University of Alberta

IN VITRO HEMATOPOIETIC STEM/PROGENITOR CELL PROLIFERATION AND LABELING

by

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ABSTRACT

Hematopoietic stem/progenitor cells (HSPC) play main role in constituting the whole hematopoietic system. Furthermore, since recognized in 1960s, HSPC are utilized to protect patients from severe chemo and radio therapy. As time goes, they are also used to treat hematopoietic disorders such as leukemia. Bone marrow, peripheral blood and umbilical cord blood are now the three sources of HSPC. Umbilical cord blood seems optimal because it is easy to obtain, no risk to graft donor and low probability of infection transmission. However, low number of HSPCs in umbilical cord blood is the main limitation. My research focuses mainly on *in-vitro* proliferation of HSPCs. In addition, I also worked on labeling HSPC *in-vitro* for tracking these cells after transplantation. The experimental results indicated that HSPCs are effectively labeled and their proliferation rate is significantly enhanced *in-vitro*.

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LIST OF ABBREVIATION

HSPC	Hematopoietic stem/progenitor cells
HSC	hematopoietic stem cells
CFU-S	colony-forming units in the spleen
FACS	fluorescence-activated cell sorting
G-CSF	granulocyte colony-stimulating factors
CB	umbilical cord blood
CD	cluster of differentiation
PROML 1	prominin 5 transmenbrane glycoproteions
KDR	Vascular growth factor receptor
MACS	Magnetic-activated cell sorting
PVP	polyvinylpyrrolidone
Ang1	angiopoietin-1
Opn	Osteopontin
PI3K	phosphatidylinositol-3-OH kinase
Pten	Phosphatase and tensin homolog
BFU-E	burst-forming units–erythroid
CFU-E	colony-forming unit-erythroid
CFU-GM	colony-forming unit-granulocyte, macrophage
CFU-G	colony-forming unit-granulocyte
CFU-M	colony-forming unit-macrophage
CFU-GEMM megakaryocyte	colony-forming unit-granulocyte, erythroid, macrophage,
CLP	common lymphoid progenitors
CMP	common myeloid progenitors
Eo-CFC	eosinophil–colony-forming cells

G-CFC	granulocyte–colony-forming cells
GM-CFC	granulocyte macrophage–colony-forming cells
GMP	granulocyte-macrophage progenitors
M-CFC	macrophage–colony-forming cells
Mast-CFC	mast–colony-forming cells
Meg-CFC	megakaryocyte–colony-forming cells
MEP	megakaryocyte-erythroid progenitors
HSCT	Hematopoietic stem cell transplantation
UCBT	umbilical cord blood transplantation
FITC	fluorescein-isothiocyanate
BM	bone-marrow
PB	peripheral blood
Flt3L	Flt-3 ligand
SCF	stem cell factor
TPO	thrombopoietin
HIFU	high intensity focused ultrasound
LIPUS	low intensity pulsed ultrasound
TGF-β1	transforming growth factor-beta 1
MSC	mesenchymal stem cells
LP	leukapheresis product
MNC	Mononuclear cells
IMDM	Isceve's Modified Dulbecco's medium
BGS	bovine growth serum
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxhenyl)-2 -(4sulfophenyl)-2H-tetrazolium
CNT	carbon nanotubes

mCNT	magnetic carbon nanotubes
NIR	near-infrared
MRI	Magnetic resonance imaging
XPS	x-ray photoelectron spectroscopy
ATCC	American Type Culture Collection
DMEM	Dulbecco's Modified Eagle Medium
FBS	fetal bovine serum

CHAPTER 1 :LITERATURE STUDY OF HEMATOPOIETIC STEM CELLS

1. Introduction

Hematopoiesis is a remarkable self-regulated production of all types of blood cells which occurs in the yolk sack prenatally, then liver, and eventually the bone marrow. In normal adults, hematopoiesis is maintained homeostatically by multipotent hematopoietic stem cells (HSC). All mature blood cells turn over on a daily basis in normal adult. Therefore, in order to keep blood cell homeostasis, HSC are constantly generated in the bone marrow where they can produce multipotent progenitors and lineage-committed progenitors that ultimately differentiate into all types of blood cells (1, 2). As Figure 1.1 shows, the hematopoietic system is organized hierarchically with a series of cell populations arranged in rank from stem cells. As the study of HSC gets mature, people realized that HSC have tremendous biomedical application potentials for the treatment of hematological disorders and cancers (3, 4). In this study, I would to first discuss the properties and potential clinical usage of HSC.

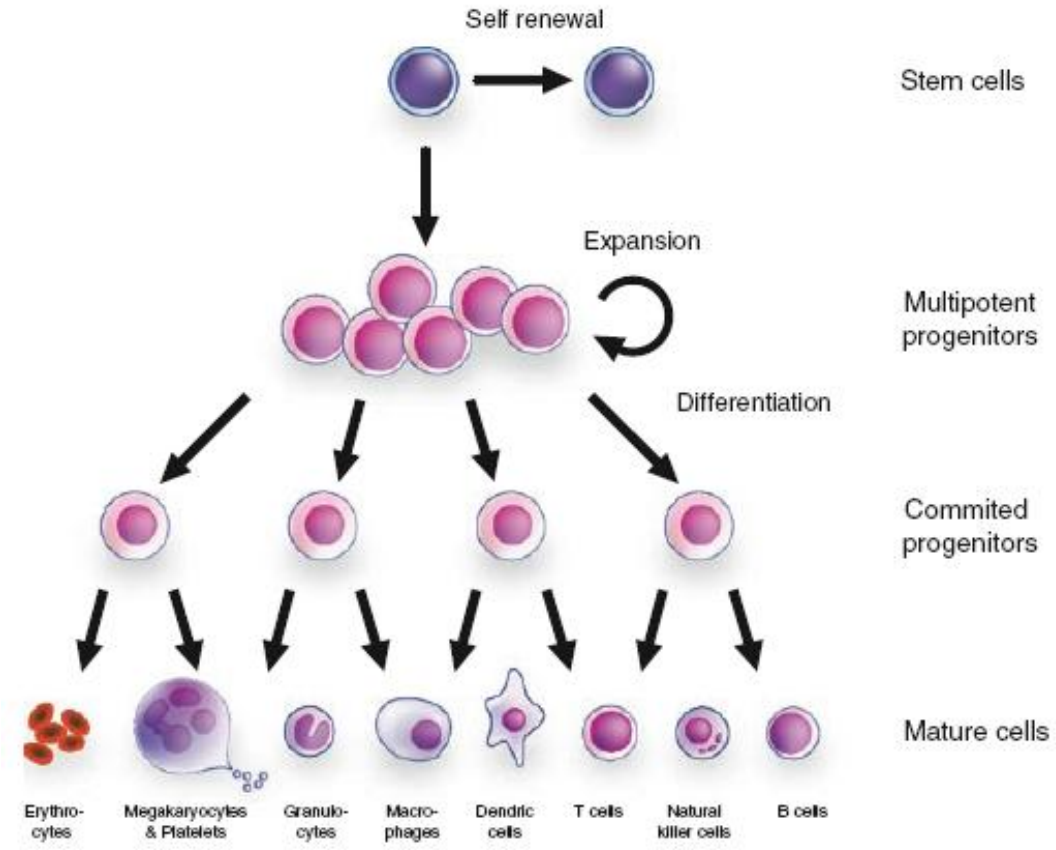


Figure 1.1 The hematopoietic system (5).

2. The definition and identification of HSC

2.1 Stem cell definition and classification

Stem cells are unspecialized cells with capacities of self-renewal and differentiation. Self-renewal refers to generate one or two daughter cells identical to their parental cells via cell division in order to maintain an undifferentiated stem cell pool(6). Differentiation refers to a process of generating more specialized cells to perform particular roles within tissues (1).

This definition was initially framed by J. E. Till and E. A.McCulloch, based on their discovery and characterization of colony-forming units in the spleen (CFU-S) (7). When primitive bone marrow progenitors are injected into lethally irradiated mice, they are able to form colonies of myeloid, erythroid, and megakaryocytic cells on the spleen of the recipient mice in 8-13days after transplantation. Ever since then, CFU-S has been considered equivalent to HSC until 1990. It is found that multipotent progenitors and erythroid/megakaryocytic progenitors are highly enriched in day 12 CFU-S and day 8 CFU-S, respectively (8, 9). Nonetheless, many concepts built upon detailed analysis of CFU-S apply to stem cells.

In general, stem cells classified by their differentiation capacities which mean how many types of cells they can give rise to(10). Totipotent stem cells produced by the fusion of egg and sperm can differentiate into embryonic and extraembryonic cell types. Pluripotent stem cells which only exist at the earliest stage of embryonic development, like embryonic stem cells, can form nearly all cell types of body(11). Multipotent or unipotent stem cells are found in many tissues and organs including bone marrow, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, and retina. Multipotent stem cells can generate several cell types within a given tissue or organ while unipotent stem cells can only give rise to one cell type.

In mammal, there are two broad types of stem cells: embryonic stem cells and adult stem cells, based on their existence in biological development process. Embryonic stem cells are isolated from the inner cell mass of blastocysts and pluripotent while adult stem cells are found many tissues and mulipotent or unipotent (12). Adult stem cells, like embryonic stem cells, have at least two things in common. First, they can make identical copies of themselves through the lifespan of an organism which is referred to as long-time self renewal (1, 2). Second, they can differentiate into at least one cell type with mature morphology and specialized function that is appropriate for the tissue. Typically, instead of producing mature cells directly, adult stem cells give rise to one or more intermediate cell types which are called progenitor cells. Progenitor cells are partially differentiated and differentiate into mature cells along a specific committed pathway. At the same time, these progenitor cells lose their self-renewal capacity (1, 2, 11).

2.2 Identification of HSC

HSCs were identified because of clinical need for cells capable of reconstructing hematopoietic system after people exposed to lethal doses of irradiation. Lead coverage of hematopoietic tissues during irradiation and intravenous infusion of bone marrow after irradiation can prevent death (13, 14). In irradiated mice,

injected bone marrow was able to survive, divide and repopulate the hematopoietic tissues(15).

As time elapsed, bone marrow was found to contain hematopoietic progenitor cells that gave rise to visible colonies of myeloid, erythroid, megakaryocytic cells within the spleen of recipient mice(7). As colony-forming unit-spleen (CFU-S), these cells can differentiate into lymphocytes and form more CFU-S in serial transplantation(16). At the same time, Siminovitch et al proposed the functional criteria for HSC definition: self renewal as well as multilineage differentiation. They also proposed that a population of radiation protective cells fell in these criteria of HSC (16).

Extensive efforts were involved in purification and characterization of HSC. Mice hematopoietic stem cells were first isolated in 1988 with the use of a variety of phenotypic cell markers(17). With the invention of fluorescence-activated cell sorting (FACS) and knowledge of cell surface markers, it turned out the ability to isolate various populations of progenitors to form the blood system. As the first-identified and best characterized stem cell type, HSC are often served as the model for the whole stem cell biology research (18).

3. Purification of hematopoietic stem cells

3.1 Source

It is indicated in figure 1.2 that there are three mainly sources of HSC: bone marrow, peripheral blood and umbilical cord blood (CB) (19, 20). In adult, HSC are mainly found in bone marrow which includes femurs, hip, ribs, sternum, and other bones. They can be directly obtained from the hip using a needle and syringe as well as obtained from cytokines such as granulocyte colony-stimulating factors (G-CSF) pre-treated peripheral blood stream. G-CSF can induce HSC to mobilize to blood from bone marrow compartment. Umbilical cord blood is another source of HSC. Non-invasive procurement and immense abundance are two main advantages of umbilical cord blood. Now, in several countries around the world like Canada, China, USA and UK, umbilical cord blood are preserved in either public bank for general use or private bank for private use (19, 20).

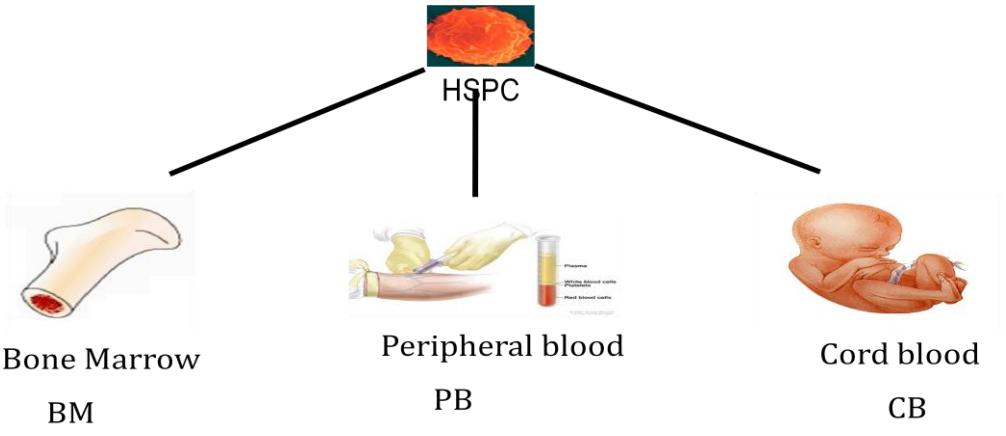


Figure 1.2 sources of human HSCs: bone marrow, peripheral blood and cord blood

3.2 Cell makers for human hematopoietic stem cell enrichment

CD34, as a cluster of differentiation (CD) molecule, is the universal marker of human HSC since primitive human HSC are highly enriched in the CD34+ population rather than the CD34- population in bone marrow. At the same time, CD34 expression will be down regulated as cell differentiation goes (21, 22). Human CD34 is highly conserved and play an important role in cell adhesion and in homing process (23, 24). Human HSC express low or undetectable level of CD38 and also negative for markers of lineage commitment (25). Therefore, human HSC are described as $CD34^{+}$, $CD38^{lo/-}$, lin^{-} human HSC. However, there may be some human HSC do not express CD34. Xenograft repopulation assays are to transplant human stem cells into fetal sheep or immune-depressed mice for stem cell function identification. Human Lin-CD34- cells contain stem cells capable of long-term repopulation and mutilineage differentiation after inoculated into a fetal sheep xenograft model (26). Moreover, these cells are capable of repopulation in secondary recipient. This result may indicate that CD34- cells are more primitive than CD34+ stem cells and stay higher level in the hierarchy of hematopoietic system(27). However, CD34 is still a marker of activated and cycling stem cells and CD34+human HSC can generate CD34- cells(28).

CD133, the human homolog of prominin 5 transmenbrane glycoproteions (PROML 1), co-expresses with CD34, c-kit and other cell surface markers on hematopoietic stem and progenitor cells derived from bone marrow and cord blood (29). It provides an alternative to CD34 for human HSCs enrichment. Vascular growth factor receptor 2 (KDR) and $C1qR_p$ are also alternative markers of bone marrow-repopulating cells (30, 31).

3.3 Purification methods

There are two commonly used methods to enrich human HSC: Magnetic-activated cell sorting (MACS) and Fluorescence-activated cell sorting (FACS), both of which are working based on cell surface markers. Prior to undergo these two methods, samples of bone marrow and cord blood have to run density gradient centrifugation in order to get rid of red blood cells and collect the mononuclear cells. Ficoll and Percoll are two popular media for density gradient centrifugation. Ficoll is a neutral, highly branched, high-mass, hydrophilic polysaccharide of which Ficoll PM400 with an average molecular weight of 40KD is the most extensively used one(32). Percoll is composed of colloidal silica coated with non-dialyzable polyvinylpyrrolidone (PVP)(33).

Magnetic-activated cell sorting

Magnetic-activated cell sorting (MACS) is to separate cells based on their surface CD molecules (34). MACS kit is composed of magnetic microbeads, columns and strong magnetic separators. When MACS kit is used to isolate cells, a heterogeneous mixture of biological cells should be incubated with magnetic microbeads coated with specific antibodies to the antigens expressed on the wanted cells first. Afterwards, the cells suspension is transferred into a column placed in magnetic separator. In this step, the magnetic microbeads labeled cells are trapped in the column while the other cells flow out of the column. When we use this method to enrich CD34+ human stem/progenitor cells (HSPC), mononuclear cells are incubated with microbeads and then transferred into the column. CD34+HSPC stay in the column. We collect these cells, count cell number, calculate the recovery rate, test the purity through flow cytometry and then run our experiments.

Fluorescence-activated cell sorting

Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. It provides a method for separating a heterogeneous mixture of biological cells into two or more containers because each cell has different light scattering and fluorescent characteristics(35). Prior to doing cell sorting via FACS, cells need to be incubated with fluorescent molecules conjugated antibodies which are used to identify specific types of cells. Afterwards, cells will be absorbed into FACS and

sorted based on their difference of cell morphology and fluorescent signals. Compared to MACS, FACS approach is less time consuming and more effective which means we can get better enriched cells through FACS.

4. Self-renewal and differentiation

Patients suffering from leukemia and lymphoma have been successfully treated with bone marrow or cord blood transplantation. Prior to transplantation, patients have to take chemical or radioactive treatment to eliminate dysfunctional or malignant blood cells and to suppress the immune system. The entire hematopoietic system of the patients will be gradually reconstituted with transplanted bone marrow or cord blood cells if immunological reactions are under control. Engrafted HSC are responsible for the long-term reconstitution. As mentioned in figure 1, engrafted HSC give rise to all types of hematopoietic cells such as erythrocytes, granulocytes and megakaryocytes through differentiation. They also build up and maintain the size of stem cell pool in the bone marrow of the recipients through self-renewal. Cell division is the only way of HSC to complete the above two processes. There are three types of cell division in HSC: symmetric self-renewal, asymmetric self-renewal and symmetric differentiation. Symmetric self-renewal is the division that two daughters are identical to parental stem cells. Asymmetric self-renewal is the division that one daughter cell is stem cell while the other one is lineage-committed progenitor cell.

Symmetric differentiation is the division that two daughter cells are both progenitor cells(5). However, how do HSC determine which way to go during cell division? What regulates the HSC? There are several key factors playing important roles in the fate determination of HSC including stem cell microenvironment growth factors, cytokines and intrinsic mechanisms (27). All these factors work at the molecular level. I would like to address self-renewal and differentiation at the molecular level respectively.

4.1 Regulation of Self renewal

As a type of cell division, self-renewal is related with stem cell microenvironment, cytokine networks and molecules inside the HSCs. For instance, in adult bone marrow, HSC tend to stay in contact with osteoblastic and endothelial vascular cells which were named as osteoblastic niches and vascular niches (36-38). These two niches offer HSC the microenvironment to maintain their number and regulate their self-renewal by expressing or secreting specific molecules. In osteoblastic niche, HSCs bind with osteoblast via adhesion molecules such as N-cadherin. The close contact between each other maintains HSC to stay in G0 quiescent state and to self-renew. This close contact is regulated by activation/deactivation of adhesion molecules. One of such candidates is angiopoietin-1 (Ang1) that expressed in osteoblast. It interact with Tie2, a type of receptor tyrosine kinase expressed in HSCs in bone marrow. The Tie2/Ang1

interaction activates N-cadherin and enhances the interaction of HSC and the osteoblastic niche (39). Another candidate is Osteopontin (Opn) that expressed by osteoblast(40). CD34+ human HSC were prevented from binding with Opn by incubation with β1-integrin-blocking antibody first. It was also reported that in Opn knockout mice, transplanted mice stem cells preferred to stay randomly in the bone marrow rather than in the osteoblastic niche. These data indicated that Opn played an important role in the adhesion between HSC and osteolastic niche to further keep HSC in G0 state.

In order to divide, HSC have to enter cell cycle from G0 state. As described above, there are three types of cell division. How do HSC control which way to go? It is proposed that self-renewal is also controlled by internal signal transduction and transcriptional activities(5).

phosphatidylinositol-3-OH kinase (PI3K) family play a crucial role in regulating a broad range of cellular activities. Akt, a serine-threonine kinase, is a very important downstream effector. In response to PI3K activation, Akt is phosphorylated to further activate a large number of target molecules which would result in the mediation of cellular functions such as proliferation, differentiation, chemotaxis, survival, trafficking, and glucose homeostasis(41). Phosphatase and tensin homolog (Pten) inactivates PI3K to negatively regulate PI3K-Akt signaling. Pten-/- HSC preferred to enter cell cycle rather than to stay in

stem cell niches in the bone marrow after transplanted into lethally irradiated mice (42). Another important signaling passway involved in HSC self-renewal is Jak2/Stat5 signaling transduction. Continuous activation of Jak2/Stat5 signaling transduction results in myeloproliferative diseases (43, 44).

Transcription factors directly modulate the gene expression in response to extrinsic and intrinsic factors. In HSC, combinations of specific transcription factors determine the time and degree for HSCs to self-renew and to differentiate(27). GATA2 and HoxB4 are two important transcription factors for the development of HSC. GATA2, a member of the GATA family, regulates the proliferation and maintenance of hematopoietic stem and progenitor cells (45). GATA2-null mutant mice die of severe hematopoietic defects during gestation at embryonic day 10–11. What is more, the size of functional stem cell pool in the Gata2+/− heterozygous mutant is reduced due to apoptosis caused by decreased expression of anti-apoptotic gene Bcl-xL (46). HoxB4, a member of Hox gene family encoding DNA-binding transcription factors, plays a crucial role in the self-renewal of HSC. HoxB4 overexpression is reported to enhance self-renewal rather than differentiation without causing leukemic transformation (47).

Other factors such as polycomb group family also play a role in HSC development. Bmi-1 is a member of polycom group family expressed in HSC. Bmi-1-/- mutant nude mice has small and hypoplastic bone marrow, thymus, and spleen, thus die

in less than two months after birth (48). Hyplasia of hematopoietic system in Bmi-1 -/- mice is demonstrated from impaired generation of self-renewing HSC. Therefore, Bmi-1 is critical for HSC self-renewal (48).

4.2 Differentiation

Differentiation of HSCs is also named as lineage commitment because HSC give birth to mature hematopoietic cells through differentiating into lineage committed progenitor cells. These progenitor cells then give rise to more restricted progenitors or immature hematopoietic cells that finally differentiate into or grow into all types of hematopoietic cells. Figure 1.3 gives us a well-described diagram of the lineage commitment of HSC. However, what controls this process? Ogawa et al examined the colony composition of single progenitors in methylcellulose culture and found that the type and number of lineages are both stochastically determined during the differentiation of HSC(49). It is suggested that intrinsic factors are the main regulators on the differentiation of HSC at a molecular level (50).

PU.1 and GATA1, as transcription factors, appear to be necessary for HSC differentiation. PU.1 plays a very important role in the development of granulocytic, monocytic, and lymphoid cells. PU.1 mutation severely impairs the generation of neutrophils and macrophages and leads to fetal death(51). GATA1

is expressed in erythroid cells, megakaryocytic cells, eosinophils, dendritic cells, and mast cells of hematopoietic lineage and promotes hematopoietic differentiation into all the above cells. For example, in the erythroid differentiation, GATA1 expression can be detected from common myeloid progenitors, erythoid progenitors and mature erythoid cells (52, 53). It is also reported that there are physical interaction and cross-antagonisms between PU.1 and GATA1. High level of PU.1 expression suppresses erythoid maturation and leads to erythroleukemia (54). At the same time, GATA1 expression in myelomonocytic cells forces them into erythroid, megakaryocytic, and eosinophilic differentiation(55). There also are some other transcription co-factors such as FOG1, SCL/TAL1-E2A heterodimer interacting with GATA1 to regulate the erythoid differentiation (56, 57). It seems to utilize multiple transcription factors to mediate one lineage differentiation, which makes it more difficult to analyze the effect of each transcription factor during the HSC differentiation.

