Culture of Hematopoietic Stem Cell

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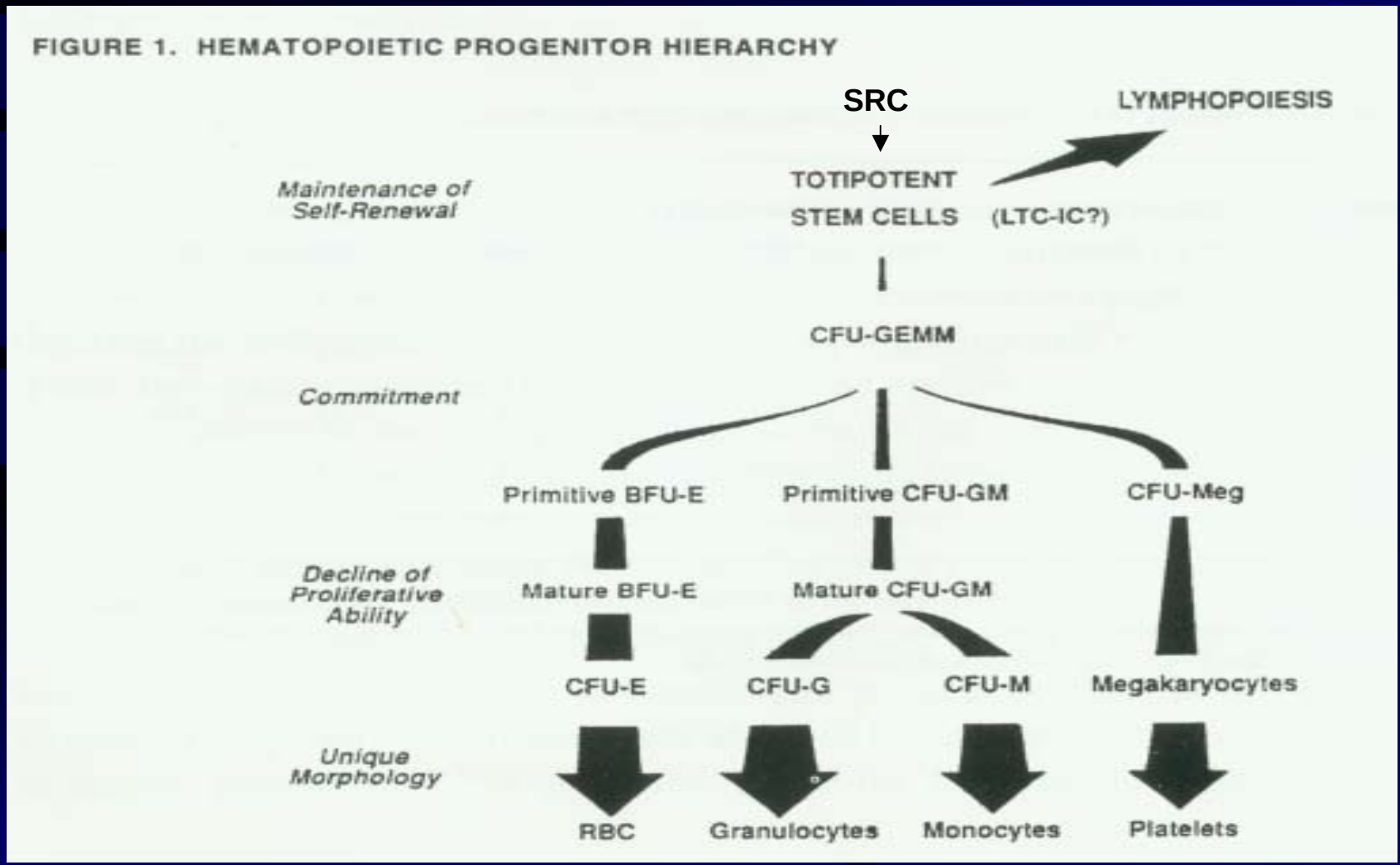
Concept of HSC

- Pluripotent stem cell
- Committed stem cell- HSC/ progenitor cell

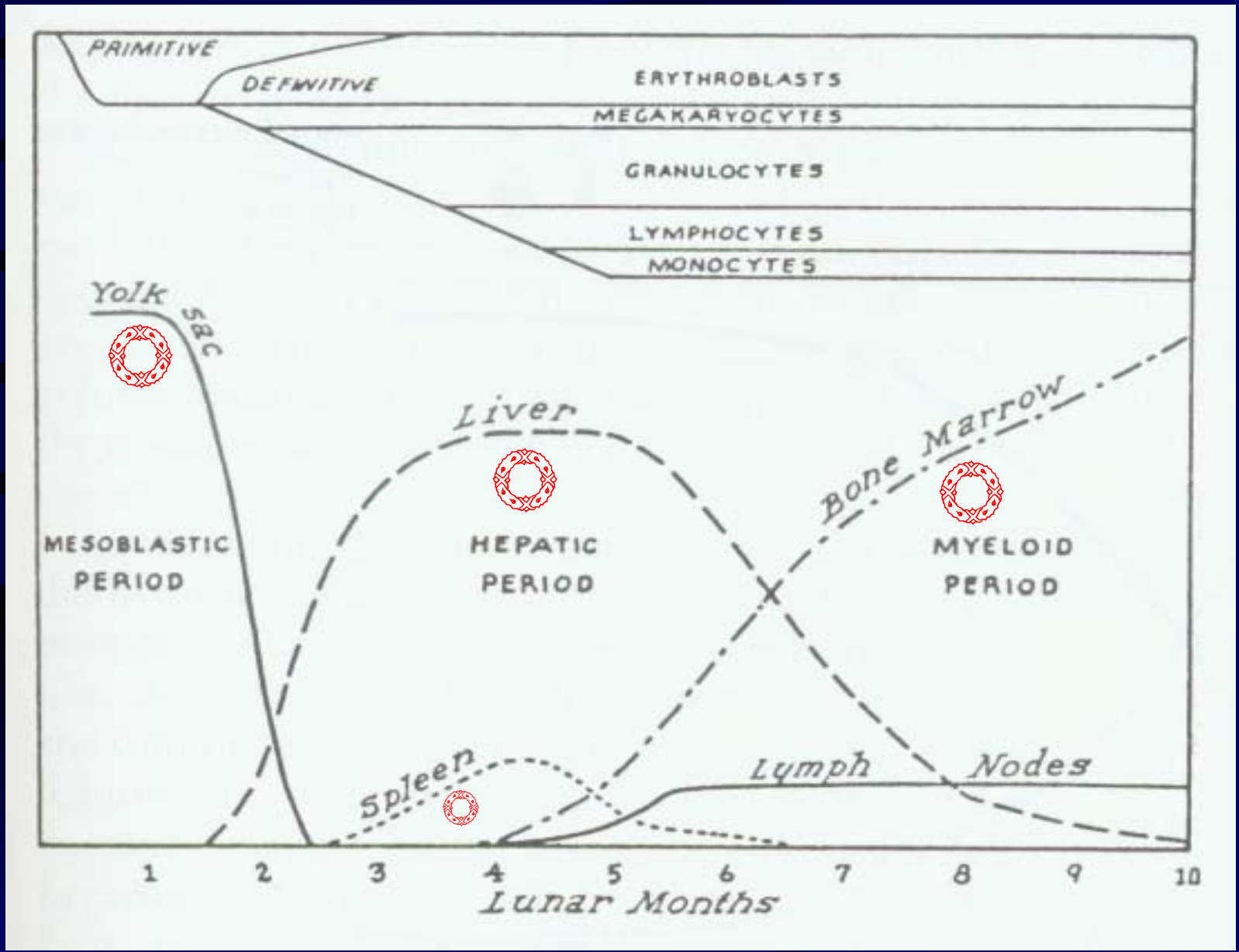


- SRC:
  - Severe combined immunodeficiency (SCID) mice repopulating cell
- LTC-IC:
  - Long -term culture-initiating cell
- CFU-GEMM
  - Colony forming unit- granulocyte, erythroid, macrophage, megakaryocyte
- CFU-GM
  - Colony forming unit- granulocyte, macrophage
- BFU-E
  - Burst forming unit- erythroid
- CFU-Meg
  - Colony forming unit- megakaryocyte

Culture of HSC

- HSC source
- Type of input HSC
  - Total cell or MNC or selected cell
- Medium
  - Liquid or semisolid
  - Serum-containing or serum-free
  - Growth factors (cytokines)
- Duration
  - Short-term or long-term
- Method
  - *In vitro (Ex vivo)* or *in vivo*
- Aim
  - HSC assay or maintenance/ expansion

Stages of hematopoiesis in the embryo and fetus



Hematopoietic Stem Cell (HSC)

Sources :

- Yolk sac
- Fetal liver
- Fetal spleen
- Umbilical cord blood
- **Bone marrow**
- Peripheral blood
  - G-CSF/GM-CSF mobilized

Type of input HSC

associated with input cell no.

