the serum-free medium to support HSC survival and activity were SCF (50 ng/mL), FL (50 ng/mL), IL-3 (25 ng/mL) and TPO (50 ng/mL); this cytokine-supported serum-free medium was introduced only into the ECS side of the bioreactor. Change of media in the reservoir and ECS was conducted every two/three days with a fully replacement by fresh medium. The evaluation of hematopoietic cell populations can be performed as early as the end of one-week culture. Long-term cultures can also be supported in the HFBR co-culture system.

4.3.6 Evaluation of cell coverage

We evaluated the coverage and homogeneity of stromal cell attachment and cell growth by cutting across the cross section of the HFBR (Figure 4.5 A). Each section was identified as the top, middle and bottom sectors of the bioreactor setup and labeled as section 1, 2 and 3 respectively (Figure 4.5 B). For evaluation under light microscopy, fibers were cryo-frozen in the freezing medium and carefully

sectioned onto microscopic slides. The cryo-freeze and sectioning steps were performed on the Cryostat (Leica Microsystems). For evaluation under SEM, fiber sections were first fixed in 2% paraformaldehyde. Fixed samples were then dehydrated using a graded series of ethanol and finally gold sputter-coated before viewing under the field emission SEM (SEM; FEI Company, Hillsboro, OR, USA).

Fig 4.5 Cross section of HFBR cartridge. (A) Cutting position of HFBR cartridge; (B) Three different sampling positions.

4.3.7 Cell culture monitoring

Metabolites such as glucose, lactate, glutamine and ammonia can be continuously monitored through periodic sampling from the media reservoir or ECS. A standard biochemical analyzer can provide accurate readings for a number of metabolites and measurements of dissolved oxygen, various ions and CO₂ measurements. This serves as a good analytical tool to monitor physiological status of the cell culture. Metabolite assays can also be used as a cheaper alternative to take periodic measurements. However the accuracy and stability of standard

metabolite assays do not perform as well as the readings obtained from the commercial biochemical analyzer. Careful calibration and procedural sampling tests must be taken to ensure consistency of the method used.

4.3.7.1 Glucose

Glucose concentration in culture media was determined with a glucose assay reagent (Sigma-Aldrich) *via* phosphorylation of glucose to glucose-6-phosphate, followed by its oxidation to 6-phosphogluconate in the presence of oxidized nicotinamide adenine dinucleotide (NAD) over a 15 min incubation period. This oxidation caused an equimolar reduction of NAD to nicotinamide adenine dinucleotide (NADH), resulting in an increase in absorbance at 340 nm which was directly proportional to glucose concentration. Glucose consumption was calculated based on the difference in glucose levels to a reading taken from the previous day.

4.3.7.2 Lactate

Lactate concentration in medium is measured with Lactate colorimetric assay kit (Sigma Aldrich). Lactate concentration was determined by an enzyme assay, which results in a colorimetric (450 nm) product, proportional to the lactate present. Samples were mixed with lactate assay reagent and incubated for 30 minutes at room temperature, and the absorbance at 450 nm were measured for calculation the concentration of samples.

4.3.7.3 Protein

Concentration of solubilized protein was assessed using a dye-binding assay (Bio-Rad Laboratories), causing a differential color change of the dye in response to various protein concentrations. Subsequent to a 5 mins incubation period, the colored reaction solution was measured spectrophotometrically at a wavelength of 595 nm. Relative protein concentrations were determined based on BSA standard curve. Protein production was calculated based on the difference in protein levels to a previous day reading.

4.3.7.4 Ammonia

Ammonia concentration was measured with Ammonia colorimetric assay kit (BioVision). Ammonia was converted to a product that reacted with OxiRed probe to generated color ($\lambda=570\text{nm}$) which can be easily quantified by plate reader. Medium samples collected from HFBR were centrifuged to remove insoluble material. Samples were diluted 10 times with PBS, 2 μL samples were mixed with 50 μL reaction mix solution and incubated for 60 min at 37°C, protected from light. After incubation, O.D. 570 nm was measured for ammonia concentration calculation by standard curve.

4.3.7.5 PH

PH values for medium samples from HFBR were measured with pH meter (PL-600 Lab pH meter). Sensor was immersed into the samples after rinsing with

distilled water, shaking the vessel few minutes, measurements were recorded when reading was stable.

4.3.8 Cell harvesting from HFBR

Non-adherent mature cells and committed progenitors of the hematopoietic lineages can be easily harvested from the HFBR *via* a gentle flushing procedure. This process was performed several times with fresh medium *via* the two horizontal side ports while the two lateral end ports remained in a closed position. Adherent and more primitive hematopoietic cells were harvested *via* enzymatic digestion using StemPro Accutase (Life Technologies), which was incubated for 30 min in 37°C for two rounds. Subsequently, dislodged cells were retrieved by gentle flushing action between two side-port syringes.

To separate cells from HS-5 cells, cell suspension were reseeded into plates and left in the incubator for 1 h. Suspension cells were then collected for analysis. Growth and viability of cells were assessed by the enumeration of live cells performed *via* trypan blue exclusion and manual counting using a hemocytometer.

4.4 CELLULAR ANALYSIS

4.4.1 Cellular growth

Cell number and cell viability were evaluated by manually counting under haemocytometer and microscope (Zeiss) with Trypen Blue (sigma), which only

stained dead cells blue. Fold of increase was defined as a measure describing how much a quantity changes going from an initial cell number to a final cell number.

$$\text{Fold of increase} = \frac{\text{Final cell number}}{\text{Initial cell number}}$$

4.4.2 Cell morphology

To observe cell morphology, harvested cells were collected by cytopsin. 100 μ L cell suspension solution in PBS was loaded into the cytopsin well, and centrifuged at 100g for 3mins. The filter paper and cuvette were gently removed from cytopsin without damaging the sample on cytopsin. The slide was air-dried for 30 mins at room temperature before staining. Samples were stained with KaryoMax Giemsa stain solution (Life Technologies Corporation) following manufacturer's protocol. Slides were dipped in coplin jars containing pre-made solutions, rinsed and air-dried in the oven.

4.4.3 Flow cytometric and cell cycle analysis

Cells were analyzed for immunophenotype and surface adhesion makers *via* flow cytometry using the Epics-Altra System (Beckman Coulter, San Jose, CA) flow cytometer with 488nm Argon laser. Cells were incubated with antibodies at 4°C for 30 mins in PBS containing 2% (v/v) FBS, and 0.1% (w/v) sodium azide (Sigma-Aldrich). Cells were washed twice before analyzing with flow cytometer. Surface marker expression of CD34, CD38, CD45, CD31, CD71 and GPA, and integrin expressions of α 2, α 5, and α 6 were interrogated using antibodies conjugated with either fluorescein isothiocyanate (FITC), phycoerythrin (PE), or

Peridinin-chlorophyll-protein (PerCP). Negative (unstained cells) and isotype controls were included to confirm specificity. Data acquisition using the Epics-Altra was typically performed immediately or within three days following fixation, and a total number of 30,000 ungated events was recorded for each sample. Data analysis and off-line color compensation for the FITC-PE, PE-PC5, PE-FITC, and PC5-PE fluorescent panels were performed using FlowJo (Tree Star, Inc., Ashland, OR).

Cell cycle analysis was performed on K562 cells after 2 days and 4 days of culture on NFS and in standard 2D TCP (control). Cells were fixed in 70% ethanol prior to staining in 50 µg/mL propidium iodide (PI) solution. A total of 30,000 events were acquired for each sample. All data analysis was performed in FlowJo software (Tree Star, Inc., Ashland, OR).

4.4.4 Clonogenic assay

The clonogenic potential of suspension and adherent hematopoietic cells harvested from TCP and HFBR were evaluated using the CFU assay. Harvested cells were plated (1×10^4 cells/mL) in 1% methylcellulose (Miltenyi Biotec, USA) culture medium with 30% FBS and with the following mixture of growth factors (50ng/ml SCF, 20ng/ml GM-CSF, 20ng/ml G-CSF, 20ng/ml IL-3, 20ng/ml IL-6, 3U/ml Erythropoietin). Colonies were scored after 14 days incubation in a humidified incubator at 5% CO₂. All assays were performed in duplicates.

4.4.5 Gel electrophoresis and western blot analysis

From each media sample, 50 mg of proteins were loaded onto each lane of the gel in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). Protein samples were mixed with 2× SDS–PAGE sample buffer which consisted of 0.125M Tris–HCl at pH 6.8, 4% SDS, 20% (v/v) glycerol, 10% b-mercaptoethanol, and 0.005% (w/v) bromophenol blue. The sample mixture was heated at 95°C for 10 min for protein denaturation and placed in ice prior to gel loading. A protein ladder consisting of a range of proteins from 10–250 kDa was included in each gel. One dimensional SDS–PAGE was performed in the OmniPAGE mini vertical gel electrophoresis unit (Clever Scientific, Warwickshire, UK) initially at 90 V then a constant voltage of 150 V. Gels were stained with silver staining and visualized in the Chemidoc XRS (Bio-Rad) gel documentation imaging system.

To perform Western analysis, gels were blotted onto 0.2 mm nitrocellulose membranes. Protein blotting was performed in the OmniPAGE mini (Clever Scientific) at a constant current of 100mA for up to 120 min. The blotted membrane was then air-dried for at least 1 h at room temperature prior to immunostaining. Western blot analysis of the growth factor IL-6 (Abcam, Cambridge, UK) was performed. Culture media were used as negative control 1 and K562 cell lysate was used as negative control 2. Protein membranes were then incubated overnight at 48°C in the primary antibody and 1 h at room temperature for the secondary antibody. The primary antibody dilution for IL-6 was 1:1,000. Secondary antibodies were conjugated to horseradish peroxidase (HRP) and a

dilution of 1:3,000 was used. Proteins were detected using ECL chemiluminescent substrate (GE Healthcare, NJ). Western blot images were captured in a gel documentation imaging system.

4.4.6 Cytokine Arrays

The identification of secreted cytokines and their expression levels from different stromal conditioned medium was measured using a cytokine array kit, ARY005 (R&D system, USA) as previously described [163]. Cytokines produced by HS-5 stromal cells in both TCP and HFBR under various culture conditions *i.e.* 10% serum, serum-free and co-culture, were collected and evaluated. The medium was concentrated with concentrator (Corning, USA). The cytokine array membranes were incubated overnight in different stromal conditioned medium (DMEM with 10% FBS medium from TCP and HFBR, serum free medium from TCP and HFBR, and serum free medium after co-culture of Lin⁻ cells in HFBR) with the same amount of total protein in it. The arrays were then incubated in HRP-labeled streptavidin for 1 hour and were visualized by enhanced chemiluminescence (ECL) (Advansta, USA) in black box (Bio-rad, USA). Estimation of the cytokine expressions was based on a relative ratio to the controls. There were a total of six positive control antibodies (A1, A2, A19, A20, E1, E2) and two negative control antibodies (E19, E20) in the array membrane (Table 4.1). The relative cytokine concentration was calculated by the following equation:

$$\bar{C}_{rx} = \sum \frac{C_x - \bar{C}_n}{\bar{C}_p - \bar{C}_n} / n$$

$\bar{C}_{rx}$ ----Mean value of integrated intensity for cytokine x.

C_x ----Measured reading from cytokines array for cytokine x.

$\bar{C}_n$ ----Mean value of two negative controls on cytokines array.

$\bar{C}_p$ ----Mean value of six positive controls on cytokines array.

n---- Number of spots for cytokine x on cytokine array, normally it is 2.

Table 4.1 Cytokines table for cytokine array membrane.