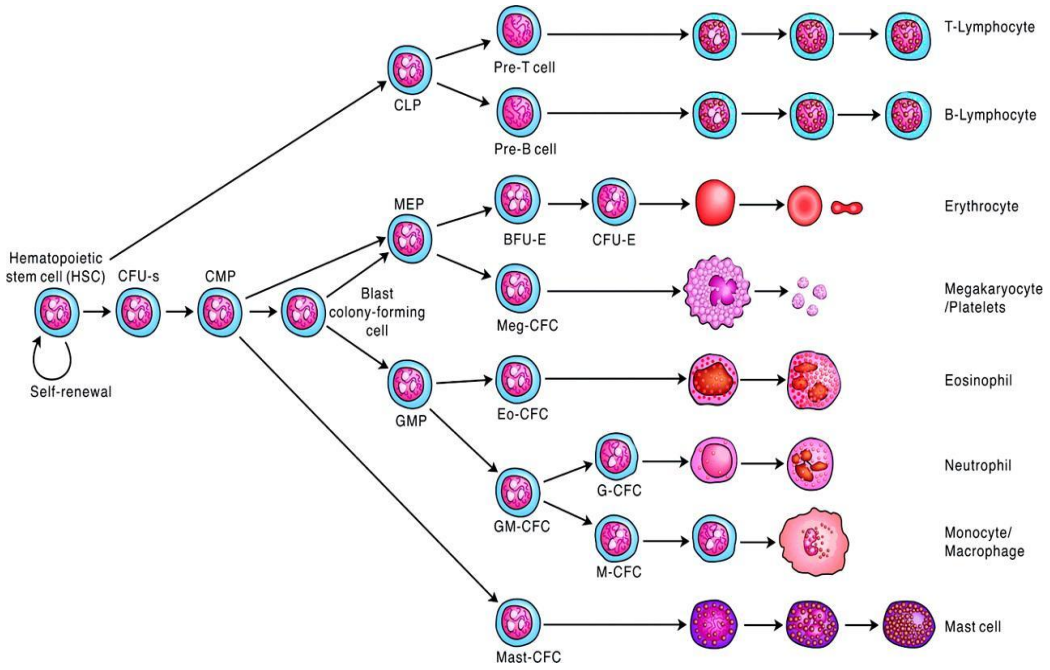


Figure 1.3: lineage commitment of HSCs (58). Abbreviations: BFU-E, burst-forming units–erythroid; CFU-S, colony-forming units–spleen; CLP, common lymphoid progenitors; CMP, common myeloid progenitors; Eo-CFC, eosinophil–colony-forming cells; G-CFC, granulocyte–colony-forming cells; GM-CFC, granulocyte macrophage–colony-forming cells; GMP, granulocyte-macrophage progenitors; HSC, hematopoietic stem cell; M-CFC, macrophage–colony-forming cells; Mast-CFC, mast–colony-forming cells; Meg-CFC, megakaryocyte–colony-forming cells; MEP, megakaryocyte-erythroid progenitors.

5. Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) is to transplant HSC or blood derived from bone marrow or umbilical cord blood(59). HSCT is now utilized to treat patients with leukemia or other types of hematopoietic diseases. Bone marrow transplantation has been used since 1950s. As time goes, umbilical cord blood is recognized as a better source for HSCT because it is easy to procure, no risk to graft donor and a reduced probability of infection transmission (60-62).

What is more, HLA matching is less restrict since umbilical cord blood transplantation (UCBT) offers a reduced risk of severe Graft-Versus-Host disease. However, the disadvantage of umbilical cord blood is the low number of CD34+ HSPC which limits the success of transplantation and delays the reconstitution of the hematopoietic system (63). Thus, it is critical to improve the number of HSPC. It is also reported that HSPC do not home or engraft to the bone marrow environment after transplantation with absolute efficiency (64). Therefore it is important to label HSPC and track them after transplantation, which could offer us an opportunity to understand HSPC homing and engraftment. In order to solve these two problems, I did two projects during my master. One is using low intensity pulsed ultrasound stimulation (LIPUS) to enhance HSPC proliferation *in vitro*. In this project, we found that LIPUS had a significant positive effect on *in vitro* HSPC proliferation without adverse effect on cell differentiation capacity. This technology would be important for USCT and future stem-cell based therapy. The other one is labeling HSPC with fluorescein-isothiocyanate (FITC) conjugated magnetic CNT. We found that the labeling reached 100% efficiency without compromising cell viability and differentiation capacity. It has a potential to use these labeled HSPC to investigate the homing and engraftment *in vivo* in the future(65). It also could be an effective way to delivery genes or other molecules into HSPC. These two projected will be discussed in detail in the following two chapters.

CHAPTER 2 :LOW-INTENSITY PULSED ULTRASOUND STIMULATION ENHANCES HEMATOPOIETIC STEM/PROGENITOR CELL PROLIFERATION IN VITRO

1. Introduction

The tremendous potential of hematopoietic stem/progenitor cells (HSPC) for reconstituting the hematopoietic system leads to the development of HSPC transplantation as a clinical strategy in the treatments of hematological disorders and cancer (3, 4). Although the transplants were initially carried out using bone-marrow (BM) cells (66), cells from the peripheral blood (PB) or umbilical cord blood (CB) are now commonly used (63, 67). HSPC directly obtained from the patient (autologous HSPC) are used to rescue the patient from the effects of high doses of chemotherapy or used as a target for gene-therapy vectors. HSPC obtained from another person (allogeneic HSPC) are used to treat hematological malignancies by replacing the malignant hematopoietic system with normal cells (68). CB has been identified as a rich source of HSPC and provides an alternative to BM or mobilized PB transplantation due to its widespread availability, and decreased immunological rejection by the patients (69, 70). However, the major restriction of CB transplantation is the low number of HSPC in each CB unit, which limits its use in clinical transplantation (71, 72). A large amount of HSPC is

needed in HSPC transplantation, and gene and stem cell-based therapies due to the fact that the cell dose infused is a pivotal item on the engraftment rate and clinical outcome (67). Therefore, the *ex vivo* expansion of HSPC is an attractive strategy to increase the number of self-renewing HSPC for clinical transplantation, and gene and stem cell therapies. Several culture conditions, different cytokine combinations inducing a significant expansion of HSPC have been proposed (73, 74). There is a general agreement on the key role played by Flt-3 ligand (Flt3L) and thrombopoietin(TPO) in the regulation of the early stages of hematopoiesis (75). The combination of early acting cytokines, like stem cell factor (SCF), allows a wider HSPC amplification (76). However, the maintenance of primitive HSPC with long-term engraftment potential is still controversial (77) and the characterization of the optimal conditions for *in vitro* culture of HSPC is challenging. Therefore, it is clear that more effective strategies for the expansion of CB or PB HSPC are needed and would considerably improve the therapeutic potential of the clinical transplantation.

Ultraousnd is defined as sound wave or pressure with a frequency greater than the upper limit of human hearing that refers to 20 kHz usually (78). Based on the differences of frequency and intensities, ultrasound can be divided into three types (79): 1) diagnostic ultrasound with low intensity and a frequency from 3 to 5 MHz which is used in sonography to produce pictures of fetuses in the human womb; 2) disruptive ultrasound with low frequency (20 to 60 kHz) and high

intensity such as high intensity focused ultrasound (HIFU) which is used to treat benign and malignant tumors and to break calculi such as kidney stones and gallstones (80); 3) therapeutic ultrasound with low intensity (0.01 to 2W/cm^2) and a frequency between 1 to 3MHz such as low intensity pulsed ultrasound (LIPUS).

LIPUS, as a type of therapeutic ultrasound, is found to enhance bone formation and wound healing (81). It is also reported that LIPUS can be used to induce dental itssue growth in human to repair root resportion (82). LIPUS is also proved to enhance the regeneration of myofiber with a better physiologic performance after muscle laceration in animal experiment (83). LIPUS also has positive effect on soft tissue heating including inter-vertebral discs, cartilage and tendon by improving the expression of transforming growth factor-beta 1 (TGF-β1) (84). TGF-β is also involved in the regulation of chondrocyte differentiation of mesenchymal stem cells (MSCs). LIPUS treatment enhances TGF-β mediated chondrocyte differentiation of human MSCs in vitro (85). LIPUS is found to enhance proliferation of human skin fibroblasts by activating the ERK1/2 signaling pathway (86). LIPUS treatment can also increase the protein production such proteoglycan and aggrecan (87, 88). More interestingly, the optimization of the growth of the mesenchymal stem cells from human CB was recently established using ultrasound (89). However, nothing is known yet about the effect of ultrasound on the human HSPC. In this chapter, I will address our

investigation on the effect of ultrasound on the proliferation and differentiation of human HSPC from CB and PB leukapheresis product (LP).

2. Methods materials

2.1 Equipments and materials

- NUAIR class A2 Biohazard safety cabinet
- NUAIR autoflow CO_2 air-Jacked Incubator
- Motic AE31 Inverted light microscope
- Laboratory centrifuge (Cat#: 75002382, Thermo Fisher)
- Vortex mixer-touch (Cat#: 02215360, Fisher Scientific)
- Electronic Pipette-aid (Cat#: 1438678, Fisher Scientific)
- Air Displacement Single Channel Pipetters (Cat#: 21-377-328, Fisher Scientific)
- Pipetter tips: 100ul-1000ul (Cat#: 02-681-163, Fisher Scientific)

  1ul-200ul (Cat#: 07-707-504, Fisher Scientific)

  0.1ul-10ul (Cat#: 21-277-2A, Fisher Scientific)
- Ependorf microcentrifuge tubes: 2ml (Cat#: 508-GRD, Rose Scientific LTD)

  1.5ml (Cat#: 05-408-129, Fisher Scientific)

  0.5ml (Cat#: 05-408-120, Fisher Scientific)
- Disposable glass Pasteur pipets (Cat#: 13-678-20D, Fisher Scientific)

- Autoclave bag (Cat#: 01-814B, Fisher Scientific)
- 35mm cell culture dish (Cat#: 430165, Corning)
- 0.45um syringe drive filter units (Cat#: SLHAM33SS, Millipore, carrigtwohill co. Cork, Ireland)
- 0.22um syringe drive filter units (Cat#: SLGSM33SS, Millipore, carrigtwohill co. Cork, Ireland)
- Powder free nitrile gloves: Small (Cat#: 2705851, Fisher Scientific)

  Medium (Cat#: 2705852, Fisher Scientific)

  Large (Cat#: 2705853, Fisher Scientific)
- Precision 180 series water bath (Cat#: 2823, Thermal electron corporation)
- Laboratory counter (Cat#: 02-670-14, Fisher Scientific)
- Costar pipets: 5ml (Cat#: 4487, Corning)

  10ml (Cat#: 4488, Corning)

  25ml (Cat#: 4489, Corning)
- 4℃, -20℃ and -80℃ refrigerators
- Isotem Oven (Cat#: 13-247-751F, Fisher Scientific)
- Autoclave (Market force)
- Vacuum driven disposable filtration system (Cat#: SCGPU02RE, Millipore, carrigtwohill co. Cork, Ireland)
- Centrifuge tubes: 50ml (Cat#: 430290, Corning)

15ml (Cat#: 430055, Corning)

- 12cmx75mm round-bottom tubes (Cat#: 352054, BD falcon)
- Pipet fine tip sterile (Cat#: 1371127, Sam Co Scientific)
- Laboratory parafilm 4inx 125feet roll (pechiney plastic packaging, Chicago, IL)
- Kimwipes(4.4x8.4in) (Kimberly-clark professional)
- Neubauer hemacytometer (buffalo, NY, USA)
- FACscan (Becton-Dickinson, San Jose, CA, USA)

2.2 Chemicals and reagents

- Dimethy sulfoxide (C2H6OS) (Cat#: 13P231-100, Fisher Scientific)
- Antibiotics penicillin/streptomycin (Cat#: 15070, GIBCO)
- Cell culture medium: IMDM (Cat#: 12200, GIBCO)
- Serum: bovine growth serum (Cat#: SH30541.03, Hyclone)
- Antibodies: PE mouse anti-human IgG (Cat#: 555787, BD phamingen)

  PE mouse anti-human CD34 (Cat#: 550761, BD phamingen)

  FITC- mouse anti-human IgG (BD phamingen)

  FITC- mouse anti-human CD14 (BD phamingen)
- 0.5M EDTA PH 8.0 (Cat#: 15575-038, GIBCO)
- Ficoll-paque (Cat#: 17-5442-02, GE Healthcare)
- Percoll (Cat#: 17-0891-01, GE Healthcare)

- MACS CD34 microbead kit (human) (Cat#: 130-046-703, Miltenyi Biotec)
- MACS multi stand (Cat#: 150.96, Miltenyi Biotec)
- Mini MACS separator (Cat#: 130-042-102, Miltenyi Biotec)
- Midi MACS separator (Cat#: 130-042-302, Miltenyi Biotec)
- MACS 25LS separation column (Cat#: 130-042-401, Miltenyi Biotec)
- MACS 25MS separation column (Cat#: 130-042-201, Miltenyi Biotec)
- 50 pre-separation filters (Cat#: 130-041-407, Miltenyi Biotec)
- Phosphate buffered saline (1x) (Cat#: 14190, GIBCO)
- 0.25% trypsin-EDTA (Cat#: 25200, GIBCO)
- 10x phosphate buffered saline (Cat#: ICN1960454,MP biomedicals)
- Paraformaldehyde, powder 95% (Cat#: 158127-500g, Sigma-Aldrich)
- Sodium bicarbonate (NaHCO3) (Cat#: s8875-500g, Sigma-Aldrich)
- 95% ethanol (biochemistry store, university of alberta)
- Trypan blue (Cat#: T6416-25g, Sigma-Aldrich)
- Methylcellulose medium with cytokines for human cells (cat#: 0444, Stem cell technologies)
- Cytokines: SCF, TPO and Flt3-ligand (Peprotech Inc, Rocky Hill, NJ, USA)
- CellTiter 96 AQueous One Solution (Promega, Madison, WI)

2.3 CD34+ Hematopoietic stem/progenitor cells (HSPC)

2.3.1. Source of CD34+ HSPC

Peripheral blood leukapheresis product (LP) was obtained with the patients' informed consent (in accordance with the institutional guidelines approved by the Human Research Ethics Board of the University of Alberta) before cryopreservation.

Umbilical cord blood (CB) was immediately collected after delivery in a sterilized tube containing heparin (1000 IU/mL), with the informed consent of the mother (in accordance with the institutional guidelines approved by the Health Research Ethics Board of the University of Alberta and the Genesis Bank, LLC, Indianapolis, USA) or frozen CB mononuclear cells were obtained from Stem Cell Technologies (Vancouver, BC, Canada).

2.3.2. Isolation of CD34+HSPC from CB and LP

Mononuclear cells (MNCs) from cord blood and LP were obtained by Percoll density gradient centrifugation and then CD34+ HSPCs were collected from mononuclear cells by immune affinity selection with MACS paramagnetic beads (Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's instructions. The purity of isolated CB and LP CD34+ cells were >95% and >91%, respectively, as determined by fluorescence-activated cell sorter (FACS) analysis. Cell viability was measured by the trypan blue exclusion assay.

The protocol of isolating CD34+HSPC from cord blood and LP was as below:

- Record the information about the cord blood and LP when they arrived at our lab for further reference. The information about cord blood included the date of birth and gestation period. The information about LP included the patient number and disease description.
- Transfer blood into 50ml centrifuge tubes and spin at 1500rpm for 5 minutes with maximal deceleration rate to obtain the buffy coat which contains most of the white blood cells and platelets(90). The following figure 2.1 showed where the buffy coat stayed after centrifugation.

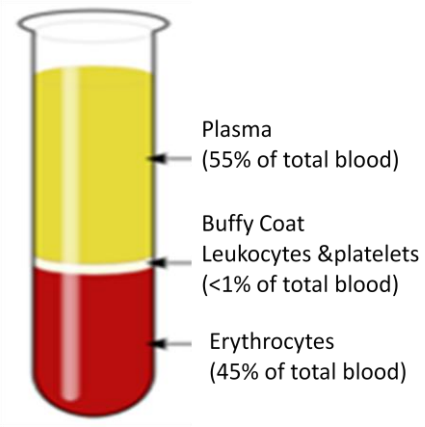


Figure 2.1 buffy coat separation of blood.

- Collect the buffy coat, make dilution with Isceve's Modified Dulbecco's medium (IMDM) and count the number of white blood cells (Note: For frozen leukapheresis production sample. We thawed it first in 37°C water

bath and diluted 10 times with IMDM. And then started Percoll density gradient centrifugation).

- Percoll density gradient centrifugation:

  - Calculate the number of 15ml centrifuge tubes (load~$30x10^6$cells/tube) and the volume of 60% percoll (4ml/tube)
  - Based on the calculation, dilute the buffy coat with IMDM. Overlay 4 mL diluted buffy coat on top of 4 mL 60% Percoll. (Note: Prepare 90% Percoll first from stock using 10x PBS, then dilute to 60% with IMDM)
  - Spin at 1800 rpm for 20 minutes with minimal deceleration rate. Collect mononuclear cells. Wash twice with 50 mL IMDM by spinning at 1500 rpm for 5 min with maximal deceleration rate. Resuspend in wash buffer (1xPBS/5 mM EDTA/0.5% BSA). Count the number of mononuclear cells and spin down at 1200rpm for 5 minutes with maximal deceleration rate. Calculate the total number of mononuclear cells. The following figure showed where mononuclear cells were after percoll density gradient centrifugation. Figure 2.2 indicated the blood separation after gradient centrifugation.

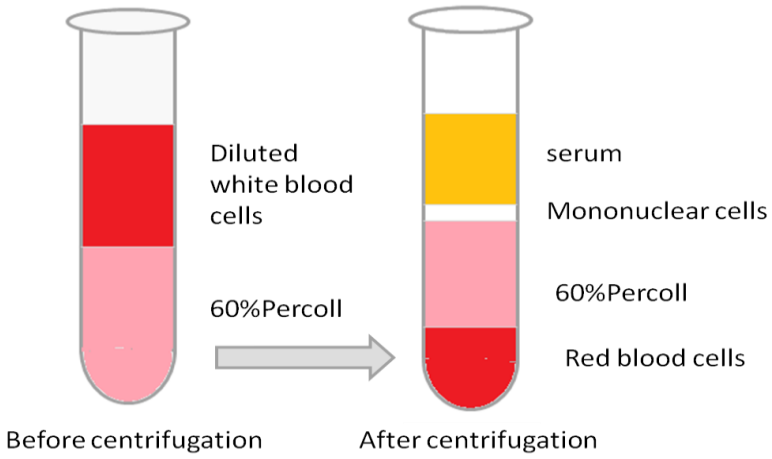


Figure 2.2: blood separation after gradient centrifugation.

- Magnetic labeling

➢ Resuspend cells in wash buffer (300 µL per 10^8 cells).

➢ Add FcR blocking reagent (100 µL per 10^8 cells).

➢ Add CD34 Microbeads (100 µL per 10^8 cells).

➢ Refrigerate for 30 minutes.

➢ Wash cells with wash buffer (1 mL per 10^8 cells) by spinning at 1200 rpm for 5 minutes and Resuspend in wash buffer again (0.5 mL per 10^8 cells).

- Column Separation

Note: Use Mini Macs column for cells $\leq 2 \times 10^8$

Use Midi Macs column for cells $> 2 \times 10^8$ to 2×10^9

- Wet the column with appropriate volume of wash buffer (500 μL for Mini Macs OR 3 mL for Midi Macs)
- Put a filter on top of the column and apply cell suspension
- Wash twice with appropriate volume of wash buffer (500 μL for Mini Macs OR 3 mL for Midi Macs)
- Collect CD34+HSPC into a separate 15ml centrifuge tube and count the number.
- Calculate the recovery rate

Recovery rate=Number of CD34+HSPC/ Number of mononuclear cells

(Note: recovery rate should not be over 2% in order to maintain high purity)

- Spin CD34+HSPC down at 1200rpm for 5minutes and resuspend in IMDM with 20%bovine growth serum and 1% antibiotics and store at 4°C for overnight. Do experiment the next day.

2.4 Stimulation with ultrasound

All cells were then maintained at $3x10^5$/well either in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% bovine growth serum (BGS) or IMDM with 20% BGS and a combination of cytokines including SCF (100ug/ml), TPO (50ug/ml) and Flt3-ligand (50ug/ml).

The LIPUS generator is invented in our group which is composed of a power-supply subsystem, an impedance-matching network, a piezoelectric transducer, and an IC capable of pulse-modulated signal generation (91). It is capable of outputting ultrasound with a frequency of 1.5MHz which is determined by the transducer, a 20% duty cycle, a pulse repetition frequency of 1 kHz and an intensity between 10 to 100mW/cm^2.

The ultrasound was applied by our invented Sonacell device through a piezoelectric transducer to a cell culture in an enclosed conventional 12-well tissue culture plate as described in figure 1. Coupling gel was used to ensure maximum and uniform distribution of the ultrasound throughout the growth medium.

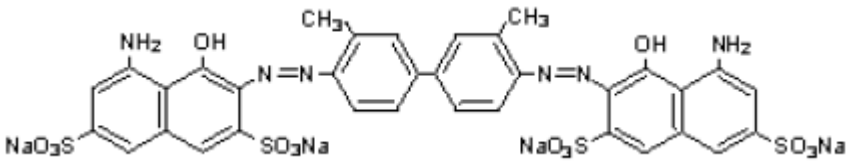
2.5 Cell proliferation

Cell proliferation and viability were assessed at day 5 by trypan blue exclusion and 3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxhenyl)-2-(4sulfophenyl)-2H-tetrazolium (MTS) proliferation assay, using CellTiter 96 AQueous One Solution.

2.5.1. Trypan blue exclusion assay

Trypan blue, derived from toluidine, is a vital stain used to dye dead tissue or cells blue. It has the chemical name 3,3'-[(3,3'-dimethyl-4,4'-biphenylylene) bis

(azo)] bis(5-amino-4-hydr oxy-2,7-naphthalenedisulfonic acid) tetra sodium salt, a molecular weight of 960.8, a molecular formula of $C_{34}H_{24}N_6Na_4O_{14}S_4$, and has the following chemical structure(92)



Trypan blue itself cannot pass through the cell membrane unless it is broken, therefore live cells or tissue with intact membrane cannot be stained while dead cells or tissue can be stained. This staining method is also named as trypan blue exclusion assay because live cells or tissue are excluded from staining. In this assay, trypan blue is prepared as 0.4% (w/w) solution with 1xPBS. Here is the protocol we followed to do this assay:

- Make a cell suspension in a known volume and mix well with pippeting
- Transfer 20ul into a 0.5ml eppendorf centrifuge tube and add in 20ul 0.4% trypan blue solution.
- Vortex for 5 second and transfer on top of the hemocytometer carefully and count the live cells under inverted microscope.
- Calculate the number of cells per ml and the total number of cells using the following formula:

$$\text{Cell number (per ml)} = \frac{\text{total number of cells counted}}{\text{total number of squares counted}} \times \text{dilution factor} \times 10^4$$

- Repeat count one or two more times to check the reproducibility.