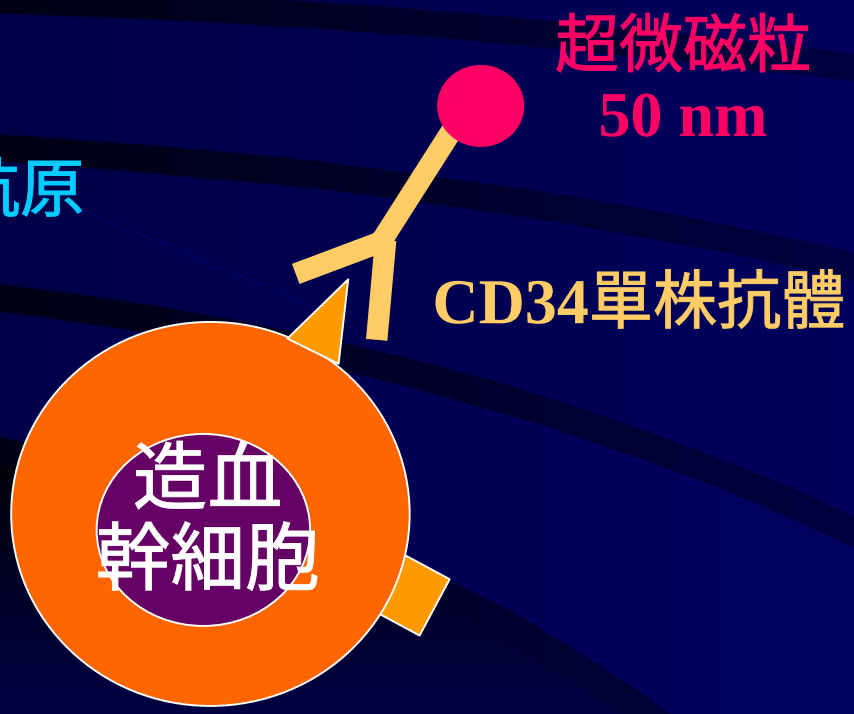
- Total cells
- Mononucleated cells (MNCs)
- Selected cells
  - Eg. CD34+cell selection

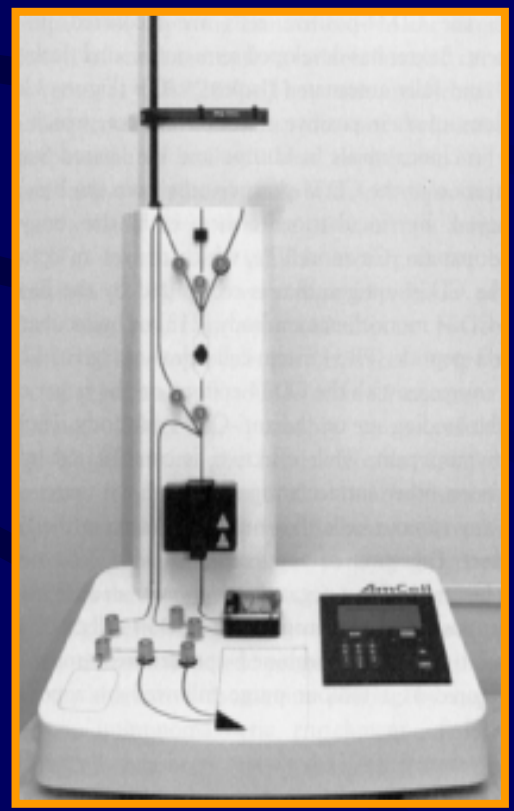
Putative HSC phenotype	
No hematopoietic lineage specific markers	M, H
Sca+*kit* +	M
CD34+KIT+	H
Rhodamine dull	M, H
CD38-Dr-CD34+	fetal marrow

M: mouse; H: human

MACS 磁性細胞篩選系統
Magnetic Cell Sorting System

CliniMACS
第三代全自動造血幹細胞篩選純化機





Medium (I)

- **Liquid-**
  - *Eg. long-term culture (LTC) medium*

    Iscove modified Dulbecco's medium (IMDM) with

    - 400 mg/ml L-glutamine,
    - 40 mg/ml myoinositol,
    - 10 mg/ml folic acid,
    - 1.6×10^{-4} M α-monothioglycerol,
    - 1.5 U/ml heparin,
    - 40 U/ml penicillin,
    - 40 mg/ml streptomycin,
    - **fetal calf serum** (FCS) 12.5%
    - **horse serum** 12.5%

Medium (II)

- **Semisolid**
  - **Agar** for late CFU-GM/CFU-E
    - Leukocyte conditioned medium (LCM)
  - **Methylcellulose** for early CFU-GM/BFU-E
    - 0.92 methylcellulose in IMDM
    - 30% **FCS**
    - detoxified bovine serum albumin (BSA) 10 mg/ml
    - Individual colony canbe easily picked up for analysis

Medium (III)

- Serum-containing
- Serum-free
  - Eg. *Medium for HSC expansion*

    IMDM with

    - inositol (40 mg/L),
    - L-glutamine (2,6 mM)
    - heparine (1,5 U/mL)
    - BSA (5 mg/mL)
    - bovine insulin (10 mg/mL)
    - cholesterol (7,5 mg/mL)
    - folic acid (10 mg/L),
    - penicilline-streptomycine (40 U/ml)
    - α-monothio-glycerol ($1{,}58 \times 10^{-4}$ M)
    - hydrocortisone hemisuccinate (10^{-6} M)
    - soybean lecithine (30 mg/mL)
    - human transferrin (30 mg/mL)

Cytokines for hematopoiesis

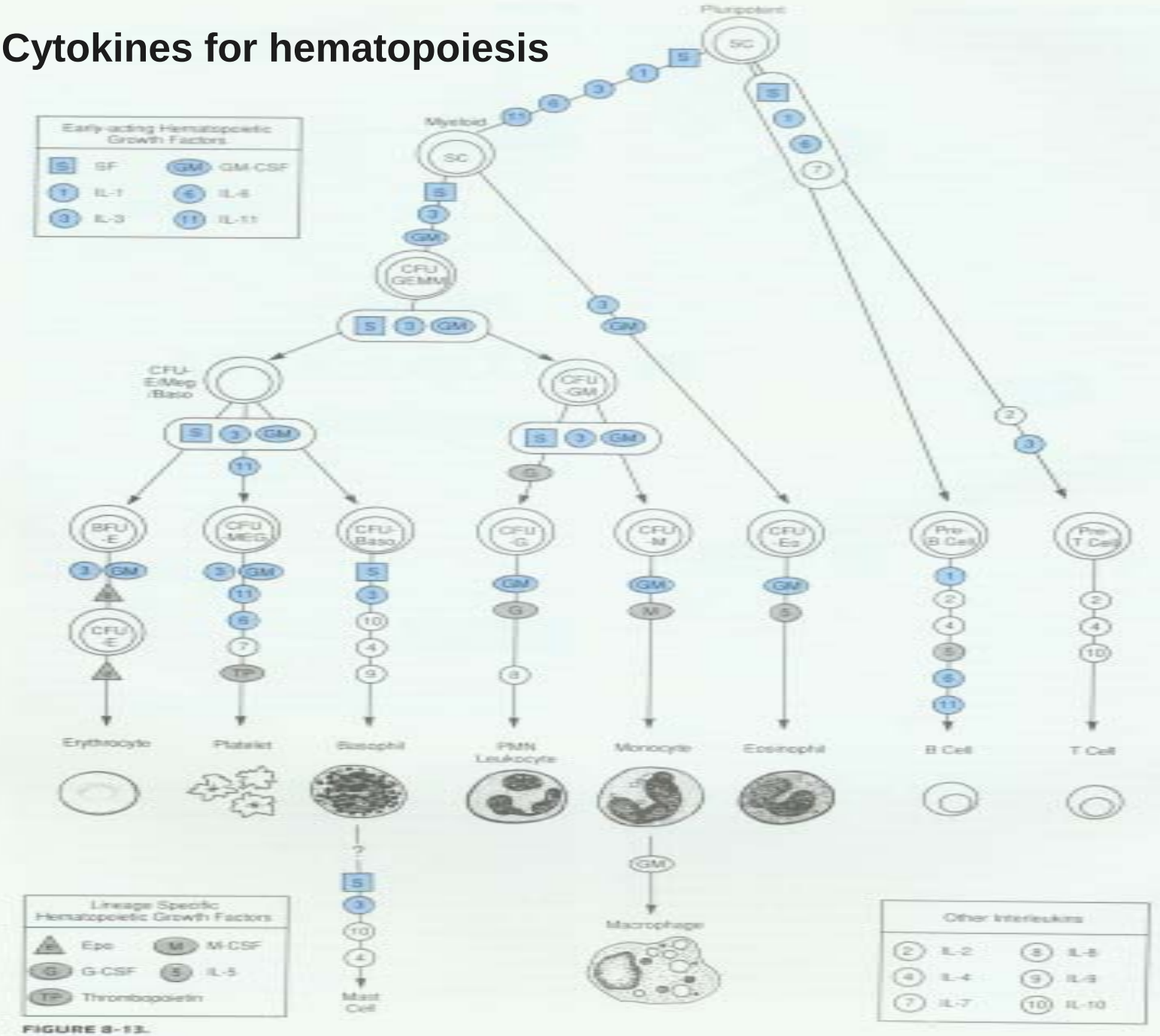


FIGURE 8-13.

