CHAPTER II

THEORY AND LITERATURE REVIEW

Hematopoietic stem cell (HSC) are characterized by their capability of self – renewal, a process which is able to generate a continuous supply of additional stem cells and the capacity to differentiate into long – term, multilineage reconstitutation of hematopoiesis. [22] Normal hematopoiesis is characterized by the generation of mature cells of all hematopoietic lineages from pluripotent stem cells which occurs in the major hematopoietic tissues such as bone marrow and spleen (Figure5). [23]

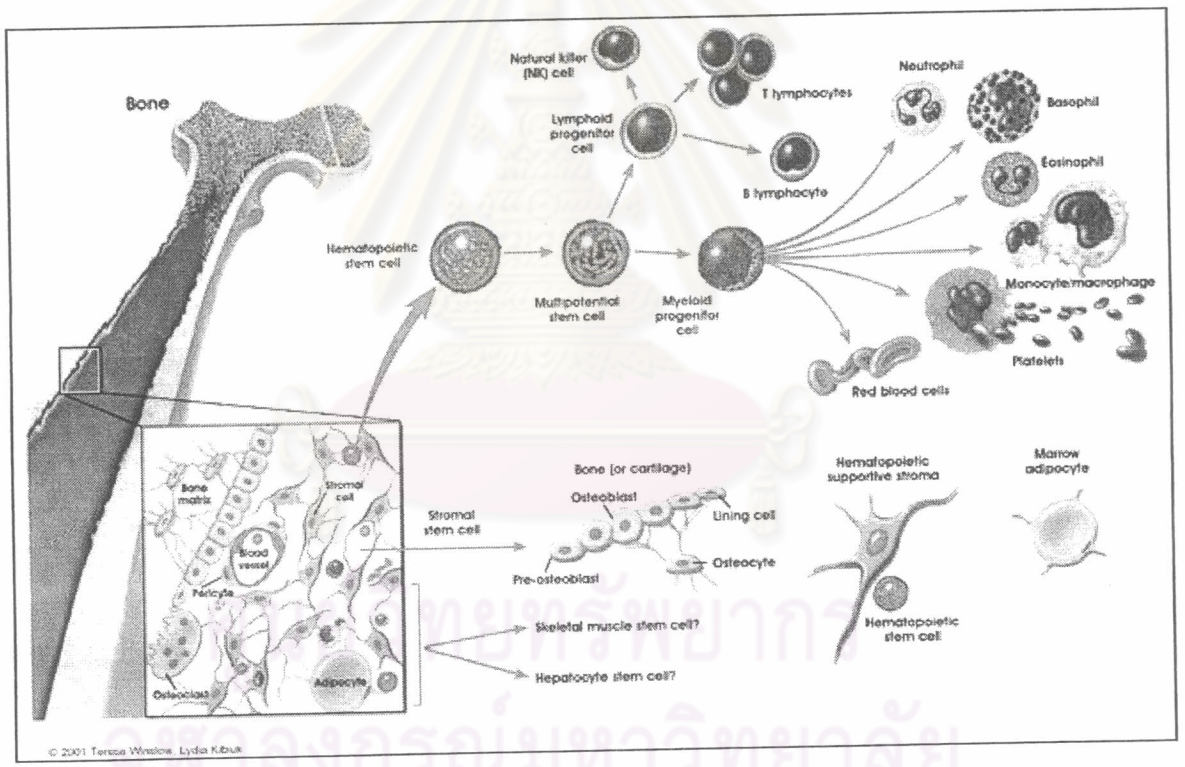


Figure 5 Commitment and differentiation in a cell lineage. Blood cell types are from pluripotent hematopoietic cells in the bone marrow (BM) . [23]

Kohler and Milstein in 1975 reported the first successful use of monoclonal antibodies(Mab) against cluster of differentiation ($CD34^+$) antigens production which were used to facilitate the identification of assay systems for hematopoietic progenitor cells. [24]They are considered to reside in a population of cells that express CD 34 antigens is a 115 Kda transmembrane surface glycophosphoprotein. Subsets of CD34 cells can be subdivided based on levels of CD34 expression and lack of expression of other antigens on the cell surface.[25] Thus $CD34^+$ cells positive selection has gained increasing importance in hematopoietic stem cell transplant setting.

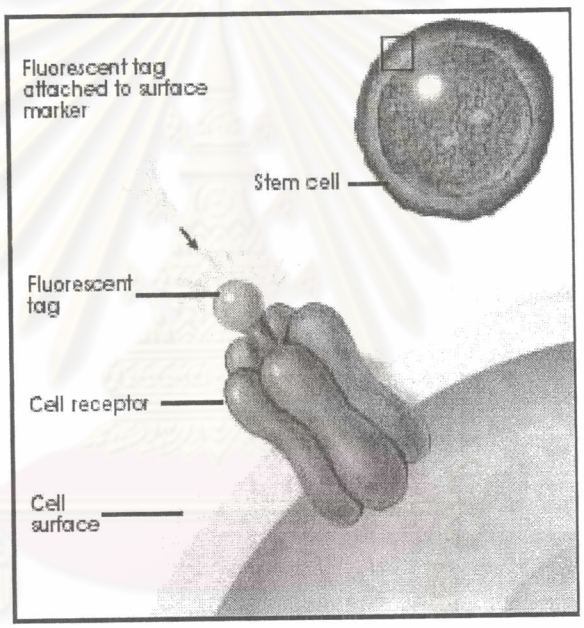


Figure 6 Human hematopoitic stem cell that express CD 34 antigens transmembrane surface glycophosphoprotein [25]

The characteristics of HSC are their capability of self – renewal and multilineage differentiation. These two reasons HSC to become the cellular element to reestablish hematopoiesis after HSC transplantation.

1. Source of HSC for transplantation

There are several potential sources of donor HSC for transplantation including adult bone marrow (BM), peripheral blood (PB), fetal liver and umbilical cord blood which are summarized in Table 3.