2.5.2. MTS assay

MTS assay is a colorimetric assay to measure the reductase activity in live cells that reduce MTS into a purple formazan product that has a maximal absorbance at 490nm. The main applications are to verify the viability and proliferation of cells (93). In this assay, cells were collected in 100ul IMDM with 20%BGS and transferred into 96-well plate. 20ul MTS solution was added into each well and then the plate was incubated in 37℃ incubator for 1.5-4hours. Finally, the plate was read at 490nm through microplate reader.

2.6 Morphology

Ultrasound-stimulated cells were spun for cytospin preparation. Cytospin preparations were stained with May-Grünwald-Giemsa that is used to visualize the morphogy of blood cells and parasites (94). Cytospots were washed with distilled water and allowed to air-dry before analysis under a microscope.

2.7 Fluorescence-activated cell sorting (FACS) analysis

Flow cytometry is a machine used to analyze microscopic particles, such as cells based on their different fluorescent properties. It has a very broad application in both clinical and research field. Clinical applications included monitoring AIDS patients, diagnosis of Paroxymal Nocturnal Hemoglobinuria and so on. Research application included cell cycle test, proliferation test, viability test and so on (95, 96). In this analysis, LIPUS-stimulated and unstimulated cells at day 5 were collected and stained with PE-anti-CD34 and FITC-anti-CD14 monoclonal antibodies. After the final wash, cells were fixed in 1% paraformaldehyde prior to FACS analysis using PE-mouse anti- human IgG and FITC-mouse -anti-human IgG as the isotype control.

2.8 Colony Forming Unit (CFU) Assay

As described previously, CD34+HSPC have the ability to differentiate into all mature hematopoietic cells. During differentiation to mature blood cells, CD34+HSPC will go through intermediate stages including multi-potential progenitors and lineage-committed progenitors. In vitro Colony-forming unit assay provides information about the frequencies and growth properties of hematopoietic progenitor cells at various developmental stages (97).

When cultured in methylcellulose supplemented with appropriate cytokines and supplements, CD34+HSPC proliferate and differentiate to form discrete colonies

containing colony-forming unit-erythroid (CFU-E) and burst-forming unit-erythroid (BFU-E); colony-forming unit-granulocyte, macrophage (CFU-GM); colony-forming unit-granulocyte (CFU-G) and colony-forming unit-macrophage (CFU-M); and colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM). Under optimal plating and culture conditions, each colony is derived from a single progenitor. Therefore, the number and types of colonies counted in a CFU assay provides information about the ability of CD34+HSPC to proliferate and differentiate (97).

In order to test the effect of LIPUS on the proliferation and differentiation of CD34+HSPC, we did CFU assay. After exposure to ultrasound for 4 days, CD34+ cells were plated into 35mm cell culture dishes in triplicate in standard semisolid methylcellulose hematopoietic progenitor culture media at concentrations of $1x10^3$/mL. These culture plates were incubated at 37°C in 5% CO_2. Colonies were identified and enumerated 14 days later.

3. Results

3.1 Effect of ultrasound simulation on fresh LP HSPC

The optimization of the growth of the CB human MSC using ultrasound was recently established (89). Here, we investigated the effect of ultrasound on

human HSPC by our invented Sonacell ultrasound device. Figure 2.3 is the picture of our invention, which produces a 1.5-MHz ultrasound wave, 20% duty cycle, with adjustable output intensity between 30mW/ cm^2 to 100mW/ cm^2. The ultrasound was applied by a piezoelectric transducer to a cell culture in an enclosed conventional 12-well tissue culture plate. First we used fresh LP CD34+ cells from cancer patients other than leukemia due to the fact that the insufficient number of mobilized peripheral blood cells collected from heavily treated cancer patients may benefit from ex vivo expansion of these cells for autologous clinical transplantation. We applied ultrasound at a frequency of 1.5 MHz and intensity of 40, 50, 60 and 70 mW/cm^2 on fresh LP CD34+ cells supplemented with only IMDM with 20 % BGS for for 10 minutes each day for 4 days. At day 5, fresh LP CD34+ cell number dropped about 3.3-fold compared the initial cell number seeded at day 1 (Figure 2.4A). However, ultrasound with the intensity of 60 mW/cm^2 resulted in a better maintenance of fresh LP CD34+ cells viability compared to the unstimulated cells at day 5. Although ultrasound stimulated cell number dropped 2-fold compared to initial cell number at day 1, it increased the number of cells 1.5-fold compared to the unstimulated cells on day 5 without any growth factors (Figure 2.4A), which was confirmed by the MTS assay. Next, to investigate whether the addition of early acting cytokines will enhance the growth-promoting effect of ultrasound, we applied ultrasound on fresh LP CD34+ cells supplemented with IMDM with 20 % BGS in the presence

of early acting cytokines (SCF(100ug/ml), TPO(50ug/ml), and FLT3(50ug/ml)) for 4 days. After 4 days of stimulation, ultrasound stimulation with all intensities significantly enhanced the proliferation of fresh LP CD34+ cells in the presence of cytokines with respect to the unstimulated cells (Figure 2.4B). 60 mW/cm^2 was the optimal ultrasound intensity for the extremely significant growth of fresh LP CD34+ cells (Figure 2.4B). These results suggest that ultrasound enhances the proliferation and maintenance of HSPC viability depending on the presence of cytokine combinations (SCF, TPO and Flt3).

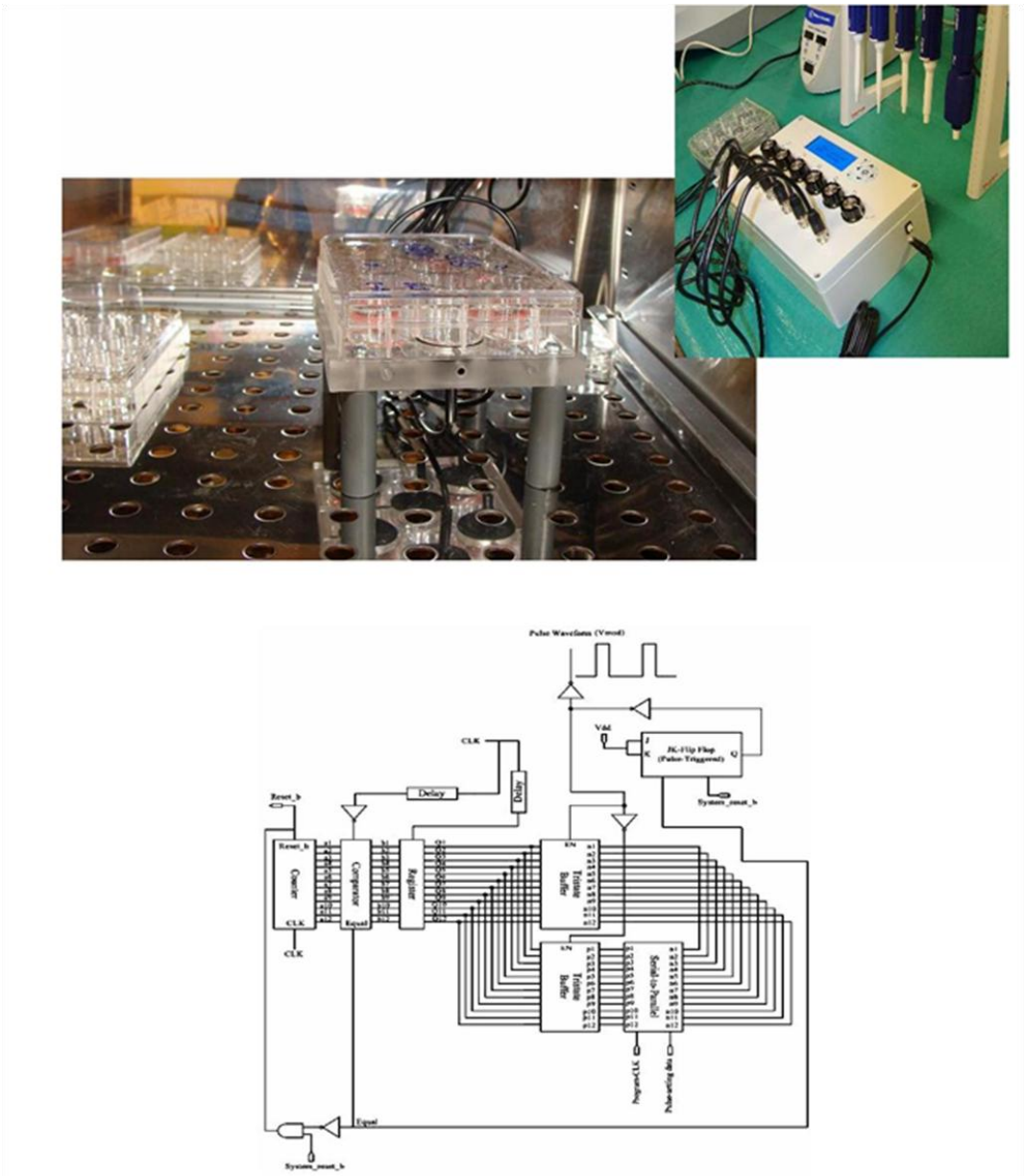


Figure 2.3. Picture of the ultrasound generate device and its experimental setup.

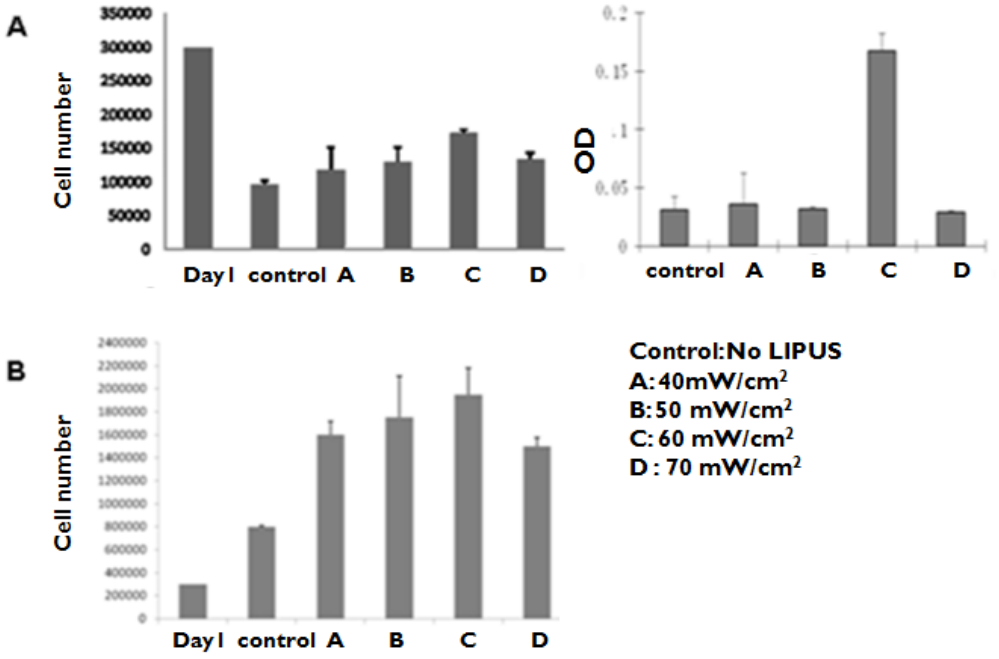


Figure 2.4. Proliferation of ultrasound-stimulated fresh LP HSPC. Proliferation of **A,** fresh LP CD34+ cells exposed to ultrasound at a frequency of 1.5 MHz and intensity of 40, 50, 60 and 70 mW/cm^2 in the absence of early acting cytokines for 4 days. Results represent the mean ±SEM of proliferation of cells obtained from three different patient cells . **B,** fresh LP CD34+ cells exposed to ultrasound in the presence of early acting cytokines (SCF: 100ug/ml, TPO: 50ug/ml , Flt3: 50ug/ml) for 4 days. Results represent the mean ± SEM of proliferation of cells obtained from three different patients.

3.2 Ultrasound stimulated fresh LP CD34+ cells did not differentiated

To determine whether differentiation was induced in LIPUS-stimulated fresh LP CD34+ cells on day 4, the surface antigen expressions of CD34 (hematopoietic stem/progenitor cell marker) and CD14 (monocyte/macrophage differentiation marker) were analyzed by FACS after 4 days of stimulation. The expression of

CD34 or the percentage of CD14-expressing cells was not affected by ultrasound stimulation, indicating that ultrasound stimulated cells still maintain their stem cell phenotype and did not differentiate compared to the control (Figure 2.5A).

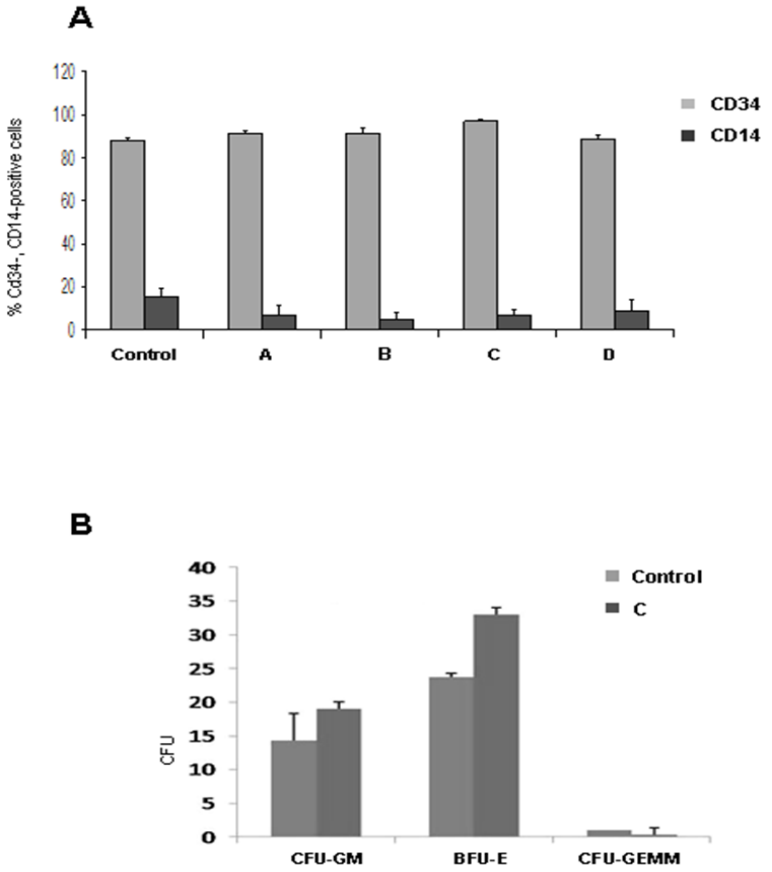


Figure2.5. Ultrasound-stimulated fresh LP CD34+ cells do not differentiate after 4 days of stimulation and gave rise to more erythroid progenitor colonies (BFU-E). A, FACS was performed at day 5 to detect% CD34 and CD14 in ultrasound-expanded HSPC. B, Colony formation unit (CFU) of ultrasound-stimulated (C: 60mW/cm^2) fresh LP CD34+ cells. Abbreviation: CFU-GM: Colony Formation Unit Granulocyte-Machrophage; BFU-E: Burst-Forming Unit-Erythrocyte; CFU-GEMM: Colony Forming Unit - Granulocyte-Erythroid -Macrophage- Megakaryocyte. Results represent the mean ± SEM of differentiation and colony formation ofcells obtained from three different patients.

3.3 Ultrasound stimulation enhances the formation of burst-forming unit-erythrocyte (BFUE) colonies from fresh LP CD34+ cells

In order to detect the *in vitro* functionality and long-term differentiation of ultrasound stimulated fresh LP HSPC, we performed a colony forming unit (CFU) assay. Fresh LP CD34+ cells were stimulated with the optimal ultrasound intensity (60 mW/cm^2) in the presence of cytokine combinations (SCF, TPO and Flt3) for 4 days. Cells were then collected and plated into methylcellulose. After 14 days, colonies were enumerated and identified. We obtained about 1.4-fold increase in overall colony number of CB CD34+ cells stimulated with ultrasound compared to the unstimulated cells (Figure 2.5B). More interestingly, a significant increase in the of primitive erythroid progenitor colonies *(*BFU-E*:* burst-forming uniterythrocyte) was observed in ultrasound-stimulated HSPC compared to the control (Figure 2.5B). These results indicate that ultrasound not only induces the growth of fresh LP CD34+ cells, but also favors the differentiation of fresh LP CD34+ cells into erythroid progenitors.

3.4 Effect of ultrasound stimulation on frozen LP CD34+ cells

Since frozen PB HSPCs are used in clinical transplantation practice, we further evaluated the growth-promoting effect of ultrasound on frozen LP CD34+ cells from 3 patients with lymphoma. We observed that ultrasound maintained the initial number of frozen LP CD34+cells obtained from these patients for 4 days, whereas the number of unstimulated cells significantly dropped (~2-fold) (Figure 2.6). In addition, the number and proliferation rate of ultrasound-stimulated cells were significantly higher (1.6-fold) than unstimulated cells at day 4 as shown by the MTS assay (Figure 2.6).

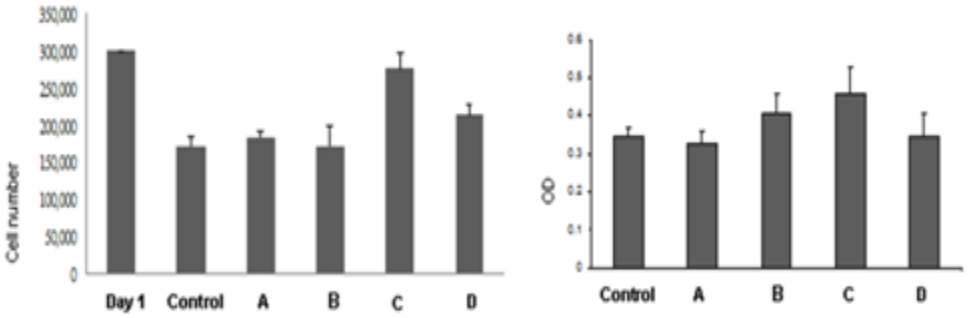


Figure 2.6. Proliferation of ultrasound-stimulated frozen LP CD34+ cells**.** Results represent the mean ± SEM of proliferation of cells obtained from three different patients. Control: no LIPUS; A: 40mW/cm^2; B: 50mW/cm^2; C: 60mW/cm^2; D: 70mW/cm^2.

3.5 Effect of ultrasound stimulation on fresh and frozen CB CD34+ cells

Although CB is a promising source of HSPC for allogeneic transplantation, graft rejection and delayed engraftment remain major limitations both of which are

related to a limited number of stem cells in the CB (71, 72). Therefore, we investigated whether ultrasound might enhance the amplification of both fresh and frozen CB CD34+ cells. CB CD34+ cells were exposed to ultrasound with intensity of 60 mW/cm^2 in the presence or absence of cytokine combination (SCF, TPO, Flt3L) for 4 days. We showed that ultrasound stimulation increases the proliferation of CB CD34+ cells from fresh (Figure 2.7A) and frozen CB units (data not shown) about 1.3-fold compared to the unstimulated cells after 4 days of stimulation. However, the proliferation of CB CD34+ cells from one fresh unit was not affected by the ultrasound stimulation (data not shown), most likely due to fact that the cells were already growing very fast without ultrasound stimulation. We also did not observed any increase in the proliferation of either fresh or frozen CB CD34+ cells without cytokine combinations using ultrasound (data not shown). In addition, ultrasound-stimulated fresh CB CD34+ cells did not differentiate because the surface expression of CD34 and CD14 did not change after 4 days of stimulation (Figure 2.7B). Both the control and LIPUS-stimulated cells retained immature blast-like morphology, as shown by a characteristically large nucleus and a thin rim of cytoplasm (Figure 2.7C). Very similar data was also obtained in frozen CB HSPCs.

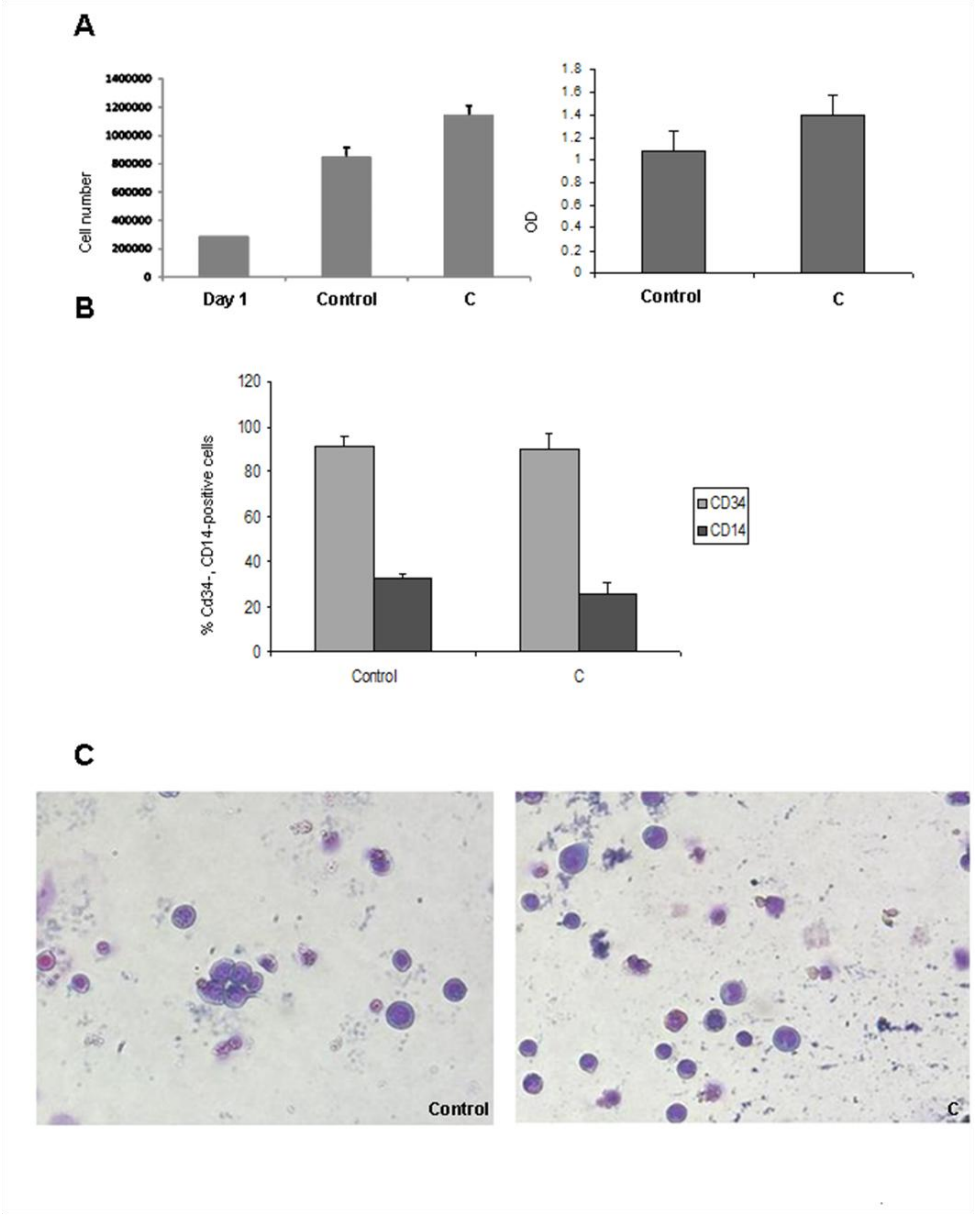


Figure 2.7. Proliferation and differentiation of ultrasound-stimulated fresh CB CD34+ cells. **A,** the number of ultrasound-stimulated fresh CB CD34+ cells **B,** % CD34 and CD14 of ultrasound stimulated fresh CB CD34+ cells., **C,** morphology of ultrasound-stimulated fresh CB CD34+ cells. Results represent the mean ± SEM of three independent experiments. Control: no LIPUS; C: 60mW/cm^2.