Assays of HSC

In vitro culture:

- Short-term culture
  - CFU-GM/CFU-GEMM assay
  - CFU-E/BFU-E assay
  - CFU-Meg assay
- Long-term culture
  - LTC-IC assay

In vivo culture:

- SRC assay

Short-term HSC culture

- Culture for 10-18 days
  - CFU-GM/CFU-GEMM /CFU-E/BFU-E assay
    - Methylcullulose
    - Cytokines:
      - » Stem cell factor (SCF) 100 ng/ml,
      - » interleukin 3 (IL-3) 10 ng/ml,
      - » GM-CSF 10 ng/ml,
      - » G-CSF 10 ng/ml and
      - » erythropoietin (EPO) 3U/ml

STEP 1 Prepare Cells

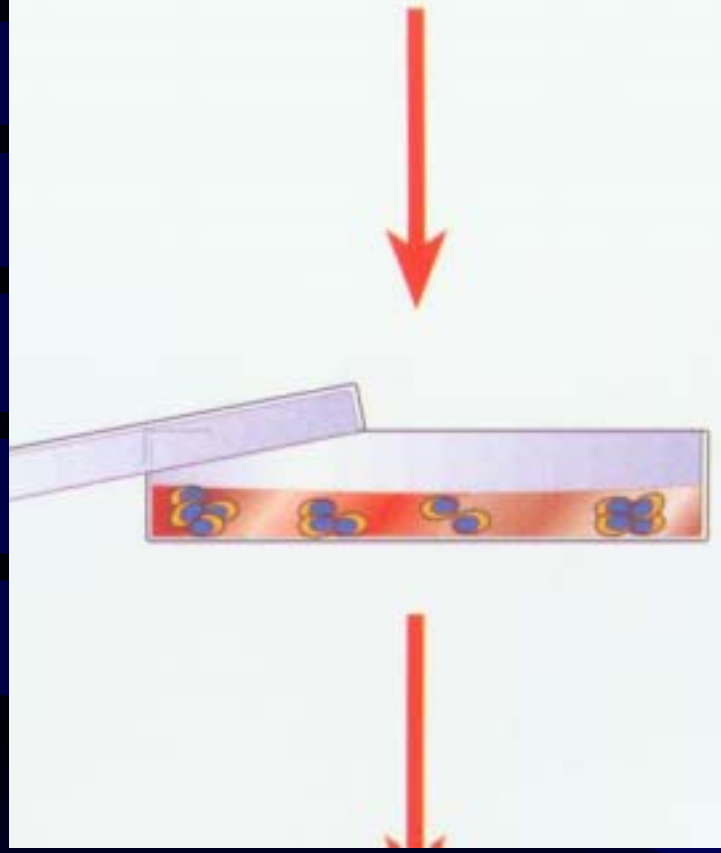
Process human cells by:

- ammonium chloride lysis
- density gradient separation
- $CD34^+$ cell enrichment with StemSep™, RosetteSep™ or FACSorting

Wash cells (e.g. in Iscove's MDM + 2% FBS), then count and adjust cell concentration.

STEP 2 Add Cells to MethoCult™

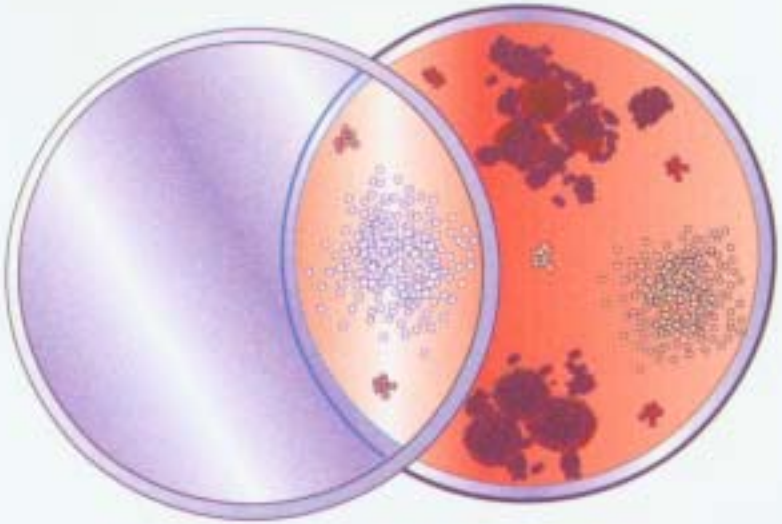
Add cells to MethoCult™ and vortex.



STEP 3 Plate & Incubate

Dispense cells into pre-tested petri dishes using syringe and blunt-end needle.

Incubate human cells for 14-16 days, murine cells for 7-14 days, in humidified incubator at 37°C and 5% CO_2.



STEP 4 Count Colonies

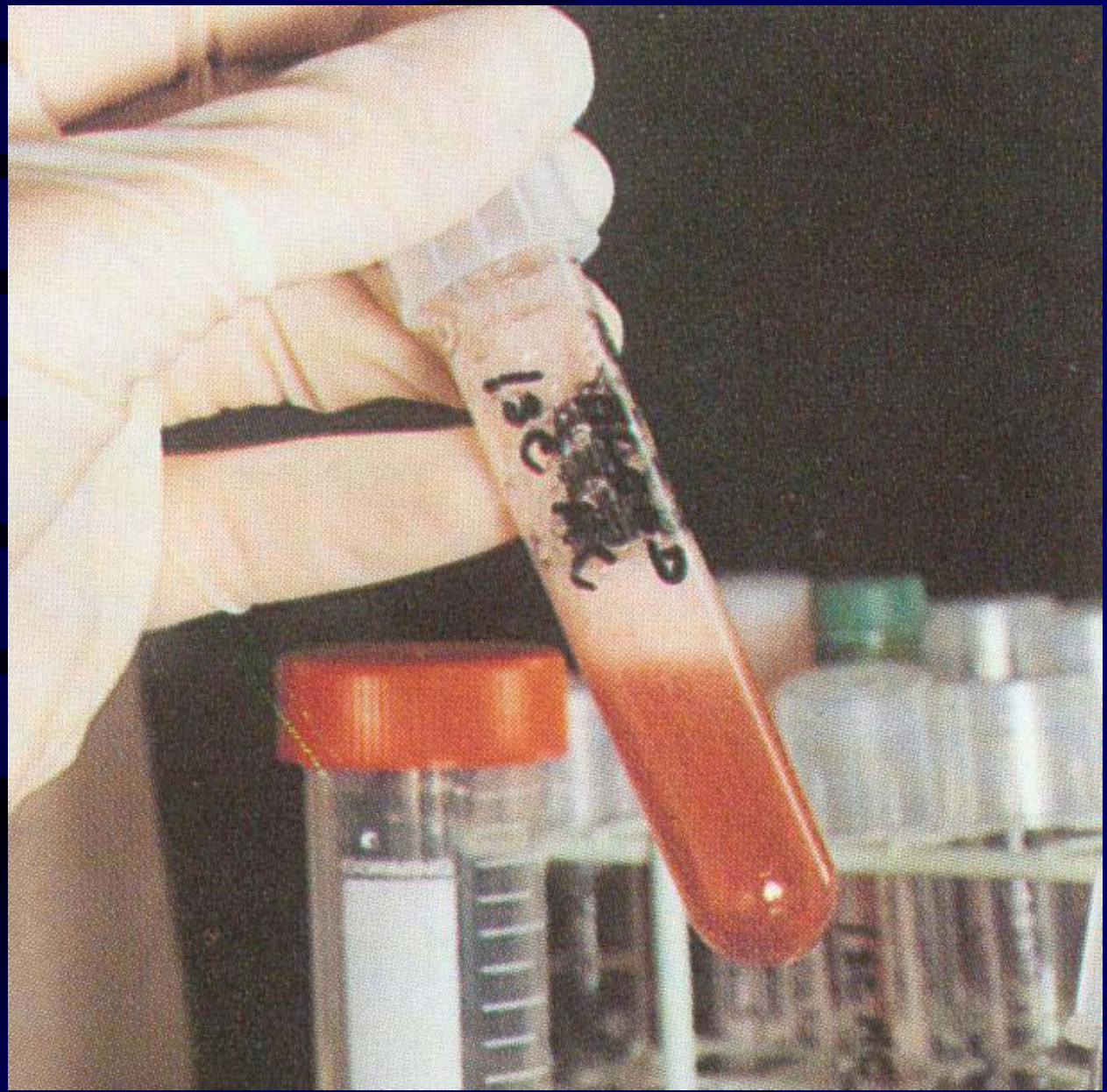
Count and evaluate colony types using inverted microscope and gridded scoring dishes. Alternatively, individual colonies may be plucked for routine staining, PCR, or cytogenetic analysis.