1.1) Adult bone marrow (BM)

Before 1985, the major source of tissue for postnatal transplan -tation of HSC has been and currently continues to be adult bone marrow cells which obtained fromHLA – matched sibling donors as the source of the graft. The advantages of HSC from BM are abundance in HSC, ethically unobjectionable and readily available for additional HSC. The alloreactive T- cell in bone marrow will readily engraft but the results are related to the risk of developing graft – versus host disease (GVHD). [26] When T – cell depletion of BM is performed before transplantation to prevent GVHD, the incidence and level of donor cell engraftment is significantly reduced. [27] However, harvesting a BM graft requires a surgical procedure which associated with significant risk and discomfort of the marrow collection procedure , including general anasthesia for the donor.

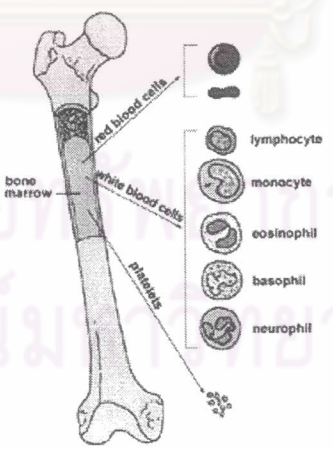


Figure 7 Type of blood cell in the bone marrow differentiated from hematopoietic stem cell. [28]

1.2) Peripheral blood (PB)

The early studies of Goodman et al in 1962 [29] demonstrated that HSC in peripheral blood which are increasing used as a source of HSC for transplantation. [30] The practical problem with this approach is difficult to collect sufficient number of stem cells by mobilization or increase the number of circulating stem and progenitor cells. Following chemotherapy PB can increase the yield of stem cells that immediately provide monocyte and neutrophil recovery and lead to more rapid engraftment than bone marrow. [31] But there is still risk of the infectious side effect after chemotherapy.

1.3) Fetal liver

The use of fetal liver as fetal donor HSC materials for transplantation has an important advantage from the potential immaturity of fetal immune system that can avoid GVHD. However, there are several limitations to use fetal liver including the practical difficulties to obtained fetal liver from human abortus which its suitable age is 12-14 weeks of gestation. Fetal liver from fetuses below 12 weeks of gestation have often failed to engraft, while older fetuses have a tendency to cause GVHD.[32] If additional HSC are required, the donor cells is not available in adult transplantation. The use of abortuses as a source of fetal liver HSC may also have infectious risk to the recipients and ethical problem.[33]

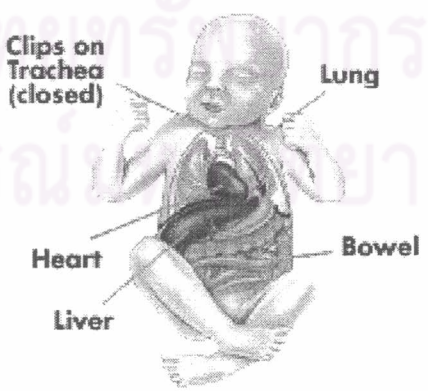
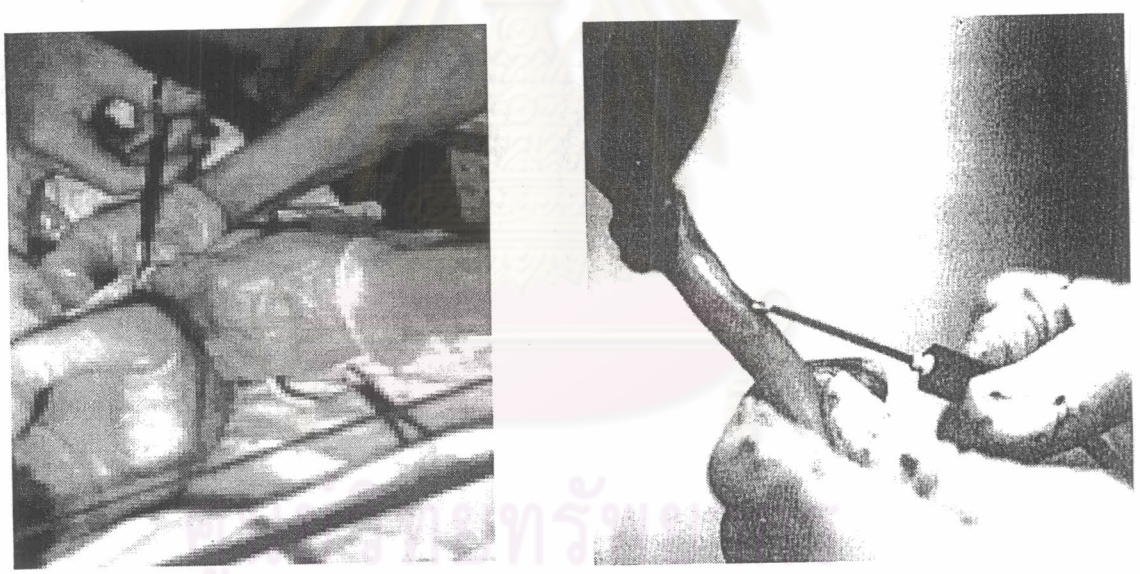


Figure 8 The position of fetal liver [34]

1.4) Umbilical cord blood (UCB)

In 1986 Broxmeyer et al, reported umbilical cord blood (UCB) as a potential source of transplantable stem and progenitor cells. [35] There has been increasing interest in the use of HSC from UCB as an alternative unrelated donors for patients lacking a HLA – matched sibling. Stem cells in UCB are unique when compared with adult tissue for greater proliferative and self – renewal capacity. Additionally, UCB contains largely virgin T – cells that have not yet encountered antigen, so this can reduce the risk of GVHD. [36] Furthermore UCB has advantages of ethically unobjectable and readily available for HSC transplantation.