We also investigated the colony formation of ultrasound-stimulated CB CD34+ cells either in the presence of cytokines or without cytokines. There was no difference in the overall colony number or the morphology in CB CD34+ cells stimulated with ultrasound in the presence of cytokines compared to the unstimulated cells (Figure 2.8A). Interestingly, we obtained morphologically different BFU-E colonies from the CB CD34+ cells stimulated with ultrasound in the absence of cytokines compared to the unstimulated cells. BFU-E colonies were bigger, much more compact and hemoglobinized in comparison to the control group (Figure 2.8B). However, there was no difference in the colony number of the cells stimulated with ultrasound without cytokine combination compared to the unstimulated cells.

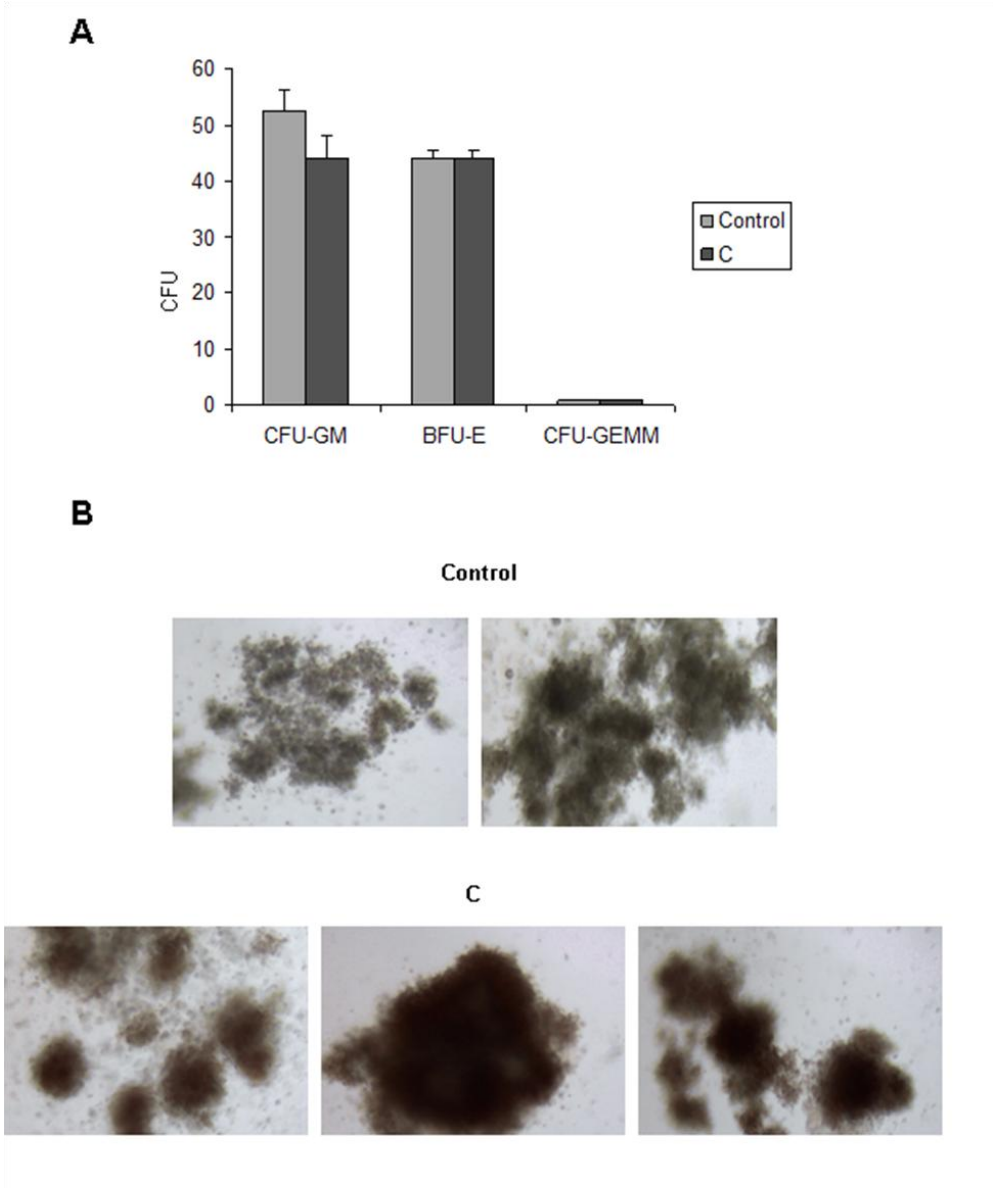


Figure 2.8 Colony formation of ultrasound-stimulated fresh CB CD34+ cells. A, CFU of ultrasound-stimulated fresh CB CD34+ cells in the presence of cytokines. Results represent the mean ± SEM of three independent experiments. B, colony morphology of ultrasound stimulated fresh CB CD34+ cells in the absence of cytokines. Control: no LIPUS; C:640mW/cm^2.

4. Disscussion

There is a mounting evidence that mechanical stimulations are critical to morphological, developmental, and functional states of living cells, and may play critical roles in controlling stem cell fate and lineage determination (98). In fact, mechanical stimulus of ultrasound exposure has been recently shown to be an effective noninvasive method for the stimulation of bioactivity in the wide variety of cells including human MSC (79, 81-85, 89, 99). In this chapter, we report for the first-time that ultrasound has a profound effect on HSPC fate in vitro. Ultrasound combined with early acting cytokines greatly enhances the proliferation of fresh LP HSPC, whereas it maintains the viability of frozen LP HSPC. Consistent with our present data, a recent study reported that ultrasound increases cells sensitivity to cytokines (88). These data suggests that the effect of ultrasound on cells is dependent on the presence of growth factors and whether the stem cells are fresh or frozen.

Ultrasound enhances the proliferation in both fresh and frozen CB HSPC to a lower extent than LP HSPC, suggesting the effect of ultrasound proliferation is also dependent on the stem cell source. This difference in ultrasound-stimulated cell growth between LP HSPC and CB HSPC could be attributed to the synergistic effect of other growth factors such as granulocyte colony-stimulating factor (G-CSF) on ultrasound-induced cell growth. G-CSF is commonly given to patient

prior to obtaining LP or mobilized PB progenitor cells (100), but not for obtaining CB. Taking such positive effects of ultrasound on fresh and frozen HSPC into consideration, further studies on its applications in combinations with other factors or its applications in semi-solid media instead of liquid system may enhance its effect and need to be further investigated. Similar to our results, different cell sources/types have been reported to respond ultrasound stimulation differently, and there are different optimal ultrasound intensities for cell proliferation and gene synthesis in rabbit and human IVD cells (88, 101). Our results also suggest that there is a different optimal ultrasound intensity and stimulation time for stem cell proliferation in comparison to the other types of cells, such as osteoblasts, chondrocytes IVD cells, and human MSC (17-23). More importantly, ultrasound also favors the differentiation of fresh LP HSPC into more eryhtroid progenitors, or induces bigger, more compact and hemoglonized BFU-E colony formation of CB HSPC. In fact, we obtained these results in the absence of cytokines without obtaining any increase in the cell growth. This could be explained by the fact that cytokines contributes to the proliferation of CB HSPC with ultrasound stimulation and the effect of ultrasound on colony formation was shadowed by cytokine combination, since cytokine stimulation of cells prior to CFU assay might greatly contributes the colony formation as well. However, in the absence of cytokine combination, the direct effect of ultrasound on erythroid progenitor colony formation of CB HSPC was visible and clear, as it

induces more compact, bigger and much better hemoglobinized colony formation. This newly discovered effect of ultrasound on HSPC proliferation, viability and differentiation may have important therapeutic impacts for clinical transplantation, and stem cell and gene therapies. We suggest that safe and non-invasive ultrasound stimulation might be used for in vitro amplification of fresh or the maintenance of frozen HSPC viability, especially prior to CB, PB transplantation or gene therapies. Since frozen samples or units are used in clinical transplantation, and the maintenance of stem cell viability in culture is crucial for successful transplantation and delivering genes into stem cells, ultrasound might overcome obstacles in clinical transplantation or gene therapies. Ex-vivo amplified hematopoietic progenitors by ultrasound may eliminate the need for repeated aphaeresis, or provide sufficient numbers of progenitor cells to support multiple cycles of chemotherapy. Furthermore, the period of cytopenias, associated with high-dose chemotherapy, may be abrogated by the infusion of ultrasound-stimulated post-progenitor cells, which rapidly differentiate into fully mature and functional erythrocytes, neutrophils, or megakaryocytes. Clinical trials have shown that the infusion of ex-vivo expanded progenitors, important for the induced cell response and function, is safe and effectively supports hematopoiesis following high-dose chemotherapy (102, 103). More importantly, patients with malignant hematologic disorders including leukemia, lymphoma and non-malignant hematological disorders

including myelodysplastic syndrome and anemia might benefit especially from enhanced BFU-E formation using ultrasound stimulation. Impaired BFU-E formation and abnormal BFU-E size distribution have been reported in MDS and acute lymphocytic leukemia, respectively (104, 105). In addition, a significant decrease has been recently observed in the number of BFU-E in LP from lymphoma patients (106). However, the favorable in vitro effect of ultrasound on proliferation, maintenance and differentiation of HSPC needs to be further investigated on the engraftment potential of HSPC in mice models.

5. Conclusion and future plan

In conclusion, we found that LIPUS treatment enhanced the proliferation of HSPC without disturbing their viability and differentiation capacity. Our work indicated that ultrasound stimulation might be a good method for improving the efficiency of clinical transplantation, stem cell and gene therapies.

In the future, we will test the homing and hematopoietic system reconstitution ability of LIPUS-treated HSPC in animals and uncover the molecular mechanism behind this effect.

CHAPTER 3 :MAGNETIC CARBON NANOTUBE LABELING FOR HAEMATOPOIETIC STEM/PROGENITOR CELL TRACKING[1]

1. Introduction

Nanotechnology, focused on assembling nanoscale elements to form complex structures, has set high expectations in biology and medicine in the last decade. Given the fact that many biological processes operate at the nanometer scale, it is not surprising that nanomaterials can provide tools to direct these biological processes (107). In recent years, it has been increasing interest in the biological and biomedical applications of nanomaterials such as carbon nanotubes (CNT) (108-114). The novel optical, mechanical, electrical or magnetic properties of CNT have stimulated extensive research, including their use in biosensing, tissue engineering and molecular delivery (109-113, 115-118). Nanospearing molecular delivery, which relies on the penetration of magnetic carbon nanotubes (mCNT) into cells by magnetic field exposure, has been receiving considerable attention for its high efficiency in gene delivery into tumor cell lines, such as breast cancer cells, MCF-7, and difficult-to-transfect cells, primary mammalian neurons and B

[1] *A version of this chapter has been published in January 2010,* ***Nanotechnology 21*** *155101*

lymphocytes (111). Nanomaterial mediated delivery using CNT can also be used for cellular labeling/tracking (119, 120). CNT coated with the peptide has been reported to be successfully delivered and imaged in living human cervical cancer cells HeLa(119). More interestingly, CNT fluorescence was detected and imaged from living Drosophila larva by using near-infrared (NIR) while not adversely affecting the viability and growth of the larva by ingestion in another study(120). Although CNT-mediated delivery has already been successfully applied in various cells, their proposed use in stem cells is more recent. CNT have been shown to efficiently label human mesenchymal stem cells and not affect biocompatibility, proliferation or differentiation properties of these cells(121). However, the uptake or labeling efficiency of CNT in hematopoietic stem/progenitor cells (HSPC) has not been addressed or explored yet (HSPC are the most common type of stem cells used for cellular therapies for many decades (100, 122).

HSPC are the very-well-characterized, rare and renewable population of cells that give rise to all of the differentiated cells of adult peripheral blood. Transplantation of HSPC obtained from peripheral blood (PB), bone marrow (BM) or from umbilical cord blood (CB) has been routinely used to rescue bone marrow function following high-dose myeloablative therapy for non-malignant and malignant hematologic disorders such as leukemia (122, 123). Nonetheless, little is understood about the biology of HSPC homing and engraftment to the BM microenvironment after transplantation (124). HSPC were reported not to

engraft with absolute efficiencies to the BM, though donor-derived HSPC have been detected in various organs following transplantation (125-127). Therefore, HSPC labeling and the consecutive non-invasive tracking *in vivo* following transplantation become important in understanding HSPC homing/engraftment and the therapeutic efficacy for future stem-cell-based therapies. Labeling HSPC prior to application in humans must be highly efficient and safe. Recent developments in nanotechnology allow the visualization of HSPC infused into a live recipient. For instance, the use of magnetic nanoparticles conjugated to the Tat protein of HIV offers the possibility of labeling highly purified HSPC subsets at 24 h incubation, and the specific homing and trafficking of the labeled cells can be monitored *in vivo* using MRI afterwards (128). However, more studies are needed to fully explore the possibilities of the CNT-mediated labeling of HSPC for cell tracking. In this chapter, we evaluate our set up of the magnetic-field driven FITC-mCNT delivery method with MCF-7 and THP-1 cells. We also investigate the uptake efficiency of FITC-mCNT into HSPC and their effect on cytotoxicity and the differentiation of HSPC.

2. Materials and methods

2.1 Equipments and materials

- NUAIR class A2 Biohazard safety cabinet

- NUAIR autoflow CO2 air-Jacked Incubator
- Motic AE31 Inverted light microscope
- Laboratory centrifuge (Cat#: 75002382, Thermo Fisher)
- Vortex mixer-touch (Cat#: 02215360, Fisher Scientific)
- Electronic Pipette-aid (Cat#: 1438678, Fisher Scientific)
- Air Displacement Single Channel Pipetters (Cat#: 21-377-328, Fisher Scientific)
- Pipetter tips: 100ul-1000ul (Cat#: 02-681-163, Fisher Scientific)

  1ul-200ul (Cat#: 07-707-504, Fisher Scientific)

  0.1ul-10ul (Cat#: 21-277-2A, Fisher Scientific)
- Ependorf microcentrifuge tubes: 2ml (Cat#: 508-GRD, Rose Scientific LTD)

  1.5ml (Cat#: 05-408-129, Fisher Scientific)

  0.5ml (Cat#: 05-408-120, Fisher Scientific)
- Disposable glass Pasteur pipets (Cat#: 13-678-20D, Fisher Scientific)
- Autoclave bag (Cat#: 01-814B, Fisher Scientific)
- 35mm cell culture dish (Cat#: 430165, Corning)
- 0.45um syringe drive filter units (Cat#: SLHAM33SS, Millipore, carrigtwohill co. Cork, Ireland)
- 0.22um syringe drive filter units (Cat#: SLGSM33SS, Millipore, carrigtwohill co. Cork, Ireland)
- Powder free nitrile gloves: Small (Cat#: 2705851, Fisher Scientific)

Medium (Cat#: 2705852, Fisher Scientific)

Large (Cat#: 2705853, Fisher Scientific)

- Superfrost microscope slides (Cat#: 12-550-15, Fisher Scientific)
- Cover slips (Cat#: 12-548-B, Fisher Scientific)
- Precision 180 series water bath (Cat#: 2823, Thermal electron corporation)
- Laboratory counter (Cat#: 02-670-14, Fisher Scientific)
- Costar pipets: 5ml (Cat#: 4487, Corning)

  10ml (Cat#: 4488, Corning)

  25ml (Cat#: 4489, Corning)

- Liquid nitrogen tank
- 4℃, -20℃ and -80℃ refrigerators
- Isotem Oven (Cat#: 13-247-751F, Fisher Scientific)
- Autoclave (Market force)
- Sonicator (Cat#: FS30H, Fisher Scientific)
- AJ100 electronic analytical balance (Mettler)
- Isotem magnetic stirrer with heating (Cat#: 14-259-79, Fisher Scientific)
- Vacuum driven disposable filtration system (Cat#: SCGPU02RE, Millipore, carrigtwohill co. Cork, Ireland)
- Centrifuge tubes: 50ml (Cat#: 430290, Corning)

  15ml (Cat#: 430055, Corning)

- 12cmx75mm round-bottom tubes (Cat#: 352054, BD falcon)
- Cell culture flasks: 25cm^2 (Cat#: 430639, Corning)

  75cm^2 (Cat#: 430641, Corning)
- Cryopreservation vials (Cat#: 0334118E, Wheaton)
- Pipet fine tip sterile (Cat#: 1371127, Sam Co Scientific)
- Laboratory parafilm 4inx 125feet roll (pechiney plastic packaging, Chicago, IL)
- Kimwipes(4.4x8.4in) (Kimberly-clark professional)
- Neubauer hemacytometer (buffalo, NY, USA)
- 3 inch Diameter x 1 inch Thick Neodymium Nd-Fe-B Magnet Disc (Applied magnets)
- Confocal microscope (Carl Zeiss LSM510, Toronto, Canada)
- FACscan (Becton-Dickinson, San Jose, CA, USA)
- Vacuum oven
- Silica gel Thin Layer Chromatography plate (Cat#: 05719857, Fisher Scientific)
- Pyrex condenser (cat#: 07-736B, Fisher Scientific)
- Pyrex round-bottom boiling flask 250ml (Cat#: 10-060-4D, Fisher Scientific)
- Chemical fume hood

2.2 Chemicals and reagents

- Dimethy sulfoxide (C2H6OS) (Cat#: 13P231-100, Fisher Scientific)
- Antibiotics penicillin/streptomycin (Cat#: 15070, GIBCO)
- Cell culture medium: RPMI 1640 (Cat#: 11875, GIBCO)

  IMDM (Cat#: 12200, GIBCO)

  DMEM (Cat#: 11965, GIBCO)
- Serum: bovine growth serum (Cat#: SH30541.03, Hyclone)

  Fetal bovine serum (Cat#: 12483, GIBCO)
- Antibodies: PE mouse anti-human IgG (Cat#: 555787, BD phamingen)

  PE mouse anti-human CD34 (Cat#: 550761, BD phamingen)
- 0.01% Poly-l-lysine solution (Cat#: p4832, Sigma-Aldrich)
- 0.5M EDTA PH 8.0 (Cat#: 15575-038, GIBCO)
- Ficoll-paque (Cat#: 17-5442-02, GE Healthcare)
- Percoll (Cat#: 17-0891-01, GE Healthcare)
- MACS CD34 microbead kit (human) (Cat#: 130-046-703, Miltenyi Biotec)
- MACS multi stand (Cat#: 150.96, Miltenyi Biotec)
- Mini MACS separator (Cat#: 130-042-102, Miltenyi Biotec)
- Midi MACS separator (Cat#: 130-042-302, Miltenyi Biotec)
- MACS 25LS separation column (Cat#: 130-042-401, Miltenyi Biotec)
- MACS 25MS separation column (Cat#: 130-042-201, Miltenyi Biotec)
- 50 pre-separation filters (Cat#: 130-041-407, Miltenyi Biotec)
- Phosphate buffered saline (1x) (Cat#: 14190, GIBCO)

- 0.25% trypsin-EDTA (Cat#: 25200, GIBCO)
- 10x phosphate buffered saline (Cat#: ICN1960454,MP biomedicals)
- Paraformaldehyde, powder 95% (Cat#: 158127-500g, Sigma-Aldrich)
- Sodium bicarbonate (NaHCO3) (Cat#: s8875-500g, Sigma-Aldrich)
- 95% ethanol (biochemistry store, university of alberta)
- Trypan blue (Cat#: T6416-25g, Sigma-Aldrich)
- Clear nail polish (superstore, canada)
- Rhodamine-Phalloidin (Cat#: 447152, Invitrogen)
- Methylcellulose medium with cytokines for human cells (cat#: 0444, Stem cell technologies)
- Carbon nanotube, single-walled, 50-70% carbon basis, diam.1.2-1.5 nm× 2-5 µm , bundle dimensions (Cat#: 519308-1G, Sigma-Aldrich)
- Thionyl chloride (SOCl2) (Cat#: 447285, Sigma-Aldrich)
- Nitric acid (HNO3) (Cat#: 258121, Sigma-Aldrich)
- Anhydrous Dimethyl formamide (DMF) (Cat#: 227056, Sigma-Aldrich)
- Anhydrous tetrahydrofuran (THF) (Cat#: 401757, Sigma-Aldrich)
- 2-(2-(2-aminoethoxy)ethoxy)ethanamine (Cat#: 40421, Sigma-Aldrich)
- Anhydrous methanol (Cat#: 322415, Sigma-Aldrich)
- Fluorescein isothiocyanate (FITC) (Cat#: F3651, Sigma-Aldrich)
- Anhydrous Dichloromethane (Cat#: 270997, Sigma-Aldrich)

Diethyl ether (Cat#: 443549, Sigma-Aldrich)

2.3Preparation of FITC-mCNT

Single-walled mCNT containing Ni and Y at the tip, with an average diameter of 1.2–1.5 nm and a length of 2–5 μm, were obtained from Sigma-Aldrich (Ontario, Canada). These specific mCNT were chosen for our experiment because they have been used in magnetic-field-driven biomolecule delivery with very high efficiency (111). Compared to the reported methods, our synthesis of FITC-mCNT is much simpler and yet returns higher chemical yields (129). As shown in figure 3.1, the mCNT were oxidized to form carboxylic acid groups on the surface (111, 130-133), which were reacted with thionyl chloride and then 2_-(ethylenedioxy)bis(ethylamine) to produce amine terminated nanotubes. The amine was then reacted with FITC to provide FITC-labeled highly water-soluble FITCmCNT. Infrared (IR), X-ray photoelectron spectroscopy (XPS) and UV–vis spectroscopy were used to validate the chemical reactions of the intermediate and the final FITC-mCNT products.