	1	2	3	4	5	6	7	8	9	10
A	Posi tive	Posi tive	C5/ C5a	C5/ C5a	CD4 0lig and	CD4 10ig and	G- CSF	G- CSF	GM - CS G	GM - CS G
B			IL- 1 α	IL- 1 α	IL- 1 β	IL- 1 β	IL- 1ra	IL- 1ra	IL-2	IL-2
C			IL- 10	IL- 10	IL- 12p 70	IL- 12p 70	IL- 13	IL- 13	IL- 16	IL- 16
D			IL- 32 α	IL- 32 α	IP- 10	IP- 10	I- TA C	I- TA C	MC P-1	MC P-1

E	Posi tive	Posi tive	RA NT ES	RA NT ES	SDF -1	SDF -1	TNF - α	TNF - α	sTR EM- 1	sTR EM- 1
	11	12	13	14	15	16	17	18	19	20
A	GR O α	GR O α	I- 309	I- 309	sIC AM -1	sIC AM -1	IFN - γ	IFN - γ	Posi tive	Posi tive
B	IL-4	IL-4	IL-5	IL-5	IL-6	IL-6	IL-8	IL-8		
C	IL- 17	IL- 17	IL- 17E	IL- 17E	IL- 23	IL- 23	IL- 27	IL- 27		
D	MIF	MIF	MIP -1 α	IMP -1 α	MIP -1 β	MIP -1 β	Serp in E1	Serp in E1		
E									Neg ativ e	Neg ativ e

4.4.7 *In vivo* repopulating capability of cells

Ex vivo expanded cells from TCP and HFBR were used to test their *in vivo* repopulating capability. Injection of expanded human HSCs was performed following the previously published protocol [164]. Briefly, NOD/SCID IL-2R $\gamma^{-/-}$ (NSG) mice were purchased from Jackson Laboratories and maintained under specific pathogen-free conditions in the animal facilities at Biological Resource Centre, Agency for Science, Technology and Research (A*STAR). To reconstitute mice, newborn pus (less than 48 hours old) were irradiated with 100 cGy using a Gamma radiation source and injected intracardially with expanded cells (1×10^5 cells/recipient). All research with human samples and mice was performed in

compliance with the institutional guidelines. Eight weeks later, mononuclear cells isolated from murine bone marrow were tested for expression of human CD45 (HI30) and CD45.1 (A20) (BioLegend, CA) by flow cytometry. Mononuclear cells isolated were stained with appropriate antibodies in 100 μ L PBS containing 0.2% BSA and 0.05% sodium azide for 30 min on ice. Flow Cytometry was performed on a LSRII flow cytometer using a FACSDiva software (BD, Franklin Lakes, NJ). 10,000 to 1,000,000 events were collected per sample and analyzed using the Flowjo software.

4.5 STATISTICAL ANALYSIS

All tests performed in triplicates were averaged and expressed as means $\pm$ standard deviation (SD). Statistical differences between sample groups were determined by independent two sample t-test and a p-value < 0.05 was considered statistically significant.

CHAPTER 5

Electrospun Nanofibers as a Bioadhesive Platform for Capturing Hematopoietic cells

5.1 INTRODUCTION

The bone marrow, a complex architecture comprising a multitude of ECM proteins and cellular components, is the main site of blood production. In this site, regulation of hematopoiesis is achieved through a series of cell-matrix and/or cell-cell interactions that signal hematopoietic cells toward specific cellular function. Adhesion of hematopoietic cells thus plays a vital role in hematopoietic development (normal and abnormal), cell migration, and cell localization. Interactions between hematopoietic cells and the ECM are essential in supporting key hematopoietic functions such as self-renewal, proliferation, and differentiation into committed progenitors [165, 166]. Clinically, CML is characterized by

aberrant expansion of immature myeloid progenitors with premature circulation into the blood stream. It has been proposed that increased proliferation and resistance to apoptosis of CML cells in the bone marrow is a consequence of altered adhesive behaviors of leukemic progenitors, which results in their lack of responsiveness to negative regulatory mechanisms[166]. Several observations *in vitro* have accounted for abnormal adhesive properties in CML progenitors [14, 167-169]. The inability for CML progenitors to adhere to bone marrow stroma[170] and ECM proteins such as fibronectin could be responsible for the uncontrolled immature cell expansion [171]. Studies in adhesive behaviors of leukemic cells are important in unveiling potential molecular mechanisms that result in altered cellular functions. However the exact mechanisms and roles of each interaction remain largely unclear. The study of hematopoietic cell interactions within the bone marrow microenvironment in the regulation of hematopoiesis and leukemia is therefore an intriguing and worthy of investigation.

Electrospun NFS are able to achieve nanometer length scales that are representative of the native ECM [172, 173] thus enabling them to create a 3D ECM-like architecture found in the bone marrow. Its large surface area to volume ratio, high porosity and good biocompatibility also make them good biomaterials for cell culture. Moreover, nanotopographical features have demonstrated the ability to modulate cell behaviors *in vitro* [174-176] and would serve as a good platform for studying cell-surface interactions on ECM-like architecture. Its material functionality for surface modification and potential use as growth factor or drug carriers make them highly desirable for many stem cell and tissue engineering

applications [176]. In this study, collagen and PLGA were chosen to fabricate biomimetic NFS; the materials chosen were due to the fact that PLGA is an FDA approved biodegradable synthetic polymer and collagen type-I is a major matrix component in the bone marrow niche that has preferential adhesion for myeloid and erythroid progenitors [177]. We hypothesized that electrospun NFS, due to its nanotopographical resemblance to the natural ECM, would create a suitable platform for studying hematopoietic cell adhesion and cell-ECM interaction *in vitro*. The proposed bio-adhesive platform and culture substrate will provide insight into interactions in the nanoscale between leukemia/hematopoietic cells with the bone marrow ECM *in vivo*; and provide us with tools to design artificially engineered microenvironments for studying normal/abnormal hematopoietic cell adhesion; and give us the potential for study the differences between hematopoiesis and leukemia.

5.2 AIM AND HYPOTHESIS

The aim of this study is to develop a 3D scaffold by electrospinning, which forms a nano-scale structure for better mimicking the native bone marrow ECM structure. As the important role that bone marrow ECM structure plays in hematopoiesis is widely accepted, NFS holds the potential in supporting HSC stemness and promoting HSC proliferation; and NFS provides a useful tool to study cell adherent properties and interaction between normal/abnormal hematopoietic cells and bone marrow ECM structure *in vitro*. NFS can be also used

as a tool for *ex vivo* study of the differences between normal and abnormal hematopoietic cells. The basic hypothesis is that our 3D material can provide an improved and nano-scale scaffold for hematopoietic cells study *in vitro*. Improved HSCs capture and proliferation on NFS is expected; it is also hypothesized that NFS can support enhanced and higher leukemic cells adherence than TCP *in vitro*.

5.3 APPROACH TO STUDY

The general approach was to first develop a 3D bio-mimetic scaffold that would resemble the bone marrow ECM microstructure using electrospinning. We chose PLGA, a biocompatible material approved by FDA. PLGA is the most popular polymer among various available biodegradable polymers because of its long clinical experience and favorable degradation characteristics. PLGA has shown immense potential and success as scaffolds for many tissue engineering applications particularly in those requiring structural integrity. PLGA is the copolymer of PLA and PGA, the biodegradation rate and mechanical strength can be manipulated by changing the ratio of LA and GA units. Table 5.1 shows the advantages of PLGA over PLA and PGA in different aspects of its physical and chemical properties. PLGA is made from two monomers, lactic acid and glycolic acid. The ratio of the monomers and the molecular weight determines its identity and properties. For example, the PLGA 50:50 we used for electrospinning is made of 50 percent lactic acid and 50 percent glycolic acid. According to different applications, the ratio of lactic acid and glycolic acid can be adjusted to change the

properties of PLGA to fulfill desired requirement. The tunable properties of PLGA make it a desirable material for tissue engineering.

However, the hydrophilicity of PLGA is not ideal for cell culture, so we combine it with collagen, a main ECM protein found in bone marrow, to improve its hydrophilicity and biochemical mimicry to the bone marrow ECM. We adjusted the ratios of concentration between collagen and PLGA to obtain a fine fiber within nano-scale diameter and without bead in scaffold. After the fabrication of collagen-PLGA NFS, we used SEM and FT-IR to characterize the properties of NFS. Then NFS were coated with fibronectin or E-selectin to further enhance its surface adhesive property. K562 cells and Lin⁻ cells were used as leukemic cell and HSC model for cell capture on NFS. Furthermore, captured cells were cultured on NFS for a short period (7-day) to evaluate its impact on cell proliferation and differentiation. These results were compared with TCP for cell capture and cell proliferation.

Table 5.1. Properties and applications of PGA, PLA and PLGA

Polymer	Crystallinity	T _g (°C)	Degradation Rate ^a	Typical applications
PGA	Highly crystalline (T _m =225~230°C)	35~40	2-3 months	Structure, soft anaplerosis
PLA (L form)	Semicrystalline (T _m =173~178°C)	60~65	>2 years	Fracture fixation, Ligament augmentation
PLA (R form)	Amorphous	55~60	12-16 months	Drug delivery system
PLGA	Amorphous	45~55	1-6 months ^b	Structure, Fracture fixation, Oral implant, Drug delivery microsphere

^a. Rate depends on molecular weights of the polymers.

^b. Rate may change according to the ratio of LA and GA.

5.3 RESULTS

5.3.1 Material characterization of NFS

SEM micrographs of 6%, 8% and 10% collagen-blended PLGA NFS are shown in Figure 5.1. Non-uniform structures with beading on fibers were formed at 6% collagen (Figure 5.1 A) but at the higher collagen concentrations, 8% and 10% (Figures 5.1 B and 5.1 C), no bead formation was observed. The 8% collagen blended PLGA nanofibers produced the most uniform diameter distribution with interconnected pores and a mean diameter of 284 ± 98 nm. Majority (87%) of the electrospun nanofibers fall in the range of 100-500 nm (Figure 5.1 D).

Fig 5.1 SEM images and diameter distribution of NFS. SEM images (A, B and C) and diameter distribution (D, at 8%) of collagen-blended PLGA (1:1) nanofibers with different concentration (w:v) at (A) 6%, (B) 8 and (C) 10% in HFP. Beads are showed in (A). No bead and uniform NFS are showed in (B) and (C). Around 80% NFS are in the range of 100-500nm (D). The fiber diameter of the scaffolds (n=10 fibers) was determined from the SEM micrographs using an image analysis software (Image J).

Chemical composition of collagen blended PLGA NFS was determined by FT-IR and the spectral images for a pure PLGA NFS *versus* a collagen blended PLGA NFS were taken as a comparison. The characteristic peak of pure PLGA NFS is at 1754 cm^{-1} representing the C=O bond while collagen blended PLGA

NFS have peaks of amide I and amide II bonds at 1644 cm^{-1} and 1545 cm^{-1} , and N-H bond at 3295 cm^{-1} representing the presence of a protein (collagen) in addition to the C=O bond peak at 1754 cm^{-1} from PLGA (Figure 5.2).

The water contact angle of pure PLGA NFS was $102.57^\circ \pm 3.01^\circ$ and the contact angle of collagen blended PLGA NFS was $59.13^\circ \pm 6.37^\circ$. Addition of collagen into the PLGA polymer increased the hydrophilicity of our collagen blended NFS.

Fig 5.2 FT-IR spectra of NFS. (A) Pure PLGA NFS, (B) Collagen-blended PLGA NFS. Pure PLGA shows characteristic peak at 1750 cm^{-1} which stands for the C=O bond. Collagen-blended PLGA NFS showed peaks at 1644 cm^{-1} , 1545 cm^{-1} which represent the amide I and amide II bonds.; and showed N-H stretch peak at 3295 cm^{-1} .

5.3.2 Capture efficiency of K562 cells and HSCs on different nanofibers

Erythroleukemia K562 cells showed lowest adhesion affinity and capacity to standard TCP surface, which also appeared to be time dependent, *i.e.* more cells adhered over longer exposure time (Figure 5.3 A). Both coated and uncoated electrospun NFS enhanced adherence of K562 cells (Figure 5.3 A) but only the uncoated NFS showed time-dependency in cell capture. After 2 hours of incubation, adhesion of K562 cells on uncoated NFS reached similar levels as coated NFS.

Fibronectin and E-selectin coated nanofibers had the highest degree of cell adhesion at short incubation period. After 1 hour of incubation, fibronectin and E-selectin coated nanofibers showed about 15% higher capture efficiency compared to TCP, which captured less than 5% cells. As a comparison, Lin⁻ cells representing normal hematopoietic progenitors also showed improved adhesion on NFS than TCP surfaces (Figure 5.3 B). Adhesion of Lin⁻ cells on TCP surfaces appeared to be also time-dependent but on NFS rapid capture of Lin⁻ progenitors was achieved as early as 1 hour at maximum capture efficiency (Figure 5.3 B). HSCs and leukemic cells showed different adherent ability on NFS in 1 hour capture period (Figure 5.3C). HSCs showed much higher capture percentage than leukemic cells on both NFS and TCP.

