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In vitro generation of immune cells from pluripotent stem cells

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1. ABSTRACT

Stem cell transplant recipients and acquired or inherited immune-deficiency patients could benefit from the infusion of B, T and/or NK cells. These lymphoid cells can be generated *in vitro* from bone marrow derived $CD34^+CD45^+$ hematopoietic stem cells (HSC). The number of cells that can be obtained in this way is limited especially in the adult. An alternative source may therefore constitute human pluripotent stem cells (PSC) such as embryonic (hESC) or induced pluripotent stem cells (hiPSC). Here, we focus on present knowledge on the generation of lymphoid cells from hESC. The two main obstacles for the generation of clinically relevant immune cells are the failure to generate from hESC long-term repopulating HSC which could be kept in culture for prolonged time; and insufficient knowledge of the selection process which generates mature T cells from CD4 CD8 double positive (DP) precursors *in vitro*.

2. INTRODUCTION

The *in vitro* generation and subsequent transfusion of immune cells could be beneficial for certain patient populations. Stem cell transplant recipients especially after receiving cord blood grafts or allogeneic purified $CD34^+$ hematopoietic precursor cells (HPC) isolated from bone marrow or mobilized blood go through a prolonged period of immune deficiency before the full repertoire of B and especially T cells is regenerated. Some patients never recover a polyclonal T cell repertoire. The length of this immune deficient period varies from months to several years, increases with age and is caused by slow recovery of thymopoiesis. In older patients, the involuted thymus is rejuvenated and incoming stem cells generate a wave of T cell production(1). Such transplant recipients may benefit from infusion of committed T cell precursors which home to the thymus and develop into mature

functional T cells(2-3). Alternatively, *in vitro* generated mature T cells could be beneficial on the condition that these cells do not induce graft versus host disease. Similarly HIV patients with low CD4 T cell numbers could benefit from such cells and regain immune competence. Hereditary immunodeficiency diseases are less common but diseases such as ADA deficiency could possibly be alleviated using such approaches. If it is feasible to generate T cells *in vitro* from ADA patients, such autologous T cells may, upon transfusion, reconstitute immune function.

$CD34^+$ HPC differentiate to most erythroid and myeloid blood cell lineages under relatively simple culture conditions consisting of a mixture of hematopoietic growth and differentiation factors(4). B and NK lymphoid cells can be generated *in vitro* using a feeder cell line such as MS5 or OP9 and growth factors such as IL-7, flt-3 ligand (FL) and stem cell factor (SCF) (5). T cell lineage differentiation however, was never observed using the approach mentioned above. For this reason, chimeric fetal thymic organ cultures in which murine severe combined immune deficiency (SCID) thymus were populated by human $CD34^+$ HPC were used to obtain T precursor and mature T cells(6). Although this technique is adequate for experimental purposes, the procedure is laborious, does not generate large numbers of T cells and is unfit for clinical use. In 2002, Schmitt *et al* demonstrated that murine HSC give rise to T cell precursors when cultured on OP9 cells which were genetically engineered to express delta-ligand-1(OP9-DL1) in the presence of IL-7 and FL(7). DL1 binds notch-1 expressed on HPC and drives them towards the T cell lineage. The natural ligand for notch1 is Delta-ligand-4 (DL4). DL4 which is functionally similar to DL-1 is expressed *in vivo* by thymic epithelium and mice that do not express DL-4 on thymic epithelium or Notch-1 on hematopoietic cells do not generate T cells(8-10). These cells differentiate *in vitro* over the course of a few weeks to mature CD8 T cells. Although few CD4 T cells were generated, the T cells generated were mature and functional.

In human, cultures of $CD34^+$ HPC on an OP9-DL1 feeder layer in the presence of IL-7, SCF and FL likewise give rise to mature T cells(11-12). However, the efficiency of this differentiation protocol varies depending on the source of the stem cells used. Whereas fetal liver, fetal bone marrow and cord blood $CD34^+$ cells generate T cell precursors at high efficiencies, $CD34^+$ cells obtained from bone marrow or mobilized blood especially from patients treated with chemotherapy were much less capable of generating large numbers of T cells(13). This is partially due to a lower T cell precursor frequency in bone marrow derived $CD34^+$ cells, but is especially caused by a lack of progression of T-lineage committed precursors towards the more mature stages (unpublished data, (14)). Since these are the only sources available in adult patients, generation of T cells from HPC *in vitro* is problematic in most patients. For this reason other sources of stem cells may be preferable.

Recently, Yamanaka *et al* showed first for murine and subsequently for human fibroblasts that the induced expression of oct-4, sox2, klf4 and myc is able to transform these cells into pluripotent stem cells (PSC) (15-16). These cells are called induced pluripotent stem cells (iPSC) and are very similar to hESC. The first derivations required stable insertion of multiple copies of these constructs including the proto-oncogene myc into the host DNA, which may seriously compromise clinical use. However, subsequent research showed that transient expression of these factors is sufficient. Methods which ensure transient induction without altering or damaging chromosomal DNA include protein transduction, episomal transduction and transduction with removable transposons and or addition of small molecules (reviewed in(17)). Using these techniques, autologous PSC can be generated from fetal, adult and even elderly patients.

The group of Zuniga-Pflucker showed that murine ESC could generate T cell precursors when cultured on OP9-DL1 feeder layer(18). This protocol seems to generate T cells which were able to produce interferon-gamma after CD3 stimulation, demonstrating their functionality. This finding opens up new avenues for the generation of T cells from PSC such as hESC or hiPSC from patient fibroblasts instead of bone marrow or mobilized HPC.

First attempt to generate immune cells from hESC using the protocol of Zuniga-Pflucker failed(19). However, a multistep approach in which the physiologic differentiation pathways are respected, especially the prior generation of hematopoietic stem cells (HSC) or multipotent HPC and the subsequent differentiation of these cells to lymphoid cells, was proven to be more successful in humans. HSC are defined as long-term self-renewing multipotent cells. However, the hematopoietic precursor cells obtained from hESC cultures are not always tested for these properties and therefore we will use HSC/HPC to denote this fact. In this review, we will discuss the generation of HSC/HPC from hESC, the generation of T cells from HSC/HPC, the quality of the T cell repertoire generated, and finally the first attempt to reproduce T lymphopoiesis from hiPSC.

3.GENERATION OF HPC/HSC FROM hESC

3.1.Generation of HSC in the embryo

After birth, all blood cells are generated from HSC which are included in the $CD34^+CD90^+CD38^-$ population present in bone marrow(14). Unlike murine HSC which are partly contained in the $CD34^-$ fraction(20), human HSC are confined to the $CD34^+$ population and reports demonstrating that HSC activity is present in the $CD34^-$ fraction have been difficult to reproduce in the human(21-22) Similarly, reports claiming the presence of bipotent hematopoietic and endothelial precursor cells in the adult are clouded by technical difficulties such as the discrimination between tissue macrophages and endothelium and the existence of such a cell postnatally is controversial(23-25) No other cells present in the human body have been shown to generate blood cells. Long-term cultured (and possibly altered) cells such as mesenchymal stem cells were shown to generate blood cells in the mouse.