2.3.1. Oxidation of the carbon nanotubes.

The first oxidation step was carried out as described previously (111, 130-133). Briefly, the purchased carbon nanotubes (200 mg) were refluxed with 0.5 M HNO3 (100 ml) for 48 h to introduce carboxylic groups. After refluxing, the solution was diluted with deionized water, filtered over a 0.2 µm polycarbonate

filter (Millipore) and washed several times with deionized water. The sample was collected and dried overnight in a vacuum oven at 80 ℃ to give mCNT 2 (170 mg) (figure 3.1).

2.3.2. Reaction with thionyl chloride to give SWNT-COCl.

A suspension of mCNT 2 (100 mg) in 20 ml of SOCl2, together with five drops of dimethylformamide (DMF), was stirred at 70 ℃ for 24 h. The mixture was cooled and centrifuged at 2000 rpm for 30 min. The excess $SOCl_2$ was decanted and the resulting black solid was washed with anhydrous THF (3 × 20 ml) and dried overnight in a vacuum oven at 80℃ to give mCNT 3 (78 mg) (figure 3.1).

2.3.3. Coupling with 2-(2-(2-aminoethoxy)ethoxy)ethanamine.

The mixture of mCNT 3 (50 mg) and anhydrous 2-(2-(2-aminoethoxy)ethoxy)ethanamine (120 ml) was heated at 100 ◦C for 100 h. During this time, the liquid phase became dark. After cooling, the mixture was poured into methanol (100 ml) and centrifuged to give a black solid, which was washed several times with methanol. The resulting solid was dried overnight in a vacuum oven at 80 ◦C to give mCNT 4 (42 mg) (figure 3.1).

2.3.4. Labeling with FITC.

A suspension of the mCNT 4 (25 mg) and FITC (5 mg) in anhydrous DMF (10 ml) was stirred in the dark for 5 h. Then the reaction mixture was poured into anhydrous ethyl ether (40 ml) and centrifuged to give a black solid, which was washed with methanol until TLC (10% MeOH in dichloromethane) showed no free FITC left. The product was dried overnight in a vacuum oven at 80 ℃ to give mCNT 5 (23 mg) (figure 3.1).

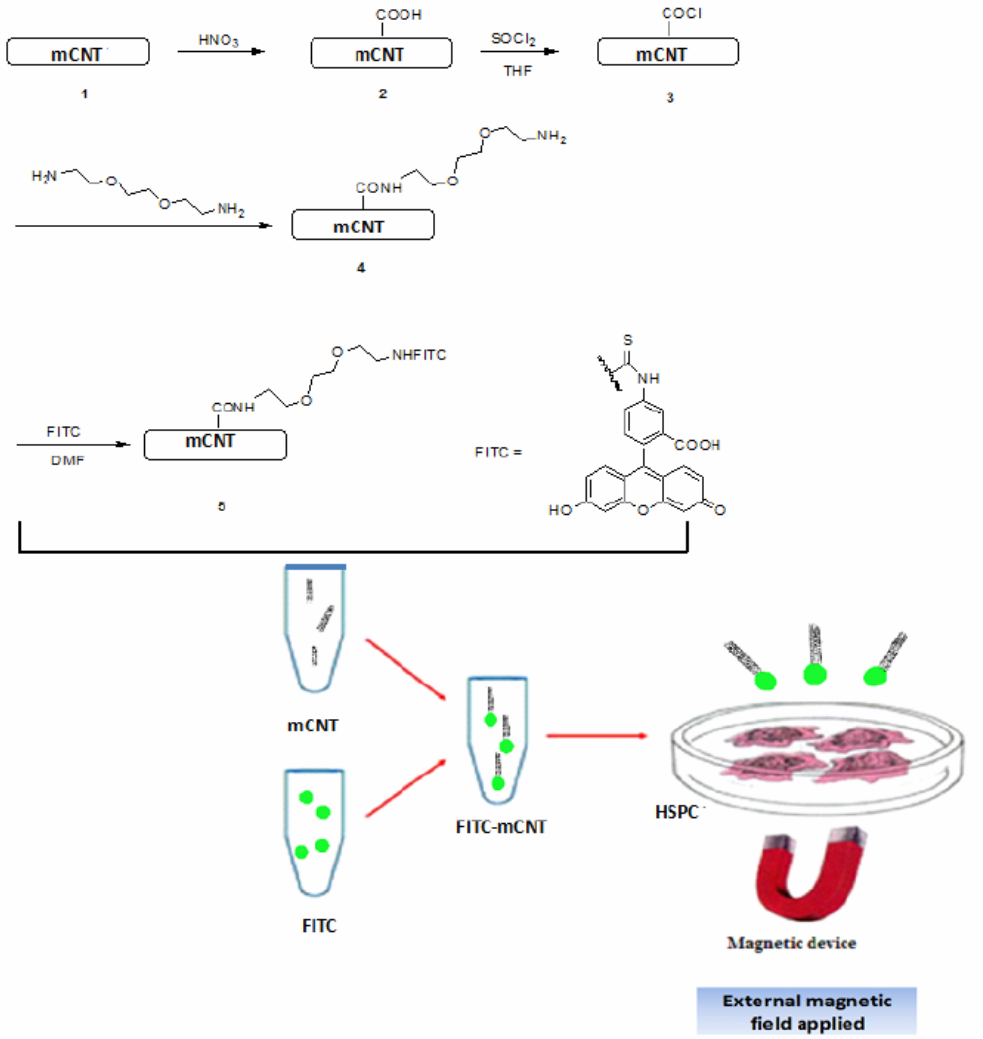


Figure 3.1. Schematic illustration of FITC-mCNT synthesis and magnetic-field-driven FITC-mCNTs into HSPCs. The mCNTs (1) were oxidized to form carboxylic acid groups on the surface (2). These nanotubes were reacted with thionyl chloride (3) and then 2_-(ethylenedioxy)bis(ethylamine) to produce amine-terminated nanotubes (4). The amine was then reacted with FITCs to form highly water-soluble FITC-mCNTs (5). Freshly synthesized fluorescent and magnetic FITC-mCNTs were then used in magnetic-field-driven HSPC uptake experiments.

2.4 Cell lines and CD34+ Hematopoietic stem/pro genitor cells (HSPC)

There were three different types of cells that we used in this project including MCF-7, THP-1 and CD34+HSPC. MCF-7 is adherent human breast epithelial adenocarcinoma cell line which is bought from the American Type Culture Collection (ATCC). THP-1 is a suspended peripheral blood acute monocytic leukemia cell line which is also bought from ATCC**.** CD34+HSPC is normal cells isolated from cord blood or patient leukapheresis product (LP)

2.4.1. Cell culture and cryopreservation

- Adherent cell line *in vitro* culture

MCF-7 was cultured in Dulbecco's Modified Eagle Medium (DMEM) added with 10% fetal bovine serum (FBS) and 1% antibiotics and incubated in 37℃ incubator supplied with 5% CO2. During cultivation, we needed to check their morphology

under the reverted microscope everyday and renew culture medium 2 to 3 times per week. When cells covered 90% of the bottom of culturing dishes or flasks, we needed to subculture at the ratio of 1:3 to 1:6.

Here is the subcultivation protocol (take 75cm2culturing flask as example):

- Remove culture medium to the liquid waste bottle.
- Briefly rinse the cell layer with 0.25% (w/v) Trypsin - 0.53 mM EDTA solution to remove all traces of serum which contains trypsin inhibitor
- Add 3.0 ml of Trypsin-EDTA solution to flask and put the flask back into the incubator. Check cells under an inverted microscope every 5minutes until cell layer is dispersed.

  Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.
- Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting
- Transfer the cell suspension to one centrifuge tube and spin at 1200rpm for 5minutes. Discard the supernatant.
- Resuspend the cell pellet in fresh growth medium. Add appropriate aliquots of the cell suspension to new culture flasks
- Incubate at 37℃ incubator supplied with 5%CO2.

- Suspended cells in *vitro* culture

THP-1 was cultured in RPMI-1640 medium added with 10% fetal bovine serum (FBS) and 1% antibiotics and incubated in 37℃ incubator supplied with 5% CO2. During cultivation, we needed to check their morphology under the reverted microscope everyday and renew culture medium 2 to 3 times per week. It was recommended by ATCC to keep THP-1 at the concentration of $3x10^5$to $1x10^6$/ml during cultivation. When they grew over $1x10^6$/ml, we needed to subculture them at appropriate ratio. Since THP-1 was suspended in the medium, there were two methods of subcultivation: one is adding fresh complete growth medium to the flask and the other is replacing the medium. In our lab, we prefer to add fresh medium in the first several subcultivations and replace medium when the flask reached its maximum load.

Here is the protocol for replacement of medium (take $75cm^2$culturing flask as example):

- Transfer cell suspension into centrifuge tubes and spin at 1200rpm for 5minutes. Discard the supernatant.
- Resuspend the cell pellet in fresh growth medium. Add appropriate aliquots of the cell suspension to new culture flasks. Add appropriate volume of fresh growth medium to the flask as well to keep the concentration at $3~4x10^5$/ml.

- Incubate at 37℃ incubator supplied with 5%CO2.

- Cryopreservation of cells.

Cell lines came in one vial when we purchased from ATCC. We needed to culture them first and make our own stock for future use. The cryopreservation protocol was the same for both adherent and suspended cell lines. Here was what we do to cryopreserve our cell lines:

- Collect cells when cells were in good condition and spin down at 1200rpm for 5 minutes. Discard the supernatant.
- Resuspend the pellet in appropriate volume of fresh growth medium. Add DMSO to the cell suspension to reach 5% (v/v) and mix gently with pippeting.

Note: sometimes we use serum supplemented with 5% (v/v) DMSO to cryopreserve some sensitive cell line.

- Transfer cell suspension to cryopreservation vials and store in liquid Nitrogen tank.

2.4.2. Source and isolation of CD34+ hematopoietic stem/progenitor cells (HSPC)

- Source of CD34+ HSPC

Cord blood was collected immediately after delivery in a sterilized tube containing heparin (1000 IU ml−1), and with the informed consent of the mother (in accordance with the institutional guidelines approved by the Health Research Ethics Board of the University of Alberta).

Peripheral blood leukapheresis product (LP) was obtained with the patients' informed consent (in accordance with the institutional guidelines approved by the Human Research Ethics Board of the University of Alberta) before cryopreservation.

- Isolation of CD34+HSPC from cord blood and LP

Mononuclear cells (MNCs) from cord blood and LP were obtained by Percoll density gradient centrifugation and then CD34+ HSPCs were collected from mononuclear cells by immunoaffinity selection with MACS paramagnetic beads (Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's instructions. The purity of isolated CB and LP CD34+ cells were >95% and >91%, respectively, as determined by fluorescence-activated cell sorter (FACS) analysis. Cell viability was measured by the trypan blue exclusion assay.

The protocol of isolating CD34+HSPC from cord blood and LP was as below:

- Record the information about the cord blood and LP when they arrived at our lab for further reference. The information about cord blood included the date of birth and gestation period. The information about LP included the patient number and disease description.
- Transfer blood into 50ml centrifuge tubes and spin at 1500rpm for 5 minutes with maximal deceleration rate to obtain the buffy coat which contains most of the white blood cells and platelets(134).
- Collect the buffy coat, make dilution with Isceve's Modified Dulbecco's medium (IMDM) and count the number of white blood cells (Note: For frozen leukapheresis production sample. We thawed it first in 37°C water bath and diluted 10 times with IMDM. And then started Percoll density gradient centrifugation).
- Percoll density gradient centrifugation:
    - Calculate the number of 15ml centrifuge tubes (load~$30x10^6$cells/tube) and the volume of 60% percoll (4ml/tube)
    - Based on the calculation, dilute the buffy coat with IMDM. Overlay 4 mL diluted buffy coat on top of 4 mL 60% Percoll. (Note: Prepare 90% Percoll first from stock using 10x PBS, then dilute to 60% with IMDM)

- Spin at 1800 rpm for 20 minutes with minimal deceleration rate. Collect mononuclear cells. Wash twice with 50 mL IMDM by spinning at 1500 rpm for 5 min with maximal deceleration rate. Resuspend in wash buffer (1xPBS/5 mM EDTA/0.5% BSA). Count the number of mononuclear cells and spin down at 1200rpm for 5 minutes with maximal deceleration rate. Calculate the total number of mononuclear cells.

- Magnetic labeling

  - Resuspend cells in wash buffer (300 μL per 10^8 cells).
  - Add FcR blocking reagent (100 μL per 10^8 cells).
  - Add CD34 Microbeads (100 μL per 10^8 cells).
  - Refrigerate for 30 minutes.
  - Wash cells with wash buffer (1 mL per 10^8 cells) by spinning at 1200 rpm for 5 minutes and Resuspend in wash buffer again (0.5 mL per 10^8 cells).

- Column Separation

Note: Use Mini Macs column for cells $\leq 2 \times 10^8$

Use Midi Macs column for cells $> 2 \times 10^8$ to 2×10^9

- Wet the column with appropriate volume of wash buffer (500 μL for Mini Macs OR 3 mL for Midi Macs)
- Put a filter on top of the column and apply cell suspension
- Wash twice with appropriate volume of wash buffer (500 μL for Mini Macs OR 3 mL for Midi Macs)
- Collect CD34+HSPC into a separate 15ml centrifuge tube and count the number.
- Calculate the recovery rate

  Recovery rate=Number of CD34+HSPC/ Number of mononuclear cells

  (Note: recovery rate should not be over 2% in order to maintain high purity)

- Spin CD34+HSPC down at 1200rpm for 5minutes and resuspend in IMDM with 20%bovine growth serum and 1% antibiotics and store at 4°C for overnight. Do experiment the next day.

2.5 Magnetic-field-driven cellular uptake of FITC-mCNT

2.5.1. Seeding cells into 35mm cell culture dishes

For adherent cells like MCF-7, we collected the cells using 0.25% trpsin-EDTA solution and counted the number via hemacytometer under the reverted Microscope. Based on our counting, we diluted the cells into $3x10^5$/ml with DMEM supplied with 10% FBS and 1%antibiotics. At last, we transfer 1ml of diluted cell suspension into 35mm cell culture dishes and incubate overnight in the incubator set up with 37 ℃ and 5%CO_2. We started the magnetic-field-driven uptake experiment the next day.

For suspended cells like THP-1 and CD34+HSPC, we counted the cell number on the same day when we did the magnetic-field-driven uptake experiment and diluted into $3x10^5$/ml with 1xPBS and put 1ml into 35mm cell culture dishes which were pretreated with 10ug/ml poly-l-lysine for 30minutes. And then we put the cell culture dishes into the incubator sep up with 37℃ and 5%CO2 for 45minutes before starting the magnetic-field-driven uptake experiment.

2.5.2. Preparation of FITC-mCNT and mCNT solution

On the day before we started the experiment, we measured the exact amount of chemically functionalized carbon nanotubes through electronic balance and transferred into new 50ml disposable centrifuge tubes. And then

we put in each tube 50ml serum-free cell culture medium and sonicated for 30minutes in order to have well distributed carbon nanotube solution. After sonicaiton, we kept the carbon nanotube solution at 4℃ overnight.

2.5.3. The magnetic-field-driven delivery experiment

As figure 3.2 shows, culture dishes with cells attached to the bottom were vertically placed into a beaker containing 50 ml serum-free medium with different concentrations of FITC-mCNT or mCNT (10, 20 and 40 μg ml−1) and then the beaker was placed on a magnetic stirrer set up at 1200 rpm for some time which is 3minutes for MCF-7 or 10minutes for THP-1 and CD34+HSPC (111).

Culture dishes were then transferred to an Nd–Fe–B permanent magnet for some time which is 3minutes for MCF-7 or 10minutes for THP-1 and CD34+HSPC. The uptake experiment was terminated by washing the cells with PBS and adding new cell culture medium. The cells were incubated for 1, 3, 6, 24 and 48 h at 37℃, 5% CO2 for uptake efficiency of FITC-mCNT.

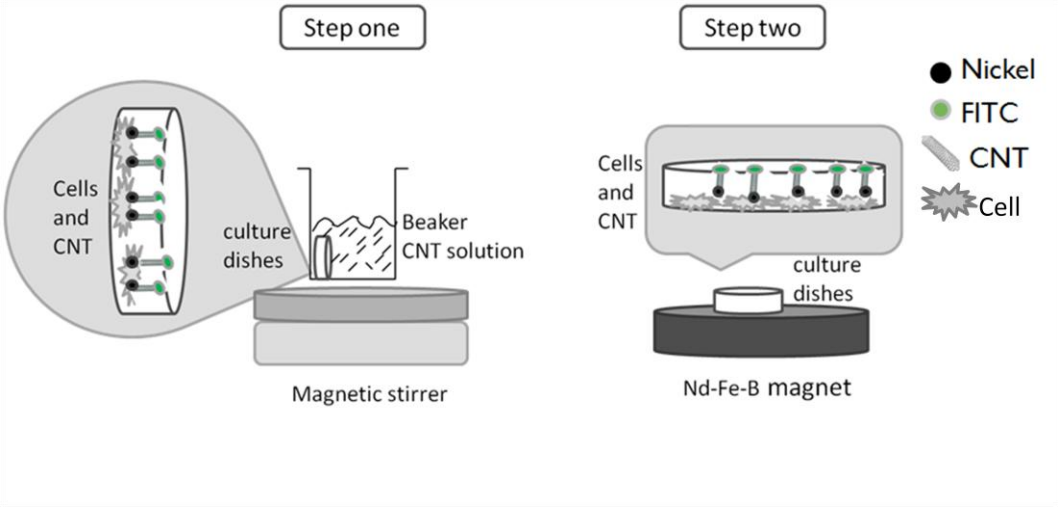


Figure 3.2 schema of magnetic-filed driven delivery method.

2.6 Uptaking efficiency test--Flow cytometry analysis

Flow cytometry is a machine used to analyze microscopic particles, such as cells based on their different fluorescent properties. It has a very broad application in both clinical and research field. Clinical applications included monitoring AIDS patients, diagnosis of Paroxymal Nocturnal Hemoglobinuria and so on. Research application included cell cycle test, proliferation test, viability test and so on(95, 135)

Sample preparation for flow cytometry analysis:

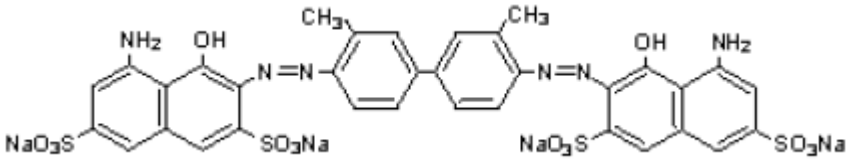
- Collect cells into sterile Falcon plastic tubes. (size 12cmx0.75cm)
- Spin down at 1200rpm for 5minutes and discard the supernatant.

- Add 1ml 1xPBS into the tube and resuspend the cells. Spin down at 1200rpm for 5minutes and discard the supernatant.
- Repeat step 3 one or two more times if necessary
- Resuspend cells in 400ul 1% paraformaldehyde solution in 1xPBS.

Cell uptake test and analysis were carried out via FACscan which was bought from Becton-Dickinson, San Jose, CA, USA.

2.7Cell viability test--Trypan blue exclusion assay

Trypan blue, derived from toluidine, is a vital stain used to dye dead tissue or cells blue. It has the chemical name 3,3'-[(3,3'-dimethyl-4,4'-biphenylylene) bis (azo)] bis(5-amino-4- hydroxy-2,7-naphthalenedisulfonic acid) tetra sodium salt, a molecular weight of 960.8, a molecular formula of $C_{34}H_{24}N_6Na_4O_{14}S_4$, and has the following chemical structure(136)



Trypan blue itself cannot pass through the cell membrane unless it is broken, therefore live cells or tissue with intact membrane cannot be stained while dead cells or tissue can be stained. This staining method is also named as trypan blue exclusion assay because live cells or tissue are excluded from staining. In this

assay, trypan blue is prepared as 0.4% (w/w) solution with 1xPBS. Here is the protocol we followed to do this assay:

- Make a cell suspension in a known volume and mix well with pippeting
- Transfer 20ul into a 0.5ml eppendorf centrifuge tube and add in 20ul 0.4% trypan blue solution.
- Vortex for 5 second and transfer on top of the hemocytometer carefully and count the live cells under inverted microscope.
- Calculate the number of cells per ml and the total number of cells using the following formula:

  $$\text{Cell number (per ml)} = \frac{\text{total number of cells counted}}{\text{total number of squares counted}} \times \text{dilution factor} \times 10^4$$

- Repeat count one or two more times to check the reproducibility.

2.8 Confocal microscopy

Compared with ordinary light microscopy, confocal microscopy is an optical imaging technique of obtaining micrograph with better optical resolution and contrast by removing out-of-focus light in specimens that are thicker than the focal plane through point illumination and a spatial pinhole(137). It enables the reconstruction of three-dimensional structures from the obtained images. This technique is popularly applied in life sciences and material science. In this project,

we used confocal microscopy to take pictures of the cells contained FITC-mCNT and reconstructed 3-D structure of the cells so as to verify the location of FITC-mCNT.

Sample preparation and observation under confocal microscope

- Sterilize cover slips using 95% ethanol and put it into 35mm cell culture dishes before seeding cells.
- Seed MCF-7, THP-1 or CD34+HSPC according the protocol described previously
- Do magnet-filed-driven cell uptake experiment according the protocol described previously.
- Fix cells with 1ml 4% paraformaldehyde and store overnight at 4℃.
- Take pictures under an inverted confocal laser scanning microscope (Carl Zeiss LSM510, Toronto, Canada) equipped with imaging software (LSM 5 Image Browser, Carl Zeiss).

In this project, we did 3-D structure reconstruction with MCF-7 and THP-1 since CD34+HSPC was too small and fragile. In order to do 3-D structure reconstruction, we marked the cell outline with Rhodamine-Phalloidin. Phalloidin is a specific antibody to F-actin which usually stays underneath the cell membrane. Hence, Rhodamine-Phalloidin can be used to mark the cell outline.

- Take cells fixed with 4%paraformaldehyde and gently rinse with twice with 1xPBS to get rid of the paraformaldehyde
- Incubate cells Incubate cells in Rhodamine-phalloidin (Molecular Probes) diluted 1:100 in PBS for 15 minutes
- Rinse 3 times in 1xPBS, 5minutes per each.
- Mount the cover slips onto microslides with mounting media containing DAPI which is one kind of cell nucleus dye.
- Seal the cover slips with clear nail polish

2.9 *In vitro* differentiation ability test of CD34+HSPC --Colony-forming unit assay (CFU assay)

As described previously, CD34+HSPCs have the ability to differentiate into all mature hematopoietic cells. During differentiation to mature blood cells, CD34+HSPCs will go through intermediate stages including multi-potential progenitors and lineage-committed progenitors. *In vitro* Colony-forming unit assay provides information about the frequencies and growth properties of hematopoietic progenitor cells at various developmental stages (138).