Fig 5.3 Comparison of capture percentage of UCB-HSCs and leukemic cells (K562) by different substrates. E-Selectin coated NFS (black); Fibronectin coated NFS (grey); None-coated NFS (line) ; TCP (dots). *Represents a statistic difference ($p < 0.05$). (A) Capture efficiency on different substrates for leukemic cells (K562 cell). (B) Capture efficiency on different substrates for UCB-HSCs (Lin⁻ cell). (C) Comparison of capture percentage of HSCs and leukemic cells on different substrates at 1h incubation time. (Legend: E-SL=E-selectin coated NFS; FN=fibronectin coated NFS; NON= Non-coated NFS; TCP=tissue culture polystyrene)

5.3.3 Cell morphology on NFS versus TCP surfaces

The SEM micrographs of K562 cells and Lin⁻ cells on NFS and TCP substrates revealed some morphological differences due to adhesion on different substrate types (Figure 5.4). Both K562 cells (Figure 5.4 A) and Lin⁻ progenitor cells (Figure 5.4 C) cultured for 7 days on NFS showed good adherence with cellular extensions between neighboring cells on nanofiber surfaces while those cultured on TCP substrates retained its rounded morphology (Figure 5.4 B and 5.4

D). However, the cellular extensions between cells adhering on NFS were not as prominent due to cell shrinkage during the sample dehydration procedure, which may have resulted in the disconnections in the cell-cell interaction (Figure 5.4 A). Close cell-cell interactions between Lin⁻ cells on NFS surfaces were more evident (Figure 5.4 C). Compared to the captured population on NFS, un-captured K562 cells (Figure 5.4 B) and Lin⁻ cells (Figure 5.4 D) cultured for 7 days on TCP remained singly suspended; it was also more difficult to retain these cells on TCP surfaces during sample preparation for SEM.

Fig 5.4 SEM images of captured HSCs/leukemic cells on NFS and TCP. (A) Captured leukemic cell on NFS cultured for 7 days. (B) Captured leukemic cells on TCP cultured for 7 days. (C) Captured UCB-HSCs on NFS cultured for 7 days. (D) Captured UCB-HSCs on TCP cultured for 7 days.

5.3.4 Short-term *in vitro* culture

The efficacy of NFS in supporting leukemic cell growth was evaluated over a short-term (7-day) culture and compared to the results in normal hematopoietic progenitors. Captured K562 cell population cultured on coated and uncoated NFS exhibited lower cell proliferation (2-3 fold expansion) compared to TCP (4 fold expansion) after one week culture, but no significant differences were found between coated and uncoated nanofibers (Figure 5.5). On the other hand, proliferation of Lin⁻ cells showed a significantly greater increase in cell number on coated NFS compared to TCP and uncoated nanofibers. In particular, E-selectin coated NFS yielded highest total cell number than all other substrates while fibronectin coated NFS preferentially induced CD34⁺ cell expansion. Overall matrix protein coated NFS significantly promoted growth of normal hematopoietic Lin⁻ cells *in vitro*.

Fig 5.5 Cell proliferation of leukemic cells, UCB-HSC total cells and CD34⁺ cells on different substrates cultured for 7 days. E-Selectin coated NFS (black); Fibronectin coated NFS (grey); None-coated NFS(line) ; TCP (dots). *Represents a statistic difference ($p < 0.05$).

5.3.5 Immunophenotype of captured and un-captured cells

From the FACS results, no differences were observed in the expression of integrin- $\alpha 2$ and integrin- $\alpha 5$ between captured K562 cells and un-captured K562 cells and only a slightly higher expression of integrin- $\alpha 6$ was found on captured K562 cells (72.6%) *versus* the un-captured population (63.3%) (Figure 5.6). No differences in surface markers (CD13, CD71 and GPA) of K562 cells cultured on various NFS substrates (fibronectin, E-selectin and uncoated NFS) were observed thus far between the captured and un-captured populations, even after 7 days in culture; both K562 cell populations expressed >90% CD31, CD71 and GPA markers. Typical hematopoietic morphological features of capture *versus* un-

captured K562 cells retained similar signatures (Figure 5.8 A and 5.8 B). Most of cells can be identified as lymphocytes and granulocytes. Cultured K562 cells also maintained good viability with > 90% viable cells in NFS captured and TCP surfaces (Figure 5.8C and 5.8D) under live/dead cells staining images.

For captured and un-captured Lin⁻ cells on NFS, similar differences were observed as in K562 cells. There was no difference in integrin- α 2 and integrin- α 5 expression between captured and un-captured Lin⁻ cells (Figure 5.7). However, slightly higher expression of integrin- α 6 was shown in captured Lin⁻ cells than un-captured Lin⁻ cells on NFS (11.74% vs. 5.66%) (Figure 5.7). CD34 is a typical surface marker for HSCs which represents the progenitor hematopoietic cells. It was shown that CD34 expression in captured Lin⁻ cells is higher than the un-captured Lin⁻ cells on NFS (19.57% vs 9.28%), which confirmed that NFS can capture more progenitor cells than TCP (Figure 5.3 B).

Fig 5.6 Expression of integrin families between captured and un-captured leukemic cells on NFS by FACS.

Fig 5.7 Expression of CD34/CD38 and integrin families on UCB-HSCs between captured and un-captured cells on NFS.

Fig 5.8 Morphological appearances by Giemsa stain and cell viability by live/dead staining of captured and un-captured K562 cells on NFS. (A) Captured K562 cells on NFS cultured for 7 days. (B) Un-captured K562 cells on NFS cultured for 7 days. (C) Cell viability of captured K562 cells on NFS after 7 days culture. (D) Cell viability of un-captured K562 cells after 7 days culture. Green represents live cells and red stands for dead cells. Cell identified as 1: lymphocytes; 2: granulocytes; 3: monocytes.

5.3.6 Cell cycle analysis

The NFS captured K562 cell population shifted toward G0/G1 phase after 2 days and 4 days in culture on NFS surfaces while the un-captured K562 cell population did not increase in G0/G1 phase (Figure 5.9). About 55% of the un-

captured cell population remained in active cycling (G2-S phase) while fewer and fewer cells in the captured cell population remained in active cycling (48.1% and 35.7% at day 2 and 4 respectively). An increase in the sub-G1 population was also noted in both captured and un-captured cell population.

Fig 5.9 Cell cycle analysis of captured and un-captured K562 cells on NFS at day 0, day2 and day4.

5.4 DISCUSSION

In the native hematopoietic microenvironment, adhesive properties of hematopoietic cells and their interaction with the ECM is often a requirement for cell homing and migration. Thus, the hematopoietic niche not only provides a physical support for hematopoietic cells, but is also involved in the regulation of cell function *e.g.* self-renewal and differentiation. In a traditional 2D TCP, suspended cell cultures provide limited/no cellular interaction with ECM proteins as in its natural microenvironment. Without the appropriate cues controlling cell-cell and cell-ECM interactions, HSCs often do not maintain their long-term self-renewal ability *in vitro* and will not be able to differentiate into mature cells [166, 178]. Similarly, in abnormal hematopoiesis, leukemic cells that reside within the bone marrow niches are highly dependent on surrounding stromal cells and ECM to support its survival, proliferation and differentiation. Adhesive properties and interactions of leukemic cells with its surrounding ECM are therefore important in understanding leukemia biology.

To find a suitable material for studying hematopoietic cell adhesion with ECM interactions, we exploited the utility of nanotopography and adhesive proteins. We hypothesized that electrospun NFS provide the structural component and appropriate bio-adhesive signals to induce functional cell adhesion and enhance cell-ECM interactions of leukemic cells *in vitro*. Previous studies have already demonstrated the ability of NFS in capturing and modulating cell behaviors of normal hematopoietic cells *in vitro* [175, 179]. In this study, we demonstrated

that nanotopographical cues can also be modulated to improve leukemia cell adhesion; coated and uncoated NFS showed higher cell capture efficiency compared to TCP surfaces (Figure 5.3). The blended form of a synthetic (PLGA) and natural (collagen) polymer produces good hydrophilicity and improved functional resemblance to the bone marrow microenvironment since collagen type-I is found as a principal functional and structural ECM protein in the bone marrow.

Fibronectin and E-selectin were chosen as coatings for our NFS as both are important adhesive proteins that play important roles in the bone marrow microenvironment [180]. E-selectin, expressed on bone marrow endothelial cells, are known to be responsible for adhesion and homing of hematopoietic progenitors to the bone marrow [180] while the ECM protein fibronectin was found to be involved in a plethora of functions including hematopoietic progenitor trafficking [181], cell cycle activation [182], anchorage and proliferative stimuli for hematopoietic cell differentiation [183]. Our results showed that both Lin⁻ cells and K562 cells were capable of adhesion to fibronectin and E-selectin coated nanofibers and the presence of these matrix proteins accelerated both normal and leukemic cell adhesion on NFS; K562 cells reached its maximum capture potential in 1 hour while Lin⁻ cells achieved its maximum potential in 30 minutes. A previous study conducted by Kun Ma *et al.* showed that E-selectin could significantly increase capture capacity of bone marrow HSCs on NFS compared with un-modified nanofibers and TCP [179]. Our findings are comparable to those previously reported [179, 184]. Coating of ECM proteins will not only help to improve cell adhesion but also serves to improve overall biocompatibility of the

material itself. However, another technique that can be implemented is melt electrospinning, which electrospins a polymer melt. This technique can further improve the biocompatibility of NFS and is highly favored in tissue engineering applications. Melt electrospinning produces nanofibers that are more biocompatible than the traditional solution electrospinning method as it eliminates the use of any potentially cytotoxic solvents during the process.

Proliferative behaviors of hematopoietic cells in the presence of key adhesive proteins such as fibronectin have been extensively studied on both normal and abnormal hematopoietic cells. Surface immobilized functional moieties of fibronectin namely the RGD and CS-1 peptides have shown to improve *ex vivo* expansion and engraftment of cord blood expanded CD34⁺ cells [185]. Our study showed that fibronectin coated NFS improved total cell expansion by ~2-fold compared to the control culture and non-coated NFS culture, with preferential increase in CD34⁺ cell expansion while E-selectin coated NFS favored total cell expansion but did not appear to retain CD34⁺ cells as well as fibronectin coated NFS. So far our experimental observations of hematopoietic cell proliferation in NFS has been consistent with observations made by others [186]. Improved *ex vivo* expansion can also be efficiently achieved in stroma-supported co-culture systems. The direct cell-cell contact triggers signals and pathways that influence regulatory functions as such homing and engraftment in HSCs. Stromal cells provide both biochemical and cell signals to support the culture of hematopoietic cells *in vitro*; the co-culture model is a widely used platform for *ex vivo* expansion studies.

The influence of adhesive proteins and its interaction with surface integrins can impact leukemic cell function such as proliferation. Previously, it has been shown that activation of $\alpha 5/\beta 1$ integrin mediated adhesion to fibronectin decreased proliferation of both primary CML progenitors and K562 cells *in vitro*. However the adhesion of BCR/Abl+ cells to fibronectin could also selectively protect a subpopulation from DNA damage-induced apoptosis. In this study, the captured K562 cell population adhering to coated and non-coated NFS exhibited lower cell proliferation (~50% reduction) compared to a normal suspension culture on TCP. More specifically, the lower cell numbers was likely a consequence of the cell cycle shift toward the quiescent state (G0/G1 phase). More cells moved to a non-cycling status under the influence of nanotopographical features and adhesive proteins as the shift toward quiescence was only observed in captured K562 cells but not in the un-captured cell population. However, additional adhesive protein coatings did not appear to impact proliferation/growth inhibition of K562 cells on NFS. In this case, proliferative nature of the captured K562 cell population, which preferentially adhered to nanotopographical surfaces, appeared to have an inherent growth kinetics affected by its adhesive nature to structural features and remained unaffected by the presence of adhesive proteins. These intriguing observations prompt for future studies in investigating the impact of nanotopographical features on leukemia cell preservation and drug resistance. We propose that electrospun NFS could act as an artificial niche-like platform where leukemia stem cells can be captured rapidly *in vitro* and studied in parallel to its adhesive properties. This

projects a new avenue for NFS as a tool to study correlations between biomechanical responses and leukemia cell behaviors.