(A) (B)

Figure 9 A-B) Collection of Umbilical cord blood samples [38]

Table 3 : Source of stem cells for transplantation [39]

Tissues	Stem cell content	T-lymphocyte content	Special features
Bone marrow	+++	+	Contain fibroblst and stroma – forming cell
Peripheral blood	+	+++	Insufficient numbers for practical application unless circulating stem cell number are increased by mobilization
Fetal liver	+++	+	High stem cell content but small volume restricts use to infant
Cord blood	+++	+++	Stem cell content similar to adult bone marrow. Small total volume restricts use to infants and children

2. Postnatal HSC transplantation

Over the last 15 years, postnatal hematopoietic stem cells transplantation is an effective and curative treatment for a number of patients with life threatening hematologic disease, such as leukemia, aplastic anemia, lymphoma, malignant tumors or some inherited disease (Thalassemia major, Severe combined immunodeficiency (SCID) , Inborn errors of metabolism, etc).[40] The choice of transplant of a patient depends upon a number of factors, including type of disease and availability of a suitable donor.

2.1) Type of transplantation

2.1.1) Syngeneic transplantation

In syngeneic transplantation, donor HSC is taken from an identical or monozygotic twin. Each people usually have different sets of proteins that were called human leucocyte – associated (HLA) antigens on the surface of their cells that allow white blood cells (WBC) to distinguish the body's own cells from those of another person. However, because identical twins have the same gene materials and set of HLA antigen.(perfect HLA match) As a result, with syngeneic HSC transplantation the recipient's body usually accepts the graft. These syngeneic HSC transplantation is also rare since identical twins occur only 0.3 percent of all birth or one birth out of every 270.

2.1.2) Allogeneic Transplantation

Allogeneic transplantation, which donor HSC came from a person other than the patient or and identical twin, is much more common, Usually the patient's sibling or parent will serve as a donor, but unrelated donors are sometimes used and depends on how closely the HLA - antigens of the donor match those of recipient. [41] The higher the number of matches leads to the greater chance the patient will accept graft and reduce GVHD in patient's body. This GVHD reaction is a potential complication of allogeneic transplantation. In addition, high dose chemotherapy is required for pretransplant condition to prepare recipients which means more of the recipient's disease cells can be destroyed. However only 30 to 40 percent of patients have an HLA – matched sibling or HLA – compatible marrow of unrelated donor. [42]

2.1.3) Autologous Transplantation

Autologous Transplantation is when patients receive their own stem cells to fight disease. There is virtually no risk of GVHD for autologous transplantation. But the patients disease stem cells must be removed before donor HSC harvesting.[43] There are generally 3 treatment in autologous transplant as shown in figure 11.

1) The eradication of the recipient's diseased marrow by chemotherapy.

2) Collecting and storage of normal stem cell.

3) The eradication of the recipient's system so that the incoming donor graft will be accepted by immunosuppression.

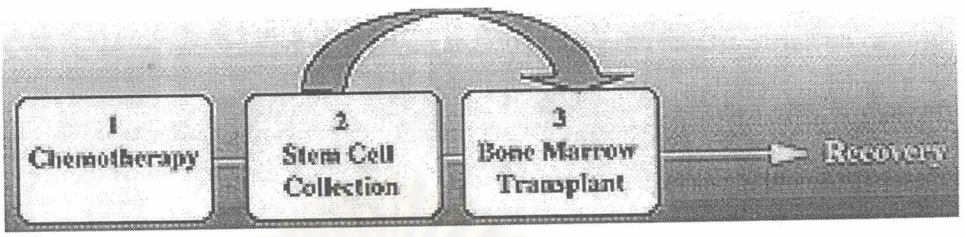


Figure 10 Procedure of autologous transplantation [44]

3. Prenatal Transplantation

In prenatal transplantation, the HSC will be transplanted into a preimmunocompetent fetal recipient which may allow the creation of a permanent hematopoietic chimera without the risk of marrow ablation, rejection and GVHD as shown in table 4.[45] Therefore, there is reason to be optimistic that in utero transplantation is an alternative for the treatment of congenital hematologic disorders that can be diagnosed early in gestation.

Table 4 Advantages of In utero HSC Transplantation [45]

Advantage	Effect
Immunologic Tolerance	No HLA – Restriction / Immunosuppression
BM space available	No myeloablation
Sterile isolation	No post – transplant isolation
Proliferative environment	Potential competitive advantage over normal cells
Clinical Disease	Avoids complicating morbidity/suffering

Tolerance induction by foreign HSC antigen was first experimentally tested in early gestation by Billingham and Medawar in 1953.[46] There is a rapid expansion and proliferation of the developing fetal hematopoietic compartment. Due to

immunogically immature available cavity in the marrow microenvironment in which HSC incoming graft can be lodged, therefore, Histocompatibility matching, immunosuppression and myeloablation which are required for optimal postnatal bone marrow transplantation, may not be necessary for prenatal transplantation.[47]

4. Recovery of hematopoiesis after HSC transplantation

4.1) Infusion and homing

During fetal development, hematopoiesis first appears in the yolk sac, subsequently the liver, spleen and finally the bone marrow. There is an associated exponential expansion of the hematopoietic compartment with continuous formation of new microenvironment sites for homing and engraftment.

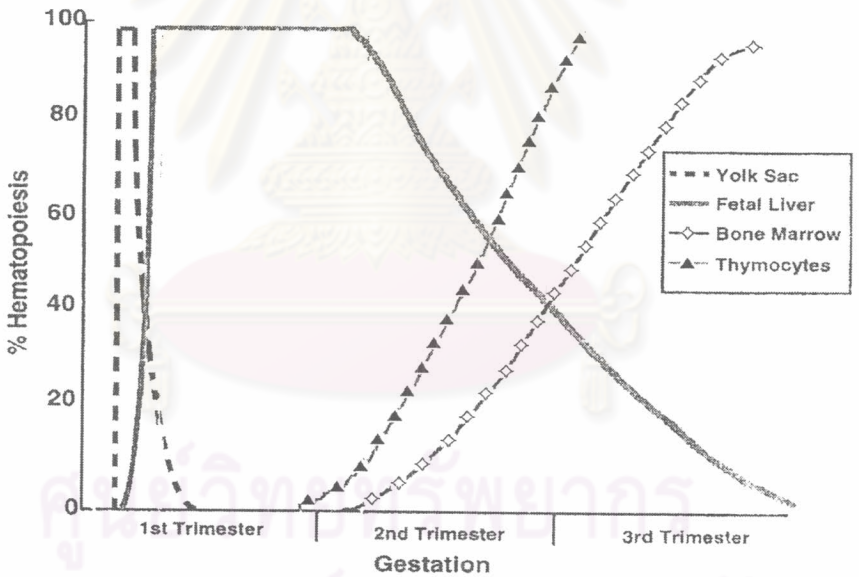


Figure 11 Schematic of normal hematopoietic and immunologic ontogeny [48]