When cultured in methylcellulose supplemented with appropriate cytokines and supplements, CD34+HSPCs proliferate and differentiate to form discrete colonies containing colony-forming unit-erythroid (CFU-E) and burst-forming

unit-erythroid (BFU-E); colony-forming unit-granulocyte, macrophage (CFU-GM); colony-forming unit-granulocyte (CFU-G) and colony-forming unit-macrophage (CFU-M); and colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM). Under optimal plating and culture conditions, each colony is derived from a single progenitor. Thus, the number and types of colonies counted in a CFU assay provides information about the ability of CD34+HSPCs to proliferate and differentiate (138).

In order to test the effect of carbon nanotube uptake on the proliferation and differentiation of CD34+HSPCs, we did CFU assay with CD34+HSPCs incubated with 40ug/ml FITC-mCNT.

- Do magnetic-field-driven cell uptake of FITC-mCNT according the protocol described previously
- After 1hour, 3hours and 6hours of incubation in 37℃ incubator, CD34+HSPCs were collected and counted.
- CD34+ HSPCs were plated into 35mm cell culture dishes in triplicate in standard semi-solid methylcellulose haematopoietic progenitor culture media (human MethoCult GF; Stem Cell Technologies, Vancouver, BC, Canada) at concentrations of $1x10^3$/ml.
- These culture plates were incubated in 37℃ incubator supplemented with 5% CO2. Colonies were identified and enumerated 14 days later.

3. Results

3.1 High cellular uptake of FITC-mCNTs by MCF-7 and THP-1 cells

Highly efficient magnetic-field-driven gene delivery using mCNT into difficult-to-transfect primary neurons and B lymphocytes has been recently achieved (111). FITC-mCNT with both fluorescent and magnetic properties were synthesized and freshly used for our uptake experiments as summarized in figure 3.1. Compared to the reported method using 1,3-dipolar cycloaddition as the key step to make watersoluble CNT with a yield of only 10% (129), our synthesis of FITC-mCNT has much higher yield. In order to test our fluorescent and magnetic FITC-mCNTs for labelling MCF-7, MCF-7 cells were exposed to solutions of FITC-mCNT or mCNT alone (40 μg ml−1) in the presence of a magnetic field. The uptake efficiencies of these cells were determined using FACS 1, 3 and 6 h after the uptake. As indicated in figure 3.3, in MCF-7 cells, the FITC-mCNT uptake began efficiently (35% FITC uptake) 1 h after exposure and reached its maximum efficiency (100% FITC uptake) after 3h. It also kept the same efficiency after 6h. We also took confocal images to confirm the labeling and to see the localization of FITC-mCNTs in MCF-7 cells. As figure 3.4 showed, FITC-mCNTs crossed the cell membrane and entered the cytoplasm and even into the nucleus. What is more, we tested the cytotoxicity of FITC-mCNT on MCF-7 cells via typan blue exclusion

staining. As expected, there was no cell viability compromise 1h, 3h and 6h after FITC-mCNT labeling (Figure 3.5).

We also tested our fluorescent and magnetic FITC-mCNTs for labeling THP-1 cells which were intrinsically more difficult to transfect than attached cells such as MCF-7 cells. According to our FACS results of this study, the FITC-uptake rate reaches 100% even after 1hr as shown in Figure 3.6. Mean fluorescence at all time points was increased compared with the control. It reached maximal level after a 6-hour incubation period. Confocal microscopy observation indicated that FITC-mCNTs were effectively taken up by THP-1 cells (Figure 3.7). In addition, we also demonstrated FITC-mCNTs were not toxic to THP-1 cells (Figure 3.8).

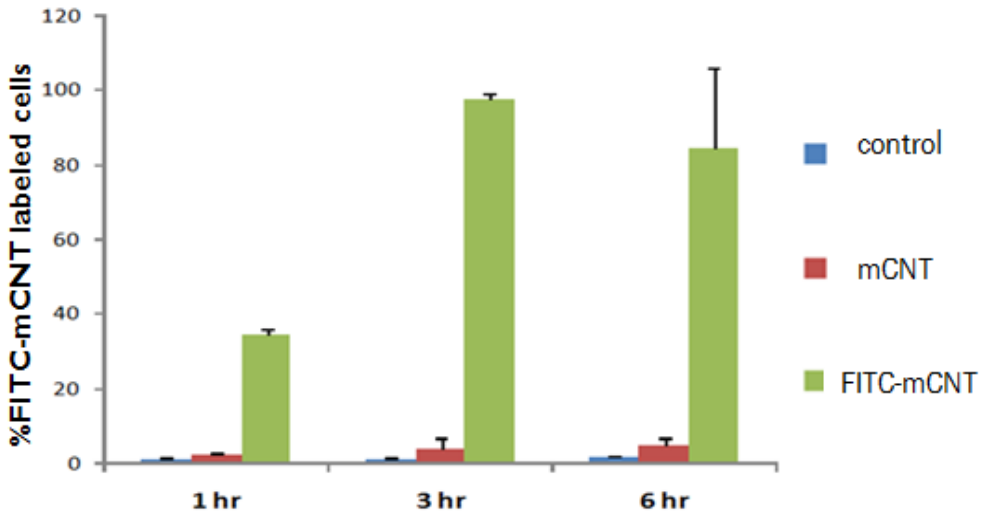


Figure 3.3. (Color) The percentage of FITC-expressing cells was quantified by flow cytometry (FACS). After three hours, the FITC-uptake rate reaches 100%.

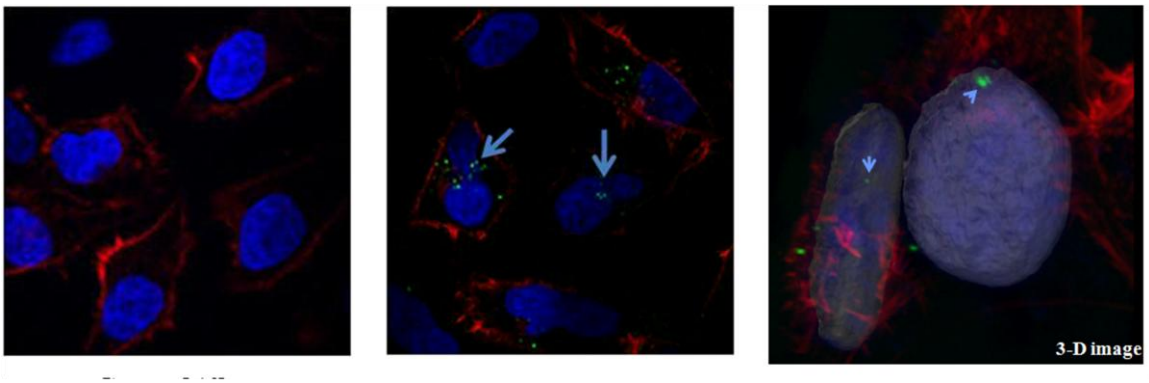


Figure 3.4. FITC-mCNT localized in the cytoplasm and nucleus of MCF-7 cells.

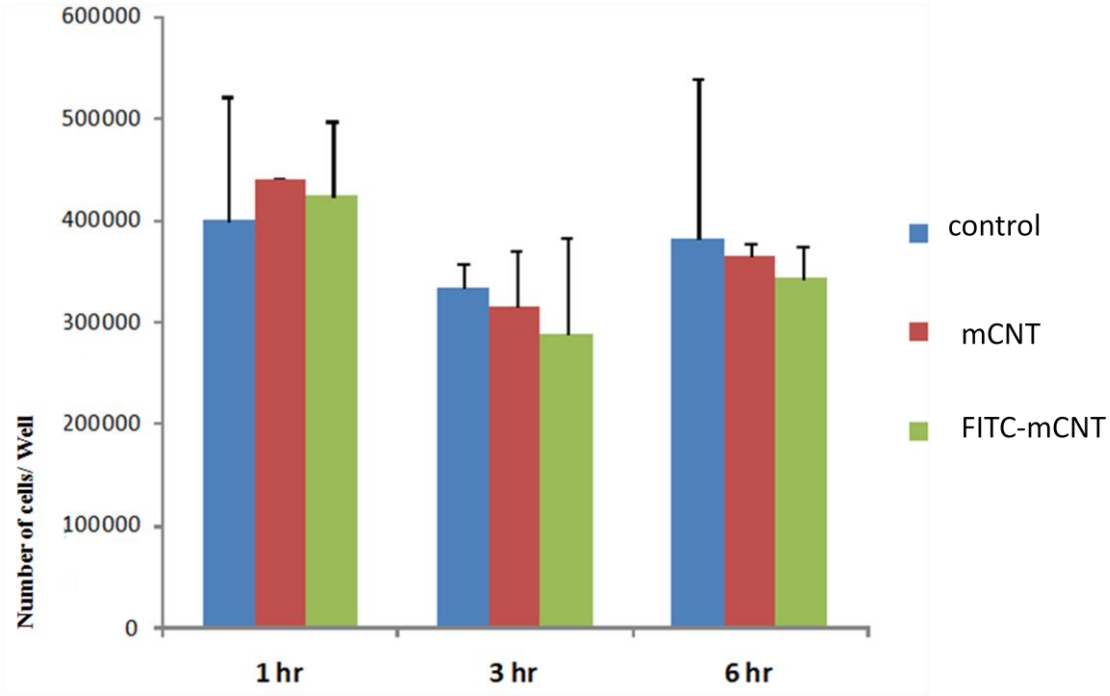


Figure 3.5. FITC-mCNT labeling did not affect the viability of MCF-7 cells after 1h,3h and 6h.

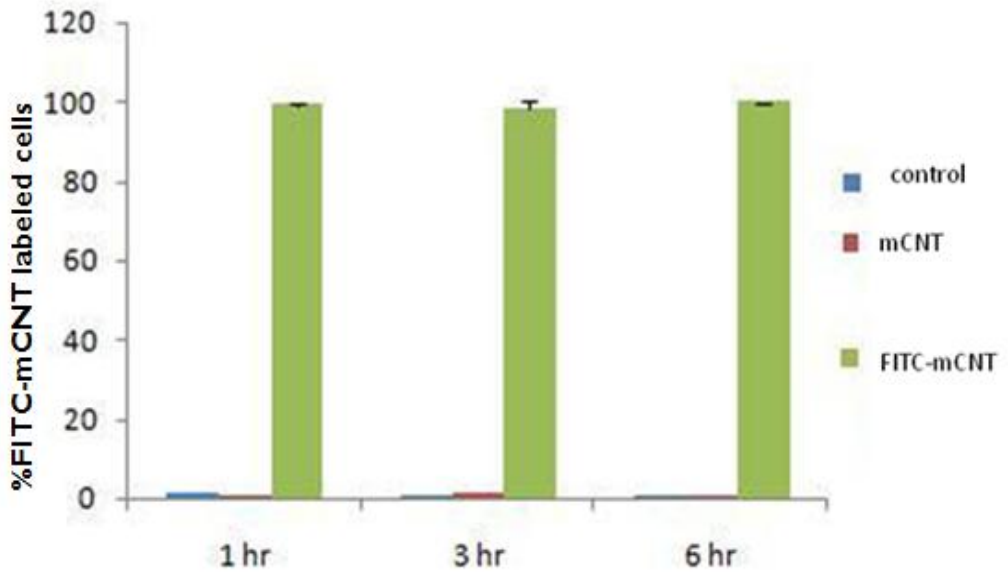


Figure 3.6. (Color) The percentage of FITC-expressing THP cells was quantified by flow cytometry (FACS). After one hour, the FITC-uptake rate reaches 100%.

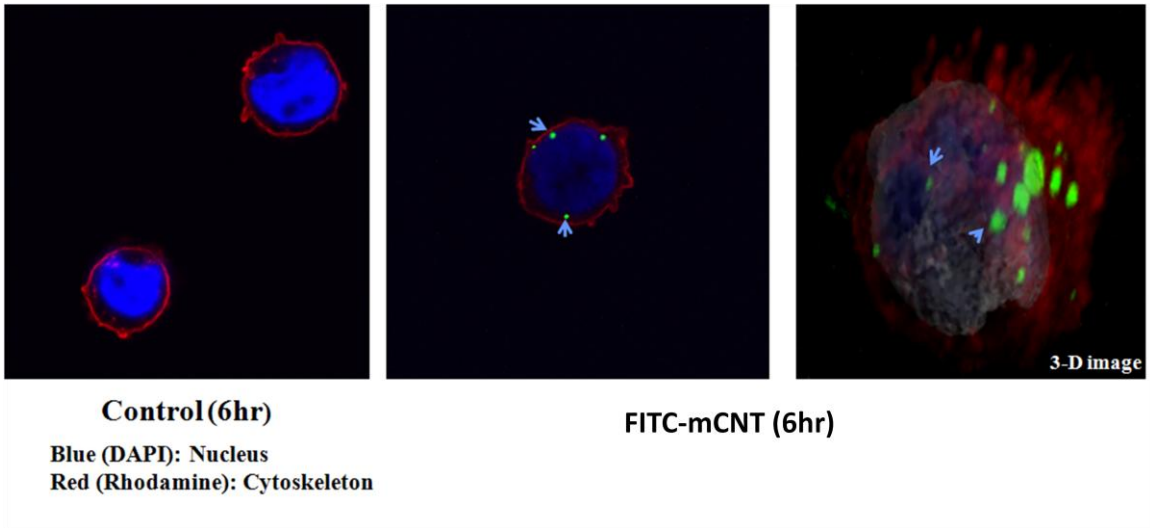


Figure 3.7. FITC-mCNT localized in the cytoplasm and nucleus of THP-1 cells.

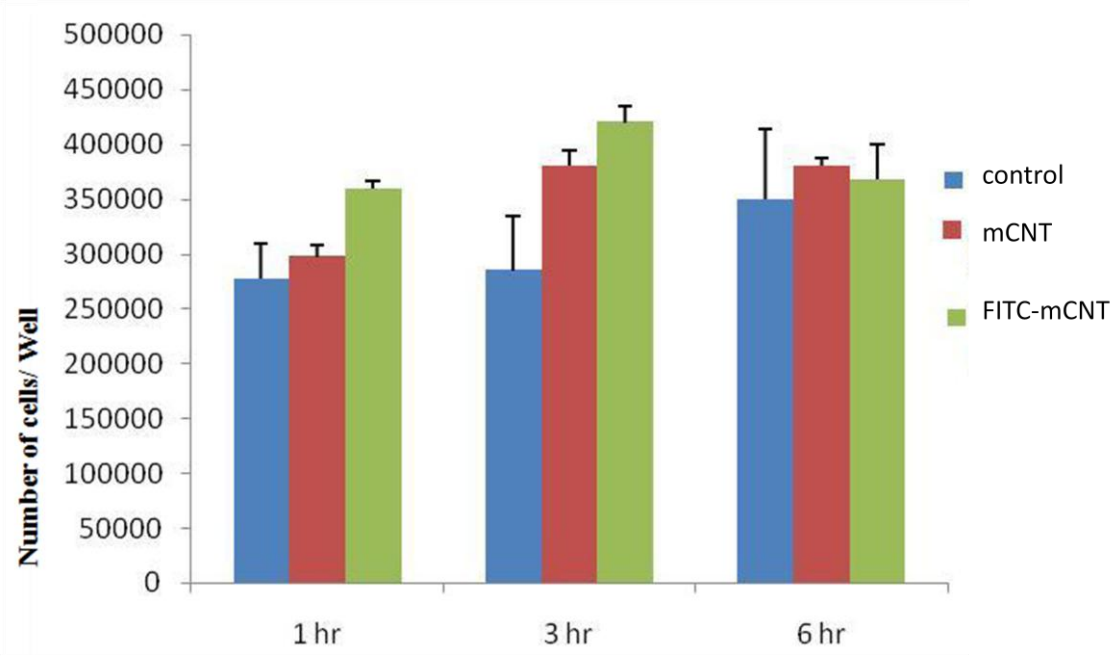


Figure 3.8. FITC-mCNT labeling did not affect the viability of THP-1 cells after 1h,3h and 6h.

3.2 High cellular uptake of FITC-mCNT by CD34+ cells

After the evaluation of our delivery method with both attached and suspension cells, we tested FITC-mCNTs for labelling HSPCs. CD34+ cells obtained from LP were exposed to solutions of different concentrations of FITC-mCNT or mCNT alone (10, 20 and 40 μg ml−1) in the presence of a magnetic field. The uptake efficiencies of these cells were determined using FACS 1, 3 and 6 h after the uptake. In LP CD34+ cells, the FITC-mCNT uptake began efficiently (45% FITC uptake) 1 h after exposure even at the lowest FITC-mCNT concentration (10 μg

ml−1) and reached its maximum efficiency (83% FITC uptake) after 6 h (figure 3.9(A)). FITC reached 83%, 90% and 100% in LP CD34+ cells at 6 h after uptake of FITC-mCNT with 10, 20 and 40 μg ml−1 concentrations, respectively. Although LP CD34+ cells were ∼100% FITC-positive at 3 and 6 h after uptake of FITC-mCNT (40 μg ml−1), the mean fluorescence (MF) was the highest at 6 h after FITCmCNT uptake (figure 3.9 (B)), which indicates more uptake of FITC-mCNT into CD34+ cells as time increases. Therefore, we further investigated the internalization of the highest concentration of FITC-mCNT (40 μg ml−1) into these cells at 24 and 48 h after uptake. LP CD34+ cells were still about 98% FITC-positive 24 and 48 h after FITC-mCNT uptake, though their MF significantly dropped compared to that at 6 h (figure 3.9 (C)), most likely due to FITC degradation over time.

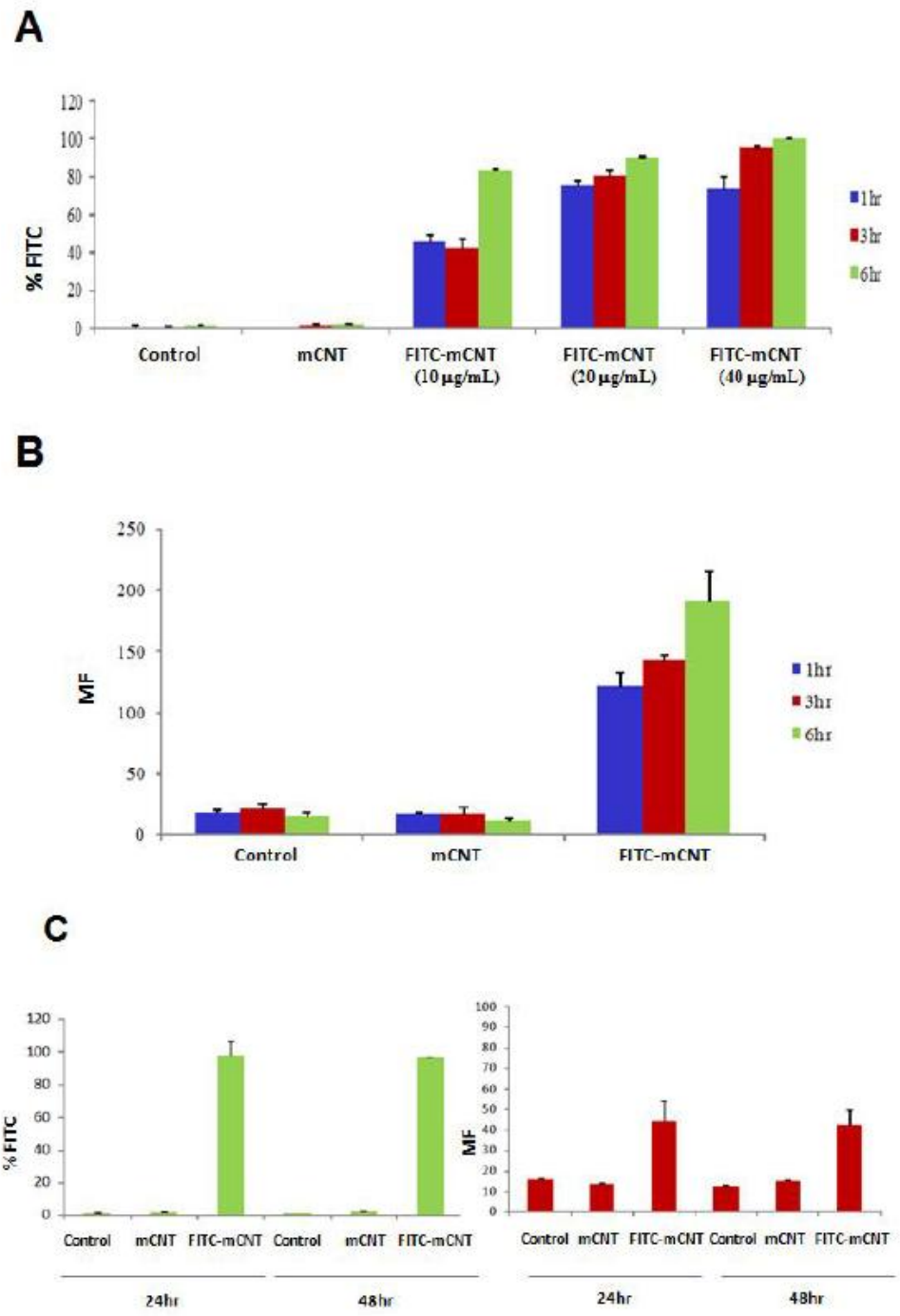


Figure 3.9. Efficient uptake of FITC-mCNTs in LP HSPC as shown by FACS analysis. (A) % FITC of LP CD34+ cells exposed to increasing concentrations of FITC-mCNTs or mCNTs (10, 20 and 40 μg ml−1) at 1, 3 and 6 h after exposure. (B) Mean fluorescence of LP CD34+ cells exposed to FITC-mCNT (40 μg ml−1) at 1, 3 and 6 h after delivery. (C) % FITC and MF of LP CD34+ exposed to FITC-mCNT (40 μg ml−1) 24 and 48 h after delivery.

To confirm that even more immature HSPCs can efficiently uptake FITC-mCNT, we next studied the internalization of FITC-mCNT (40 μg ml−1) into CB-derived HSPCs containing a larger immature stem cell fraction (CD34+, CD38−phenotype) than LP HSPCs (139). The efficient uptake started after about 1 h in CB CD34+ cells as demonstrated by ∼40% FITC uptake rate shown in figure 3.10(A), which was lower compared to the result in LP CD34+ cells. FITC-mCNT uptake reached 90% about 6 h after its exposure to the cells, similar to the result in LP CD34+ cells, based on the FACS analysis (figure 3.10 (A)). Confocal analysis also confirmed the efficient internalization of FITC-mCNT into CB CD34+ cells (figure 3.10 (B)), similar to confocal data obtained in THP-1 and MCF-7 cells. FITC-mCNT uptake was saturated at 6 h after its exposure to CB CD34+ cells similar to LP CD34+ cells (figure 3.10 (B)). However, we did not observe a fluorescence signal by confocal analysis 1 h after FITC-mCNT exposure to cells, most probably due to a weak fluorescence signal. The HSPC that were not exposed to FITC-mCNT (or the control) did not show any evidence of green fluorescence by confocal analysis, which is consistent with the FACS data in figure 3.10 (B). These data suggest that FITC-mCNTs are efficiently internalized by HSPC in a time and concentration-dependent manner, regardless of the HSPC source.