The expression of integrins- $\alpha 2$ and - $\alpha 5$ is generally high on cultured K562 cells 95% on both captured and non-captured K562 cell populations. As we observed in this study and with others, high expressions of integrins do not represent functional adhesive nature especially on leukemic cells. It has also been observed that the inability of leukemic hematopoietic cells to interact with the bone marrow stroma and ECM counterparts alters their functional behaviors. Although we have observed slightly higher integrin- $\alpha 6$ expression on NFS captured cells than un-captured K562 cells, this result remained inconclusive. Surface markers of K562 cells and their hematopoietic signatures remained unchanged after 7 days in culture under NFS demonstrate that there is no impact of nanotopography on leukemia cell differentiation.

5.5 CONCLUSION

A collagen-blended-PLGA 3D bio-mimetic scaffold was successfully fabricated and characterized. The electrospun NFS showed effectively enhanced the capture of abnormal leukemia cells and normal HSCs but had a different impact in supporting cell growth. It is shown that NFS intended to restrain leukemic cells growth when K562 cells were captured by NFS. However, NFS showed to generate higher growth of Lin⁻ cells. NFS has therefore proved ability to support the normal hematopoietic cells growth but inhibit leukemic cells proliferation.

NFS is good for capturing hematopoietic cells, both normal and abnormal, however, the electrospinning NFS is limited in the sense that it can only serve as a single layer fiber scaffold. It can be used for coating surfaces of interest to improve hematopoietic cell adhesion but will not be easy to create 3D structures.

The mimicry of bone marrow microstructure by NFS is restricted by its structure, since the HSC niche is a highly vascularized structure. Chapter 6 discusses another alternative method to mimic the bone marrow structure using a bioreactor.

CHAPTER 6

Development of a Hematopoietic Co-Culture in a Perfused Hollow Fiber Bioreactor System as an in vitro Bone Marrow Model

6.1 INTRODUCTION

Hematopoiesis, the process of blood formation, requires an intricate balance in HSC self renewal and differentiation which critically depends on its specific microenvironment called the stem cell niche [94, 95, 187]. Failure in homeostasis results in leukemia where hematopoietic progenitors no longer differentiate and acquire instead an uninhibited capacity to proliferate. Though hematopoietic malfunction is often a consequence of genetic aberration, alternations in the

hematopoietic microenvironment further contributes to the progression and/or sustenance of many hematological diseases.

The importance of the microenvironment for HSC function was revealed when a mutation in the gene encoding membrane-bound SCF present in a *Sl/Sl^d* mice (steel-Dickie mice), the HSC niche was altered which eventually resulted in failure of bone marrow and HSC maintenance *in vivo* [188, 189]. This study suggests that regulatory signals involve not only direct cell- cell contact, typically between stem cells and niche cells, but also indirect interactions mediated by the ECM or cytokines [190]. Besides functional hematopoietic cells residing in the bone marrow, the niche also consists of stromal cells which synthesize growth, differentiating, and inhibiting factors, along with ECM components to provide cues and signals needed for the maintenance and differentiation of hematopoietic stem and progenitor cells [132, 191-193]. Significance of supporting stromal cells in maintaining long-term hematopoietic cultures *in vitro* was first demonstrated in a feeder-layer- based co-culture technique by Dexter *et al.*. This co-culture was established by inoculating mouse bone marrow cells onto a confluent irradiated stromal cell layer which will support hematopoietic cell culture. This technique proved to be superior in maintaining HSC stemness as opposed to cells cultured in the absence of a feeder layer or additional cytokines which terminally differentiated within the first 2 weeks of culture [194, 195] as a consequence of *in vitro* spontaneous differentiation [196]. However, static co-culture systems often suffer from decreased viability at 8–12 weeks and reduced cell proliferation over time [197]. Culture improvements such as the utility of perfusion can be made to

optimize conditions for furthering cellular growth. For large-scale expansion, bioprocess stability and scalability become yet more critical factors. This area has consequently led to the development of bioreactor systems in various configurations; perfusion chambers, stirred reactors, packed and fluidized bed reactors have been explored for *ex vivo* expansion [147, 198]. Many scientists believed that the best approach in understanding hematopoietic biology and designing an ideal *in vitro* system for hematopoiesis is to replicate the bone marrow microenvironment. HFBR was mainly used to produce expensive biochemicals such as hormones [199], growth factors [200] and monoclonal antibodies[201]. More recently, HFBR found its application in the area of tissue engineering. HFBR has been successfully used to culture a variety of cell lines, such as liver cells [202] and kidney cells [203]. Pan demonstrated that CD34⁺ progenitors were successfully cultured and transduced in HFBR without losing CD34 identity while maintaining viability [204]. To this end, our goal is to develop an *in vitro* culture system that can replicate key aspects of the cell-stroma microenvironment found in the bone marrow. The bone marrow, as depicted in Figure 6.1 A, consists of a vast vasculature of medullary sinuses surrounded by a packed density of hematopoietic and supporting cells. One method, which we believe holds much promise in replicating cellular densities and fluid dynamicity inside the bone marrow, is the HFBR. The discovery of HFBR system addressed many drawbacks in conventional static culture systems such as constraints in growth space and non-homogenous distribution of dissolved oxygen, pH, glucose, and cytokines [4]. The HFBR is a continuous perfusion system that ensures a

constant media exchange and optimized oxygen delivery (Figure 6.1 B). With a high surface area to volume ratio and 3D structural contact, it can model *in vivo* tissue conditions and replicate cell stroma and ECM interactions found in the stem cell niches (Figure 6.1B). Furthermore, this system facilitates bioprocess scalability replacing the need for hundreds of tissue culture flasks while concentrating the secreted products by hundred times or more [205, 206].

Fig 6.1 Schematic diagrams of the human bone marrow vasculature (A) (Adapted from [207]) and the co-culture technology used in the hollow fiber bioreactor system (B). Stromal cells acting as feeder layers grow in the extracapillary space outside the fiber wall. Culture medium flows inside the fiber and due to the semi-permeable nature of the fiber, nutrients and metabolic waste are allowed to diffuse across the fiber.

6.2 AIM AND HYPOTHESIS

The aim of this study is to develop a culture methodology to support co-culture of hematopoietic cells in the HFBR system. Initially, as a hematopoietic cell culture model we utilized the K562 erythroleukemia cell line. The influences

of 3D structure inside the HFBR on leukemic cell proliferation and differentiation were investigated. Since HFBR allows large number of cells culturing in a small volume, cytokine concentration secreted by stromal cells was investigated for its potential usage in normal HSCs expansion *in vitro*. The hypothesis is that HFBR provides a cell-dense 3D and bone-marrow-like microenvironment, which could promote more cell-cell and cell-ECM support, and deliver a cytokine rich environment generated by stromal cells. Normal hematopoietic cell fate decision is highly affected by cell-cell interaction and cytokine. We used the leukemia cell line as a hematopoietic model to evaluate expansion capability and impact on cell differentiation in a HFBR system. Moreover, this study will show if 3D culture environments own outstanding advantages over normal TCP culture system.

6.3 APPROACH TO STUDY

First, we evaluated long-term stromal cell culture in the HFBR for up to 28 days; the stroma culture was characterized for glucose uptake and protein production. Subsequently, the erythroleukemia cell, K562 cell line was used as a hematopoietic cell model to establish a hematopoietic co-culture in HFBR. K562 cells were introduced into the ECS of HFBR and co-cultured with stromal cells for 14 days. To assess bioprocess efficacy of the HFBR as a large-scale co-culture system for hematopoietic/ leukemic cells, the culture was evaluated for its ability to expand cells *in vitro* and support hematopoietic differentiation. The leukemic co-

culture model can also be used as an *in vitro* model to evaluate erythroleukemia cells for CML.

6.4 RESULTS

6.4.1 Growth Kinetics of HS-5 Cells

Growth kinetics of HS-5 cells on TCP culture were per normal growth *in vitro* with a steady exponential increase during the first 16 days of culture before reaching a plateau when cells are space-inhibited for further expansion. The calculated average population doubling time was 2.86 days and the total harvested cell number was 4.5×10^7 cells (Table 6.1). Growth kinetics of HS-5 cells in HFBR cannot be readily characterized, however, under the same culture volume provided in the ECS (15 mL), final cell harvest in the HFBR system achieved a significantly higher total cell number with a minimum harvest of 4.4×10^8 cells. Initial seeding density in the HFBR is also significantly higher (1.0×10^8 vs. 1.5×10^6 in TCP). This is due to the high surface area to volume ratio ($2,100 \text{ cm}^2$ in a 15mL reactor) compared to conventional TCP which enables HFBR to support large cell number expansion. However, a complete harvest was not readily achievable due to the high packing density of cells and fibers. Use of collagenase improved cell dissociation but we believe that some cells remain trapped between fibers. Besides achieving a large cell harvest, good cell viability (>95%) was accomplished at the end of 28-day culture.

Table 6.1 Comparison of HS-5 stromal cell growth in TCP and HFBR systems.

System	Surface area (cm ²)	Total cells inoculated (×10 ⁶)	Inoculum density (cells/cm ²) (×10 ⁶)	Growth period (days)	Total cells harvested (×10 ⁶)	Final cell density (cells/cm ²) (×10 ⁶)
TCP	75	1.5	2	28	45.14	60.2
HFBR	2,100	100	4.76	28	442.24	21.1

6.4.2 Glucose Consumption

No significant changes in glucose levels of the TCP culture was observed during the first 2 days but an exponential decrease began after day 2 and a final concentration plateau was reached on day 10. The final glucose concentration maintained in TCP culture was 0.95 ± 0.08 mg/mL (Figure 6.2A). In the HFBR, a more gradual decrease in glucose levels was achieved and reached steady state in 4 days (Figure 6.2A). The final concentration at bioprocess stability was 1.5 ± 0.3 mg/mL (from day 4 to 20). In comparison, average physiological blood glucose level is around 1 g/L glucose and typical cell culture formulations contain glucose of 1 g/L up to 10 g/L. Average total cell glucose consumption in TCP culture was 45 mg/day while those in the HFBR was 640 ± 220 mg/day. Daily glucose consumption in our HFBR system is considered to be under “good” conditions of a healthy cell culture per manufacturer’s recommendation (<1,000 mg/day). The focus on bioprocess monitoring should be emphasized to ensure a healthy cell culture in the bioreactor [208] as high glucose consumption induces high lactate

production; the exposure to high amounts of lactate has been reported to inhibit cell growth [209]. The advantage of perfusion in the HFBR system allows one to tune process operations in line with the needs to the culture and ensures an overall superior bioprocess stability than flask cultures.

Fig 6.2 Comparison of glucose concentration (A) and glucose consumption (B) in medium of TCP and reservoir and ECS of hollow fiber system. A steady decline in glucose concentration was seen in TCP culture. Similar trend was also observed in HFBR culture until Day 22. Glucose concentration in reservoir and ECS was relatively comparable. Cells in HFBR system consumed more glucose as compared to TCP.

6.4.3 Protein Concentrations

Protein levels in TCP stroma culture averaged at 3.60 mg/ mL while that in the HFBR averaged at 3.90 mg/mL in the reservoir and 6.40 mg/mL in the ECS (Figure 6.3 A). Protein levels in the ECS were higher than that in the reservoir indicating that some of the proteins produced by HS-5 stromal cells were retained and concentrated in the ECS. Previous research has shown that polysulfone fibers which is a hydrophobic material, readily adsorbs proteins; this will therefore affect to some extent the recovery of secreted proteins. Pre-coating polysulfone fibers with another protein such as BSA or κ -casein have shown to solve this problem. The low MWCO-20 permits only small molecules < 20 kDa to diffuse freely across the membrane. Larger proteins (> 20 kDa) produced by stromal cells in the ECS

will therefore not readily diffuse through the polysulfone membrane; these include growth factors such as IL-6 and LIF. Our protein gel (Figure 6.3 B) revealed that most of the proteins produced from HS-5 stromal cells and collected in TCP or ECS were above 20 kDa. Apparent bands between 28 and 36 kDa observed in protein samples from both cultures are likely growth factors typically produced by HS-5 such as IL-1 α and IL-1 β at around 31 kDa, M-CSF at 36.4 kDa, and glycosylated LIF which could range from 32 to 62 kDa. Strong bands at around 28 kDa and weak bands below 28 kDa are likely growth factors IL-11 or IL-6. Interleukin-11, a multifunctional cytokine which plays a key role in megakaryocyte maturation, has a predicted molecular weight of 19.3 kDa but under reducing conditions, appear on SDS gel at 24 – 28 kDa. Interleukin-6 which is a prototypic pleiotrophic cytokine is often variably glycosylated and will show as multiple bands between 22 and 27 kDa. Our Western blot analysis confirms the presence of various IL-6 monomers present in both TCP and HFBR cultures (Figure 6.3 C). Our observations suggest that exogenous growth factor production is preserved in the HFBR as produced in normal TCP cultures.