The mechanism of homing is a multistep process consisted of adhesion of endothelial cells to the marrow sinusoids, followed by transmigration and anchoring within the extravascular bone marrow (BM) spaces where HSC can proliferate and differentiate.[49] The presence of homing receptors on HSC facilitates reconstitution of the

HSC from the blood following the transplantation. However, The factor necessary for successful engraftment requires not only adequate numbers and quality of HSC but also appropriately localized of these cells homing within the specific bone marrow microenvironment.

4.2) Engraftment and Hematological Recovery

Following postnatal transplantation, engraftment generally occurs within 8 to 12 days after the stem cell infusion. The engraftment refers to the return of the stable reconstitution of hematopoiesis as indicated by functional levels of both myeloid and lymphoid cell and the development into red blood cell (RBC), white blood cell (WBC) and platelet in the blood. The most important cell, to return is a type of WBC known as the neutrophil. Neutrophils are important in fighting bacterial infections, so it is necessary to get the neutrophil level close to normal. The neutrophils are usually the first cells to return and reach normal values by about 1 month postnatal transplantation.[50] While following postnatal transplantation, the change observed is a fall in circulating leukocytes and platelets, that are less than 1×10^{9} / liter about 7 days posttransplantation. In the first 10 days, there are developing erythropoiesis and granulopoiesis. The blood count usually occurs between days 14 and 21 after HSC transplantation. There is often an initial monocyte recovery accompanied by the emergence of circulating colony – forming progenitors. Reticulocytes appear in the blood at about the same time but do not usually increase above normal absolute values. Slowest to recover are the platelets. Platelet transfusion support may be required for up to 1 month posttransplantation and normal counts are only achieved after several month as shown in figure 12.[51]

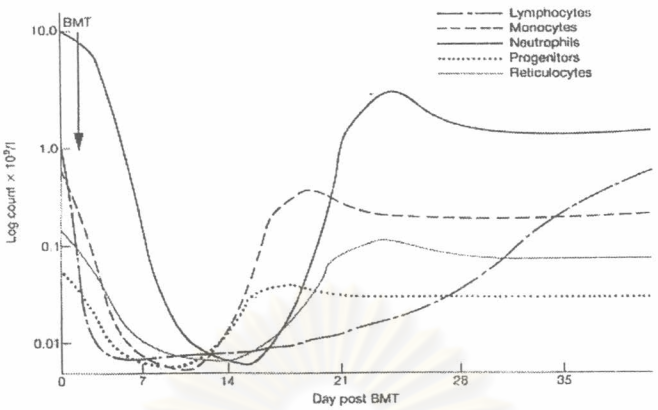


Figure 12 Pattern of hematological recovery after bone marrow transplantation [51]

5. Sheep models of in utero HSC transplantation

Animals are used to study basic transplantation biology or preclinical models for conditioning regimens and GVHD prevention and treatment. Hiscompatibility antigens systems have been determined in detail in several mammals, including the mouse, dog, pig, sheep and primate. Rodent are sensitive to many of the cytokines and growth factor but human cells are generally non – responsive to rodent growth factor. [52] Large animal models, such as dogs have been widely used for many years in the development of preparative regimen for transplantation.[53] Swine have similar skin response to human and are useful in GVHD studies. Non- human primates are much closer to the human both in their growth factor response and the surfaces phenotype of their lymphoid and myeloid cell.[54]

The fetal sheep model is also useful for widely xenogeneic transplantation. There are three reasons for study in human- sheep model.

1) The sheep has two naturally occurring alleles at the β - hemoglobin locus (A and B) that can be used as definitive markers of successful engraftment and chimerism.
2) Gestation in the pregnant ewe is sufficiently long (until term =145 days) to allow experimental manipulation.

3) The immune status of the fetal lamb is relatively permitting accurate selection of preimmune recipients.

The most successful human – sheep models are in utero HSC transplantation model. Transplantation of allogeneic, fetal liver – derived HSC into normal sheep fetuses in early gestation results in a high rate of sustained multilineage hematopoietic chimerism.[55] Chimerism in the human – sheep model is caused by the long term engraftment of pleuripotent HSC from donor cells.[56] Although chimerism has been achieved in the normal animal model, the levels of engraftment are much lower and well below that might be expected to be therapeutic for most hematologic disease.

6. Methods for the detection of donor cell engraftment

Numerous approach to the analysis of donor cell engraftment are reported in *in vitro* scientific studies. Different methods have been applied for detecting engraftment : Cytologenetic markers, HLA-types, restriction fragment length polymorphism (RFLP), fluorescence *in situ* hybridization (FISH) and PCR technique.

6.1) Flow Cytometry and FACSort

An alternative method that is widely used for investigation of donor cell chimerism. Flow Cytometry analysis by differentiation antigen was associated with human hematopoietic subpopulations. Flow Cytometry designed for applications ranging from routine clinical to advanced research. It analyzes cells as they pass one at a time through a focused laser beam. Flow Cytometry can measure up to five parameters – forward light scatter, side light scatter and three fluorescence parameters. In addition, it allows identifying and sorting a subpopulation from sample.