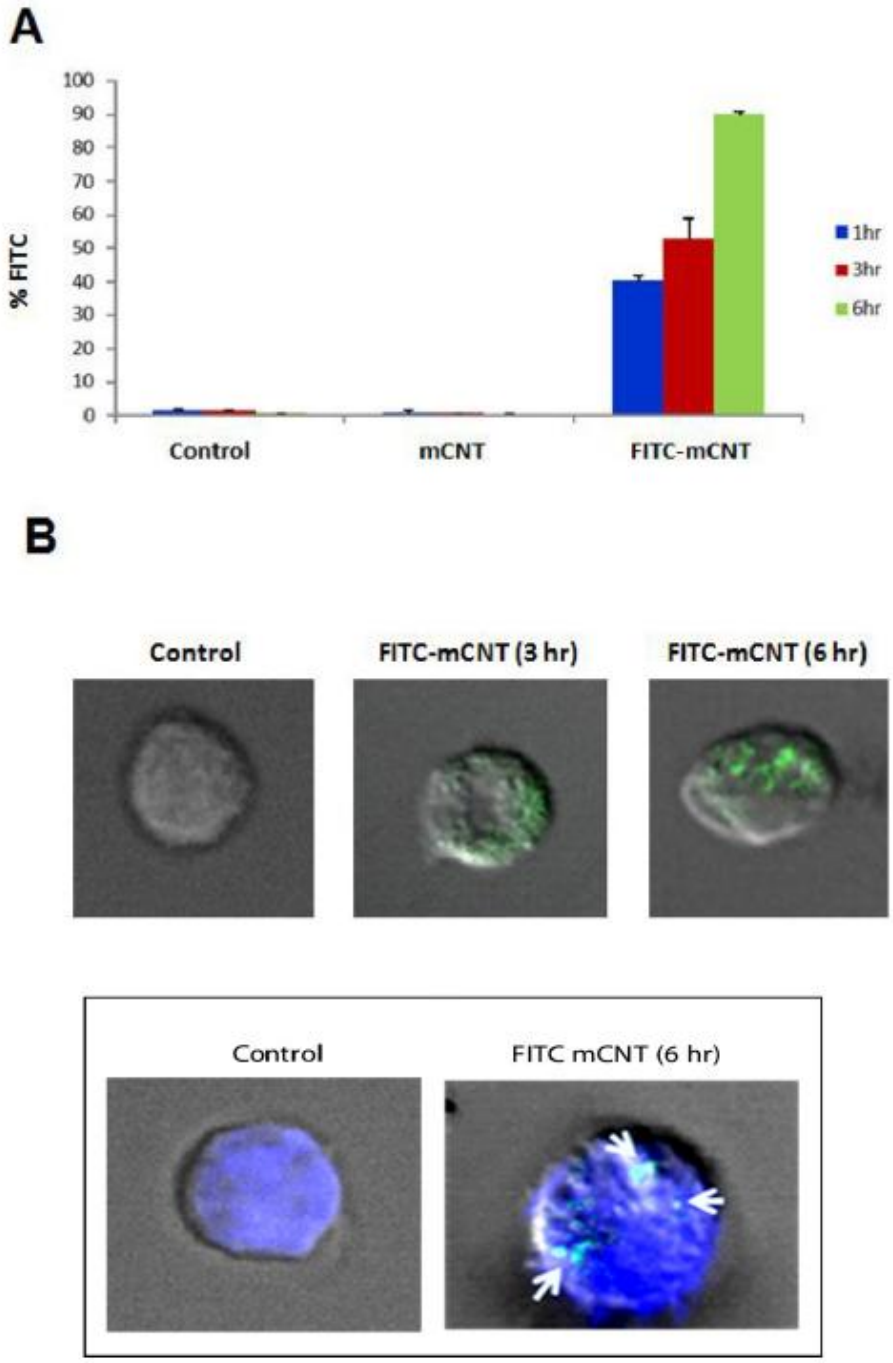


Figure 3.10. FITC-mCNTs are also taken up effectively into CB HSPC. (A) % FITC of CB CD34+ cells labelled with FITC-mCNT (40 μg ml−1). (B) Confocal microscopy of FITC-mCNT-labelled CB CD34+ cells at 3 and 6 h after delivery. Blue (DAPI): nucleus.

3.3 FITC-mCNT uptake does not affect CD34+ cell viability

To investigate the cytotoxicity of mCNT, we studied the HSPC viability after FITC-mCNT uptake by trypan blue exclusion assay. With the exposure of FITC-mCNT to various concentrations (10, 20 and 40 μg ml−1), the viability of LP CB CD34+ cells was not compromised compared to the control even 6 h after delivery when the uptake rate reached its peak in these cells (figure 3.11(A)). As expected, the viability of CB CD34+ cells was also not affected by the uptake of FITC-mCNT (figure 3.11(B)). There was no difference observed between the viability of LP and CB HSPCs using FITC-mCNT or mCNT alone in comparison with the control at 24 or 48 h after uptake.

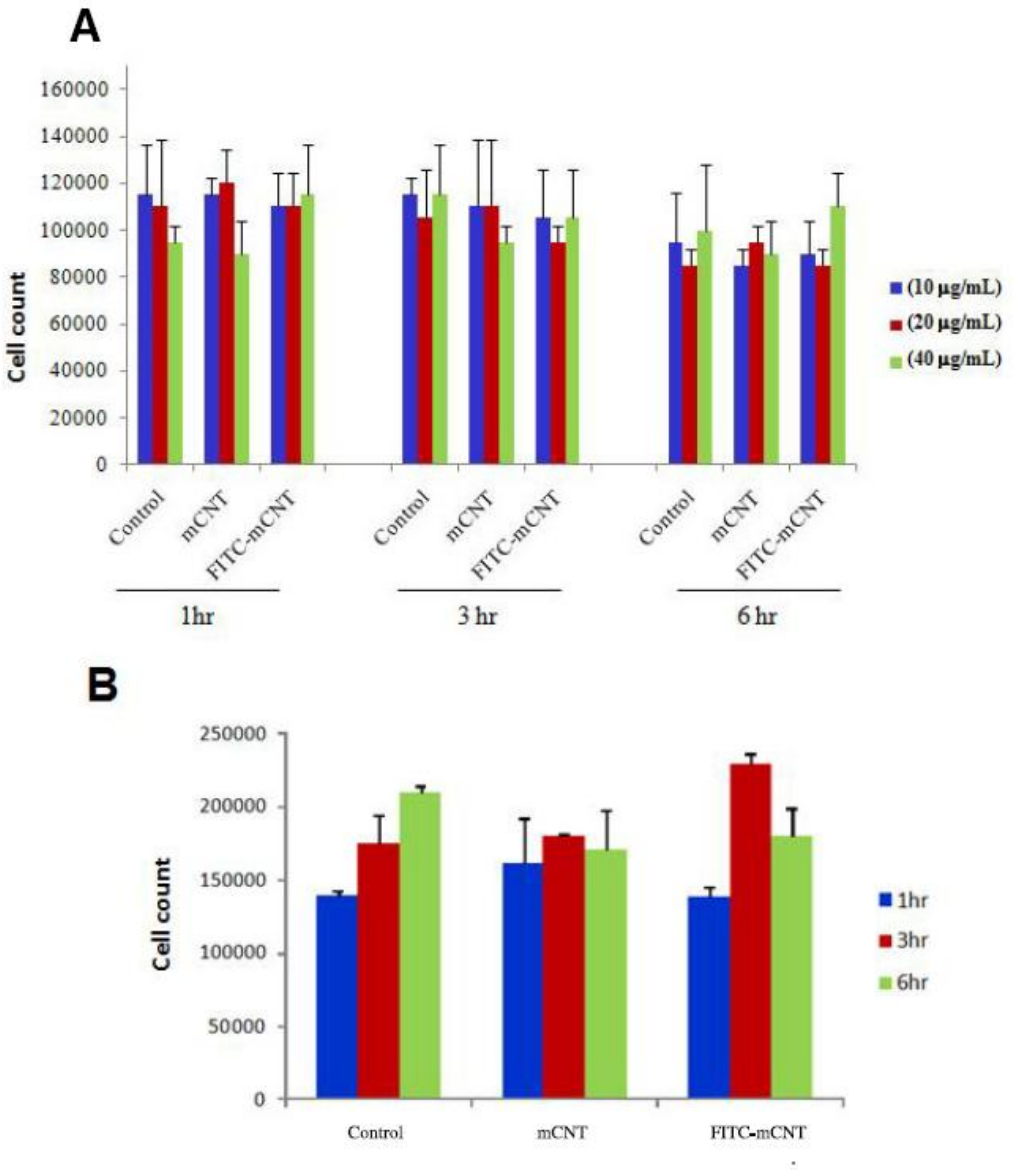


Figure 3.11. FITC-mCNTs show no adverse effect on cell viability of HSPC cells. Count of FITC-mCNT-labeled (A) LP CD34+ cells and (B) CD34+ cells.

3.4 Differentiation of HSPC was not compromised by efficient FITC-mCNT uptake

It has been reported that CNT has no adverse effect on macrophages, was not cytotoxic and has no significant effect on adipogenic, osteogenic or chondrogenic differentiation of hMSC (121). To investigate the long-term cytotoxicity effect of our magnetic-field-driven FITC-mCNT uptake and their impact on the differentiation of HSPCs, we performed a colony unit assay (CFU) on FITC-mCNT-labeled HSPCs 1, 3 and 6 h after uptake. After 14 days, colonies were identified and enumerated. No evidence was observed of cytotoxicity nor was the differentiation affected in FITCmCNT-labeled HSPC because there was no difference in overall colony number or type (CFU-GM: colony-forming unit of granulocyte/macrophage; BFU-E: burst forming unit of erythrocyte; CFU-GEMM: colony-forming unit of granulocyte macrophage-erythroid-megakaryocyte) between the FITCmCNT-labeled and the control HSPC (figure 3.12). These observations suggest that FITC-mCNT internalization is not only efficient and safe, but it also does not alter the HSPC's properties.

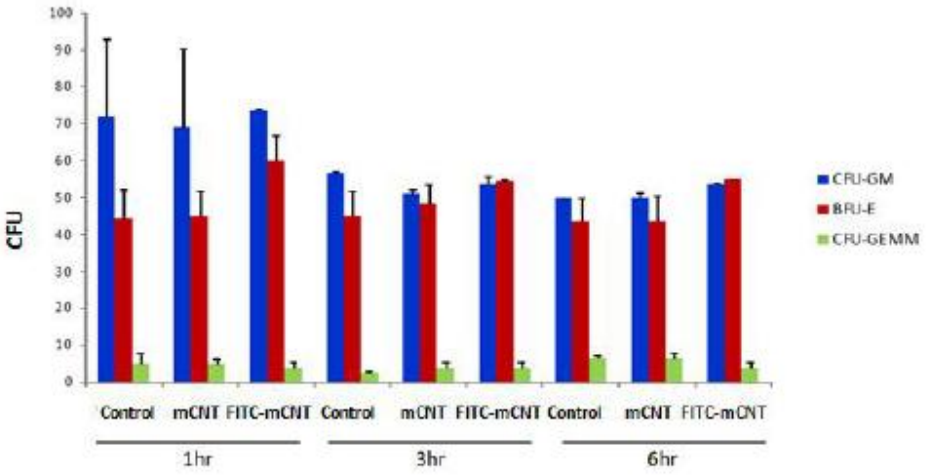


Figure 3.12. Internalization of FITC-mCNTs did not affect the differentiation of HSPC. Colony-formation unit of FITC-mCNT-labelled HSPCs at 1, 3 and 6 h after the internalization by clonogenic assay.

4. Discussion

CNT have been proposed recently as new labels for cellular imaging and vehicles for delivering biomolecules into cells due to their nanoscale tip sizes (about 1.2 nm) that can lead to enhanced cell membrane penetration and low cytotoxicity (111, 119-121). To date, only one study reported their effects on hMSC and suggested their use for tissue repair and regeneration. In this chapter, we report that FITC-mCNTs are efficiently taken up by attached cells, suspension cells and even CB and LP HSPCs. The delivery method is fast and safe and could be used for tracking the movement of the transplanted stem cells. The experimental results are in line with our expectations and previous findings by other researchers. This efficient mCNT delivery method does not depend on a targeted cell type because this method employs a physical magnetic force for delivery, unlike a biological mechanism like liposome or viral. In contrast to our findings, it has been shown that ferumoxides particles were taken up only by CB cells, but not PB cells (140), indicating that our magnetic-field-driven FITC-mCNT can enter target cells regardless of cell type and source. Similar to the effect of COOH-functionalized single-walled CNT in hMSC (121), our FITC-mCNT labelling or cell uptake has no unfavorable side-effects on viability or differentiation of

HSPC. However, the magnetic-field-driven internalization of FITC-mCNT is much more rapid (began 1 h after the delivery) than internalization of CNTs into hMSC, which occurred at 24 h of incubation. Compared with other nanomaterial uptake using native or modified super magnetic iron oxide (SPIO) (141), FITC-mCNT labelling or uptake is more efficient for stem cell labelling. Although the modification of these SPIO with an alternative strategy using HIV-Tat peptide led to highly efficient internalization of these particles into HSPC (128), concerns have been raised with respect to the biosafety of a xenogenic protein and the requirement of long term incubation for internalization. In clinical trials involving HSPC, the safe labeling of stem cells should be performed within a period of less than 24 h after their isolation from patients. Our rapid FITC-mCNT labeling of HSPC might offer a solution for the difficulty of tracking the movement of transplanted HSPC in patients.

Magnetic resonance imaging (MRI) provides a noninvasive *in vivo* method to studying the fate of transplanted cells labeled with magnetic nanoparticles. HIV-Tat peptide conjugated SPIO magnetic-particle-labeled human HSPC subsets have been reported to be tracked using the non-invasive MRI technology (128). However, the detection of mCNT by standard MRI would not be possible due to its low resolution. It was recently reported that CNT functionalized with paramagnetic contrast agents (gadolinium chelates) could be detected *in vivo* with an MRI scanner (142). Therefore, mCNT modified with a contrast agent such

as gadolinium chelates could be used for *in vivo* detection and tracking of HSPC using MRI. Another possibility of detecting or tracking our mCNT-labeled HSPC *in vivo* might be through an optical cell tracking device due to the fact that CNT possess an optical transition in the near-infrared (NIR). The ability of NIR imaging to detect single-walled CNT in organisms and biological tissues has been recently demonstrated (120). In addition to their use for HSPC labeling and tracking, mCNT could also be used as a perfect platform to deliver genes into hard-to-transfect HSPC. The genetic modification of HSPC offers enormous potential for the treatment of genetic diseases of haematopoiesis owing to the ability to provide permanent correction (143). However, transfection efficiency in HSPC is very low, which results from the low division frequency of the target cells and the quiescent nature of the most primitive HSPC (140, 144). Though viral vectors are capable of efficiently transporting recombinant DNAs into a cell, the undesirable consequences of a viral integration process (i.e. haphazard activation or silencing of host genes), as well as the immunogenicity of viral particles, have recently raised safety concerns about viral vectors (145). Hence an improved, safe and efficient non-viral gene delivery technique using mCNT might overcome these obstacles for stem-cell based gene therapies.

5. **Conclusion**

In this chapter, we demonstrated for the first time an efficient and safe mCNT-mediated labeling method for HSPC while not compromising viability or differentiation. Although still in the exploratory stage, mCNT-mediated labeling of HSPC described in this paper has exceptional long-term potential and would be an important technology with wide applications for *in vivo* labeling/tracking of HSPC and gene delivery into HSPC. The proposed technology can also be applied for gene therapy to cure cancer, hepatitis, AIDS, Alzheimer's disease and Parkinson's disease.

REFERENCE

1. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell 2000 Jan 7; 100 (1): 157-168.

2. Weissman IL, Anderson DJ, Gage F. Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Annu Rev Cell Dev Biol 2001; 17: 387-403.

3. Ball ED. Ex vivo purging and cell expansion. J Hematother Stem Cell Res 1999 Oct; 8 (5): 457-458.

4. Leger CS, Nevill TJ. Hematopoietic stem cell transplantation: a primer for the primary care physician. CMAJ 2004 May 11; 170 (10): 1569-1577.

5. Hideo Ema TK, and Hiromitsu Nakauchi. Principles of Hematopoietic Stem Cell Biology. 2010.

6. http://stemcells.nih.gov/info/basics (2008) Stem cell basics. In Stem cell information. NationalInstitutes of Health, Bethesda.

7. Till JE ME. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 1961; 14 (213): 22.

8. Yang L BD, Adolfsson J, Nygren J, Mansson R, Sigvardsson M, Jacobsen SE. Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable ofrapidly reconstituting and rescuing myeloablated transplant recipients. Blood 2005 (105): 2717-2723.

9. Pronk CJ, Rossi DJ, Mansson R, Attema JL, Norddahl GL, Chan CK, et al. Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. Cell Stem Cell 2007 Oct 11; 1 (4): 428-442.

10. Schöler HR. The Potential of Stem Cells: An Inventory In Nikolaus Knoepffler, Dagmar Schipanski, and Stefan Lorenz Sorgner. Humanbiotechnology as Social Challenge 2007; Ashgate Publishing, Ltd. p. 28. ISBN 0754657558.

11. Vats A, Bielby RC, Tolley NS, Nerem R, Polak JM. Stem cells. Lancet 2005 Aug 13-19; 366 (9485): 592-602.

12. http://en.wikipedia.org/wiki/Stem_cell.

13. Jacobson LO. Marks EK RMGEZR. Effect of spleen protection on mortality following X-irradiation. 1 Lab Clin Med 1949: 34:1538-1543.

14. Lorenz E UD, Reid TR, Shelton E. Modification o f irradiation injury i n mice and guinea pigs by bone marrow injections. 1 Natl Cancer Inst 1951: 12: 197-201.

15. Nowell pc CU, Habermeyer JG, Roan PL. Growth and continued function of ratmarrow cells in X-irradiated mice. Cancer Res 1956: 16:258-261.

16. Siminovitch L, McCulloch EA, Till JE. The Distribution of Colony-Forming Cells among Spleen Colonies. J Cell Physiol 1963 Dec; 62: 327-336.

17. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science 1988 Jul 1; 241 (4861): 58-62.

18. Morrison SJ, Uchida N, Weissman IL. The biology of hematopoietic stem cells. Annu Rev Cell Dev Biol 1995; 11: 35-71.

19. http://www.isscr.org/public/adultstemcells.htm.

20. http://en.wikipedia.org/wiki/Hematopoietic_stem_cell.

21. Andrews RE SJ, Bernstein ID. Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of CD33 and CD34 antigen and light scatter. . J Exp Med 1989: 169:1721-1731.

22. Krause DS, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility. Blood 1996 Jan 1; 87 (1): 1-13.

23. Simmons DL, Satterthwaite AB, Tenen DG, Seed B. Molecular cloning of a cDNA encoding CD34, a sialomucin of human hematopoietic stem cells. J Immunol 1992 Jan 1; 148 (1): 267-271.

24. Healy L, May G, Gale K, Grosveld F, Greaves M, Enver T. The stem cell antigen CD34 functions as a regulator of hemopoietic cell adhesion. Proc Natl Acad Sci U S A 1995 Dec 19; 92 (26): 12240-12244.

25. Terstappen LW, Huang S, Safford M, Lansdorp PM, Loken MR. Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. Blood 1991 Mar 15; 77 (6): 1218-1227.

26. Zanjani ED, Almeida-Porada G, Livingston AG, Flake AW, Ogawa M. Human bone marrow CD34- cells engraft in vivo and undergo multilineage expression that includes giving rise to CD34+ cells. Exp Hematol 1998 Apr; 26 (4): 353-360.

27. Bonnet D. Hematopoietic stem cells. Birth Defects Res C Embryo Today 2003 Aug; 69 (3): 219-229.

28. Dao M AJ, Nolta JA. . Reversibility of CD34 expression on human hematopoietic stem cells that retain the capacity for secondary reconstitution. . Blood 2003: 101:4201-4208.

29. Amy H. Yin SM, Esmail D. Zanjani, Graca Almeida-Porada, Makio Ogawa, Anne G. Leary, Johanna Olweus, John Kearney, and David W. Buck, . AC133, a Novel Marker for Human Hematopoietic Stem and Progenitor Cells. Blood 1997: 90: 5002-5012.

30. Ziegler BL, Valtieri M, Porada GA, De Maria R, Muller R, Masella B, et al. KDR receptor: a key marker defining hematopoietic stem cells. Science 1999 Sep 3; 285 (5433): 1553-1558.

31. Danet GH, Luongo JL, Butler G, Lu MM, Tenner AJ, Simon MC, et al. C1qRp defines a new human stem cell population with hematopoietic and hepatic potential. Proc Natl Acad Sci U S A 2002 Aug 6; 99 (16): 10441-10445.

32. http://www.gelifesciences.com/aptrix/upp01077.nsf/Content/cellpreparation_home~products_cell~density_gradient_media~ficoll_pm400?OpenDocument&moduleid=167163.

33. http://www.gelifesciences.com/aptrix/upp01077.nsf/Content/cellpreparation_home~products_cell~density_gradient_media~percoll?OpenDocument&moduleid=167165.

34. http://www.miltenyibiotec.com/en/NN_21_MACS_Cell_Separation.aspx.

35. http://en.wikipedia.org/wiki/Fluorescence-activated_cell_sorting#Fluorescence-activated_cell_sorting.

36. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 2003 Oct 23; 425 (6960): 841-846.

37. Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003 Oct 23; 425 (6960): 836-841.

38. Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 2005 Jul 1; 121 (7): 1109-1121.

39. Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 2004 Jul 23; 118 (2): 149-161.

40. Nilsson SK, Johnston HM, Whitty GA, Williams B, Webb RJ, Denhardt DT, et al. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood 2005 Aug 15; 106 (4): 1232-1239.

41. Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. Annu Rev Cell Dev Biol 2001; 17: 615-675.

42. Yilmaz OH, Valdez R, Theisen BK, Guo W, Ferguson DO, Wu H, et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature 2006 May 25; 441 (7092): 475-482.

43. Schwaller J, Parganas E, Wang D, Cain D, Aster JC, Williams IR, et al. Stat5 is essential for the myelo- and lymphoproliferative disease induced by TEL/JAK2. Mol Cell 2000 Sep; 6 (3): 693-704.

44. Kato Y, Iwama A, Tadokoro Y, Shimoda K, Minoguchi M, Akira S, et al. Selective activation of STAT5 unveils its role in stem cell self-renewal in

normal and leukemic hematopoiesis. J Exp Med 2005 Jul 4; 202 (1): 169-179.

45. Tsai FY, Keller G, Kuo FC, Weiss M, Chen J, Rosenblatt M, et al. An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature 1994 Sep 15; 371 (6494): 221-226.

46. Rodrigues NP, Janzen V, Forkert R, Dombkowski DM, Boyd AS, Orkin SH, et al. Haploinsufficiency of GATA-2 perturbs adult hematopoietic stem-cell homeostasis. Blood 2005 Jul 15; 106 (2): 477-484.