However, it is not clear whether this capacity is acquired *in vitro* and whether the uncultered initiating cell present in the bone marrow can generate blood cells (26).

Crucial to the understanding of hematopoiesis is the realization that in the embryo, in contrast to the postnatal period, hematopoiesis is not initiated from multipotent self-renewing HSC(27). During fetal life the earliest blood cells generated are nucleated red cells. These cells contain fetal haemoglobin (epsilonzeta chains). Besides nucleated red cells, foamy macrophages and some megakaryocytes are also observed in blood islands of the yolk sac (YS). At that time, no HSC activity can be observed(28). This activity is usually tested by the ability of YS cell suspensions to reconstitute irradiated mice. These earliest blood cells arise from a vascular endothelial growth factor receptor 2 $(VEGFR2)^+$ mesenchymal precursor which arises in the primitive streak and migrates to the YS(29). In zebrafish it was shown that $VEGFR2^+$ endothelial cells give rise to these primitive blood cells(30). These cells are notch as well as runx1 independent.

A second "attempt" by the fetus to generate blood cells results in an erythromyeloid precursor(27, 31). Similar to primitive hematopoiesis, blood is generated from precursors which are not HSC, since the cells do not generate lymphoid cells nor do these cells have reconstitutive potential in irradiated animals beyond three months after transplantation(28, 32). These cells probably originate in the YS since these cells can be obtained from YS tissue harvested prior to the establishment of circulation. These erythromyeloid precursors give rise to enucleated red cells which contain adult type haemoglobin and besides macrophages also myeloid cells are generated. These cells are notch independent but runx1 dependent(33).

The first transplantable HSC arise during the third "attempt" in the embryo proper and their generation is runx1 as well as notch1 dependent(34). These cells are generated from VE-cadherin$^+$ runx1$^+$ endothelial cells of the aorta and possibly other arteries. The cells generated are phenotypically different from the adult HSC. In the adult mouse the long-term repopulating and quiescent HSC is a $CD41^-CD150^+CD48^+CD34^-$ lineage$^-$ sca-1$^+$ kit$^+$ (LSK) and $CD45^+$ cell(35). The first stem cells generated in the aorta are $CD41^+$ $CD150^-CD48^-CD34^+$ and ckit$^+$ and $CD45^-$(36). This may indicate that the hematopoietic cells as they are generated from endothelium are immature and need passage to the fetal liver (or bone marrow in the experimental animal) before these cells acquire the full functionality of self renewal and multipotency(37). Alternatively, it may be that these differences in markers represent less relevant functional aspects such as activation state or proliferative state with no repercussions for the basic stem cell functions of self renewal and multipotency. Adult HSC are progeny of the third wave, as lineage tracing studies in which restricted labeling of VE-cadherin progeny in the aorta-gonado-mesonephron (AGM) region from E10–11.5 resulted in labeled HSC postnatally(38).

In conclusion, hemogenic endothelium generates blood cells by transformation rather than by asymmetrical cell division(30, 39-40). This process is complex and, depending on the circumstances, oligopotent precursors or multipotent HSC with self renewal activity ensue. Few of the stimuli which direct this process one way or the other are known at the molecular level. Phenomena as complex as the presence of a heartbeat were shown to have a profound influence on the nature of the hematopoietic cells generated(41).

3.2.Generation of HSC/HPC from hESC

When put under conditions which induce differentiation, hESC generate hematopoietic precursors and end-stage blood cells. In general, two different methods have been used to generate blood cells from hESC. In the embryoid body (EB) method, hESC cells are cultured in clumps which are not allowed to adhere to the culture dish by using low adherence plastic or by culture in methylcellulose(42). These cell clumps initiate differentiation in the presence of serum and generate cystic bodies consisting of endoderm, mesoderm and ectoderm and subsequently more differentiated progeny. Such EB cultures can be performed under serum free conditions and the required growth and differentiation factors can be added simultaneously or sequentially(43-44). This allows a careful dissection of the stimuli which are required to obtain HSC/HPC. In the feeder method, hESC colonies are cultured on feeder layers such as OP9 cells or stromal cells derived from fetal liver, AGM or fetal placenta(45). These cultures are performed in the presence of serum without any addition of growth factors. Differentiation can be followed microscopically in such cultures or cells can be dissociated using trypsin and analyzed by flowcytometry.

When differentiation is monitored for the expression of hemoglobin genes, independent of the method used, fetal hemoglobin expression is observed first, rapidly followed by expression of adult hemoglobin chains(46-47). This suggests that during differentiation cultures a built-in development plan is started up which begins with a wave of primitive hematopoiesis, followed by a wave of definitive hematopoiesis. Likewise, the generation of HSC/HPC cells is initiated at a fixed amount of time after the start of the culture. Once this wave has generated a number of HSC/HPC, further generation of HSC/HPC stops and the precursors present in the cultures differentiate to erythroid or myelo-monocytic cells. At present, no culture conditions have been described which generate hematopoietic precursors continuously. This limitation has to be taken into account when the generation of large numbers of HSC/HPC is attempted for clinical purposes(48).

The earliest precursor which arises in EB and gives rise to hematopoietic cells has a $VEGFR2^+$ kit^- $CD34^-$ phenotype (see Figure 1 and table 1)(49). These cells include blast colony forming cells (BL-CFC), cells which proliferate and differentiate into colonies containing adherent VE-cadherin positive cells as well as blood cells. Because BL-CFC generate endothelial and hematopoietic cells in the same assay, these cells are also called hemangioblasts, suggesting that these cells are bipotent. Additional hemotopoietic activity was observed in the