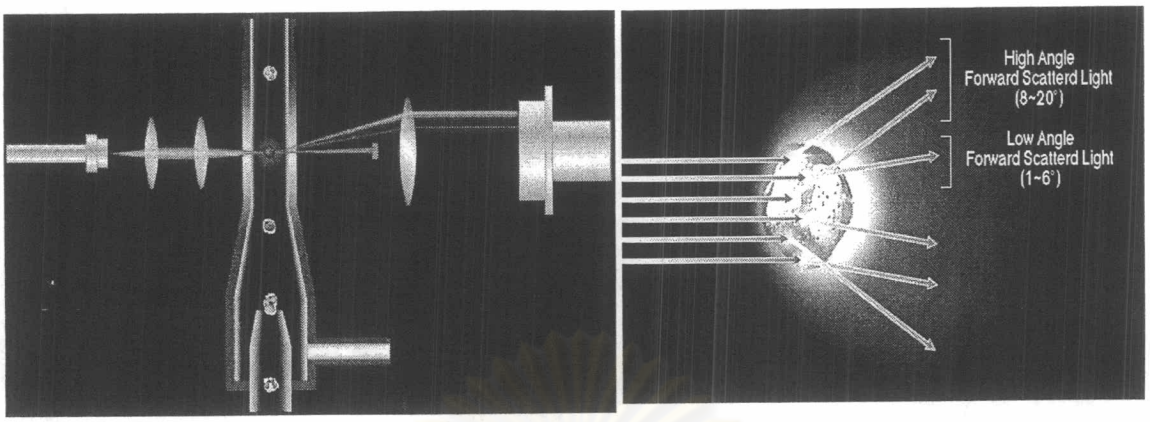


Figure 13 shows analyzed cell pass through a focused laser beam by Flow Cytometry can measure up to five parameters – forward light forward light scatter, side light scatter and three fluorescence parameters, allows to identify subpopulation from sample.[57]

Principle of operation of Flow Cytometry is to analyze cells as they flow in a moving fluid stream past a fixed laser beam. As a cell passes in front of the laser, several measurements are made based on the physical characteristics of the cell. These characteristics which pertain to how the cell scatters the laser light and emit fluorescence, provide information about the cell's size, internal complexity and relative fluorescence intensity. This information is collected and transmitted to the computer.

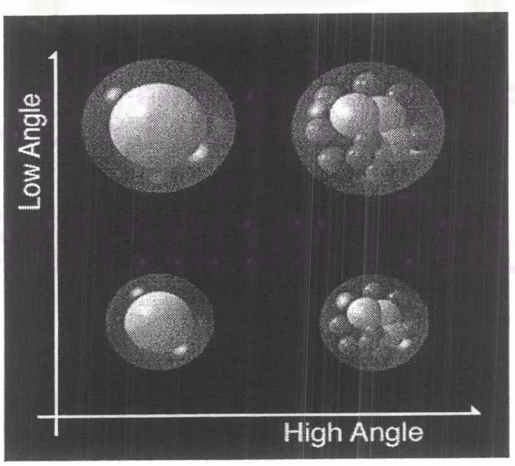


Figure 14 shows the cell scatters the laser light and emit fluorescence, provide with information about the cell's size, Internal complexity and relative fluorescence intensity. [57]

Optimization is the instrument adjustment procedure that optimally sets the detectors , amplifiers, threshold and compensation for specific samples. The procedure depends on the application as well as the number of the fluorochrome used. For immunophenotyping applications, an forward light scatter (FSC) and side light scatter (SSC) plot are viewed to ensure that all cell populations of interest are on scale for these parameters. PBMC isolated from whole blood displays FSC and SSC through logarithmic amplification, that show WBC populations such as monocyte, lymphocyte and granulocyte.The gate will be drawn around the population that was stained with the monoclonal antibodies as shown in Figure 15.

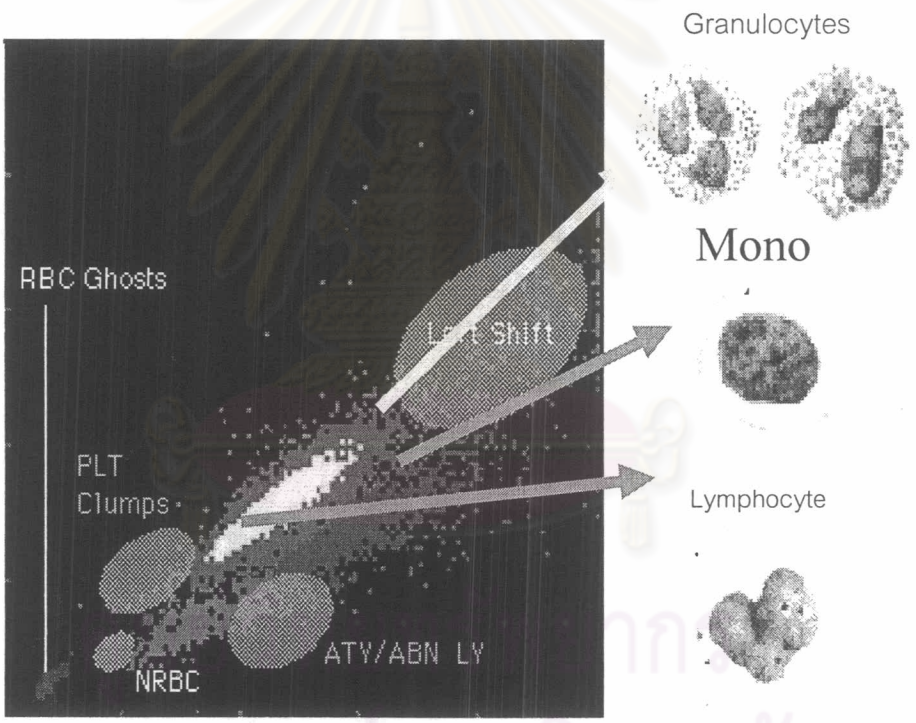


Figure 15 Show WBC population such as monocyte, lymphocyte and granulocyte.[57]

FACSort (fluorescence activated cell sorter) is ideal for sorting cells for verification of morphology or molecular studies, or for sorting viable cells that can be returned or used functional assays. FACSort uses a mechanical device called a catcher tube , positioned within the sheath stream in the upper portion of the flow cell. As a process through the laser, the FACSort electronics quickly decide if it is a cell of interest

(target cell) based on the sort gate characteristics. The electronics wait a fixed period of time to allow the cell to reach the catcher tube before triggering the catcher tube to swing into the sample stream to capture the cell. Figure 17 a-b illustrate the process of sorting a cell. Figure 17-a shows the catcher tube in its resting position in the sheath stream and figure 17-b shows the catcher tube positioned in the sample core stream ready to capture a target cell.