MNC separation
Ficoll-Hypaque

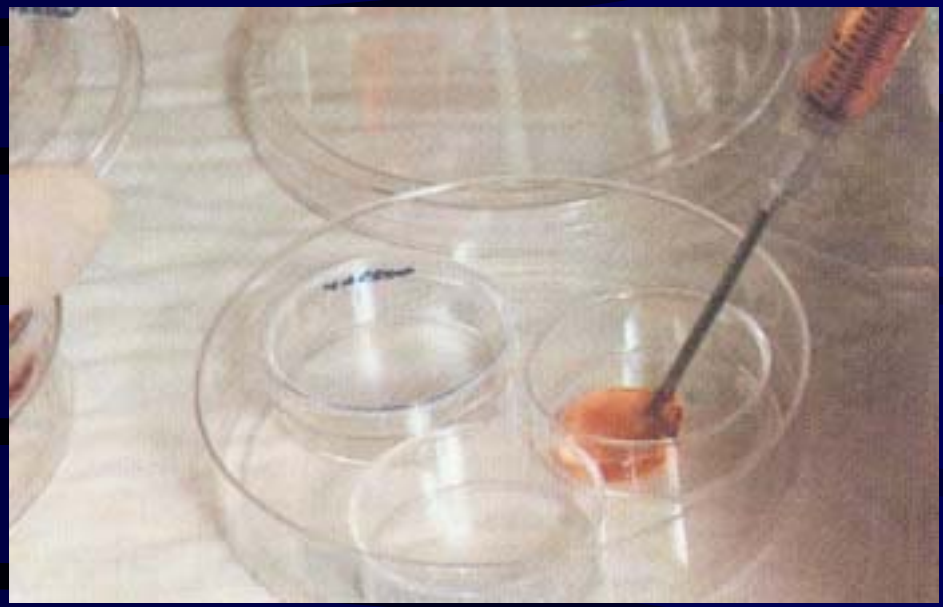
Take pre-frozen Methylcullulose medium out from freezer

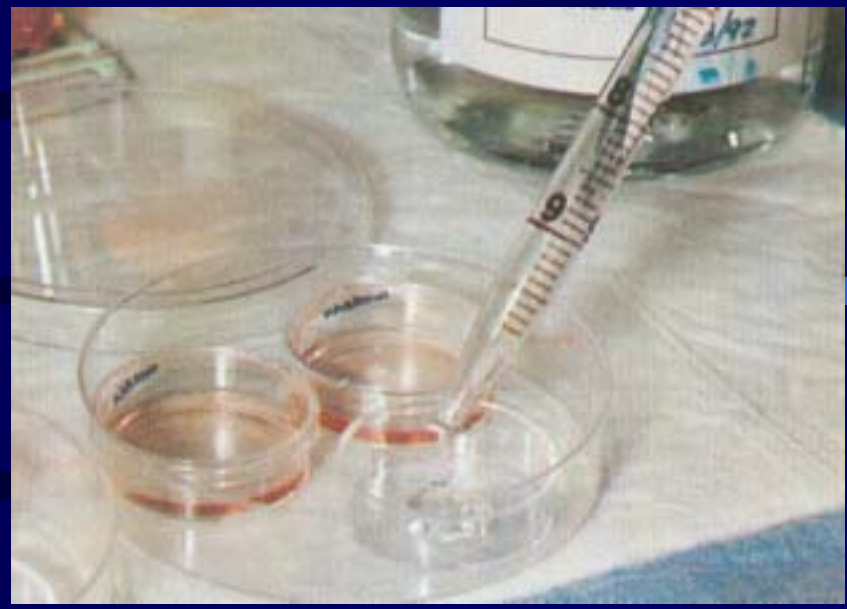
TABLE 2: RECOMMENDED PLATING CONCENTRATIONS PER 1.1 ML

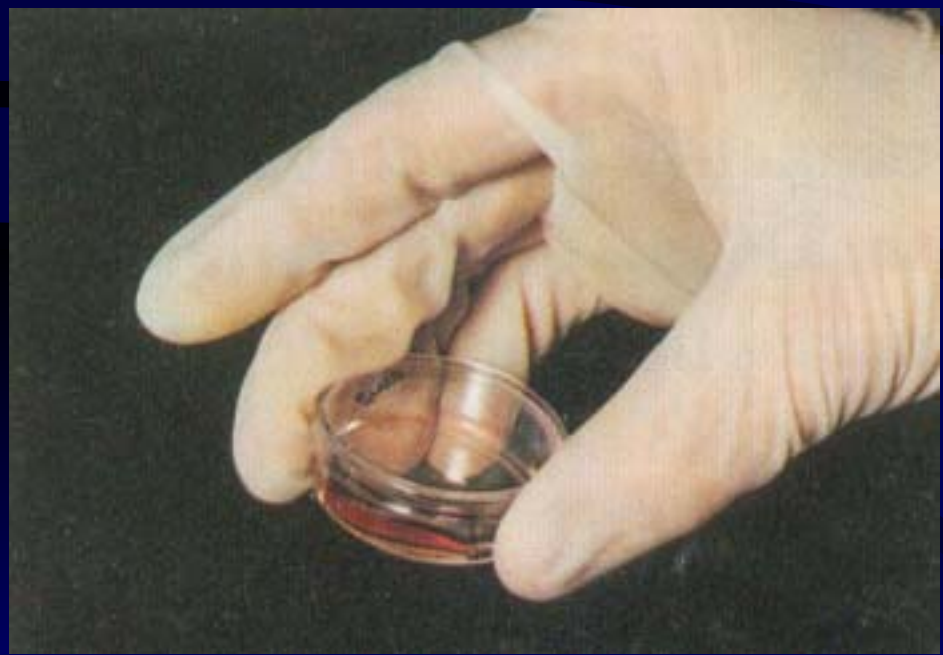
NORMAL MARROW	2×10^5 BUFFY COAT CELLS
	or
	1×10^5 LIGHT DENSITY CELLS
NORMAL BLOOD	4×10^5 LIGHT DENSITY CELLS