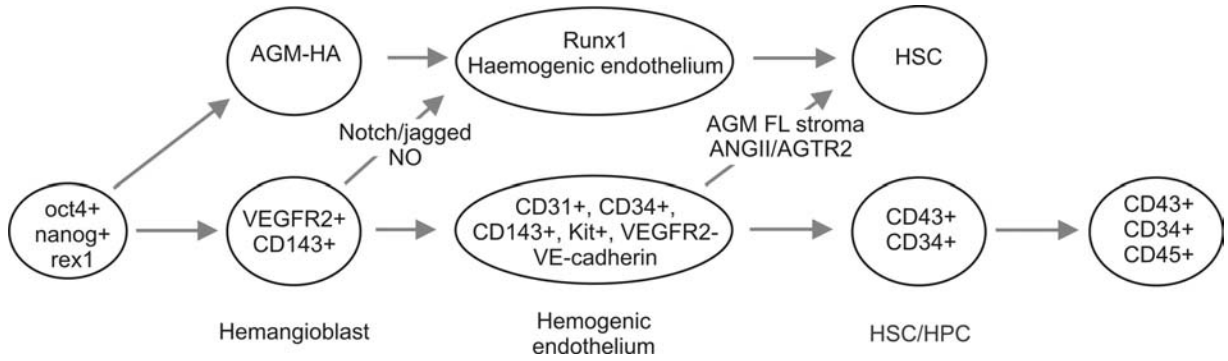

Figure 1. (lower part) hESC express oct4, nanog and rex1. Upon differentiation expression of these markers is rapidly lost. The hemangioblast is a bipotent cell which is characterized by the co-expression of CD143 and VEGFR2. The hemangioblast gives rise to $CD31^+$ endothelial cells. Some of these endothelial cells transform into $CD43^+$ hematopoietic precursor cells(HSC/HPC) which have little long-term reconstitutive potential. Further differentiation leads to $CD45^+$ precursor cells which are T/myeloid committed. (upper part) Potential strategies to differentiate hESC cells into long-term repopulating HSC: to induce AGM-like hemangioblast (AGM-HA), to exploit signals associated with AGM hematopoiesis such as jagged-1 or NO, and finally to characterize fetal liver or AGM mesenchymal stroma-associated signaling molecules which may induce maturation of hematopoietic precursors to long-term repopulating HSC.

Table 1. Flowcytometric markers for hemogenic precursors derived from hESC

Phenotype	Comments	Ref
$MIXL1^{gfp+}$	Primitive streak like cells	(109)
$Brachyury_{ic}$ +	Brachyury positive cells, primitive streak-like cells	(49)
$CD143^+$	Contains BL-CFC	(44)
$VEGFR2^+kit^-$	Contains BL-CFC	(49)
$VEGFR2^+PDGFRA^-$	Contains BL-CFC	(54, 110)
$VEGFR2^+ckit^+$	Similar to $CD45^-$ PFV	(49-50)
$CD45^-$ $VEGFR2^+$,$CD31^+$, VE-$cadherin^+$ ($CD45^-$ PFV)	Generates hematopoietic and endothelial colonies at low frequencies	(55)
$CD34^+CD43^+CD45^-$	Generates hematopoietic CFC, on stroma: pro-B cells, T cells and NK cells	(47, 57-58)
$CD34^+CD43^+CD45^+$	Generates neutrophils, eosinophils, monocytes, dendritic cells	(47, 57-58)

$VEGFR2^-$ kit^- cells, however this may have been caused by the use of a weak staining antibody, since the $VEGFR2^-$ sorted cells clearly expressed VEGFR2 when assayed by RT-PCR. Both primitive and definitive hematopoiesis is observed. Also included in this phenotypic population is the cardiovascular progenitor(50). This precursor forms colonies which contain endothelial cells as well as cardiomyocytes. Although both precursor types are present in this phenotypically defined cell population, the conditions for outgrowth are different. They seem to arise from different cells rather than a single precursor. Notch stimuli, however, can direct haemangioblasts to the cardiogenic lineage(51). BMP4 is required for the generation of both hemato-endothelial and cardiogenic precursors from hESC. BMP4 induces the expression of Wnt3a and activin/nodal. These factors are known to induce $brachyury^+$ primitive streak (PS)--like cells. BMP4 has also an effect on differentiation which is not mediated by Wnt or activin/nodal: it inhibits anterior streak formation and promotes differentiation to mesenchymal precursors of the circulatory system, hematopoiesis, AGM and extraembryonic mesoderm. This is evidenced by the induction besides brachyury of posterior mesenchymal markers such as mesp1, hoxb1, cdx1 (see table 2)(52-53).

Although BMP4 is required during the early phase of differentiation, it may inhibit blood formation by inducing differentiation towards extra-embryonic tissues if not washed away or counteracted by the addition of other growth factors such as FGFs. Activin is required for the differentiation of $brachyury^+$ mesenchymal precursors to $VEGFR2^+$ cells. The generation of cardiovascular precursors is inhibited by the action of canonical wnt, added exogenously and/or produced by the PS- like cells whereas it is required for the generation of hemangioblasts and for primitive hematopoiesis(50). BMP4 and wnt3a are specific inducers of cdx1 and cdx4, which are required for hematopoietic development(52).

The group of Peault studied angiotensin converting enzyme (ACE) or CD143 as a marker for early hemogenic precursors(44). In contrast to VEGFR2, virtually all hemogenic activity was included in the $CD143^+$ fraction. No activity was seen in the $CD143^-$ population, although antibody staining for CD143 using the BB9 antibody is dim. When cells are double-stained for VEGFR2 and CD143 markers, a diagonal staining pattern is seen, suggesting that both markers are upregulated simultaneously.

VEGF is required for the survival and differentiation of $VEGFR2^+$ precursors (table 3). Only in the presence of VEGF do $VEGFR2^+$ cells generate hematopoietic colonies. Other factors such as activin or BMP4 are no longer required at this stage. Canonical wnt is required especially for primitive erythropoiesis. In contrast, retinoic acid (RA) drives $VEGFR2^+$ BL-CFC to definitive hematopoiesis as evidenced by an increased number of myeloid cell-containing CFU and an increased percentage of $CD45^+$ cells(54). In addition, cultures treated with RA

Table 2. Common PCR markers for monitoring of differentiation from ESC

PCR markers	Cell type
Oct4, nanog, rex1	Pluripotent stem cell
Cdx2, HCG	throphectoderm
brachyury, MIXL1	Primitive streak-like/panmesendodermal markers
NeuroD, Pax6, sox1	neurectoderm
Sox17, foxa3, pdx1	endoderm
FoxA2	mesendoderm
mesp1, hoxB1	posterior mesoderm
cer1, chrd	anterior mesoderm
Meox1, mesp2, tbx6,fst, dll1	paraxial mesoderm
Isl1 , Nkx2.5, tbx5	cardiac
Tal/scl, Runx1, gata1 GATA2, LMO2	hemogenic endothelium
c-myb, PU.1, hoxB4	Hematopoietic