Fig 6.3 Total protein concentrations found in TCP and HFBR culture (A). Protein production started to decrease after 10 days of TCP culture whereas in HFBR culture, production of protein changes depending on its feeding rate. Gel electrophoresis of protein samples (B). Lanes 1 – 3 from TCP on Days 8, 14, and 21; Lanes 4 – 6 from HFBR on Days 8, 14, and 21. Western blot analysis of IL-6 of HFBR and TCP media samples (C). Negative controls were normal culture media (Neg 1) and K562 cell lysate (Neg 2).

6.4.4 Co-Culture of Erythroleukemia Cells

In vitro proliferation of human K562 erythroleukemia cells in TCP was first evaluated in the presence of HS-5 stromal cells and in a stroma-free culture (control). Maximum growth of K562 cells in TCP on stroma supported *versus* stroma-free culture were 43- and 52-fold, respectively, but this comparison showed no statistical difference (Figure 6.4A). Proteins secreted from stromal cells, both soluble and ECM proteins, may induce enhancing or inhibitory effects on hematopoietic and leukemia cells. In one study, conditioned media collected from HS-5 stromal culture imposed inhibitory effects on proliferation of K562 cells *in vitro* [210], while in another study, a direct contact culture improved short-term proliferation and viability of primary AML cells *in vitro* by protection from apoptosis [211]. The competing effects between secreted soluble factors and stromal contact could have resulted in a net no-change effect of myeloid leukemia cell proliferation in our TCP experiments. Stromal cell numbers remained the same after 14 days of co-culture thus the ratio of stroma to leukemic cell contact becomes limiting as K562 cell number increases. This limitation is a main disadvantage of 2D TCP cultures. In contrast, co-culture established in HFBR supported a prolonged continuous growth of K562 cells *in vitro*, reaching a fold increase of 3,130 (initial inoculum of 5×10^5 cells) after 2 weeks in culture compared to 43 folds in the TCP co-culture system (Figure 6.4 B). The average population doubling time of K562 cells cultured in the HFBR system was estimated at 29 h, which corresponds well to the normal doubling time of K562 cells of 24–30 h [212] while that observed in TCP which also averaged at 30–31 h

lasted only 1 week. Very little/slow growth was observed on week 2, as K562 cells cease to proliferate on TCP *in vitro*. This demonstrates an advantage of the HFBR system in sustaining normal and healthy cell division continuously throughout a 2-week culture period whereas in TCP culture, it is limited by an availability of space and sub-optimal physiological conditions slowed growth rates and resulted in an eventual cease of growth. Furthermore, low MWCO fibers allow the retention of many key proteins within the ECS (where K562 cells reside) while maintaining a continual exchange of nutrients and metabolites; this ensures a well balanced physiological culture environment. We believe that the retention of key soluble proteins and growth factors within the ECS significantly improved the cultivation of K562 cells *in vitro* whereas in TCP culture, these viable proteins are continuously being removed during each change of culture media. Greater stromal contact is another reason in supporting sustained cell proliferation.

Fig 6.4 Total cell expansion and cell viability of K562 cells co-cultured in the presence of HS-5 stromal cells. Cells were co-cultured in TCP and HFBR systems for 14 days; harvested K562 cells were compared with control *i.e.* suspension culture in the absence of stromal cells in TCP system (A). Expansion in HFBR is significantly higher than both TCP cultures (* for P-value<0.05). Harvested cells in HFBR co-culture were sorted into two populations: loose and adherent (B). Adherent cells showed significantly higher proliferation than loosely adherent cells (* for P-value <0.05). Cell viability for control and TCP were determined using trypan blue exclusion (C).

Harvested K562 cells from HFBR system were classified into two populations: loose and adherent. Loose cells were loosely adhering onto the HFBR co-culture, which can be removed by flushing medium in a continuous repeated

motion through the side ports of the cartridge. The adherent population was defined as cells that would only detach by the action of Accutase. In this study, adherent K562 cells made up 68% of the total cell harvest accounting for over 2,000-fold increase in cell numbers thus suggesting that stromal cell surface is the predominant site of K562 cell proliferation. Our observations are therefore in congruent with the findings of others and support the notion of cell-stromal contact enhanced leukemic survival and proliferation. Due to the large surface area to volume ratio provided in the HFBR system, cell-stromal contact is maximized. This provides not only fertile grounds for a long-term stable co-culture but also the opportunity for interrogation of stromal effects due to its biomimicry in cell densities and 3D cell–cell contacts found in the bone marrow. A manual evaluation by trypan blue exclusion showed high cell viability of >90% in both TCP and HFBR co-cultures (Figure 6.4C).

6.4.5 Morphological Observations

The cytoplasm and progressive condensation of nuclear chromatin were used as morphological evidence of cytoplasmic and nuclear maturation respectively. Early myeloid progenitors (myelocytes and myeloblasts) make up the majority of K562 cell population, and this was indicative of the K562 cell population pre-culture (Figure 6.5A). These progenitors have a diameter of about 15–20 μm with a round/ovoid nucleus and a small cytoplasm to nucleus ratio. Erythroid progenitors, namely proerythroblasts and orthochromatic erythroblasts (labeled 3 in Figure 6.5A–D), tend to have denser nuclei structure, indicating chromatin condensation.

They also tend to have more rounded nucleus with a dark to light blue cytoplasm. Monocytes can vary in appearances and sizes which can range from 20 to 40 micrometer, and often have a lobed nucleus. The appearance of monocytes was greater after 14 days in co-culture (Figure 6.5B–D). Neutrophilic granulocytes tend to have segmented nucleus (Figure 6.5B). Megakaryocytes and its early progenitors which are generally larger cells with a variable size range of 30–100mm appeared more prominent in HFBR cultures (Figure 6.5 C and D). They also have multiple nuclei and a cytoplasm full of granulation. The morphological analysis reflect the presence of monocyte, erythrocyte and megakaryocyte lineages, signifying the differentiating capacity of the K562 cell line to a diverse myeloid population was more readily achieved in the HFBR system *in vitro*.

Fig 6.5 Morphological appearances of K562 cells from day 0 to day 14. Appearances of K562 cells at the start of the culture—Day 0 (A), 14 days in 2D co-culture (B), adherent population in HFBR culture after 14 days (C), and the loose population in HFBR culture after 14 days (D). Distinct populations of myelocytes [1], myeloblasts [2], erythroblasts [3], monocytes [4], neutrophilic granulocyte [5], and megakaryocytes [6] were identified in these images. Scale bar is 50 mm.

6.4.6 Immunophenotypic Analysis

Previous studies have shown that K562 cells can exhibit both erythroid and megakaryocyte markers in absence of any inducing agent [213] and this is parallel to our results shown in Figure 6.6. Surface marker expression of K562 cells at the start of a culture, Day 0, showed high percentages of Gly-A, CD71 and CD41 positive cells. Culture of cells in TCP, with or without stroma, displayed no

considerable difference in the percentage of Gly-A (late erythroid marker) positive cells but a significant increase in CD13 positive cells was observed (Figure 6.6 A and B) in stroma supported culture. This result suggests that stroma cell contact may be responsible for proliferation and differentiation of myeloid/monocytic progenitors, and could attribute from growth factors produced by HS-5 stromal cells, such as IL-1, IL-6, G-CSF, and LIF, which showed to induce differentiation to myeloid and macrophage/monocyte lineages [214]. Besides the effects of endogenously produced factors, emerging evidence showed that cell–cell interactions, playing an essential role in HSC regulation of normal hematopoiesis, may be as equally important for stromal cell-mediated inhibition in leukemic cell differentiation [215]. A difference in CD13 expression between the loose and adherent cell population (38.09% and 99.86% respectively) found in HFBR culture (Figure 6.6C) lean toward this hypothesis. Loosely adherent cells which do not benefit from direct cell–cell contact with HS-5 stromal cells tend toward a more mature cell population which may have lost their adhesive properties. Thus a low percentage of CD13 surface expression in loosely adherent cells is preserved; their profile resembles suspension culture in TCP control (27.63%) (Figure 6.6B) but loosely adherent cells from the HFBR culture show slightly greater numbers of CD13 positive cells due to the presence of exogenously secreted growth factors supporting myeloid/monocytic proliferation. In normal hematopoiesis, bone marrow stromal cells express adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) [216] that mediate the binding of hematopoietic progenitors. This type of interaction is responsible for homing and localization of primitive

progenitors into their hematopoietic niches [217] and in maintaining HSC self-renewal properties *in vitro* [216]. As progenitors commit toward specific lineages, variable expression and functionalities of integrin and adhesive protein receptors would occur [218]. In erythroid maturation, variable integrin levels and adhesive capacities of erythroid progenitors to fibronectin were observed until a final loss of attachment at terminal erythroid differentiation [219]. An *ex vivo* study performed by Zweegman *et al.* [220] showed that stromal contact inhibited megakaryocyte maturation and non-adherent populations tend toward granulocytic and erythroid lineages. Distinction between loose and adherent cell populations is useful for evaluating the roles of cell stroma contact on hematopoietic and leukemic cell differentiation. Although this can still be studied on TCP cultures, limitations in cell–cell contact and cellular densities may impact cell fate decisions on specific cell type lineages.

In our study, direct contact culture on TCP did not alter erythroid and megakaryocytic differentiation as observed in standard suspension culture. Both TCP cultures (stroma free *vs.* stroma-supported) showed similar trends in CD41, CD71, and Gly-A expressions (Figure 6.6). A closer analysis in the subpopulations of erythroid lineages revealed that erythroid maturation is mildly induced on TCP cultures leading to a decrease in Gly-A⁺/CD71⁺ (CFU-E and pronormoblasts) by ~20% and a corresponding increase ~25% in Gly-A⁺/CD71⁺ (reticulocyte or mature erythroid cells) population. In contrast, co-culture established in the HFBR did not induce erythroid maturation, that is, the same percentages of CFU-E and pronormoblasts (Gly-A⁺/CD71⁺) at ~90% were

maintained as those observed at the start of the culture (Day 0) with very few mature erythroid cells (Gly-A⁺/CD71⁺) present. We believe that the tight cell–cell contact due to a high packing density in the HFBR system inhibited erythroid maturation. Overall, co-cultures in both TCP and HFBR favored monocytic progenitor cell proliferation and supported the survival of both erythroid and megakaryocytic cell lineages.

Fig 6.6 Surface marker expression of cells expanded in TCP and HFBR co-culture. Plots from FACS analysis of K562 cells with monoclonal antibodies directed against various hematopoietic cell surface markers (blue peaks for Day 0; orange peaks for Day 14) (A). An isotype control was included in each experiment to identify background fluorescence (red peaks). Number of cells analyzed (events) was plotted on the y-axis; intensity of staining was plotted on the x-axis. Percentage of cells expressing each marker in TCP culture (B). Percentage of cells expressing each marker in HFBR culture (C).

6.5 DISCUSSION

In this experiment, we used a CML cell line, K562 cells as a hematopoietic and leukemia cell model cultured in parallel under different culture platforms in TCP and HFBR. The main motivation was to evaluate efficacy of our novel HFBR co-culture platform in supporting hematopoietic cell cultures and its potential in *ex vivo* expansion. In this study, notable differences were observed between the two culture systems.

The first observation was the fact that HFBR supported significantly higher cell number expansion than TCP culture. Total K562 cell number harvested from HFBR was significantly higher than from TCP, demonstrating its great potential in *ex vivo* expansion strategies. Improved expansion of the K562 cell population could have attributed from enhanced direct contact with the stromal cells in collaboration with the 3D structure of HFBR that better mimic the cellular compartment of human bone marrow. Moreover, as shown in the flow cytometry results, cells harvested from HFBR and TCP had similar expression of CD41, CD42 and GPA, which were surface markers for mature erythrocyte and megakaryocyte. The HFBR culture platform showed the ability to support superior cell growth and induce multi-lineage cell differentiation.