Mix
Vortex

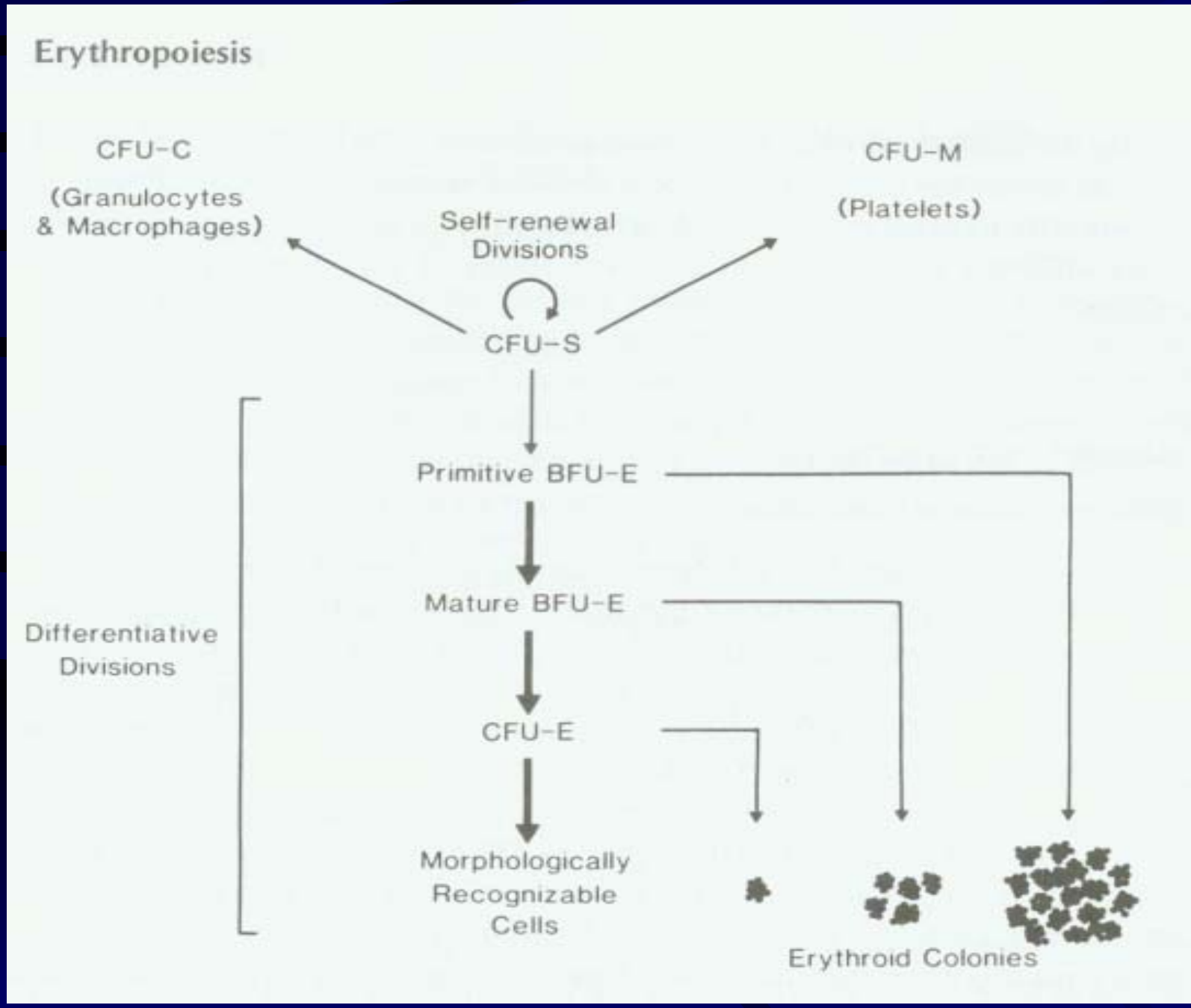






Plating->Incubation at 37^{o}C with 5% CO_2

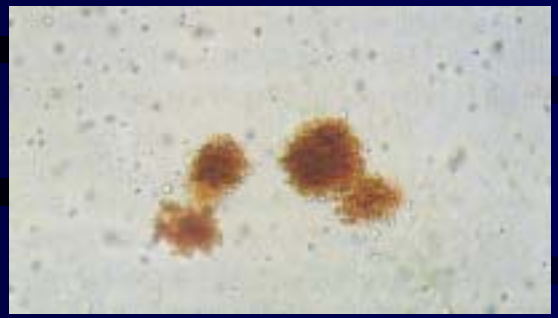
counting


Erythropoiesis
CFU-C
(Granulocytes
& Macrophages)
CFU-M
(Platelets)
Self-renewal
Divisions
CFU-S
Primitive BFU-E
Mature BFU-E
CFU-E
Morphologically
Recognizable
Cells
Differentiative
Divisions
Erythroid Colonies

Erythroid colonies

read by inverted microscope

CFU-E (Day 10)



Mature BFU-E (Day 10)

Primitive BFU-E (Day 18)

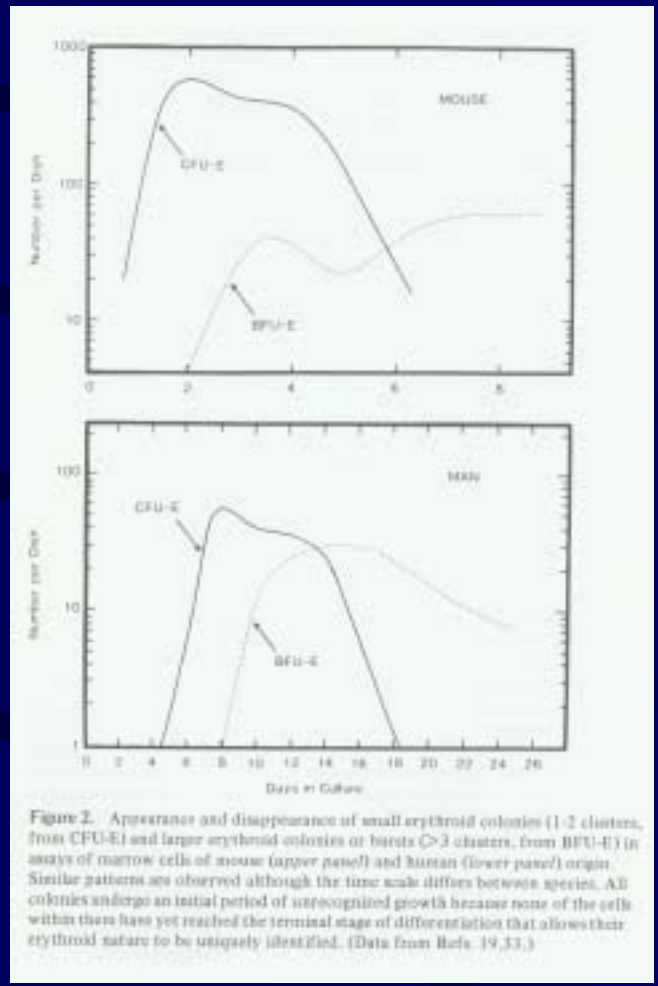
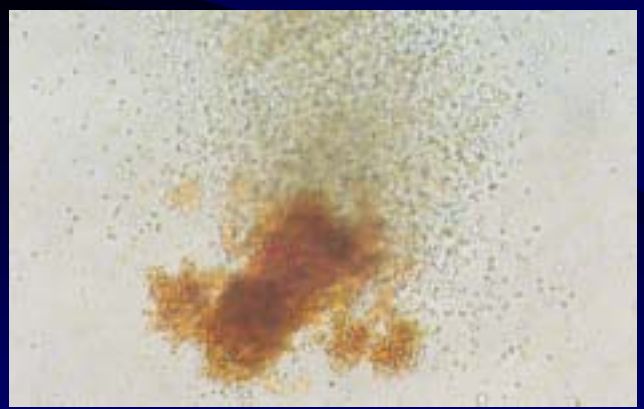


Figure 2. Appearance and disappearance of small erythroid colonies (1-2 clusters, from CFU-E) and larger erythroid colonies or bursts (>3 clusters, from BFU-E) in assays of marrow cells of mouse (*upper panel*) and human (*lower panel*) origin. Similar patterns are observed although the time scale differs between species. All colonies undergo an initial period of unrecognized growth because none of the cells within them have yet reached the terminal stage of differentiation that allows their erythroid nature to be uniquely identified. (Data from Refs. 19,33.)

Granulopoietic colonies

CFU-GM (Day 14)

CFU-GM (Day 18)



CFU-GEMM (Day 18)

Megakaryopoiesis

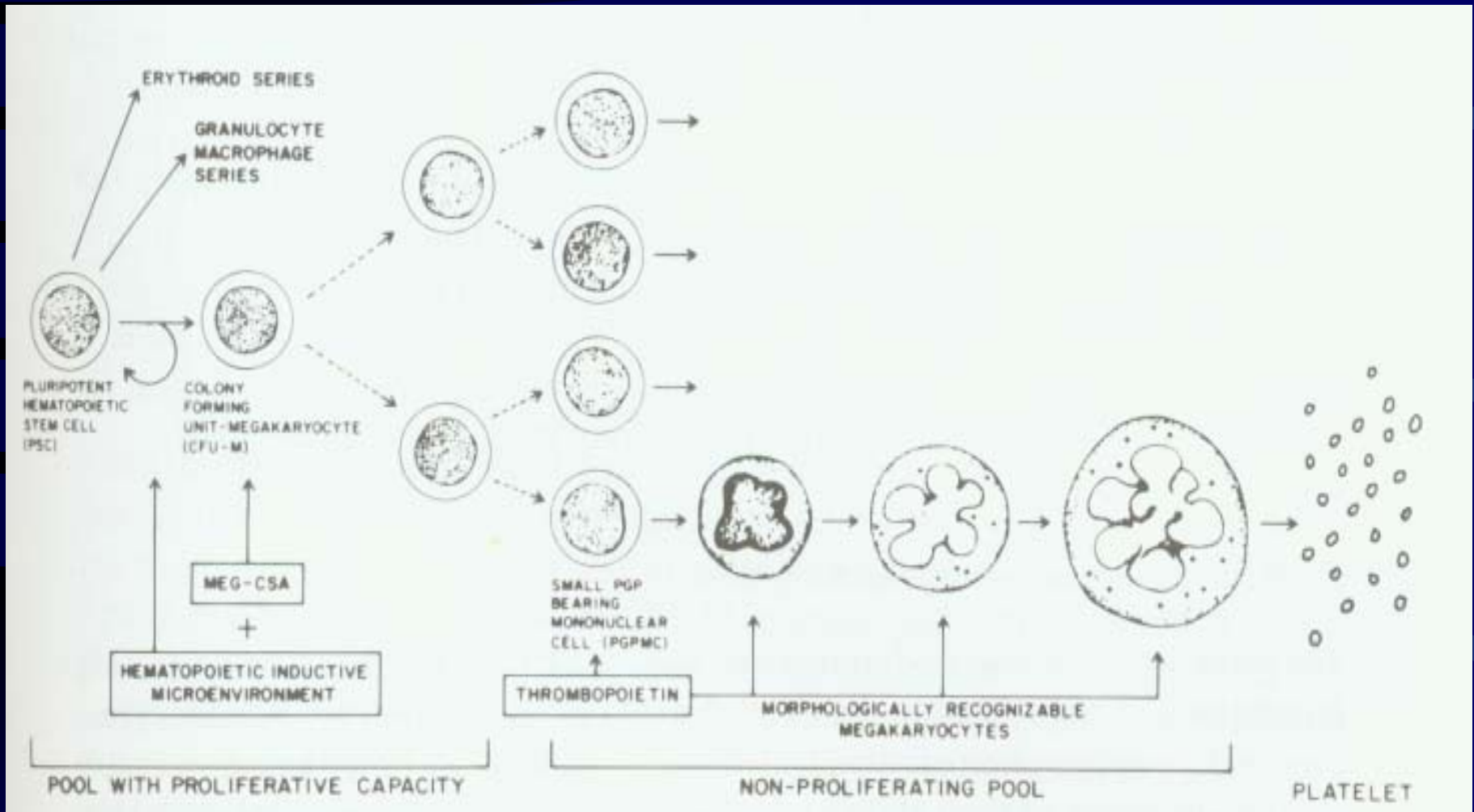


Figure 1. Proposed schema of human megakaryocyte maturation.