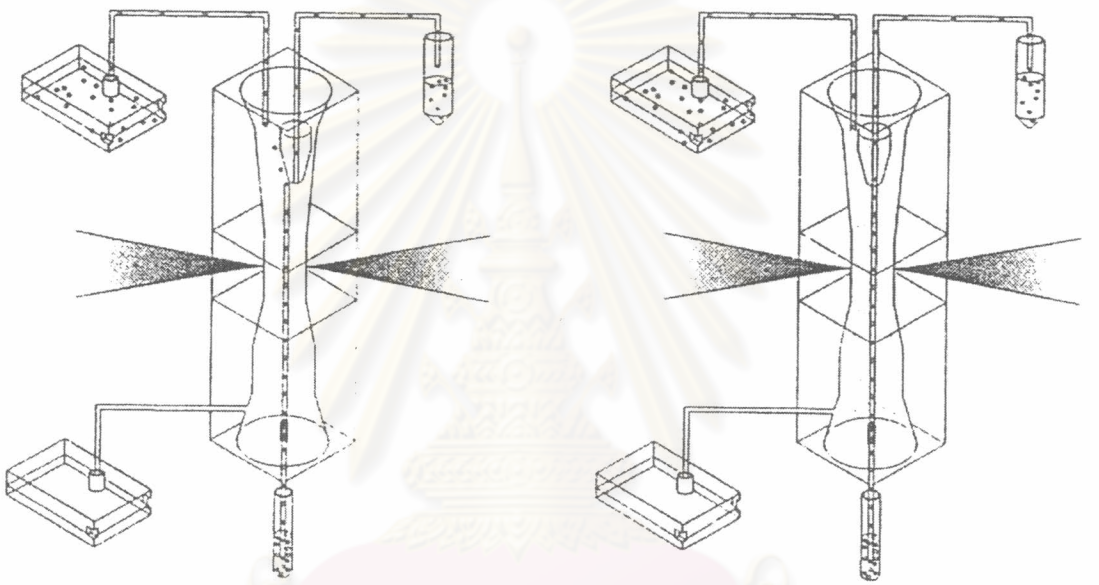


Figure 16-a Catcher tube in sheath stream

16-b Catcher tube in sample stream [58]

Because the catcher tube is positioned in the sheath stream while it waits for a target cell, it continuously collects sheath fluid, in addition to the sorted cells, resulting in a dilute sample. After sorting, the collection tubes must be centrifuged to concentrate the cells.

6.2) Fluorescence *in situ* hybridization (FISH)

The fluorescence in situ hybridization technique, using chromosome – specific probes, is a simple and quantitative way to detect mixed chimerism following HSC transplantation. The use of FISH is growing rapidly in genomics, cytogenetics, prenatal research, tumor biology, radiation labels, gene mapping, gene amplification and basic biomedical research. The hybridization reaction identifies or labels target genomic sequences so that their location and size can be studied. DNA or RNA sequences from appropriate chromosome specific probes are first labeled with reporter molecules, which are later identified through microscopy. The labeled DNA or RNA probe is then hybridized to the metaphase chromosome or interphase nuclei on a slide. After washing and signal amplification, the specimen is screened for the reporter molecules by fluorescence microscopy.

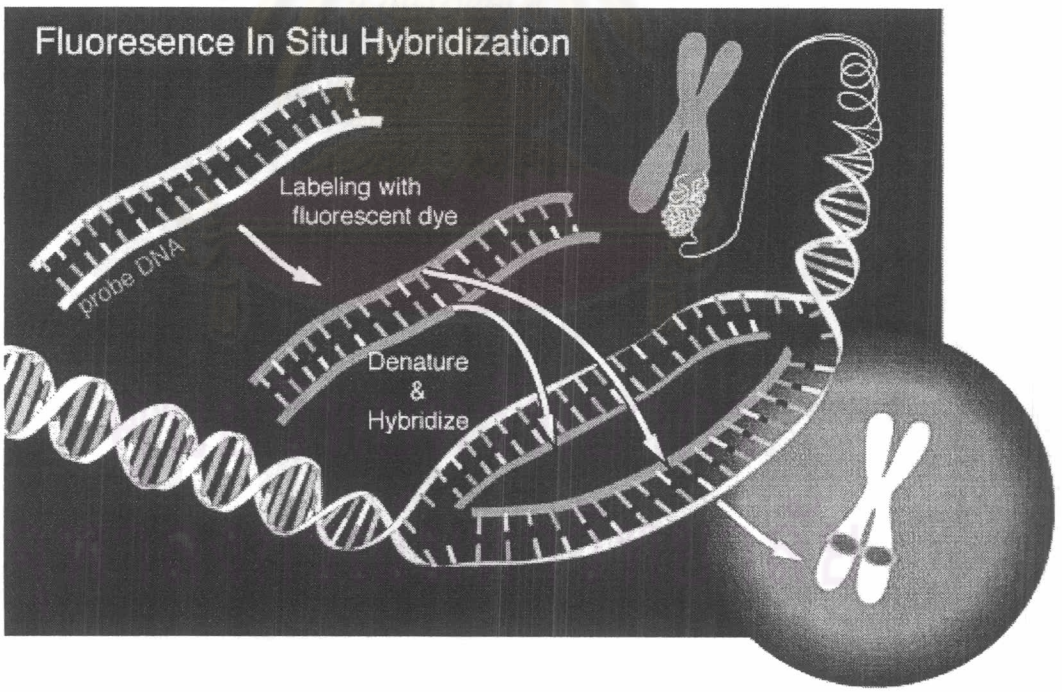


Figure 17 Fluorescence *in situ* hybridization (FISH) [59]

FISH allows very precise, spatial resolution of morphological and genomic structures. The technique is rapid, simple to implement and offers great probe stability. The genome of a particular species, entire chromosome, chromosome- specific regions,

or single copy unique sequences can be identified, depending on the probe used. There are three main types of probes.