Table 3. Factors influencing the generation of HSC/HPC from ESC

Factor	Biologic effects	inhibitor	references
BMP4	Induces primitive streak- like cells, posterior type	noggin	(53), (111), (52), (112), (113)
Activin/nodal	High concentrations induce endoderm Increases VEGFR2$^+$ cells Increase in CFC (primitive and definitive)	SB-431542	(53), (111, 113-114)
TGFbeta1 TGFbeta3	Enhances definitive hematopoiesis(feeder layer) Blocks endothelial development (EB)	SB-431542	(74), (115)
Wnt 1(hu)/Wnt3a(m)	Required for Brachyury expression Blocks definitive hematopoiesis No effect on primitive erytropoiesis Increases %CD31, CD34 and CD45 positive cells	DKK1,	(53), (56), (116)
FGF2	Increases VEGFR2$^+$ cells Proliferation and cell expansion	SU5402	(111)
VEGF	Proliferation of VEGFR2$^+$ bipotent cells	avastin	(111)
Retinoic acid (RA)	induces PS-like cells, paraxial type Enhances definitive hematopoiesis	LE540	(54)
Jagged, delta-ligand	Arterial specification; Runx1 , gata2 induction; Increases cardiac differentiation of VEGFR2$^+$ cells	DAPT	(51, 117-119)
Angiotensin II	Increases BL-CFC formation	Captopril losartan , PD123-319	(44)
NO or SNAP (NO donor)	Induction of runx1 and myb expression in haematopoietic precursors, increases CFU activity	L-NAME	(41, 108, 120)
IL-3, SCF, FL	Early proliferation and survival factor of hemogenic cells		(37, 121)
TPO	Increases BL-CFC in mouse		(122-124)

displayed increased expression of RUNX1, GATA2, MYB and HOXB4. Before the appearance of VEGFR2$^+$ cells, RA favors the induction of paraxial type PS-like cells, the precursors of skeletal cells rather than haemangioblasts. Angiotensin II is generated from angiotensin I by ACE/CD143 expressed on the membrane of early hematopoietic precursors. It expands CD143$^+$ precursors and increases BL-CFC activity. It binds the angiotensin receptor 2 (AGTR2) and AGTR1 which are both expressed on the hematopoietic precursor. Stimulation through the AGTR1 favors endothelial differentiation, whereas stimulation through the AGTR2 favors definitive hematopoiesis.

After acquisition of CD143 and VEGFR2, cells become VEGFR2 bright and acquire expression of kit, CD31, CD34 and VE-cadherin more or less at the same time (see table 1). The group of Bhatia described an adherent CD45$^-$ PFV population present in late EB which is positive for Pecam (CD31), flk-1(VEGFR2) and VE-cadherin(55). This population also expresses high levels of CD34 but is CD45$^-$. These cells differ from human umbilical vein endothelial cells in the absent or low level expression of vWF and eNOS. A similar population obtained in feeder cultures was described by Kaufman *et al.*(56) Single PFV cells gave rise to hematopoietic as well as mature endothelial cells. These cells were enriched for markers of hemogenic endothelium (RUNX1 and SCL/Tal), for endothelial markers (TIE2) and for hematopoietic markers (MYB, MPL). Single cells gave rise to colonies in 3.2% of the cases and were either of hematopoietic or endothelial origin. Less than 0.1% of the colonies contained endothelial as well as hematopoietic cells, indicating that these precursors are more differentiated than the BL-CFC. BL-CFC are hemato-endothelial precursors, may contain other cell types such as smooth muscle cells and can be induced by environmental factors such as notch stimulation to adopt other cell fates(51).

Vodyanik *et al*, using OP9 feeder layers were the first to describe an early CD45$^-$ purely hematopoietic population(57). This CD43$^+$CD34$^+$CD45$^-$ population contains myelo-monocytic precursors, red cell precursors and NK precursors. This population is CD34^{high} and is probably derived from the CD34^{high} endothelial cells described earlier. These cells can generate CD19$^+$ pro-B cells. A molecular expression analysis could demonstrate expression of genes associated with self renewal and especially high expression of HOXB3 and HOXB4 (not by the CD45$^+$ cells), suggesting that in contrast to mouse ESC-

derived HSC/HPC HOXB4 is not deficient in these cells(58). This may be the reason why overexpression of HOXB4 in human ESC-derived HSC/HPC does not convert these cells in transplantable HSC, as in the mouse(59-61).

HSC self-renewal genes such as *EZH2, MEIS1, MLL* , survival genes (*ARHGAP1, ETV6,* and *HLF*), and especially the *AP-1* genes, encoding fos and jun proteins essential for growth factor responsiveness, were expressed at much lower levels compared to fetal liver $CD34^+CD38^-$ cells. In the EB system, similar $CD34^+$ precursors can be generated but also here precursor B cell were obtained and full differentiation was arrested at the pro-B cell stage(44). The $CD43^+CD34^+$ cell population is in fact heterogeneous for CD45 expression. About half of the cells express low levels of CD45. These cells seem to be myeloid committed, since it was no longer possible to generate $CD19^+$ cells from this population. Myeloperoxidase and PU.1 were expressed at higher levels in the $CD45^+$ than in the $CD45^-$ fraction. After addition of myeloid growth factors to these cells, large numbers of neutrophils, eosinophils, dendritic cells and monocytes could be obtained (62).

At which moment the cells become responsive to hematopoietic growth factors is not clear. CD117 (kit) is already expressed on the endothelial precursor and the IL-3 receptor (CD123) is expressed at high levels on $CD34^+CD43^+CD45^-$ cells. Mpl, the receptor for TPO, is detectable by RT-PCR at a very early stage. Using these growth factors, Taoudi *et al.* were able to expand early VE-cadherin$^+$ $CD45^+$ hematopoietic cells isolated from the AGM and at the same time these cells matured from nonfunctional to repopulating HSC(37).

3.3.Characterization of HPC/HSC

The $CD43^+CD34^+$ cells are the best candidate HSC generated from hESC cells. As stressed previously, various precursors are generated during fetal life which are limited in multipotency. These precursors seem to be generated also in hESC cultures. For this reason, it is paramount that multipotency and self renewal capacity is tested. This $CD43^+CD34^+$ population contains CFU-Mk, CFU-GEMM,CFU-GM, CFU-G, suggesting that within this population individual cells are present which are multipotent. Since this population, when cultured on MS5 cells give rise to $CD56^+$ NK cells and pro-B cell phenotypes it can be argued that adult type multipotent HSC are present. It was shown recently, that this population can also generate mature T cells (see below). However, it has not been shown formally that a single cell can generate all hematopoietic lineages and that a measurable frequency of such multipotent cells are present within the $CD34^+CD43^+$ population.

3.4.SCID repopulating cells (SRC)

Definitive proof for the presence of HSC is usually obtained in transplantation experiments(28). In mice, competitive repopulation experiments can be performed in which two populations that can be discriminated by a fluorescent marker or an allotypic membrane marker (e.g. CD45.1/CD45.2) are compared in repopulating activity. Subtle differences in cell-autonomous factors important for self-renewal can be observed in this way(63-64). In addition, single cells of the adult HSC, $CD150^+CD48^-$ Lin^- $sca1^+$ kit^+ (LSK) cells are able to repopulate a mouse and reconstitute all lineages, demonstrating not only self-renewal capacity but at the same time multipotency(35).