HFBR also showed several bioprocess advantages over TCP. The volume of medium needed for TCP and HFBR makes a difference. The volume of the bioreactor cartridge is similar with the total volume of TCP. For our experiment,

we changed the medium in cartridge and TCP every 2 days with fresh medium. However, due to the large number of stromal cell seeded in the bioreactor cartridge, extra nutrients must be provided, the medium used for HFBR reservoir started from 100ml, then was increased to 200ml, and finally maintained at 400ml. Furthermore, the outstanding advantage of HFBR over TCP is large surface area (2100 cm^2 vs 75 cm^2), which means surface area in one single HFBR cartridge is equivalent to 28 flasks, but within small volume (15 mL). Although the HFBR is more bulky compared to one flask, it is much more convenient than handling 28 flasks. The HFBR is able to support a large number of cells culture in a small volume with enough medium in the reservoir to support cells. Moreover, the HFBR is a continuous culture system, which requires significantly less labor compared with same number of cells cultured in many different TCP flasks.

Unfortunately, due to the design and setup of the HFBR, it is impossible to observe the cells in HFBR under microscope. To monitor the culture, glucose concentration was taken as an indicator to monitor cell growth and cell health, which is commonly used in cell culture bioprocess [221, 222]. Our results indicate that the glucose concentration in TCP is slightly higher than in HFBR, but still in a similar range, which suggests that the HFBR provides an equally healthy culture environment as those presented in TCP culture. Yet the advantage in using the HFBR is its ability to sustain a high density culture over TCP.

In the present paper, we described the cell culture and bioprocess strategies to set up a hollow fiber bioreactor system as an *ex vivo* cell culture platform

capable of providing stromal cell support and a 3D culture environment. This protocol is intended for use hollow fiber bioreactor as a co-culture, *ex vivo* system with bone marrow mimicry properties, it is not exclusively for HSCs culture, but also can be used for various applications depends on the goal of research.

6.6 CONCLUSION

This study premieres the utility of a HFBR modeled as a bone marrow mimetic *in vitro* system for leukemic cell proliferation and differentiation. Our unique HFBR co-culture approach enables the extension of the well established Dexter's culture in process scalability and bio-mimicry. Localization of leukemic cells to stromal cells, which is significantly enhanced in the HFBR, resulted in sustained cell growth over a 2-week culture as opposed to a TCP culture where cell growth ceased after one week. Dense cellular contacts in the HFBR not only inhibited spontaneous erythroid differentiation but is likely to support better leukemic cell survival and provides closer cell physiological conditions mimicking the bone marrow. Thus we believe that the HFBR is a better *in vitro* model for testing leukemic cell survival and drug resistance than standard TCP cultures. Our data further suggest that the stromal cell surface remains the predominant site of K562 cell proliferation along with diversity in multi-lineage myeloid differentiation. With the advantage of achieving high cell densities and 3D cellular contact, the HFBR system recreates a closer mimicry of the cell-stroma

microenvironment which can provide significant improvements to *in vitro* studies of stroma-dependent leukemic and hematopoietic cell functions.

CHAPTER 7

The Hollow Fiber Bioreactor as an ex vivo Expansion Platform for Stroma-Supported, Serum-Free Human Umbilical Cord Blood Hematopoietic Stem Cell Culture

7.1 INTRODUCTION

The HSC niche has drawn great attention because of its important function in regulating HSC functions such as quiescence, self-renewal and differentiation [129, 130, 223]. The HSC niche not only provides cytokines and proteins to support HSC activities and maintain the HSC pool, but also involves functional, mirco-anatomical dimension and signaling pathways in determining HSC fate, which are linked to normal tissue homeostasis and/or dysfunction of the blood system [31, 95, 207, 224, 225]. As a key cellular composition of the HSC niche,

stromal cells are capable of supporting long-term survival of HSCs *in vitro*. Many stromal cell supported HSC co-culture systems have been developed by different groups [226-228], confirmed that stromal cells play an important role in HSC fate decision such as extensively enhancing HSC proliferation and self renewal *in vitro*. For instance, in an *ex vivo* expansion system with MSCs, direct cell-cell contact with MSCs showed to preserve the stemness of CD133⁺ hematopoietic progenitor cells [131]. Ito *et al.* also reported a higher CD34⁺ percentage in human bone marrow stromal cells supported co-culture system (8.0±0.4%) than a stroma-free culture system (4.6±0.1%) [229]. So far, stromal cell supported co-culture systems successfully provided support for HSCs, and deliver ECM productions to HSCs; but most of these stromal co-culture systems only provide 2D and monolayer culture environment, which is still different with native HSCs niche structure.

Another important aspect of the hematopoietic niche is the 3D microenvironment and ECM components that play important roles in HSC development. Several 3D culture systems were developed to represent and mimic the native 3D microenvironment found in bone marrow [228, 230, 231]. For instance, Ferreira *et al.* tested four different 3D scaffolds for cord blood (CB) HSC expansion, three of which (PCL, fibrin and collagen) supported CB CD34⁺ cell expansion and were additionally stimulated by stromal cell support, except 3D PLGA scaffold [228]. Jiang *et al.* investigated PET film surface-immobilized with peptides containing CS-1 binding motif (EILDVPST) and the RGD motif (GRGDSPC) for CB CD34⁺ cell expansion. Their results showed that CS-1 modified surface was observed as the material supporting highest cell expansion,

and all immobilized surfaces were more efficient in supporting CD34⁺ cell proliferation than non-immobilized surfaces [232]. These studies show that 3D architecture of the ECM and its respective components can improve HSC expansion *in vitro*.

Based on current clinical experience, the recommended minimum cell dose numbers for successful HSC transplantation are total nucleated cells 3.5×10^7 (TNC)/kg and 2.0×10^5 CD34⁺HSC/kg. Due to the large number of HSCs needed for transplantation (Range: $1.5\text{--}14.0 \times 10^6$ /kg) in clinical application [233, 234], large-scale *ex vivo* expansion systems have captured many researchers' interests [129, 230, 235-237]. Development of bioreactor systems like perfusion chambers [1], stirred bioreactors [2, 3], rotating wall bioreactors [4], membrane based bioreactors [5], and packed or fluidized bed reactors [6, 7] grew and were widely tested for *ex vivo* expansion of HSCs. Herein we propose the utility of a HFBR as a unique bio-mimicry and co-culture platform for HSC expansion. As described in our previous publication [238], several aspects in the design of the HFBR can replicate the native bone marrow microenvironment. Its large surface area to volume ratio and fiber architecture provides a 3D architecture for cell-cell and cell-ECM interaction. The membrane fibers in our HFBR has a 5 kDa MWCO, which is designed to retain as much cytokines produced by the stromal cells as possible on the ECS of the HFBR; secreted factors from the stroma would further support HSC expansion. Another primary advantage of our HFBR culture is that it overcomes many drawbacks in the standard tissue culture systems, such as constraints in

growth space and non-homogenous distribution of dissolved oxygen, pH, glucose, and cytokines.

7.2 AIM AND HYPOTHESIS

As described in our previous chapter, we have successfully used the HFBR as an *ex vivo* culture platform for supporting leukemia cell culture, which enhanced cell proliferation and cell differentiation. In this chapter, we demonstrated how we were able to utilize the HFBR as an artificial bone marrow system for *ex vivo* expansion cord blood HSCs. A detailed methodology for a stroma-supported serum free co-culture supporting *ex vivo* expansion of human umbilical cord blood HSCs was established and described in detailed in this chapter.

It is hypothesised that the stemness of HSCs is lost during *ex vivo* culture in TCP because of lack of 3D microenvironment. The HFBR provides a bio-mimetic, 3D structure for HSCs *in vitro*, which is believed to support better co-cultures in hematopoiesis and preserve the stemness of HSCs.

7.3 APPROACH TO STUDY

In this chapter, we describe the methods to develop a co-culture platform utilizing the HFBR for *ex vivo* expansion of UCB HSCs supported by bone marrow stromal cell line HS-5. More specifically, we were interested in evaluating the efficacy of the proposed culture platform in comparison to standard flask culture

practices that are typically used for *ex vivo* expansion in supporting HSC proliferation and preserving HSC stemness. Cell metabolites (glucose, lactate, ammonia and pH) were continuously measured for monitoring the health status of cells in culture and compared to the TCP culture. We also performed sectional studies of the HFBR culture to obtain a better view of the 3D structure formed by stromal cells in HFBR. Finally we evaluated the hematopoietic reconstitution of *ex vivo* expanded cells in mice.

7.4 RESULTS

7.4.1 *Ex vivo* culture of stromal cells in HFBR

Figure 4.3 A provides a general schematic of the HFBR showing the bundle of fibers enclosed around an outer casing. The *ex vivo* culture was established on the ECS side of the HFBR (Figure 4.3 B) where cells were introduced through the horizontal side ports of the HFBR (Figure 4.3 A). Medium is continuously perfused into the bioreactor through the intra-capillary space (Figure 4.3 B) *via* the lateral end ports.

Microscopic sections of the hollow fiber membranes (Figure 7.1 E-G) reveal that the top section in the HFBR generally had a higher packing cell density than the bottom sections. Figure 7.1 E-G provides a representative image of each section. Due to the orientation of the bioreactor when it is positioned in place during cell culture, cells that settled around the outer/lower sections during

initiation of the culture will have a greater tendency to fall to the bottom of the bioreactor due to gravity and will not attach onto the fibers at all. The unattached cells would eventually die and this would explain for the poorer cell viability observed in the suspension population during the start of the culture. Modifications in the design of the HFBR and/or properties of the membrane fibers can be made to improve cell attachment at the start of the culture. Pre-coating of membrane fibers in serum during the priming step may have been useful in promoting cell attachment but is insufficient to ensure homogeneity and efficacy of the cell attachment.

Electron micrographs of HS-5 stromal cells covering the membrane fibers (Figure 7.1 A-D) clearly show a healthy production of extracellular matrices surrounding the fibers. In areas of high cell density, the formation 3D cell-matrix architecture resulted (Figures 7.1 C and D) and this is generally not observed in cultures on 2D flasks. The SEM images provide evidences that the HFBR is conducive for the formation of a 3D microenvironment supporting HSCs. The density and complexity of this 3D architecture is a better mimic of the natural bone marrow microenvironment than cultures in 2D flasks. Therefore, the HFBR has a great potential as an *in vitro* bone marrow model for producing and/or studying hematopoiesis.

Fig 7.1 SEM and cross-section images of HFBR cartridge. (A-D) SEM images of fiber from HFBR cultured with stromal HS-5 cells. (E-G) Cross section images of HFBR cartridge from top, middle and bottom.

7.4.2 HFBR supported more progenitor cell proliferation compared to TCP

Cells harvested from TCP and the HFBR were distinguished as two populations: adherent and suspension cells. In the adherent hematopoietic

population, the total number of cells harvested from the HFBR and TCP were similar, 2.40×10^7 and 2.36×10^7 respectively (Figure 7.2 A). However, in the suspension population, a higher number of hematopoietic cells were harvested from the HFBR (2.78×10^7) than TCP (1.14×10^7) (Figure 7.2 A). Non-stroma supported *ex vivo* culture (the control) produced the least number of cells. Compared to this control culture, co-culture in HFBR produced 9.33 fold more cells and TCP produced 6.31 fold more cells respectively. Overall, cell viability was $> 80.00\%$ for all cell populations in the TCP and HFBR, the only cell population with slightly inferior cell viability were the suspension population in the HFBR (76.58%).