CFU-Meg assay

Eg.

MegaCult™-C Serum-Free Medium with Cytokines

Quantity: 2.0 mL/tube (24 tubes/rack); 50 mL bottle

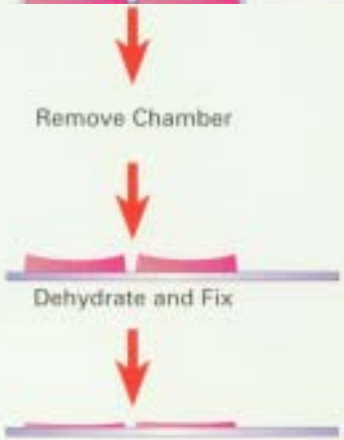
Components: Final concentration of media components following the addition of cells (0.1 mL) and collagen (1.2 mL) to MegaCult™-C medium (2.0 mL):

1.1 mg/mL	Collagen
1%	Bovine Serum Albumin
10 µg/mL	rh Insulin
200 µg/mL	Human Transferrin (Iron saturated)
10^{-4} M	2-Mercaptoethanol
2 mM	L-glutamine
50 ng/mL	rh Thrombopoietin
10 ng/mL	rh IL-6
10 ng/mL	rh IL-3
	Iscove's MDM

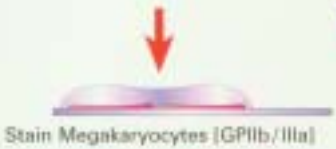
CFU-Meg assay

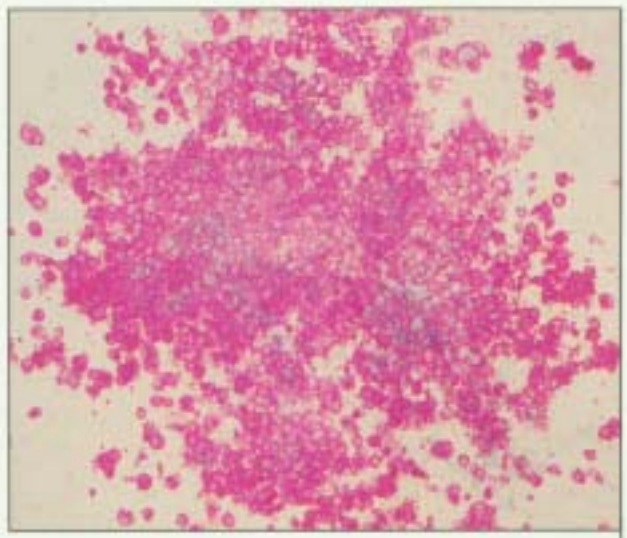
STEP 1 Set up and Culture

STEP 2 Dehydrate and Fix



STEP 3 Stain and Count





Long term culture of HSC

- Medium
  - Serum-free
  - Serum-containing
- Feeder

Feeder of HSC culture

- Served as stromal cells in BM
- Necessary in HSC long-term cuture
  - Irradiated allogeneic human BM cells
    - hydrocortisone necessary
  - Murine cell line
    - Eg.MS-5
      - hydrocortisone harmful

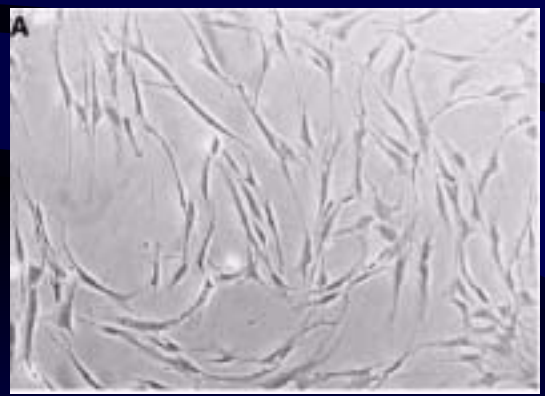
BM-derived MSC

More than 20 years ago, Friedenstein and then others grew adult stem cells from bone marrow called mesenchymal stem cells or marrow stromal cells (MSCs).

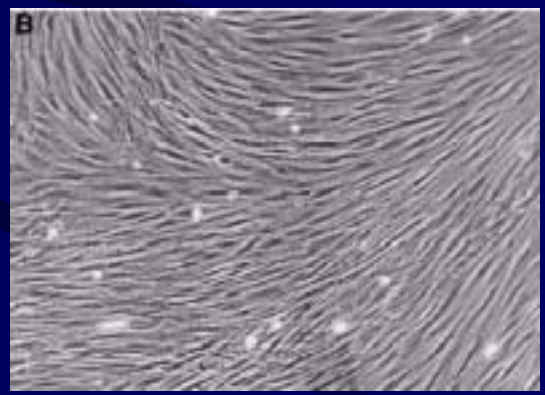
Friedenstein A. :Stromal mechanisms of bone marrow: cloning *in vitro* and retransplantation *in vivo*. In: Thienfelder S, Rodt H, Kolb HJ (eds). *Immunology Of Bone Marrow Transplantation*. Berlin: Springer Verlag, **1980**, 19-20.

Function:

1. In vivo: Support HSC growth
   in vitro: Feeder layer of HSC growth (in LTC-IC assay etc.)



7 days BM culture



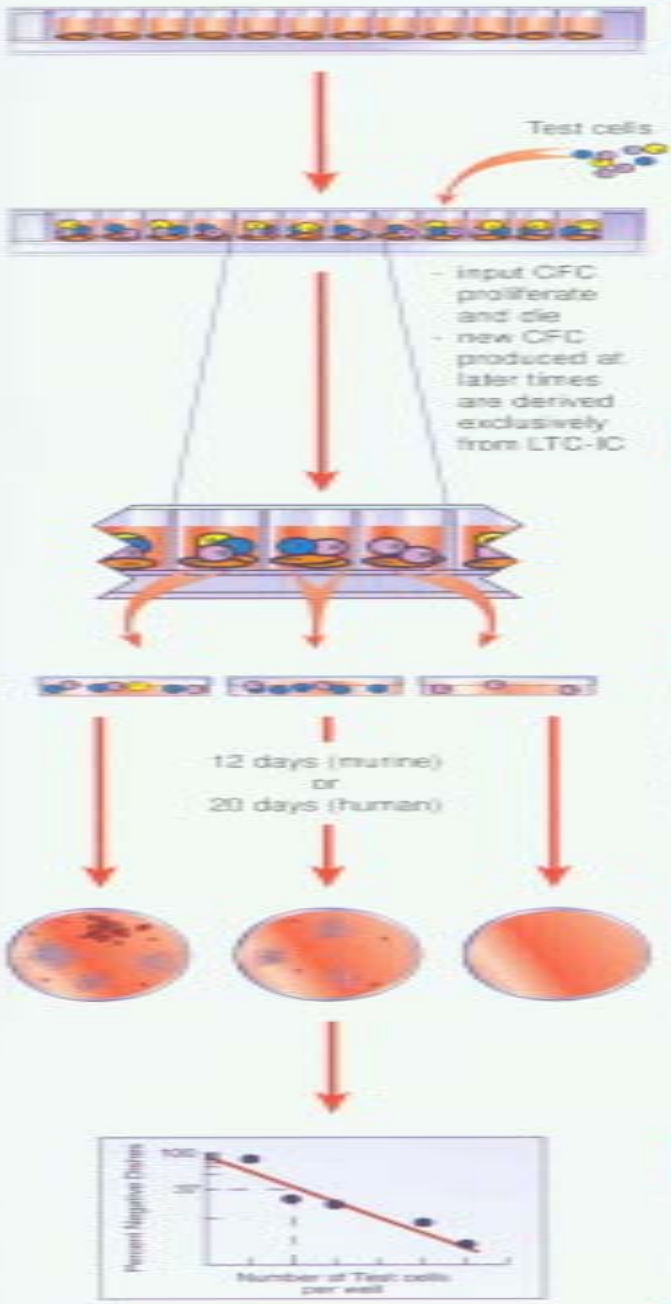
21 days BM culture

Long-Term Culture-Initiating Cell (LTC-IC)

- Both adherent and non-adherent cells Cell that possessed hematopietic ability after 4-week culture
- Duration of culture: 4-8 weeks
- Bulk culture
- Limited dilution analysis

- **Sutherland HJ**, Lansdorp PM, Henkelman DH, Eaves AC, Eaves CJ. (1990): Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers. Proceedings of the National Academy of Sciences of the United States of America 87: 3584-3588.