In the human, such assays did not exist until the advent of SCID mice. The group of Dick described the NOD/SCID repopulating assay(65). In this assay, NOD/SCID mice were injected with a candidate stem cell population and 8 weeks later repopulation of these mice with human cells was measured by DNA analysis. Flow cytometric analysis could demonstrate multilineage engraftment of erythroid, megakaryocytic, myeloid, B and NK cells. Later modifications also allowed the demonstration of T lymphopoiesis from human precursors in the murine thymus(66). The cells that engraft were $CD34^+CD38^-$ cells and about 1/600 of these cells could reconstitute. In addition, it was shown by retroviral marking that only few cells were transduced, suggesting that human SCID repopulating cells (SRC) were not cycling upon addition of a cytokine cocktail to the cultures. Based on these data, it was argued that this activity was mediated by human long-term repopulating self-renewing HSC and the assay became the gold standard for human HSC activity.

Since the original description of the SRC assay, a number of modifications were introduced such as the use of SCID/NOD/gamma$_c^{-/-}$ mice, which lack functional activity of gamma chain cytokines including IL-15 and IL-2 and therefore are deficient in NK cells and macrophage function. These mice display less xenogeneic activity resulting in higher percentages of chimerism and more lengthy engraftment of short-term repopulating $CD34^+CD38^+$ cells(67-68). In addition, intrafemoral injection instead of intravenous injection, by itself, results in increased engraftment. In these models, $CD34^+CD38^+$ engraft for a limited period and secondary transplants are advised to demonstrate self-renewal of HSC(68).

Recently, the SRC assay was validated in baboons and patients(69-70). These studies show that the presence of SRC (and SRC of secondary transplants) does not correlate in primates with long-term engraftment but rather with short-term engraftment. When a retrovirally marked hematopoietic precursor population was transplanted in SCID/NOD mice and in irradiated baboons, it was found that clones with identical integration sites were found in the short-term repopulating cells after transplantation in baboons and in the reconstituting cells of NOD/SCID mice, but long-term engraftment of baboons was associated with different clones than the ones found in SCID/NOD mice. Similarly, Delaney *et al* could show that *in vitro* expanded HSC using DL1 generated superior engraftment in the SRC assay than uncultured cells. These same expanded HSC were transplanted in patients together with fresh cord blood from another donor. In 10/10 patients the fresh cord blood engrafted in the long term, whereas the

expanded cord blood were only superior in the very short term (first weeks after transplantation).These data strongly suggest that SRC are not long-term repopulating HSC, but multipotent short-term repopulating precursors.

Finally, NOD/SCID repopulation assays are usually performed with human cells exclusively derived from hematopoietic sources. In this context, the detection of human cells points to hematopoietic outgrowth from $CD34^+$ precursors. However, when unsorted differentiation cultures of hESC are injected, many cell types are expected to grow including endothelial cells which express markers very similar to hematopoietic stem cells(71). It is therefore important to screen such mice not only with human cell specific probes and/or human markers such as hCD31 or hCD34 but also with hCD45. Expression of hCD34 on CD45 negative or weak cells does not constitute solid evidence for the presence of HSC/HPC in these mice(72-73).

With these caveats in mind, several studies could show SRC activity in differentiation cultures of hESC. Both cultures generated by the EB method as well as cultures generated by the feeder method have been injected and screened for HSC activity in the NOD/SCID model. The study by Wang *et al* was performed with EB-generated hematopoietic precursors(61). When $CD45^-$ PFV cells were injected in the bone marrow, in about half of the mice, outgrowth of hematopoietic cells was observed at 12 weeks after injection. Some of the $CD45^+$ cells expressed CD19 and CD33 suggesting lymphoid precursor activity and possibly multipotency. However, these cells showed poor trafficking since barely detectable hematopoiesis was measured in the non-injected bone marrow. The study by Ledran *et al* showed the most convincing repopulation(74). In this study, hESC were cultured on feeder cell lines of various origins. If left undisturbed for 10-18 days, hematopoietic zones were detected in the cultures. These areas consist of endothelium-lined vessels or bags filled with round $CD34^+$ cells. These structures were also described by others and were shown to contain all hematopoietic activity present in the cultures(75-76). These structures are also formed on OP9 and S17 cell lines and were shown to correlate with increased megakaryocytic and T cell precursor activity. When these cultures are harvested and the cells were injected intrafemorally in SCID/NOD/gamma$_c^{-/-}$, around 1% of human $CD45^+$ cells were recovered after eight to twelve weeks. In addition, secondary transplants gave engraftment percentages of 1-2% human $CD45^+$ cells in bone marrow and peripheral blood, suggesting again that some HSC activity is generated in these co-cultures. In the co-cultures tested, hESC were seeded on primary or established cell lines derived from the AGM region or the fetal liver. It is unclear whether AGM or fetal liver derived lines are crucial or whether more common bone marrow derived lines such as OP9 or S17, which were not tested in this study, are equally potent(77). The presented *in vitro* data, morphological aspects (hematopoietic zones), phenotype of the cells present (VEGFR2, CD34, CD45) and functionality (CFU assays), were qualitatively similar to the data obtained on OP9. Furthermore, although the authors calculated significant differences between the engraftment percentages of HSC/HPC generated on the various cell lines, the inter-experimental variation of both hESC differentiation cultures and SRC assay is so large, that differences between conditions should be interpreted carefully. For this reason, we feel that insufficient evidence has been presented that fetal mesenchymal cell lines are more effective than standard lines such as OP9 in generating HSC and that possibly a membrane molecule or secreted factor important in the generation of HSC is uniquely produced by these fetal mesenchymal lines.

EB derived cells in the absence of feeder lines are less potent in generating HSC/HPC than hESC generated on stromale lines. Recently, a head to head comparison was reported by the group of Peault(73). $CD143^+$ cells were cultured in serum free medium or on stromale lines (fetal liver stroma or human mesenchymale cells) and subsequently tested *in vitro* and *in vivo*. In contrast to serum free cultures, stromal hESC cultures generated CFU identical to cord blood $CD34^+$ CFU including expression of adult hemoglobin, and upon injection in the fetal liver of immune-deficient animals, generated hematopoietic activity lasting at least 12 weeks. In the thymus of some of these mice, $CD4^+CD8^+$ T precursor cells were observed indicating T precursor potential of the injected human cells.

4.GENERATION OF LYMPHOID CELLS FROM HPC/HSC

YS-like precursors do not generate lymphoid cells *in vivo* although they may generate lymphoid cells under non-physiological conditions such as culture on AGM-derived stroma(78). hESC derived HSC/HPC, however, can generate T, B and NK cells.