47. Sauvageau G, Thorsteinsdottir U, Eaves CJ, Lawrence HJ, Largman C, Lansdorp PM, et al. Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Genes Dev 1995 Jul 15; 9 (14): 1753-1765.

48. Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, et al. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 2003 May 15; 423 (6937): 302-305.

49. Ogawa M. Differentiation and proliferation of hematopoietic stem cells. Blood 1993 Jun 1; 81 (11): 2844-2853.

50. Mayani H, Dragowska W, Lansdorp PM. Lineage commitment in human hemopoiesis involves asymmetric cell division of multipotent progenitors and does not appear to be influenced by cytokines. J Cell Physiol 1993 Dec; 157 (3): 579-586.

51. Scott EW, Simon MC, Anastasi J, Singh H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science 1994 Sep 9; 265 (5178): 1573-1577.

52. Kuhl C AA, Iborra F, Nieswandt B, Porcher C, Vyas P GATA1-mediated megakaryocyte differentiation and growth control can be uncoupled and mapped to different domains in GATA1. Mol Cell Biol 2005: 25:8592-8606.

53. Suzuki N SN, Ohneda O, Obara N, Imagawa S, Pan X, Motohashi H, Yamamoto M. Identification and characterization of 2 types of erythroid progenitors that express GATA-1 at distinct levels. Blood 2003: 102:3575-3583.

54. Moreau-Gachelin F WF, Molina T, Denis N, Titeux M, Grimber G, Briand P, Vainchenker, W TA. Spi-1/PU.1 transgenic mice develop multistep erythroleukemias. Mol Cell Biol 1996): 16:2453-2463.

55. Kulessa H FJ, Graf T. GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts, and erythroblasts. Genes Dev 1995: 9:1250-1262.

56. Wadman IA, Osada H, Grutz GG, Agulnick AD, Westphal H, Forster A, et al. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. EMBO J 1997 Jun 2; 16 (11): 3145-3157.

57. Tsang AP, Fujiwara Y, Hom DB, Orkin SH. Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. Genes Dev 1998 Apr 15; 12 (8): 1176-1188.

58. Metcalf D. Concise review: hematopoietic stem cells and tissue stem cells: current concepts and unanswered questions. Stem Cells 2007 Oct; 25 (10): 2390-2395.

59. Cutler C, Antin JH. Peripheral blood stem cells for allogeneic transplantation: a review. Stem Cells 2001; 19 (2): 108-117.

60. Ende M, Ende N. Hematopoietic transplantation by means of fetal (cord) blood. A new method. Va Med Mon (1918) 1972 Mar; 99 (3): 276-280.

61. Wagner JE, Kurtzberg J. Cord blood stem cells. Curr Opin Hematol 1997 Nov; 4 (6): 413-418.

62. Gluckman E, Broxmeyer HA, Auerbach AD, Friedman HS, Douglas GW, Devergie A, et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J Med 1989 Oct 26; 321 (17): 1174-1178.

63. Wagner JE, Barker JN, DeFor TE, Baker KS, Blazar BR, Eide C, et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and nonmalignant diseases: influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. Blood 2002 Sep 1; 100 (5): 1611-1618.

64. Camargo FD CS, Drew E, McNagny KM, Goodell MA. Hematopoietic stem cells do not engraft with absolute efficiencies. blood 2006: 107 501-107.

65. Hilal Gul WL, PengXu, JamesXing and Jie Chen. Magnetic carbon nanotube labelling for haematopoietic stem/progenitor cell tracking. Nanotechnology 2010: 21 155101.

66. Thomas ED, Lochte HL, Jr., Cannon JH, Sahler OD, Ferrebee JW. Supralethal whole body irradiation and isologous marrow transplantation in man. J Clin Invest 1959 Oct; 38: 1709-1716.

67. Bensinger WI, Storb R. Allogeneic peripheral blood stem cell transplantation. Rev Clin Exp Hematol 2001 Jun; 5 (2): 67-86.

68. Bordignon C. Stem-cell therapies for blood diseases. Nature 2006 Jun 29; 441 (7097): 1100-1102.

69. Tse W BKaLM. New insights into cord blood stem cell transplantation. Curr Opin Hematol 2008; 15:279-84.

70. Cardoso AA LM, Batard P, Hatzfeld A, Brown EL, Levesque JP, et al. . Release from quiescence of CD34+ CD38- human umbilical cord blood stem cells reveals their potentiality to engraft adults. Proc Natl Acad Sci USA 1993; 90:8707-11.

71. Bradley MB, Cairo MS. Cord blood immunology and stem cell transplantation. Hum Immunol 2005 May; 66 (5): 431-446.

72. Rubinstein P. Why cord blood? Hum Immunol 2006 Jun; 67 (6): 398-404.

73. Conneally E, Cashman J, Petzer A, Eaves C. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. Proc Natl Acad Sci U S A 1997 Sep 2; 94 (18): 9836-9841.

74. Kaushansky K. Thrombopoietin and the hematopoietic stem cell. Blood 1998 Jul 1; 92 (1): 1-3.

75. Shah AJ, Smogorzewska EM, Hannum C, Crooks GM. Flt3 ligand induces proliferation of quiescent human bone marrow CD34+CD38- cells and maintains progenitor cells in vitro. Blood 1996 May 1; 87 (9): 3563-3570.

76. Petzer AL, Zandstra PW, Piret JM, Eaves CJ. Differential cytokine effects on primitive (CD34+CD38-) human hematopoietic cells: novel responses to Flt3-ligand and thrombopoietin. J Exp Med 1996 Jun 1; 183 (6): 2551-2558.

77. Devine SM, Lazarus HM, Emerson SG. Clinical application of hematopoietic progenitor cell expansion: current status and future prospects. Bone Marrow Transplant 2003 Feb; 31 (4): 241-252.

78. Matre K, Eik-Nes SH. [Biological effects of ultrasound and potential hazards in diagnosis. Review of the literature]. Tidsskr Nor Laegeforen 1983 Aug 10; 103 (22): 1497-1501.

79. Doan N, Reher P, Meghji S, Harris M. In vitro effects of therapeutic ultrasound on cell proliferation, protein synthesis, and cytokine production by human fibroblasts, osteoblasts, and monocytes. J Oral Maxillofac Surg 1999 Apr; 57 (4): 409-419; discussion 420.

80. Therapeutic Ultrasound: A Promising Future in Clinical Medicine<http://www.acoustics.org/press/135th/crum.htm>.

81. Rubin C BM, Ryaby JP, Hadjiargyrou M. The Use of Low-Intensity Ultrasound to Accelerate the Healing of Fractures. JBJS 2001; 89: 259-70.

82. El-Bialy T, El-Shamy I, Graber TM. Repair of orthodontically induced root resorption by ultrasound in humans. Am J Orthod Dentofacial Orthop 2004 Aug; 126 (2): 186-193.

83. Yi-Sheng Chan K-YH, Chia-Hua Kuo, Shin-Da Lee, Su-Ching Chen,Wen-Jer Chen, And Steve Wen-Neng Ueng. Using Low-Intensity Pulsed Ultrasound To Improve Muscle Healing After Laceration Injury: An In Vitro And In Vivo Study. Ultrasound In Med & Biol 2010; 36 (5): 743-751.

84. Khanna A, Nelmes RT, Gougoulias N, Maffulli N, Gray J. The effects of LIPUS on soft-tissue healing: a review of literature. Br Med Bull 2009; 89: 169-182.

85. Ebisawa K, Hata K, Okada K, Kimata K, Ueda M, Torii S, et al. Ultrasound enhances transforming growth factor beta-mediated chondrocyte differentiation of human mesenchymal stem cells. Tissue Eng 2004 May-Jun; 10 (5-6): 921-929.

86. Zhou S SA, Seufferlein T, Li Y, Zhao J and Bachem MG. Molecular mechanism of low intensity pulsed ultrasound in human skin fibroblast. J Biol Chem 2004; 279: 54463-54469.

87. Parvizi J, Wu CC, Lewallen DG, Greenleaf JF, Bolander ME. Low-intensity ultrasound stimulates proteoglycan synthesis in rat chondrocytes by increasing aggrecan gene expression. J Orthop Res 1999 Jul; 17 (4): 488-494.

88. Toru Iwashina JM, Takeshi Miyazaki, Takuya Watanabe, Sadahiro Iwabuchi, Kiyoshi Ando, Tomomitsu Hotta and Daisuke Sakai. Low-intensity pulsed ultrasound stimulates cell proliferation and proteoglycan production in rabbit intervertebral disc cells cultured in alginate. Biomaterials 2006; 27: 354-361.

89. Jong Hyun Yoon EYR, Sue Shin, Nam Hee Jung, Eun Young Song, Dong Soon Lee, Kyou Sup Han, Joung Sung Kim, Byoung Jae Kim and Hye Won Jeon, et al. Introducing pulsed lowintensity ultrasound to culturing human umbilical cord-derived mesenchymal stem cells. Biotechnol Lett 2009; 31:329-35.

90. Hong S, Kang UJ, Isacson O, Kim KS. Neural precursors derived from human embryonic stem cells maintain long-term proliferation without losing the potential to differentiate into all three neural lineages, including dopaminergic neurons. J Neurochem 2008 Jan; 104 (2): 316-324.

91. Woon Tiong Ang CS, Wing Hoy, Tarek El-Bialy, Ying Yin Tsui, and Jie Chen,. Design and Implementation of TherapeuticUltrasound Generating Circuit for Dental Tissue Formation and Tooth-Root Healing ,. IEEE TRANSACTIONS ON BIOMEDICAL CIRCUITS AND SYSTEMS, FEBRUARY 2010; VOL. 4, NO. 1, .

92. Furuta A, Jankowski RJ, Pruchnic R, Yoshimura N, Chancellor MB. The potential of muscle-derived stem cells for stress urinary incontinence. Expert Opin Biol Ther 2007 Oct; 7 (10): 1483-1486.

93. Cory AH OT, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer communications July 1991; 3 (7): 207-212.

94. National Committee for Clinical Laboratory Standards V, PA. Laboratory Diagnosis of Blood-Borne Parasitic Diseases. Approved Guideline M15-A. NCCLS 2000.

95. Wittwer MBaC. Flow Cytometry: Principles and Clinical Applications in Hematology. Clinical Chemistry 2000; 46: 1221-1229.

96. Li B, Shi M, Li J, Zhang H, Chen B, Chen L, et al. Elevated tumor necrosis factor-alpha suppresses TAZ expression and impairs osteogenic potential of Flk-1+ mesenchymal stem cells in patients with multiple myeloma. Stem Cells Dev 2007 Dec; 16 (6): 921-930.

97. Kassem M, Abdallah BM. Human bone-marrow-derived mesenchymal stem cells: biological characteristics and potential role in therapy of degenerative diseases. Cell Tissue Res 2008 Jan; 331 (1): 157-163.

98. Guilak F, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS. Control of stem cell fate by physical interactions with the extracellular matrix. Cell Stem Cell 2009 Jul 2; 5 (1): 17-26.

99. Walton TJ, McCulloch TA, Bishop MC. Aggressive renal cell carcinoma in a 27-year-old kidney transplant. Nephrol Dial Transplant 2005 May; 20 (5): 1018-1019.

100. Copelan EA. Hematopoietic stem-cell transplantation. N Engl J Med 2006 Apr 27; 354 (17): 1813-1826.

101. Kobayashi Y, Sakai D, Iwashina T, Iwabuchi S, Mochida J. Low-intensity pulsed ultrasound stimulates cell proliferation, proteoglycan synthesis and expression of growth factor-related genes in human nucleus pulposus cell line. Eur Cell Mater 2009; 17: 15-22.

102. Brugger W, Heimfeld S, Berenson RJ, Mertelsmann R, Kanz L. Reconstitution of hematopoiesis after high-dose chemotherapy by autologous progenitor cells generated ex vivo. N Engl J Med 1995 Aug 3; 333 (5): 283-287.

103. McNiece I JR, Bearman SI, Cagnoni P, Nieto Y, Franklin W. Ex vivo expanded peripheral blood progenitor cells provide rapid neutrophil recovery after highdose chemotherapy in patients with breast cancer. Blood 2000; 96:3001-7.

104. Brada S dWJ, Hendriks D, Esselink M, Ruiters M, Vellenga E. . The supportive effects of erythropoietin and mast cell growth factor on CD34+/CD36- sorted bone marrow cells of myelodysplasia patients. . Blood 1996; 88:505-10.

105. Praloran V, Klausman M, Naud MF, Harousseau JL. Blood erythroid progenitors (CFU-E and BFU-E) in acute lymphoblastic leukemias. Blut 1989 Feb; 58 (2): 75-78.

106. Villaron EM AJ, Lopez-Holgado N, Sanchez-Guijo FM, Alberca M, Blanco B, et al. In leukapheresis products from non-Hodgkin's lymphoma patients, the immature hematopoietic progenitors show higher CD90 and CD34 antigenic expression. Tranfus Apher Sci 2007; 37:145-56.

107. Bogunia-Kubik K, Sugisaka M. From molecular biology to nanotechnology and nanomedicine. Biosystems 2002 Mar-May; 65 (2-3): 123-138.

108. Wei W, Sethuraman A, Jin C, Monteiro-Riviere NA, Narayan RJ. Biological properties of carbon nanotubes. J Nanosci Nanotechnol 2007 Apr-May; 7 (4-5): 1284-1297.

109. Klumpp C KK, Prato M and Bianco A. Functionalized carbon nanotubes as emerging nanovectors for the delivery of therapeutics. Biochim Biophys Acta-Biomembr 2006 1758 404-12.

110. Pantarotto D, Singh R, McCarthy D, Erhardt M, Briand JP, Prato M, et al. Functionalized carbon nanotubes for plasmid DNA gene delivery. Angew Chem Int Ed Engl 2004 Oct 4; 43 (39): 5242-5246.

111. Cai D MJ, Qin ZH, Huang Z, Huang J, Chiles TC, Carnahan D, Kempa K, Ren Z. Highly efficient molecular delivery into mammalian cells using carbon nanotube spearing. Nat Methods 2005; 2 449-54.

112. Liu Z WM, Holodniy M and Dai H J. siRNA delivery into human T cells and primary cells with carbon-nanotube transporters Angew Chem Int Edn 2007; 46 2023-7.

113. Kam N W S LZAaDHJ. Carbon nanotubes as intracellular transporters for proteins and DNA: an investigation of the uptake mechanism and pathway. AngewChem Int Edn 2006; 45 577-81.

114. Shao N LSX, Wickstrom E and Panchapakesan B. Integrated molecular targeting of IGF1R and HER2 surface receptors and destruction of breast cancer cells using single wall carbon nanotubes. Nanotechnology 2007; 18 315101.

115. Bekyarova E, Ni Y, Malarkey EB, Montana V, McWilliams JL, Haddon RC, et al. Applications of Carbon Nanotubes in Biotechnology and Biomedicine. J Biomed Nanotechnol 2005 Mar 1; 1 (1): 3-17.

116. Davis J J CRJaHHAO. Protein electrochemistry at carbon nanotube electrodes. . J Electroanal Chem 1997; 440 279-82.

117. Besteman K, Lee, J. O., Wiertz, F. G. M., Heering, H. A. & Dekker, C. . Enzyme-coated carbon nanotubes as single-molecule biosensors. . Nano Lett 2003; 3, 727-730

118. Mattson MP HR, Rao AM. Molecular functionalization of carbon nanotubes use as substrates for neuronal growth. J Mol Neurosci 2000; 14:175-182.

119. Chin SF, Baughman RH, Dalton AB, Dieckmann GR, Draper RK, Mikoryak C, et al. Amphiphilic helical peptide enhances the uptake of single-walled carbon nanotubes by living cells. Exp Biol Med (Maywood) 2007 Oct; 232 (9): 1236-1244.

120. Leeuw TK, Reith RM, Simonette RA, Harden ME, Cherukuri P, Tsyboulski DA, et al. Single-walled carbon nanotubes in the intact organism: near-IR imaging and biocompatibility studies in Drosophila. Nano Lett 2007 Sep; 7 (9): 2650-2654.

121. Mooney E, Dockery P, Greiser U, Murphy M, Barron V. Carbon nanotubes and mesenchymal stem cells: biocompatibility, proliferation and differentiation. Nano Lett 2008 Aug; 8 (8): 2137-2143.

122. Lu L, Shen RN, Broxmeyer HE. Stem cells from bone marrow, umbilical cord blood and peripheral blood for clinical application: current status and future application. Crit Rev Oncol Hematol 1996 Mar; 22 (2): 61-78.

123. Kolb HJ, Simoes B, Schmid C. Stem cell transplants for patients with relapsed/refractory leukaemia. Curr Opin Hematol 2009 Nov; 16 (6): 444-452.

124. Lapidot T. Mechanism of human stem cell migration and repopulation of NOD/SCID and B2mnull NOD/SCID mice. The role of SDF-1/CXCR4 interactions. Ann N Y Acad Sci 2001 Jun; 938: 83-95.

125. Camargo FD CS, Drew E, McNagny KM, Goodell MA. . Hematopoietic stem cells do not engraft with absolute efficiencies. . Blood 2006; Jan 15;107(2):501-7.

126. Korbling M, Katz RL, Khanna A, Ruifrok AC, Rondon G, Albitar M, et al. Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. N Engl J Med 2002 Mar 7; 346 (10): 738-746.

127. Muller P, Pfeiffer P, Koglin J, Schafers HJ, Seeland U, Janzen I, et al. Cardiomyocytes of noncardiac origin in myocardial biopsies of human transplanted hearts. Circulation 2002 Jul 2; 106 (1): 31-35.

128. Lewin M, Carlesso N, Tung CH, Tang XW, Cory D, Scadden DT, et al. Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. Nat Biotechnol 2000 Apr; 18 (4): 410-414.

129. Bianco A, Kostarelos K, Partidos CD, Prato M. Biomedical applications of functionalised carbon nanotubes. Chem Commun (Camb) 2005 Feb 7 (5): 571-577.

130. Hirsch A. Functionalization of single-walled carbon nanotubes. Angew Chem Int Ed Engl 2002 Jun 3; 41 (11): 1853-1859.

131. Baker SE, Cai, W., Lasseter, T. L., Weidkamp, K. P., Hamers, R. J. Covalently Bonded Adducts of Deoxyribonucleic Acid (DNA) Oligonucleotides with Single-Wall Carbon Nanotubes: Synthesis and Hybridization. Nano Lett 2002; 2(12), 1413-1417.

132. Balasubramanian K, Burghard M. Chemically functionalized carbon nanotubes. Small 2005 Feb; 1 (2): 180-192.

133. Shi Kam NW, Jessop TC, Wender PA, Dai H. Nanotube molecular transporters: internalization of carbon nanotube-protein conjugates into Mammalian cells. J Am Chem Soc 2004 Jun 9; 126 (22): 6850-6851.

134. http://en.wikipedia.org/wiki/Buffy_coat.

135. http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-and-Tissue-Analysis/Flow-Cytometry/Cell-Health-and-Viability-Assays-for-Flow-Cytometry.html

136. http://en.wikipedia.org/wiki/Trypan_blue.

137. Handbook of Biological Confocal Microscopy (3rd ed.). Berlin: Springer ISBN 038725921X 2006.

138. HUMAN COLONY-FORMING CELL ASSAYS Using MethoCult Technical manual version 3.1.0, Stem Cell Technologies, Vancour, Canada.

139. Tse W BKDaLMJ. New insights into cord blood stem cell transplantation. Curr Opin Hematol 2008; 15 279-84.

140. Daldrup-Link HE RM, Oostendorp RA, Jacobs VR, Simon GH, Gooding C, Rummeny EJ. Comparison of iron oxide labeling properties of hematopoietic progenitor cells from umbilical cord blood and from peripheral blood for subsequent in vivo tracking in a xenotransplant mouse model. Acad Radiol 2005; 12(4):502-10.

141. Bulte JW, Kraitchman DL. Iron oxide MR contrast agents for molecular and cellular imaging. NMR Biomed 2004 Nov; 17 (7): 484-499.

142. Richard C, Doan BT, Beloeil JC, Bessodes M, Toth E, Scherman D. Noncovalent functionalization of carbon nanotubes with amphiphilic gd3+ chelates: toward powerful t1 and t2 MRI contrast agents. Nano Lett 2008 Jan; 8 (1): 232-236.

143. Bank A. Hematopoietic stem cell gene therapy: selecting only the best. J Clin Invest 2003 Nov; 112 (10): 1478-1480.

144. Keller H, Yunxu C, Marit G, Pla M, Reiffers J, Theze J, et al. Transgene expression, but not gene delivery, is improved by adhesion-assisted lipofection of hematopoietic cells. Gene Ther 1999 May; 6 (5): 931-938.

145. Uchida N, Sutton, R.E., Friera, A.M., He, D., Reitsma, M.J., Chang, W.C., Veres, G., Scollay, R., and Weissman, I.L. HIV, but not murine leukemia virus, vectors mediated high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. Proc Natl Acad Sci USA, 1998; 95: 11939-11944.

PUBLICATION

Journal paper

1. "Magnetic carbon nanotube labelling for haematopoietic stem/progenitor cell tracking". Hilal Gul, Weibing Lu, **Peng Xu**, James Xing and Jie Chen, 2010 ***Nanotechnology*** **21** 155101
2. "Low-intensity pulsed ultrasound stimulation enhances hematopoietic stem/progenitor cell proliferation in vitro", Hilal Gul-Uludag, **Peng Xu**, Woon T. Ang, Xiaoyan Yang, Min Huang1, James Xing, Beihua Kong, Eric Swanson, and Jie Chen, in manuscript.

Conference papers

1. A novel gene delivery system using magnetic nanodarts" Weibing Lu, Gul, H., **Peng Xu**, W.T. Ang, J. Xing, Jian Zhang and Jie Chen, *IEEE/NIH lifescience systems and application workshop 2009*, Page(s): 173 – 175, Digital Object Identifier: 10.1109 / LISSA.2009.4906738
2. "Systematic study of enhanced cytotoxicity effects of gold-based nanoparticles in targeted cancer radiotherapy", Kun Song, **Peng Xu**, Yongde Meng, Jie Chen, Xiaoyan Yang, W. Roa, Beihua Kong and James Xing, *IEEE/NIH lifescience systems and application workshop 2009*, Page(s): 33 – 35, Digital Object Identifier: 10.1109/LISSA.2009.4906702

3. "Ultrasound treatment enhances proliferation of hematopoietic stem/progenitor cells: implications for clinical transplantation, gene and cellular therapies", Hila Gul-Uludagl, Peng Xu, Woon Ang, Xiaoyan Yang, Min Huang, James Xing, Beihua Kong, Eric Swanson, Jie Chen, Poster presentation at *International Society for Cellular Therapy 2010 Annual Meeting* in Philadelphia, Pennsylvania, Tuesday May 25, 2010.

4. "Highly efficient labeling of hematopoietic stem/progenitor cells using magnetic carbon nanotubes: implication for stem cell tracking", Hilal Gul, Weibing Lu, Peng Xu , James Xing and Jie Chen, Oral presentation at *International Society for Cellular Therapy 2010 Annual Meeting* in Philadelphia, Pennsylvania, Tuesday May 25, 2010.