Figure 1: Limiting Dilution LTC-IC Assay



STEP 1

Establish Feeders

Use irradiated marrow cells or suitable cell line (e.g. M2-10B4) **or MS-5**

= feeder cell

STEP 2

Add Serial Dilutions of Test Cells

= LTC-IC
= CFC
= mature cell

STEP 3

Maintain Cultures

With weekly 1/2 medium changes
for ≥ 4 weeks for murine LTC-IC
for ≥ 5 weeks for human LTC-IC

STEP 4

Harvest and Plate

Harvest all cells from each well, including adherent and non-adherent cells, and transfer into individual methylcellulose cultures

STEP 5

Score Dishes

Record growth (≥1 colony) or no growth (no colonies)

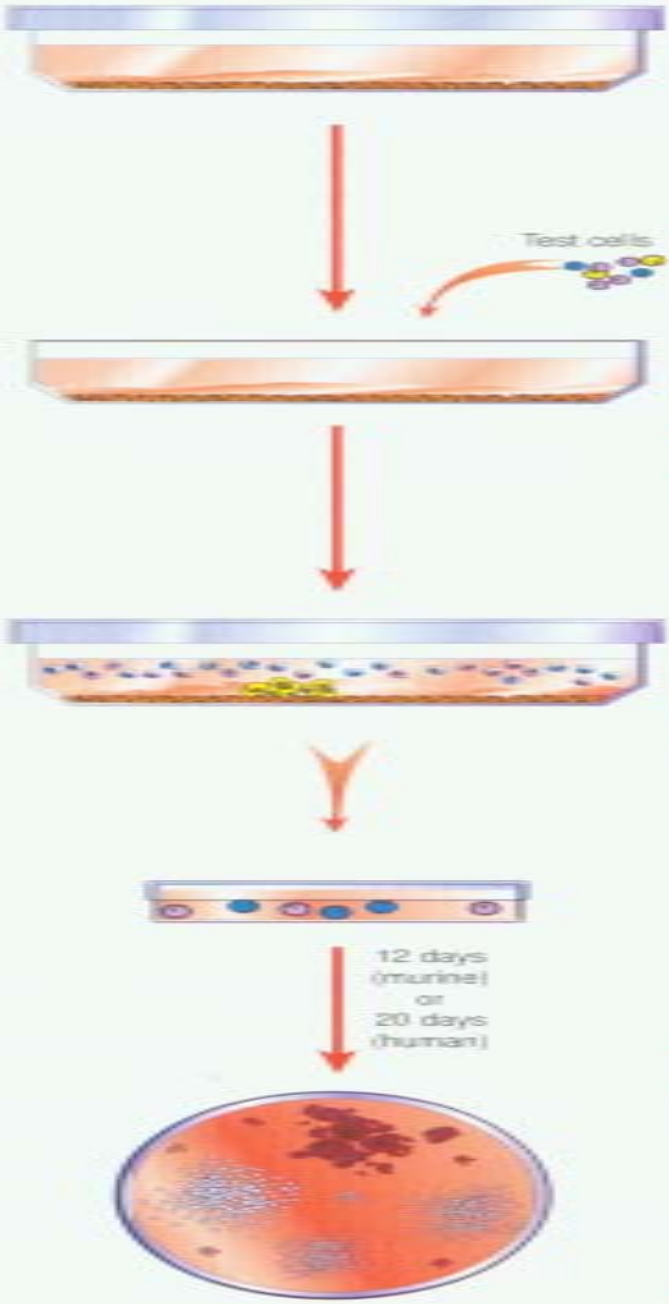
***Poisson* statistical analysis**

STEP 6

Calculate LTC-IC Frequency

Use L-Calc™ - a statistical software program for limiting dilution analysis

Figure 2: Bulk Culture LTC-IC Assay



STEP 1

Establish Feeders

Use irradiated marrow cells or suitable cell line (e.g. M2-10B4)

= feeder cell

STEP 2

Add Known Number of Test Cells

= LTC-IC

= CFC

= mature cell

STEP 3

Maintain Cultures

With weekly 1/2 medium changes

for ≥ 4 weeks for murine LTC-IC

for ≥ 5 weeks for human LTC-IC

STEP 4

Harvest, Count and Plate

Harvest entire dish, including adherent and non-adherent cells, and plate appropriate aliquot in methylcellulose cultures

STEP 5

Count Total Number of Colonies

Calculate number of LTC-IC in test cell suspension based on the average output of CFC per LTC-IC as determined by a limiting dilution assay

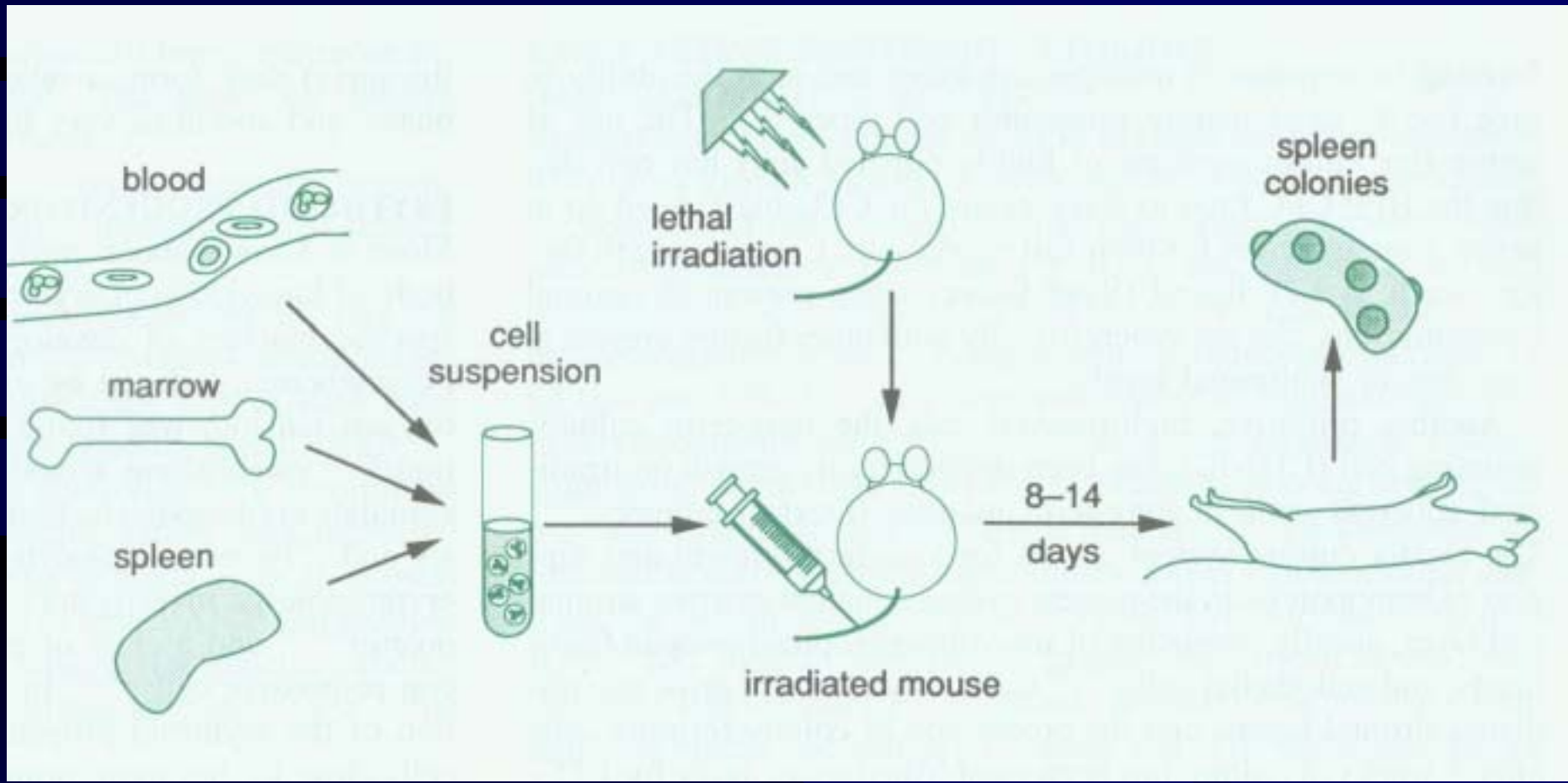
Ex vivo expansion of HSC

- Culture
  - Short-term *vs* Long-term
    - Change medium every 3-5 days
- Medium
  - Serum-free vs Serum-containing
- Cytokines cocktail
  - Stem cell factor (S)+IL-3+IL-6+G-CSF(G) +Epo(E) +Tpo
- Bioreactor