4.1.Generation of B cells

Murine ESC readily generate B cells on OP9 stromal cells: the appearance of $CD19^+$ pro-B cells is preceeded by generation of $sIgM^+$ B cells, which are LPS responsive (45, 79-81). The addition of FL and IL-7 is crucial to obtain B cells and cultures should be kept for 30-40 days for full B cell maturation to occur. No reports claiming the generation of human $sIgM^+$ B cells were published. The SRC assay is in principle a well suited assay to detect B cell precursor activity of hESC derived HPC/HSC, since B cells are usually detectable in the bone marrow of human $CD34^+$ transplanted NOD/SCID mice. B cells increase in relative percentage with time after transplantation. Ledran *et al.* reported the presence of small numbers $CD2^+CD19^+$ B cells in the peripheral blood and Wang *et al* showed robust $CD19^+CD45^+$ outgrowth in the injected bone marrow(61, 74). However, these cells were not further studied and it is unclear whether the cells had rearranged heavy chain and/or kappa lambda light chain loci, expressed and/or secreted IgM and IgD, and whether they responded to classical B cell proliferation stimuli such as CD40 and IL-4.

Two papers report the generation of $CD19^+$ cells in differentiation cultures of hESC. Zambidis *et al*, using the EB method to generate HSC/HPC and subsequent OP9

cultures to differentiate the cells to B and NK cells, could generate few weakly staining CD19 cells, which expressed low levels of RAG, and scant surrogate light chains(44). Vodyanik using the OP9 co-culture system and subsequent culture on MS5, could generate a robust CD19 population which expressed surrogate light chains and CD79a(47). No further characterization of these cells was reported in either of these studies. $CD19^+$ cells were generated from the $CD34^+CD43^+CD45^-$ fraction whereas $CD34^+CD43^+CD45^+$ cells were devoid of B cell precursor activity.

4.2.Generation of NK cells

NK cells are readily generated in HSC/HPC differentiation cultures. When hESC derived $CD34^+$ HSC/HPC cells are transferred to MS5 stromal layer in the presence of SCF, FL, IL3 and IL-7 $CD56^+$ NK cells are generated which are IL-15 responsive(47). When hESC (or $CD34^+$ adult HSC) are cultured on OP9-DL1 in the presence of Il-7, SCF and FL, conditions which favor T cell development, NK cells are generated as well. However, when IL-15 is added to the cytokine mixture, T cell development is inhibited and preferentially NK lineage cells grow out (82). NK cell function of these cells was studied most extensively by the group of Kaufman(83-84). Using hESC feeder cell co-cultures, HSC/HPC cells were transferred on AFT024 stromal line in the presence of HSC growth factors such as IL-3, SCF and FL and specific NK factors such as IL-15. NK cell differentiation progressed from a $CD34^+$ precursor which acquired CD117 and subsequently lost expression of CD34 and acquired CD94 expression. Mature NK cells are $CD94^+$ $CD56^+$ $CD117^-$. hESC derived HPC/HSC, in contrast to cord blood HSC, seem to mature much faster to $CD117^-CD94^+$ mature NK cells and immature intermediate stage are no longer present 3 weeks after the initiation of the cultures. hESC derived NK cells expressed CD45 and CD56. The majority of the cells were $CD16^+$ and expressed NK receptors such as CD94, NKG2D and KIR receptors. The cells express granzyme and perforin and were cytolytic for K562 and for a panel of tumor lines. It was shown by Vodyanik that $perforin^+$ $CD56^+$ NK cells are generated from the $CD34^+CD43^+$ fraction and both the $CD45^-$ and $CD45^+$ fraction can generate NK cells(57).

4.3.Generation of T cells

"Lymphoid" differentiation cultures which usually consist of an MS5 or OP9 feeder layer supplemented with various cytokines including FL, IL-7 do not support T cell differentiation. Expression of CD3 molecules is seen, but this is due to the presence of fetal-type NK precursors or mature NK cells.

To obtain T cell differentiation, Galic *et al* used an *in vivo* model(85). The SCID-hu mouse is a SCID mouse transplanted with human fetal thymic material together with human fetal liver material. In these mice fetal liver HSC generate T cells in the transplanted thymic lobule during the lifetime of the mouse(86). After low-dose irradiation to induce apoptosis of resident thymocytes, the human thymus can be injected with T precursors and the progeny of these precursors can be studied during the weeks and months following injection. Usually GFP or HLA-A2 is used to discriminate endogenous from injected cells. Injected GFP-labeled $CD34^+$ cells derived from hESC were injected and few GFP^+ $CD3^+$ $CD4^+CD8^+$ positive cells could be detected 3 weeks after injection in about half of the injected mice. The fact that the $CD3^+$ cells expressed only low levels of CD45 compared to the endogenous thymocytes suggests aberrant differentiation. Surprisingly, hESC derived $CD133^+$ $CD34^-$ cells, which in our hands had no hemato-endothelial precursor activity also generated $CD3^+$ cells in SCID-hu mice. In a more recent paper of the same group, the differentiated progeny of hESC cultured as EB were injected in a human thymic lobule of SCID-hu mice(87). In a percentage of these thymuses, GFP positive $CD45^+$ (bright) cells were observed. Phenotypic analysis was consistent with a single wave of T cell precursor activity as first CD4 CD8 DP cells were observed and after 8 weeks only mature SP cells were observed. This suggests that T cell precursor activity was present in the EB cultures, but, unlike fetal liver $CD34^+$ cells, these EB-derived cells were unable to support continuous precursor activity. They furthermore showed that T cell precursor activity was confined to the $CD34^+CD45^+$ population, whereas the $CD34^+CD45^-$ fraction had little T cell precursor activity. The latter is not surprising since the vast majority of the $CD34^+CD45^-$ fraction consists of endothelial cells, with little haemogenic activity and the $CD34^+CD43^+CD45^-$ precursor population represents only a minor fraction of this population. It is therefore not clear from their studies whether the $CD34^+CD43^+CD45^-$ or $CD45^+$ population is the main T cell precursor population. Timmermans *et al* has reanalyzed that and found that both populations have T cell precursor activity(76). They could furthermore show that T cells generated were polyclonal by spectratype analysis, indicating that rearrangement activity of the TCRbeta genes was comparable to that of normal thymocytes.

More recently, Peeters *et al* cultured EB derived $CD143^+CD34^+$ cells on murine fetal liver stroma or human mesenchymal cells(73). These cells were subsequently injected in SCID/NOD/$gamma_C^{-/-}$ mice and reconstitution was observed. Surprisingly, $CD4^+CD8^+$ DP human cells were detected in the murine thymus of these mice 12 weeks after injection.