1) Specific DNA probes : for the detection of a specific gene
2) Multiple sequences probes : for the detection of a whole chromosome
3) A satellite probes : for the detection of sequence located in the centromeric region

The DNA probes used in this study recognize the satellite DNA of the centromeric region of the human chromosome 16 (16q11.2, locus D 16Z3). The probe detecting chromosome 16 were labeled with fluorescence haptens CEP (chromosome enumeration probe) spectrum orange.

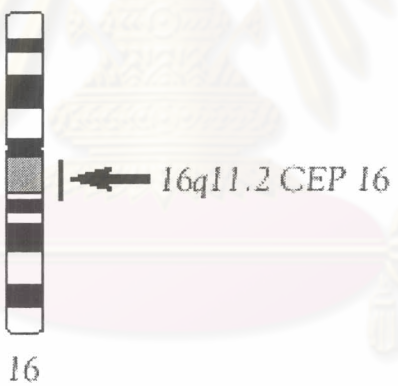


Figure 18 CEP 16 recognize the satellite DNA of the centromeric region of the human chromosome 16 (16q11.2, locus D 16Z3)[60]

LITERATURE REVIEW

In utero Hematopoietic stem cell (HSC) transplantation for cellular/genetic replacement therapy for hematopoietic and metabolic disorders is becoming increasingly attractive as prenatal diagnosis continues its rapid evolution. Development of prenatal diagnostic techniques may provide the opportunity for early detection of diseases, some of which might then be treated in utero prior to development of potentially lethal or severely debilitating pathologies. Candidate disease of prenatal replacement therapy include thalassemia, sickle cell anemia, severe combined immunodeficiency disease (SCID) and several metabolic storage disease. [61] These disease can be ameliorated and sometimes cured by postnatal bone marrow transplantation.[62] However the paucity of histocompatibility (HLA) – matched donors, degenerative pathologies of disease progression in utero, and postnatal complications which arise from untreated disease, suggest the desirability of prenatal therapy.

Owen et al [63] first observe that natural chimerism could be arised from shared placental circulation in dizygotic cattle twins. This state of mixed chimerism could persist for life and was associated with specific transplantation tolerance. Natural chimerism had been observed in other species as well as in non – human primate. It has been observed that donor hematopoiesis in some chimeric animals can actually predominate with the presistence of very high levels of donor – derived cells. This experiment of natural hematopoietic chimerism in such specific circumstances showed that allogeneic donor cells could competitively populate a hematopoietically normal recipient and stable levels of donor cell expression could be achieved.

Experimental hematopoietic chimerism after in utero transplantation has been shown in animal model . Flake AW et al [64] had studied transplantation of normal immature, fetal HSC into a preimmune fetal recipient early in gestation and permitted foreign grafts without rejection. In this experiment, β- hemoglobin locus of the sheep was used as a marker for engraftment and hematopoietic chimerism. The donor hemoglobin levels of 14 –

29 % in this study was impressived when one considered that only a fraction of the 2×10^8 to 5×10^8 nucleated cells were injected per kilogram of body weight.

Demoor et al [65] reported a case of human dizygotic twins in which the female twin exhibited an XY human chromosome by using karyotype technique in a high percentage mitoses. Sellers et al [66] had studied in utero transplantation of development fetal liver stem cells into anemic newborn mice and cured normocytic anemia in 6 of newborn mice.

Flieshchman and Mintz [67] demonstrated that in utero transplantation of normal allogeneic fetal liver cells by transplacental injection into 11 days gestation of W mutant anemic mice which had a stem cell deficiency resulted in rescue of severely anemic mice and complete reconstitution of donor hematopoiesis. Subsequently Blazer et al [68] acheieved the high levels of multilineage chimerism of donor cells with confirmation of HSC engraftment by repopulation of irradiated secondary recipients.

In large animal studies, Zanjani et al [69] reported the first successful In utero HSC transplantation of allogeneic bone marrow derived HSC from adult sheep transplanted into fetal lamb recipients. Although successful engraftment was observed in three of five lambs surviving to term out of 11 fetal lambs transplanted, all of them died from GVHD. The same experince was confirmed by Roodman et al [70] in a model of baboon bone marrow HSC transplanted into fetal baboon. Brent [71] was also able to achieve chimerism in a monkey model transplanting T cell – depleted adult bone marrow HSC into fetal recipient, but the level of engraftment was low and all chimeric fetal recipients died in utero of GVHD.

In order to eliminate the risk of GVHD, Flake et al [72] used HSC derived from preimmune fetal liver. They demonstrated in sheep model that fetal liver HSC transplanted in utero early in gestation resulted in sustained engraftment without evidence of rejection or GVHD. Pearce et al [73] also obtained similar results in two of four goat fetuses transplanted in utero with HSC from fetal goat livers. Chimerism was sustained in these animals, but they ultimately died of GVHD.

Crombleholme et al [74] showed subsequently in the same rhesus model that hematopoietic chimerism without GVHD could be achieved if transplantation was done early in gestation before 80 days of gestation (term 165 days). They also reported induction of tolerance against donor cells by using mixed lymphocyte reaction assay. The early gestation immunologic window is so profoundly permissive, it even allows induction of donor specific tolerance across species barriers. Engraftment of human HSC in xenograft models has been achieved in irradiated or genetically immunodeficient mice. Pallavicini et al [75]achieved successful mouse xenogenic chimerism in the immunologically intact anemic mouse model.

Zanjani et al[76] were able to transplant human fetal liver – derived donor HSC into fetal sheep injected with $2 - 5 \times 10^{8}$ cells per kilogram into the peritoneal cavity of fetuses exposed by the amniotic bubble technique. These xenogeneic transplants were possible because both the fetal donor HSC and fetal recipients were immunologically naïve. These in utero xenogeneic HSC transplant models have demonstrated stable multilineage hematopoietic chimerism without evidence of GVHD or graft rejection. As with all surgical procedures on the fetuses early in gestation, there was a high perioperative mortality. Three out of four fetuses were born with clear evidence of donor cell engraftment as determined by both hemoglobin and chromosome analysis.