Problems in HSCs
Ex vivo expansion

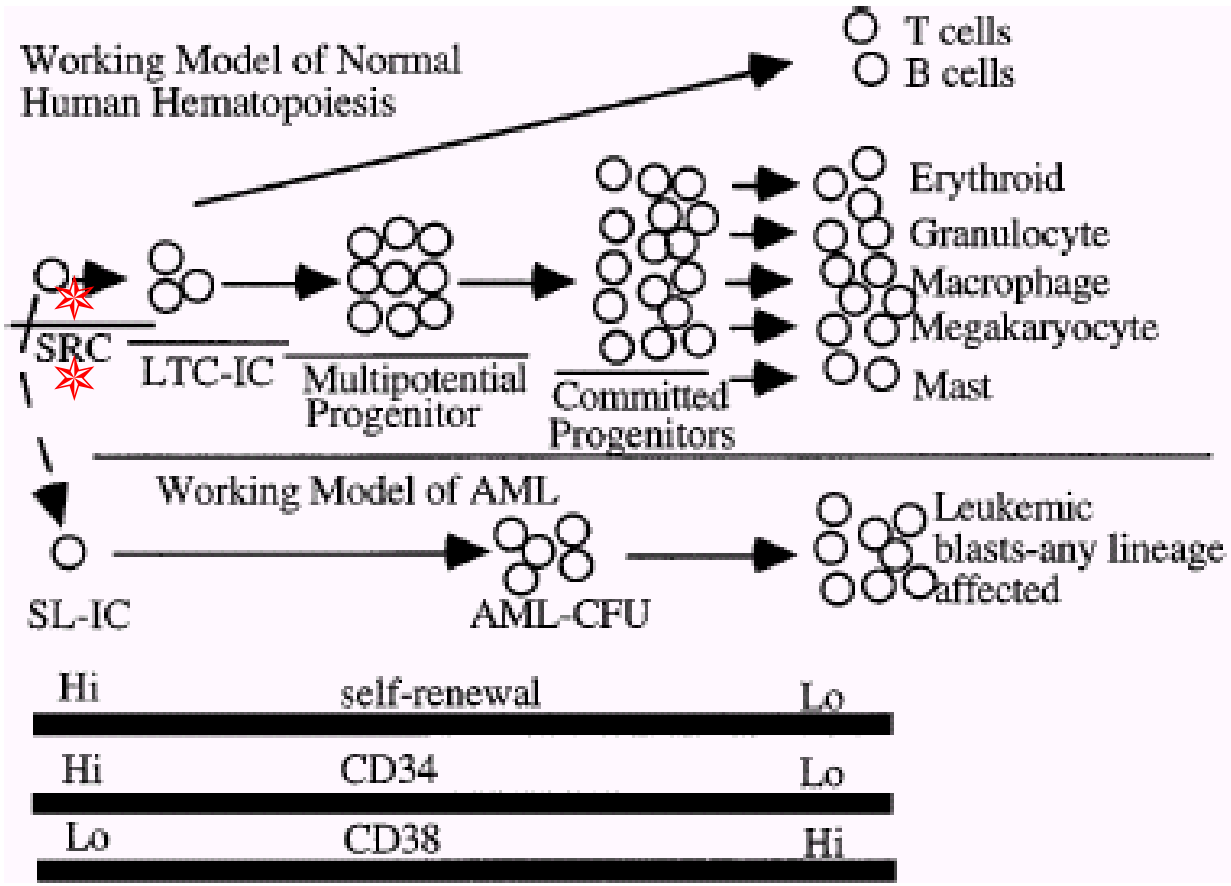
- What cells will be expanded? MNCs or CD34+cells?
  - Expansion rate are higher when **CD34-selected cells** were seeded instead of unmanipulated MNCs.
    - Poloni A. et al. Hematology & Cell Therapy. 39:49-58, 1997
- What is the best cytokines cock-tail for expansion?
  - Stem cell factor (S)+IL-3+IL-6+G-CSF(G)+Epo(E) **(5C)**or
  - S+IL-3+IL-6+G+E+**Flt3-ligand** (F) **(6C)**

In vivo HSC assay

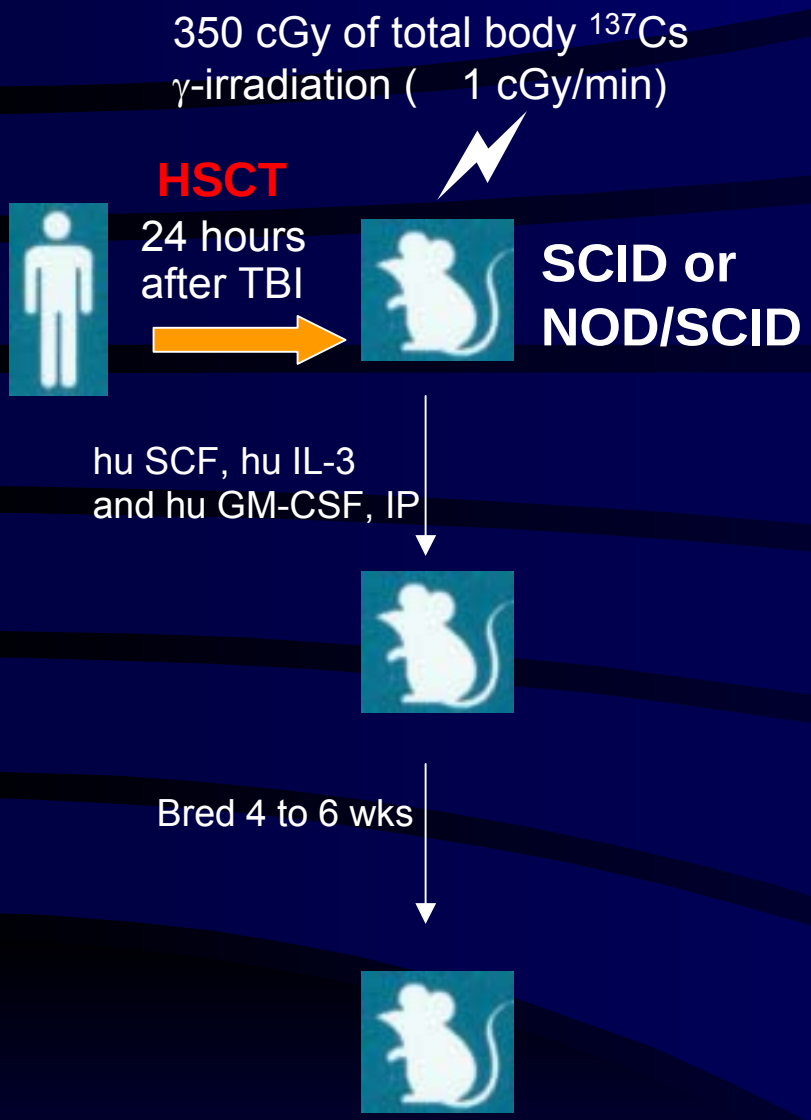


CFU-S assay

SRC assay



Dick JE. Lapidot T. Pflumio F. Transplantation of normal and leukemic human bone marrow into immune-deficient mice: development of animal models for human hematopoiesis. Immunological Reviews. 124:25-43, 1991



•Mice will be killed by cervical dislocation

•BM aspirated from 2 femurs, 2 tibiae, and 2 iliac crests will be mixed and then flushed into IMDM plus 10 % FCS for analysis

Analysis

- ***Analysis of human cell engraftment***
  - DNAs from the BM of mice were analyzed by Southern technique. hybridized with a human chromosome 17-specific α-satellite probe (p17H8)
- ***Immunophenotyping***
  - CD34, CD33, CD45
- ***CFCs and LTC-ICs assays:***
  - The specificity of these assays will be confirmed by plating human and mouse mixtures and by PCR on individual colonies using primers specific for the human dysphorin gene
- ***Quantification of SRCs:***
  - Limiting dilution analysis (LDA)
  - a transplanted mouse was scored as positive (engrafted) if any human cells were detectable in murine BM by Southern blot analysis.
  - We will assume that only one SRC is required in infused cells to generate a positive response (a engrafted mouse), and that every transplanted SRC will generated a positive response.

Application of HSC Culture

- **Mechanism of BM disorders**
  - Shih LY. Lee CT. Identification of masked polycythemia vera from patients with idiopathic marked thrombocytosis by **endogenous erythroid colony assay**. *Blood. 83(3):744-8, 1994*
  - Yao M, et al Quantitative and qualitative alterations of **long-term culture-initiating cells** in patients with acute leukaemia in complete remission. *British Journal of Haematology. 103:124-128, 1998*
- **HSC transplantation**
  - HSC no. correlates directly to engraftment kinetics
  - Yao M et al **Ex vivo expansion** of CD34-positive peripheral blood progenitor cells from patients with non-Hodgkin's lymphoma: no evidence of concomitant expansion of contaminating bcl2/JH-positive lymphoma cells *Bone Marrow Transplantation 26 : 497-504, 2000*
- **Gene therapy**
  - Larochelle A. Vormoor J. Hanenberg H. Wang JC. Bhatia M. Lapidot T. Moritz T. Murdoch B. Xiao XL. Kato I. Williams DA. Dick JE. Identification of primitive human hematopoietic cells capable of repopulating NOD/SCID mouse bone marrow: implications for gene therapy. *Nature Medicine. 2:1329-37, 1996*