Although these *in vivo* experiments showed that T cell precursor activity is present in hESC derived HPC/HSC, the approach they used cannot be applied to translational medicine. We therefore studied whether T cells could be generated from hESC using only culture systems and no *in vivo* differentiation steps(76). We used the OP9 feeder system because the HSC/HPC were well defined in this system. Hematopoietic zones generated on OP9 cells, were subsequently transferred en bloc to OP9-DL1 feeder layer that was supplemented with FL and IL-7. It was found that in the presence of DL1 $CD34^+$ cells rapidly became $CD45^{dim}$ and remained $CD34^{bright}$, which is the typical HSC phenotype seen in postnatal peripheral blood and bone marrow. In contrast to postnatal HSC, these $CD34^{bright}CD45^{dim}$ cells were negative for CD133. It was furthermore observed that these cells were highly responsive to SCF and addition of high doses of SCF increased progeny significantly. Within 10 days of culture

on OP9-DL1 T /NK lymphoid commitment was visible by the expression of CD7 and intracellular CD3epsilon. These cultures first generated mature $CD7^+CD5^-$ $CD56^+$ NK cells which were highly active and seem to lyse the OP9-DL1 feeder layer. Gradually, T cell precursors appeared: first CD7+CD5+ cells, then CD4+, CD1+ and finally CD4+CD8+ DP cells were obtained which are the hallmark of thymic TCRalphabeta differentiation pathway. About 5 weeks after the start of the OP9-DL1 culture, mature TCRgammadelta cells and CD4 and CD8 TCRalphabeta cells were observed. The cells displayed a mature CD1-CD27+ CD4 or CD8 SP phenotype and could be expanded using CD3 and CD28. T cell clones could be obtained that secrete cytokines such as interferon-gamma upon activation. The hESC derived HSC/HPC behaved similar to HSC obtained from fetal liver, in that the T cell differentiation kinetics were similar and much faster than the kinetics of cord blood or adult bone marrow HSC. In addition, mainly TCRalphabeta cells were obtained, whereas cord blood HSC initiated cultures generate mainly TCRgammadelta cells(11).

These experiments demonstrate that besides fully mature NK cells also T cells can be generated from HSC/HPC derived from hESC. It is surprising that B cell activity cannot be generated from these HSC/HPC. This could be due to notch stimulation in these cultures. Since notch1 is expressed on HSC/HPC and DL is expressed on arterial endothelium, it may be that the HSC/HPC, which under the culture conditions used are trapped within the endothelial lining, become stimulated via the notch pathway. It is known that one of the earliest effects of notch activation is the loss of B cell precursor activity, whereas erythromyeloid differentiation is not or much less affected.

By CFU-GEMM assays, it was demonstrated that within the $CD34^+$ population, single cells are present which can differentiate to erythroid, magakaryocyte and myelomonocytic lineages. It was furthermore shown that NK cell activity and T cell activity is present in the $CD34^+CD43^+CD45$+/-(47, 76) population. In addition, abortive B cell activity was demonstrated in the $CD34^+CD43^+CD45^-$ fraction(47). These data suggest that the multipotent precursor is confined to the $CD34^+CD43^+CD45^-$ population. The $CD34^+CD43^+CD45^+$ population, which has lost B cell activity, is comparable to the early thymic precursor(88). Although the data are suggestive, it has not been formally shown that a single cell can generate myeloid as well as lymphoid cells, as has been shown for adult HSC(14). Neither has it been shown that the SRC which was demonstrated by Ledran and others is confined to this same phenotypic population(61, 72-74).

5.THE QUALITY OF THE T CELL REPERTOIRE

T cell populations should be polyclonal, positively selected on the MHC molecules of the patient and negatively selected for auto-reactive cells. Only if these 3 conditions are fulfilled, can such T cell cultures be used for treatment of stem cell transplant recipients or HIV patients.

5.1.CD4/CD8-polyclonality

Similar to *in vivo* thymopoiesis, immature DP cells start expression of the TCRalphabeta receptor in OP9-DL1 cultures. These $CD3^+$ cells are polyclonal in the usage of the Vbeta repertoire(11, 76). The cells subsequently mature to $CD27^+$ TCRalphabeta cells and finally acquire the fully mature $CD1^-CD27^+$ TCRalphabeta phenotype. In contrast to the thymus, mainly CD8 SP cells are generated. These $CD8^+$ cells express the CD8alpha and CD8beta chain, are fully functional and produce cytokines such as interferon-gamma and IL-2, and are cytotoxic. Fewer but significant numbers of $CD4^+$ SP cells are generated. These cells display a helper phenotype probably initiated by ThPOK, which is expressed in these cells, and express CD40L, IL-2, IL-4 after TCR stimulation(89).

5.2.Positive selection

It is unclear at the moment, how T cells acquire the signals to become mature T cells. During *in vivo* thymopoiesis, the TCR and the CD4 or CD8 co-receptor molecules of $CD3^+$ DP T cells interact with MHC class I or class II molecules respectively on thymic epithelium. This interaction is weak, but strong enough to activate the erk pathway which via Egr1/3 upregulation activates the ID proteins(90). MiR181a, a miRNA which inhibits phosphatases lowers the TCR stimulation threshold at the DP stage(91). ID proteins counteract the E proteins, which keeps the cell in the DP state. After blocking of the E proteins, DP cells become SP CD4 or CD8 cells and simultaneously acquire function. After TCR stimulation, DP cells lose CD8 expression and in the presence of IL-7, expression of RUNX3 is upregulated. Runx3 initiates CD8 upregulation, CD4 downregulation and the CD8 cytotoxic program(92). In the absence of IL-7 and continuing TCR stimulation mediated by CD4-MHC class II interaction, ThPOK is upregulated which initiates the Th program, typical for CD4 SP T cells.

Which mechanism initiates the generation of mature T cells from DP cells in OP9-DL1 co-cultures is unclear. MHC molecules are expressed in these cultures, however no thymic epithelial cells are present. We have analyzed whether OP9 cells replace the MHC presenting function of thymic epithelial cells and whether MHC expressed on these cells is responsible for positive selection. However, murine MHC expression on OP9 cells was low to undetectable in our hands. In addition, neither forced expression of HLA-A2 by retroviral transduction of OP9-DL1, nor the increased expression of murine MHC by incubation with interferon-gamma, increased the number of mature T cells generated.

In addition, we initiated cultures in which no MHC class II expression at any one time could be measured. These cultures were set up with $CD4^+$ immature SP cells. $CD4^+$ immature SP cells are pure (TCRalphabeta en TCRgammadelta) T cell precursors and no NK cells or dendritic cells are generated from these precursors. Monitoring these cultures for MHC expression and for generation of dendritic cells from the start until the appearance of mature $CD4^+$ SP $TCRalphabeta^+$ cells, clearly demonstrated that in the complete absence of MHC

class II, mature $CD4^+$ SP T cells were generated in numbers virtually identical to cultures in which dendritic cells were present(89).

Similarly, $CD8^+$ T cells were generated in equal numbers in cultures in which MHC class I expression and the CD8 molecule were functionally blocked by monoclonal antibodies, again demonstrating that positive selection, as it happens in the thymus, is not responsible for the generation of mature CD8 and CD4 cells in these cultures (unpublished data).

Alternatively, mature CD4 and CD8 SP T cells may be generated in these cultures by the SLAM-mediated pathway by which a number of thymic populations such as NK/T cells are generated(93). Characteristic for this pathway is the fact that the presenting cell type is not thymic epithelium but thymocytes themselves such as DP cells. This pathway is dependent on Slam-associated protein (SAP)(94-97). Although the generation of NKT cells was reported in OP9-DL1 using murine precursors, we have found no evidence for the presence of NKT cells in co-cultures starting from human precursors(89, 98-99).