The number of CD34⁺ cells in each cell population (Figure 7.2 B) was enumerated based on FACS data (Figure 7.3). In the TCP co-culture, only 1.27% of the cells were stained CD34⁺ (Figure 7.3) while in the HFBR culture, 15% of the cells were found to be CD34⁺ (Figure 7.3). Thus, the total number of adherent CD34⁺ cells harvested from the HFBR (3.60×10^6) was in fact 12 fold higher than in TCP (3.00×10^5) (Figure 7.2 B). On the other hand, in the suspension cell populations, the TCP culture contained 17.07% CD34⁺ cells (Figure 7.3) while the HFBR only had 6.25% CD34⁺ cells (Figure 7.3), approximating to 1.95×10^6 (TCP) and 1.74×10^6 (HFBR) CD34⁺ cells respectively. In total, the HFBR still produced more CD34⁺ cells (5.34×10^6) than culture in TCP (2.25×10^6) and the non-stroma supported Lin⁻ *ex vivo* culture in TCP expanded the least number of CD34⁺ cells (6.07×10^5) (Figure 7.2 B). As observed from the FACS data, more CD34⁺ cells were harvested from the suspension cell population cultured in TCP whereas in the

HFBR, more CD34⁺ cells were harvested from the adherent cell population. It can also be deduced that the total number of CD34⁺CD38⁻ progenitors was greater in the HFBR culture than TCP culture.

Fig 7.2 Total cells and CD34⁺ cells harvested HFBR and TCP. (A) Total cells harvested from HFBR and TCP. (B) CD34⁺ cells harvested from HFBR and TCP. The cells harvested can be classified into two classes: suspension cells which do not have interaction with stromal cells; adherent cells which have strong interaction with stromal cells, can only be collected by the action of Accutase.

Fig 7.3 CD34/CD38 expression on cell harvested from HFBR and TCP.

7.4.3 Phenotypic expressions of cells expanded in HFBR retained higher clonogenic ability

Under stroma-free conditions, the control group produced a total number of 87.5 colonies/ 10^4 cells (Table 7.1). Adherent hematopoietic cells harvested from the HFBR produced 1.63 times more colonies while adherent hematopoietic cells from stroma-supported TCP formed fewer colonies, 0.75 times of the total colonies compared to the control group. More specifically, adherent cells from the HFBR

culture showed significantly higher number of colonies of the erythroid lineage, *i.e.* BFU-E and CFU-E (Figure 7.4). The CFU results revealed that *ex vivo* expanded cells in HFBR have better differentiation tendencies toward erythroid lineages compared to expanded cells in TCP with or without stroma support.

Table 7.1 CFU assay results for expanded cells from HFBR and TCP.

	CFU-GEMM	CFU-GM	BFU-E	CFU-E
Lin ⁻ cells	22.5±3	37.5±7	28±8	5.5±1
expanded Lin ⁻ cells	34±6	25.5±7	16±2	12±10
TCP adherent cells	15.5±5	35±8	11.5±3	4±4
HF adherent cells	22±8	24.5±9	58±6	38±9

Fig 7.4 CFU assay results for expanded cells from HFBR and TCP.

7.4.4 Equivalent reconstitution of humanized mice was achieved by expanded cells from HFBR and TCP

To determine the reconstitution abilities of expanded cells harvested from TCP and HFBR, mice were reconstituted with expanded cells, and cells were analyzed by flow cytometry after 8 weeks. The results obtained showed that the frequencies of human CD45 in TCP and HFBR groups mice were equivalent (0.21% vs 0.29%) (Figure 7.5). Furthermore, the absolute number of cells from HFBR were markedly higher than cells harvested from TCP, which means that improving reconstitution of human-blood cell lineages can be achieved by one HFBR without usage of dozens of TCP.

Fig 7.5 Human CD45/mouse CD45.1 expression in humanized mice bone marrow cells after reconstitution by expanded cells from HFBR and TCP. Mononuclear cells were isolated from murine bone marrow for FACS analysis 8 weeks later after injection of cells harvested from TCP and HFBR.

7.4.5 Secreted cytokine concentrations were intensified under serum free conditions in HFBR

In the presence of serum, HS-5 cells cultured in the HFBR did not differ significantly in cytokine profile than those in TCP culture; in fact TCP culture showed higher relative concentrations on most cytokines, except for sICAM-a, IL-8, IL-10, MIF and IL-27 (Figure 7.6 A). But both cultures produced the same cytokine signatures, *i.e.* cytokines that were identified in the TCP culture were also identified in the HFBR. Higher relative concentrations of multiple cytokines were observed in the stromal cells cultured under SFM in the HFBR than other conditioned mediums in HFBR and TCP (Figure 7.6 C); increase relative

concentration in C5/C5a, IL-1a, IL-1b, IP-10, and RANTES were noted (Figure 7.6). However, when Lin⁻ cells were introduced into the *ex vivo* culture in the HFBR, the relative intensity of all cytokine signals decreased significantly (Figure 7.6 D). The change in relative cytokine concentrations may be due to an active uptake of cytokines during Lin⁻ cells large-scale expansion and differentiation.

Fig 7.6 Cytokine array of conditioned medium from HFBR and TCP. (A) DMEM+10% FBS from TCP. (B) DMEM+10% FBS from HFBR. (C) Serum free medium from HFBR. (D) Serum free medium after co-culture of Lin⁻ cells in HFBR. (E) Cytokine array results for conditioned mediums from HFBR and TCP.

7.4.6 Metabolite profile in the HFBR as compared to TCP culture

Measurements of glucose, lactate, pH and ammonia were taken every 2-3 days during the cell culture from the reservoir bottle, which is used to monitor cell growth and culture wellness in both HFBR and TCP. Monitoring of metabolites is an important step in bioprocessing to ensure that metabolic by-products such as lactate and ammonia do not reach levels that become toxic to the cells [239]. Moreover, metabolic profiling provides an indication of cell metabolism [240] and

can potentially be used as a method for monitoring cell growth/viable cell density [241, 242].

7.4.6.1 HFBR showed lower glucose concentration than TCP

Glucose values that were representative of our HFBR culture subjected to serum dilution are shown in Figure 7.7 and compared to the culture on standard 2D TCP. Glucose concentration showed a more gradual decrease than levels observed in TCP culture, and reached steady state in 4 days (Figure 7.7 A). Glucose concentrations in the HFBR culture were generally more stable than the TCP culture, showing less variation on a day-to-day basis. The average glucose consumption rates under different serum conditions averaged between 3.5-6.5 g/L per 1×10^6 cells (Figure 7.7 B). Despite changes in serum conditions, glucose consumption remained relatively stable throughout the culture. Glucose consumption rate in 10% FBS media were slightly lower and had a higher standard deviation due to the influence of the lag phase during the start of the culture. The daily glucose consumption under different serum conditions in our HFBR system is considered to be under “good” conditions of a healthy cell culture (<1,000 mg/day per manufacturer’s recommendation) (data not shown).

Cumulative glucose consumption concentration was calculated with initial glucose concentration in both TCP and HFBR (Figure 7.7 C). HFBR showed slightly higher cumulative glucose consumption concentration than TCP. HFBR and TCP showed a continuous increase of cumulative glucose consumption

concentration during the 32-day cell culture period, which indicated that HFBR provided a safe cell growth environment as TCP culture.

Fig 7.7 Glucose concentration and metabolite analysis in HFBR and TCP. (A) Glucose concentration measurements in HFBR and TCP. (B) Specific glucose daily consumption rate during serum dilution process and co-culture period in HFBR and TCP. (C) Cumulative glucose consumption concentration in HFBR and TCP.

7.4.6.2 Similar lactate concentration was observed in HFBR as in TCP

Besides glucose concentration, lactate concentration was monitored to ensure that the cell growth environment in HFBR remained in a healthy condition. Although large glucose consumption was observed in the HFBR, lactate concentration was in similar range for the HFBR and TCP; lower lactate levels were seen (1.219 mM) in HFBR as compared to TCP culture (1.277 mM) (Figure 7.8 A); lactate is a by-product of glucose cellular metabolism, which has been shown to inhibit cell growth at high concentration [37,38]. The safe and healthy environment in HFBR is guaranteed by lactate concentration data.

Specific daily lactate production rate was calculated during serum dilution in both TCP and HFBR. Specific daily lactate production rate in HFBR was lower than in TCP during serum dilution process (Figure 7.8 B). HFBR showed increasing specific daily lactate production rate at first 14 days and reached stable specific daily lactate production rate. For TCP culture, specific daily lactate production rate was stable during serum dilution process.

HFBR and TCP showed similar cumulative lactate production. Cumulative lactate production in TCP was slightly higher than in HFBR (Figure 7.8 C), which confirmed that HFBR provided a safe microenvironment for cell growth.

Fig 7.8 Lactate concentration and metabolite analysis in HFBR and TCP. (A) Lactate concentration measurements in HFBR and TCP. (B) Specific lactate daily production rate during serum dilution process and co-culture period in HFBR and TCP. (C) Cumulative lactate production in HFBR and TCP.

7.4.6.3 HFBR and TCP showed similar ammonia concentrations

Ammonia concentrations in the HFBR were comparable to TCP culture (Figure 7.9 A). Ammonia concentrations in both TCP and HFBR showed a slow increase in first 12 days, and retained stable in following period. Specific ammonia production rate in TCP was stable and did not show any difference in lag phase of cell growth (Figure 7.9 B). There were outstanding differences for 10% FBS medium in HFBR due to lag phase of cell growth. Similar with cumulative glucose consumption concentration, cumulative ammonia production in both culture

systems showed almost the same results (Figure 7.9 C). Kinetic analysis of both HFBR and TCP proved that cell growth in HFBR was as healthy as in TCP.

Fig 7.9 Ammonia concentration and metabolite analysis in HFBR and TCP. (A) Ammonia concentration measurements in HFBR and TCP. (B) Specific ammonia daily production rate during serum dilution process and co-culture period in HFBR and TCP. (C) Cumulative ammonia production rate in HFBR and TCP.

7.4.6.4 Comparable pH values were observed in HFBR and TCP

The pH value is an important indicator for monitoring cell culture environment. Too high/ low pH conditions in the medium will be toxic to cells in culture. The pH samples were taken from the HFBR reservoir and TCP. The pH readings in both HFBR and TCP were in a similar range (7.0 - 7.4), except first 2 days (Figure 7. 10). The stable pH values ensured our HFBR system a healthy cell culture system and an overall superior bioprocess stability than TCP cultures.

Fig 7.10 PH measurements in HFBR and TCP. Similar pH values were observed in HFBR and TCP during serum dilution process and co-culture period.

7.5 DISCUSSION

In this study, isolated Lin⁻ cells from a single cord blood unit were split into two fractions and cultured in parallel under different culture platforms for a direct comparison to evaluate the efficacy of our proposed HFBR co-culture platform. We reported the notable differences that were observed between the two culture systems. The first observation was the fact that HFBR supported higher total nucleated cell and CD34⁺ progenitor cell expansion than TCP. Improved cell expansion of the primitive hematopoietic population could have attributed from the 3D architecture that the membrane fibers and stromal cells collaboratively created (Figure 7.1). We hypothesize that this unique bio-artificial architecture better mimics the cellular compartment of human bone marrow thus stimulating the growth of HSCs. Expanded HSCs in the HFBR preserved better clonogenic ability than its TCP counterparts suggesting the presence of more primitive hematopoietic cell populations. The flow cytometry data further supported these observations in showing that cells harvested from HFBR and TCP co-culture had lower expression of the committed erythrocyte and megakaryocyte markers (CD41, CD42 and GPA) (data not shown). Furthermore, the results from the *in vivo* reconstitution of humanized mice showed equivalent reconstitution ability of expanded cells from HFBR and TCP. There was elevated number of cells harvested from HFBR, which made HFBR preserve higher potential in clinical application for human patients who request higher number of cells for transplantation.

From a bioprocess standpoint, the HFBR provides several advantages over an *ex vivo* culture in TCP. Perfusion of medium allows continuous exchange of nutrients and metabolites to occur inside the bioreactor. This feature provides a more controlled and homogenous cell culture environment that will ensure healthy cell growth throughout the culture period. The tendency for extreme culture conditions (*e.g.* starvation or toxicity) is reduced. In our current culture procedure, the average lactate levels in the HFBR *ex vivo* culture were found to be similar as in TCP culture. Perfusion by way of dilution distributes the concentration gradients generated in the cell culture may have resulted in an overall similar lactate concentration with more efficient glucose metabolism. Periodic feeding in the HFBR is accomplished via a change of media in the reservoir bottle while the cell culture remains enclosed inside the bioreactor. Reduced human exposure and exposure to the external environment will help to minimize the risk of contamination. The total volume of medium required to support the same number of cells (*e.g.* at a starting cell density of 1×10^8) in TCP versus the HFBR would actually be the same (~400 mL) but the total amount of space inside the incubator taken up by standard flask cultures would be significantly greater. The amount of labor required to change all the culture flasks routinely would also be significant as compared to running a HFBR operation.