Figure 19 The microbubble technique was used to perform HSC transplant. The myometrial layers were divided with electrocautery and care was taken to preserve the amnion intact. The fetus could then be manipulated under direct vision and HSC was injected into peritoneal cavity of fetal sheep recipient. [77]

Due to the mortality associated with the microbubble technique, thus we developed an ultrasound – guidance transabdominal needle transplatation technique. By this technique, we could transplant HSC into peritoneal cavity of sheep fetus more than once. This strategy was different from previous reports and might result in enhancement of successful engraftment in fetal sheep.

Presence of recovery of human donor neutrophils, lymphocytes and platelets after transplantation in PB of newborn sheep would confirm the evidence of engraftment. There are different techniques applied to detect chimerism, such as HLA – matching, immunohistochemistry, Flow Cytometry, *in situ* hybridization, Restriction fragment length polymorphism (RFLP), Polymerase chain reaction (PCR) and more recently Flow – PCR technique. Therefore, techniques and protocols are importance to investigate of donor cell chimerism which have accurate and reproducible strategies.

Zanjani ED et al [69] published the first study adult hematopoietic cells transplanted to sheep fetuses continue to produce adult hemoglobin which can be used as definitive markers of successful engraftment and chimerism. Their results indicate that there was engraftment of the donor cells and bone marrow from 2 of 11 sheep chimeras bone marrow. There were donor cells, proliferating and differentiating in the fetal sheep environment, continue to adult hemoglobin (Hb A :$\alpha_2\beta_2$) of the donor that comprised 41% by day 21 post –transplantation. Donor cell engraftment were detected by karyotype analysis. The result showed that 58% of lymphocytes were of the donor cells. The introduction of banding chromosome technique (karyotype), allowed each chromosome to be identified on the basis of their size and the position of the centromeres.

Landegent et al [78], developed a molecular hybridization technique by using fluorescence labels that also known as Fluorescence *in situ* hybridization (FISH) techniques. This technique can be used for many purposes, including analysis of chromosomal damaged, gene mapping, clinical diagnostic, molecular toxicology, cross-species chromosome homology and other application for the detection of donor cells engraftment after HSC transplantation.

Zanjani ED et al [76] , studied engraftment and long term expression of human fetal liver – derived HSC transplanted in utero into preimmune fetal sheep (48 – 54 days of gestation). Engraftment of human donor cells was analyzed by using karyotype analysis of bone marrow and liver HSC and fluorescence *in situ* hybridization with a biotinylated DNA probe (PC 190) labeling repeated alpha satellite sequences on human chromosomes in sheep blood and marrow. Their results demonstrated that engraftment occurred in 13 of 33 recipients. There are five live born sheep that exhibited chimerism, all expressed human cells in the marrow where as three expressed them in blood. The percentage of cells with human frequency was highly significant obtained by FISH method. That the procedure of FISH technique was more sensitive for detection of human cells, whereas karyotping enumerated cells in metaphase only.

Another method which can be used for engraftment analysis is the polymerase chain reaction (PCR) which is reported in the mid 1980s by Kary Mullis. The PCR is an *in vitro* method for the enzymatic synthesis of specific DNA sequences, using oligonucleotide primers that hybridize to opposite strand and flank the region of interest in the target DNA. Since then there have been several applications reported in both basic and clinical research.

Srour EF et al [79], studied human HSC $CD34^+$ transplanted in utero into immunocompetence fetal sheep to investigate the bone marrow – populating potential of these cells by detection of human β - globin sequence. The presence of human β - globin DNA sequence in chimeric bone marrow cells and in hematopoietic colonies was detected by the polymerase chain reaction (PCR). Their results show that long – term chimerism, sustained human hematopoiesis and expression of human cells belonging to all human blood cell lineages were demonstrated in two chimeric sheep for more than 7 month post transplantation. PCR analyzed these cells contained the human β - globin gene, demonstrated the human origin of the hematopoietic colonies generated from chimeric $CD45^+$ cell. Although PCR are the most currently used method but the lower limit for detection of donor cell engraftment is the range of 3-5% of positive cell. As previously reported, significant fewer human cells are present in the circulation of the chimeric

lambs. The frequency of donor cells are rather low or below 1% at one month after transplantation which is called microchimerism, defined as a rare event of donor cells. Therefore, the method to detect this microchimerism level must be highly sensitivity and specific.

By the mid to late 1980s Flow Cytometry method was developed to be used in many areas of clinical research and various application such as in vitro diagnostic use for enumeration of leucoctye subsets, reticulocyte, platelet, classification of chromosomes, DNA cell cycle analysis and sorting cells for verification of morphology or molecular studied for research. Flow Cytometry have also been widely used to investigate lower numbers of donor cell chimerism.

Zanjani ED, Srour EF and Hoffman [80] had previously reported on the successful engraftment and long – term multilineage expression of human adult marrow cells in sheep after transplantation in utero. Their result showed the presence of cells expressing the human CD 45^+ antigen is approximately 2 % chimerism in the bone marrow but not in peripheral blood at 3, 9, 12 and 15 months after transplantation by Flow Cytometry analysis.

The process of detection of donor cell chimerism was studied. Due to the use of different techniques, which have been established independently by different investigations. The presence of significant number of cells capable of giving rise to lymphocytes expressing CD 45^+ antigen would confirm human donor cells engraftment. Thus we study *in vitro* mixed human – sheep lymphocytes experiments in different concentration series for the development of a standardized analysis of donor cells engraftment by Flow Cytometry. To determine the exact nature of sorted cells whether they were from human lymphocyte populations, we planned to perform cells sorting by FACSort and further be analyzed with FISH. But due to instrument problem from malfunction laser sorting equipment so that the procedure could not be done. In addition, we applied these techniques to investigate the donor cells engraftment of in utero human – sheep transplantation model.