It is unclear which mechanism is responsible for *in vitro* maturation of TCRalphabeta cells. It is equally unclear whether such cells could reconstitute immune function in a transplant patient. Although it was originally thought that positive selection imposed by the thymus was essential for immune function, it is now clear that besides positive selection in the thymus also peripheral selection mechanisms are important(100). T cells survive in the periphery because of continuous activation through TCR MHC interactions as demonstrated by the observation that MHC deficient mice are unable to support the survival of injected peripheral T cell populations(101). Similarly, T cells positively selected in the thymus on a different MHC than that present in the periphery, reconstitute immune function poorly(102). However, in OP9 cultures mature T cells are probably randomly generated or at least without interaction with MHC present in these cultures. When such unselected cells are injected in patients, one could envision that a fraction of these cells will be stimulated by MHC present in the peripheral lymphoid organs of the patient and survive and/or expand. Based on the number of cells that dies in the thymus by "neglect", it can be estimated that 1-5% will survive and expand. Although this process is inefficient it may be sufficient for immune reconstitution in stem cell transplant recipients.

5.3.Negative selection

T cell populations which have not been deleted of autoreactive cells may cause a graft versus host-like autoimmune disease. This has been demonstrated recently in mouse models using TCR transfer(103). When a random TCRalpha or TCRbeta chain is introduced in mature postthymic T cells, these cells cause autoimmune diseases after infusion in the mouse. This phenomenon is probably caused by the pairing of the transgenic TCR chain with the endogenous TCR chains, forming a new unselected TCR. Whether T cell populations which have been generated on OP9-DL1 are negatively selected for reactivity against MHC present on the autologous cells has not been tested. Given the observation that dendritic cells are the most powerful mediators of negative selection, and that dendritic cells are formed abundantly in OP9 cultures, it could be expected that autoreactive T cells are to a large degree eliminated in these cultures before becoming mature T cells. However, medullary thymic epithelium plays an essential role in negative selection of T cells directed against tissue-specific antigens. AIRE, expressed in medullary epithelium, is responsible for the "ectopic" expression in the thymus of these proteins and the subsequent transfer to dendritic cells for presentation to T cells(104). This is an important mechanism since mutations of this gene result in APECED, an auto-immune disorder characterized by polyendocrinopathy, candidiasis and ectodermal dystrophy. Since no medullary epithelium is present in OP9 cultures, T cells reactive to endocrine organ specific antigens may not be eliminated.

6.GENERATION OF AUTOLOGOUS LYMPHOCYTES FROM hiPSC

hESC can differentiate to HSC/HPC and subsequently to mature CD4 and CD8 positive T cells. T cells generated in this way, however, are not autologous to the patient and therefore of limited use. However, with the advent of hiPSC technology, PSC can be generated from patients. It has been reported that HSC/HPC can be generated from such cells in a similar manner than from hESC. Choi *et al* generated hiPSC lines from fetal and adult sources through reprogramming with POU5F1(OCT4), SOX2, NANOG and LIN28(105). Some hiPSC lines were reprogrammed without LIN28. When cultured on OP9 stroma, these hiPSC lines generated the whole spectrum of hematopoietic precursors including the multipotent $CD34^+CD43^+CD45^-$ cells. These cells formed CFU-GEMM. The absolute numbers and the percentages of HSC/HPC generated varied by a factor 10 but were comparable to those obtained with a panel of hESC lines. It was not reported whether lymphoid cells could be generated in these cultures. Lengerke *et al* generated hiPSC from adult fibroblast using OCT4, SOX2, KLF4, MYC, hTERT and SV40 large T. hTERT and large T were no longer detectable in the established hIPSC(106). In EB cultures, $CD34^+CD45^+$ cells were generated with kinetics comparable to those of hESC cultures. In functional assays, CFU-GEMM were formed. Although none of the studies reported so far on hiPSC, demonstrated the generation of T, NK or B cells, it seems likely that the hematopoietic precursors generated are capable of lymphoid differentiation. It was reported recently that murine iPSC can differentiate into functional T cells which can reconstitute RAG-deficient mice(107).

7.CONCLUSIONS

T cells, B cells and NK cells can be generated from hESC. It was demonstrated that T and NK cells are functional. hESC-derived mature antibody-producing B cells have not been described. It is expected that immune cells can be generated from patient-derived hiPSC using similar protocols.

All immune cells are derived from HPC/HSC. For efficient generation of immune cells, the continuous generation of hematopoietic precursors and/or the generation of long-term repopulating self-renewing HSC in hESC or hiPSC cultures is essential. However, neither is possible at the present time.

The generation of hematopoietic precursors in hESC (stromal or EB) differentiation cultures is not continuous but occurs in transient waves. After the initiation of the culture, a wave of primitive erythropoietic activity is followed by a wave of definitive multipotent activity again followed by terminal differentiation of these precursors into myeloid lineages. It is therefore essential that precursors for lymphoid cell generation are harvested at the time of peak production. Insight in the complex mechanisms which control this developmental program is currently insufficient to intervene experimentally at that level.

Cells with all the characteristics of long-term repopulating self-renewing HSC are not generated in these cultures. Instead a multipotent cell is generated with limited proliferative capacity and limited self-renewal activity. Based on present knowledge, alternative approaches for the generation of self-renewing HSC can be envisioned (Figure 1).

It is possible that cultures used today for generation of hematopoieitic cells are optimized to generate extra-embryonic mesoderm which generates YS hematopoietic precursors but not self-renewing HSC. If this is the case, cultures should be screened early for markers of splanchnopleura/AGM rather than hemangioblast markers. Alternatively, hemogenic precursors may be capable of both YS-like hematopoiesis and AGM-type hematopoiesis and the environment, rather than the precursor, is the determining factor. Such a factor could be notch ligands such as jagged-1, which are not expressed in the human YS but are expressed in the AGM region. It could be more ill defined stimuli such as mechanical forces mimicking arterial pulsations mediated at least partly by the endogenous production of NO(108). Finally, the HSC/HPC may be immature and may need passage through a hematopoietic organ such as the fetal liver environment, for the acquisition of self-renewal capacity.

Whatever the nature of the hematopoietic precursor, T cells can be generated from such cultures. The HPC/HSC generate large numbers of $CD7^{+}CD5^{+}$ precursor and mature functional T cells are produced. The process that generates functional T cells differs from positive selection of conventional T cells in the thymus since MHC nor CD8 co-receptor molecules are required. It may be that alternative ligands are involved and that a SAP-dependent mechanism is operative. Alternatively, supraphysiological notch stimulation may upregulate ID3 and generate mature cells independent of TCR specificity.

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