Previous works utilizing the HFBR for culturing hematopoietic cells have been reported. In an earlier work, Malone *et al.* expanded human T-lymphocytes for clinical use in immunotherapy using a Cellco HFBR and was able to reach therapeutic doses of $\sim 3 \times 10^{10}$ cells in about 3 months [243]. In a recent study, a

group reported the design of a novel multi-component HFBR for expansion and differentiation of HSCs into red blood cells [5]. This group demonstrated that they were able to achieve 14,288 fold expansion with 40% of the cells expressing committed erythroid lineage (GPA-positive) and enucleation. Both of these works strongly support the feasibility of HFBR for large-scale cell expansion. However, so far most studies reported expansion of a single cell population. Our group is the first to report the expansion of HSCs under a direct contact co-culture system in the HFBR. The uniqueness of this approach is the recreation of a bio-artificial 3D architecture provided by the fibers and stromal cells along with better retention of soluble proteins produced by the stromal cells. Collectively, these features provide a more bone marrow mimetic culture environment.

During the pursuit of our study, minor issues that arose were the lower cell viability and accumulation of dead cell debris at the start of the culture. Incomplete cell attachment during the initiation of the stromal cell culture resulted in excess cell debris. This was routinely removed during the first few days of culture via flushing new medium into the ECS. However, further developments in the design of the bioreactor such as improvement in fiber properties can be made to improve stromal cell attachment. Due to the limitations in the current design of HFBR, it is also not possible to routinely observe cells during the culture under a microscope. Routine cell harvest from the HFBR is not recommend for the *ex vivo* culture. Thus metabolite concentrations, such as glucose and lactate, were taken as indicators to monitor the cell status; this is widely used in many cell culture bioprocesses [221, 222]. Other metabolites that are commonly monitored include glutamine, glutamate

and ammonia. Routine monitoring of metabolites is useful to maintain a good physiochemical balance of the cell culture. Excessive accumulation of metabolite products such as lactate can result in depressed cell growth [244, 245].

Our study showed the feasibility of HFBR as a bone marrow mimetic *in vitro* system for expansion of HSCs. The results obtained from our laboratory thus far have shown promising avenues in the utility of the HFBR as an *ex vivo* system for HSC expansion with improved performance over standard cultures on TCP. HFBR supported extensive expansion of HSCs while preserved the differentiation ability of HSCs. The interaction between HSCs and stromal cells is significantly enhanced in HFBR, which covered the deficiency of TCP as a 2D monolayer culture system. Continued longer term studies utilizing this system will be pursued in the near future to evaluate its efficacy in supporting long-term cultures for both HSC expansion and directed differentiation. Our data supports the HFBR system as highly beneficial for large-scale *ex vivo* expansion and can simultaneously be tailored to induce or inhibit differentiation of HSCs, depending on the desired application. These findings open up the feasibility of using HFBR as a good *in vitro* mimicry of human bone marrow for large scale expansion of HSCs.

7.6 CONCLUSION

Our study showed the feasibility of utilizing the HFBR as a bone marrow mimetic *in vitro* system for expansion of HSCs. The HFBR is able to support large scale cell culture in a small volume reactor and negates the need for using a large

number of TCP flasks for scale up of cell culture. HFBR supported extensive expansion of HSCs while preserving the differentiation ability of HSCs. The interaction between HSCs and stromal cells is significantly enhanced in HFBR, which compensate for the deficiency in TCP as a 2D monolayer culture system. The HFBR system recreates a closer mimicry of cell-stroma microenvironment, which can provide significant improvements to *in vitro* studies of stroma-dependent hematopoietic cell functions. Our data supports the HFBR system as highly beneficial for large-scale *ex vivo* expansion and can simultaneously be tailored to induce or inhibit differentiation of HSCs, depending on the desired application. These findings open up the feasibility of using HFBR as a good *in vitro* mimicry of human bone marrow for large scale expansion of HSCs.

CHAPTER 8

Overall Conclusion and Future Work

8.1 OVER ALL CONCLUSION

The main goal in this thesis is to develop *ex vivo* systems for large-scale expansion of HSCs from cord blood and controlled differentiation of HSCs to certain lineage. Overall conclusions as a result of the efforts toward this goal are summarized in the following sections.

8.1.1 NFS for capturing adherent normal/abnormal hematopoietic cells

In this study, a collagen-blended-PLGA 3D bio-mimic scaffold, electrospinning NFS was successfully fabricated and characterised for capture leukemic cells and HSCs, and NFS was tested for supporting the proliferation of leukemic cells and HSCs. Our results demonstrate the ability of nanotopographical surfaces in enhancing leukemic cell adhesion *in vitro*, capturing functionally adherent leukemic cells more effectively than TCP surface. Nanotopographical

features showed a direct impact on cell function suggesting that an important role may be played in mechanobiology influenced by adhesion on different structural components. The NFS used in this study showed different results in supporting cell growth. It is shown that NFS could restrain leukemic cell growth when K562 cells were captured by NFS. However, NFS showed to generate higher growth of Lin⁺ cells. NFS has therefore proved ability to support the normal hematopoietic cell growth but inhibit leukemic cell proliferation.

NFS were proven with good biocompatibility, large surface-to-volume ratio, high porosity and flexibility of loading growth factors or drugs. Electrospun collagen-blended PLGA nanofiber scaffold represented a simplified way to recapitulate complex architecture of the BM niche: a collagenous matrix with ECM proteins, which allows to study pathophysiological and physiological mechanisms in the bone marrow niche. The NFS served as a *ex vivo* system of bone marrow niche allows studying mechanisms of normal and abnormal hematopoiesis in a relevant environment. Thus, electrospun NFS can serve as excellent and highly biocompatible substrates providing 3D niche-like structure of the ECM in the nanoscale, which can serve as a unique *in vitro* platform to investigate aberrant cell behaviors in hematopoiesis. Furthermore, it has the potential as a useful platform for drug screening to test new molecules for the pharmacological treatment of blood disorders.

8.1.2 HFBR for co-culture of stromal cells and leukemic cells

In this study, it is hypothesized that HFBR can support co-culture of leukemic cells and stromal cells in 3D bio-mimetic environment. This study presented the utility of a HFBR modeled as a bone marrow mimetic *in vitro* system for leukemic cell proliferation and differentiation. HFBR supported a extensive growth of stromal cells in 28 days culture period, which formed a true 3D structure together with ECM protein produced by stromal cells inside the ECS of the HFBR. Subsequently, cell-cell and cell-ECM interaction between leukemic cells and stromal cells were significantly enhanced in the HFBR, which results in a sustained cell growth in 2 weeks culture period, while the cell growth in 2D culture was limited by space of TCP and ceased after one week. HFBR supported extensive growth of leukemic cells, provided dense cellular contacts in the HFBR and not only inhibited spontaneous erythroid differentiation but supported better leukemic cell survival, thus potentially providing a closer cell physiological conditions mimicking the bone marrow. HFBR can provide a better *in vitro* model for testing leukemic cell and drug resistance than TCP. HFBR system recreates a closer mimicry of the cell-stroma microenvironment which can provide significant improvements to *in vitro* studies of stroma-dependent leukemic and hematopoietic cell functions.

LSCs have similar behavior with normal HSCs, which share the same surface markers with HSCs and reside in bone marrow niche. However, detailed cellular and molecular mechanisms for LSCs have not been elucidated because of

the low frequency of LSCs. Our study have showed that HFBR supported a extensive proliferation of leukemic cells and promoted progenitor leukemic cells growth and inhibited further cell differentiation. HFBR provides a useful *in vitro* platform for research directed towards investigation of LSCs and of the difference between LSCs and HSCs as well as for the development of novel therapeutics for leukemia.

8.1.3 HFBR for *ex vivo* expansion of UCB-HSCs

In this study, It is hypothesised that the HFBR as an artificial bone marrow system provides a bio-mimetic, 3D structure for HSCs *in vitro*, which is believed to support HSCs proliferation and preserve the stemness of HSCs. It is demonstrated that HFBR reached a large scale cell culture with 3D structure in a limited volume, which represented and mimicked native bone marrow structure *in vitro*. This unique 3D structure of HFBR supported extensive expansion of HSCs while preserving the differentiation ability of HSCs, which was confirmed by the results of CFU assay and *in vivo* reconstitution of mice. HFBR showed the potential as highly beneficial for large-scale *ex vivo* expansion, and HFBR can simultaneously be tailored to induce or inhibit differentiation of HSCs, depending on the desired application. These findings open up the feasibility of using HFBR as a good *in vitro* mimicry of human bone marrow for large scale of HSC culture for clinical applications. Moreover, the HFBR shows the potential as a useful tool for *in vitro* hematopoietic studies.

HFBR is a stable and valuable *ex vivo* system for the study of the mechanisms controlling hematopoiesis, which enables the study of the dynamics of HSCs proliferation and differentiation. HFBR works as a tightly controlled and closely monitored *ex vivo* HSCs culture platform which brings a deeper and broader insight into the influence of exogenous input on HSC expansion and commitment. The close mimicry of bone marrow niche constitutes a major technical challenge that still remains largely undeveloped, HFBR has great potential to closely replicate the complex bone marrow architecture as *in vitro* bone marrow model. Furthermore, HFBR can be used as pharmacological study tool for blood disease drug screening.

8.2 FUTURE WORK

Experimental studies performed with NFS and HFBR for capture and expansion of HSCs and leukemia cells have demonstrated the efficiency of NFS for capturing HSCs and leukemia cells and support of HFBR for expansion of HSCs and leukemia cells. NFS and HFBR show potential in more applications. The following sections will discuss possible research directions toward achieving better control of HFBR and NFS bioprocess for either *ex vivo* expansion or cell capture.

8.2.1 Future development for NFS

LSCs exist in the bone marrow as a small and rare population possessing self-renewal and proliferation ability like normal HSCs. However, no easy method

thus far is able to separate this LSC population from the leukemic cells or bone marrow cells. It is found out in our study that NFS with certain surface modification showed good ability to capture normal HSCs and leukemia cells. This shows the potential for NFS to be used as a direct separation and culture tool for HSC capture and proliferation. Thus NFS can be developed into a membrane bioreactor which allow the cord blood pass through these membranes, NFS capture HSCs in the cord blood, and mature cells without adherent properties will pass these membranes directly. Then HSCs trapped in NFS can be cultured directly without the magnetic separation process. Separation of HSCs from cord blood using magnetic beads takes 4-5 hours per time. Compared to magnetic separation, it forms a straight forward and time-saving bioprocess for HSC capture and expansion. At the same, this design can be also used for LSCs capture and separate from leukemic cells for further LSCs studies to get better understanding of LSCs.

Different surface markers and cell cycle properties were identified between captured cells and un-captured cells on NFS for both HSCs and leukemic cells in our study. The captured cells on NFS showed inhibited proliferation of leukemic cells compared to un-captured cells. Different sub-populations of the leukemic cells can therefore be captured using NFS potentially for drug tests between captured and un-captured cells.

8.2.2 Future development for HFBR

The HFBR formed a 3D bio-mimetic stroma-rich architecture and has showed successful results at supporting UCB-HSC expansion and leukemia cell

proliferation. With stromal cells and cytokine cocktail support, the HFBR promoted HSC proliferation and inhibited the differentiation of HSCs to generate a large number of expanded HSCs. This bioreactor can be used as a single unit and closed platform for large-scale expansion of HSCs in a clinical setting. Furthermore, the HFBR can be used for differentiation of HSCs toward commitment to certain desired lineages. In this case, the HFBR can be used for mature blood cell manufacture extending to many different clinical applications.

With the special structure of HFBR, it formed a close-mimicry of bone marrow vascularized microenvironment with stromal cells supporting. Using this model, we can develop an *in vitro* bone marrow model to study the cell-cell and cell-ECM interaction between HSCs/leukemic cells and bone marrow structure, which will provide insight of normal/abnormal hematopoietic process in the human body. This will offer us a better way toward understanding the bone marrow niche for future research development. More work in characterizing cellular interactions realized within the HFBR platform is necessary to validate its resemblance to the native bone marrow microenvironment.

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